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CHAPTER 2

Infectious Disease

James F. X. Wellehan Jr. • *Michael Lierz* • *David Phalen* • *Shane Raidal* • *Darrel K. Styles* • *Lorenzo Crosta* • *Alessandro Melillo* • *Petra Schnitzer* • *Angela Lennox* • *Johannes Thomas Lumeij*

THE PATHOGENESIS OF INFECTIOUS DISEASES

James F. X. Wellehan Jr.

Infectious disease is often thought of as a war against microbes. Koch's postulates, established in 1844, stipulated that a pathogen must be found in diseased but not healthy hosts, that it must be isolated in culture from a diseased host, that it should cause disease when introduced into a healthy host, and that the same organism should be isolated from the experimentally infected host after causing disease. While Koch's postulates have their use, they frequently result in a false dichotomous understanding of microbes as either pathogenic or nonpathogenic. Microbes are essential for all vertebrate life, for functions including digestion, nutrition, and defense, and disease is dependent on context. There is no such thing as a microbe that is always either a pathogen or a nonpathogen. There have been many asymptomatic human Ebola virus infections, and people have died of *Lactobacillus acidophilus* septicemia.[1,2]

Evolution and ecology are central to infectious disease. Evolution is an essential concept in biology. Indeed, when one considers definitions for life, perhaps the simplest and most elegant definition is that life consists of things that evolve. A microbe does not "want" to cause disease or not cause disease. All life on earth has been selected for billions of years to reproduce successfully, and this is all that matters from an evolutionary standpoint. If pathogenic traits provide an evolutionary advantage in a given situation, they will be selected for. If they provide a disadvantage, they will be selected against.

Multiple factors influence evolutionary rates, including generation times, fidelity of copying genes, and selective pressures. Microbes often have very short generation times. Ribonucleic acid (RNA) viruses, typically lacking proofreading, have high error rates when they make copies. As a result, evolution rates in microbes tend to be rapid, and RNA viruses are the most rapidly evolving organisms. This is useful for rapid adaptation to novel selective pressures such as immune selection and antimicrobial drug use. To compensate, the most rapidly evolving genes in vertebrates are immune related.

There are a number of important selective pressures impacting microbes in an avian host, including nutrient availability, temperature, competition with other microbes, the need to transfer to a new host, and the host immune system. A vertebrate host is a nutrient-rich environment. However, some nutrients may be sequestered; one example is iron, which is a limiting factor for the growth of many bacteria. Significant resources are spent by the host synthesizing transferrin, lactoferrin, and ferritin to make iron unavailable. Many bacterial virulence pathways have evolved to access this sequestered iron.[3,4]

Homeothermic vertebrates also provide a highly temperature-controlled environment, whereas poikilothermic hosts require the ability to survive at different temperatures. Infectious disease manifestation may be highly temperature dependent in poikilotherms.[5] In nonavian reptiles, temperature manipulation is often the most significant therapeutic approach. West Nile virus infection in alligators at avian-like body temperatures presents as hepatitis and encephalitis, as it does in a bird.[6] At cooler temperatures, alligators present with lymphohistiocytic foci in skin, known as *pix disease*, which is not life-threatening.[7] Significant temperature manipulation is not a reasonable therapeutic option in birds, unlike their closest relatives, although a fever response is clinically useful. Further investigation of the role of temperature in disease manifestation in birds is strongly indicated, especially with populations of many avian species critically declining and likely to be impacted by anthropogenic climate change.[8,9]

Fortunately for birds, they do not appear to be the most susceptible taxa to climate change. Many are familiar with the K-T extinction 66 million years ago as a result of a meteor impact at the end of the Cretaceous era. Approximately 65% of species disappeared from the fossil record at this time, including the nonavian dinosaurs. This is not the largest extinction in the fossil record; at the end of the Permian era, about 252 million years ago, approximately 95% of species went extinct as a result of the eruption of the volcanoes forming the Siberian steppes, burning extensive coal beds and releasing large quantities of carbon dioxide. This led to a global warming event that was unparalleled until now.[10,11] The dominant species in the late Permian era—carnivorous gorgonopsids and herbivorous dicynodonts—were in the lineage containing mammals, Therapsida. With highly soluble urea as nitrogenous waste requiring expensive loops of Henle, lack of a renal portal system to conserve water, and lack of an efficient unidirectional air flow respiratory system, the mammal lineage

was hardest hit and nearly went extinct when Pangaea became a hot desert. The dinosaurs, more fit to deal with this, arose out of the ashes of this extinction and dominated the planet for the next 185 million years.

Microbial competition is also a major selective pressure in a bird; many organisms want to live in such a nutrient-rich environment. The majority of antimicrobials are derived from products secreted by other microbes that help them compete for ecologic niches. Animal guts are some of the most diverse and rich ecosystems to be found anywhere. Many organisms that have evolved in such a competitive environment have resistance to many antimicrobials, the *Enterococcus* sp. being a classic example.

The need to transfer to a new host creates significant selective pressure. This often involves secretion of large amounts of microbes via respiratory discharge or diarrhea, but other routes occur, such as the simultaneous behavioral changes and salivary gland shedding of rabies, or the use of insect vectors. There are three fundamental strategies that can be taken to deal with limited host lifespans. First, a microbe may survive well in the environment. Second, a microbe may adapt to a balance with the host environment. Finally, a microbe may move quickly to a new host.

Parasites often adapt to a balance with their host. Many parasites tend to have slower generation times compared with viruses or bacteria, making rapid reproduction and moving on to a new host less of a viable strategy. Many parasites bring relatively minimal costs to their definitive hosts, as it is advantageous to preserve their habitat. Bullfrog tadpoles carrying the pinworm *Gyrinicola batrachiensis* have better feed conversion and metamorphose earlier than uninfested controls, rendering the relationship mutualistic rather than parasitic.[12] However, for parasites with indirect life cycles, causing disease in an intermediate host may be advantageous. If a dove carrying *Sarcocystis calchasi* is debilitated, it is more likely to be eaten by a hawk, which would complete the life cycle. This may also result in greater disease in accidental hosts.[13] Some parasites do survive well in the environment; this reduces the selective pressure to not harm the host. Parasites that survive well in the environment are much more likely to cause significant disease.

Most fungi survive well in the environment, resulting in little selective pressure to keep their hosts alive. They compete significantly with bacteria for the same niches; this has resulted in the production of antibacterial compounds by fungi and antifungal agents by bacteria. The fungi are some of the closest relatives of animals; fungi, choanoflagellates, and metazoa (multicelled animals) form a clade known as the Opisthokonta.[14] A bird is much more closely related to a mushroom than it is to an oak tree. Antimicrobial drugs generally exploit differences in chemistry and metabolism between pathogen and host. Because fungi and avian hosts diverged more recently, there are fewer differences to exploit, and antifungal drugs tend to have narrower therapeutic indices and use a smaller subset of mechanisms.

Bacteria constitute a large portion of the avian ecosystem. There are far more bacterial cells in a normal bird than there are bird cells. Traditional approaches to examining bacterial diversity have depended on culture; this is a poor way of assaying diversity. Culture-independent methods such as 16S polymerase chain reaction (PCR) and cloning or high-throughput sequencing methods have revealed that standard culture-based methods will detect between 1% and 10% of bacterial species present in most ecologic niches. As an understanding of further diversity has arisen, it becomes clearer that a vertebrate is a complex ecosystem.[15] This system may be very dynamic. The gut flora of chickens changes significantly in response to antibiotic and anticoccidial use. After treatment with monensin and tylosin, bacteria in the phylum Firmicutes (the "classic" gram-positives, containing organisms such as *Clostridium*, *Staphylococcus*, and *Streptococcus*) shift away from the genus *Lactobacillus* and toward the genus *Clostridium*.[16]

Ecologic disturbance may have significant negative impacts on many aspects of health. Damage to healthy gut flora by antibiotic use provides opportunity for invasive species; recent treatment with antibiotics markedly increases host susceptibility to *Salmonella*.[17] A 5-day course of ciprofloxacin will change human gut flora diversity and composition for several weeks, and the original composition may never be reestablished.[18] In many ways, the use of broad-spectrum antibiotics for a bacterial infection in a vertebrate is analogous to starting a forest fire to get rid of coyotes. The ideal treatment for a bacterial pathogen would be as narrow spectrum as possible, minimally disturbing the rest of the host ecosystem. Fidaxomicin, which targets only *Clostridium difficile* and a few very closely related species and does not even significantly impact many other *Clostridium* spp., is an excellent example. Unfortunately, current market forces have resulted in pharmaceutical companies developing antibiotics with as broad a spectrum as possible, and narrow-spectrum antibiotics are often not put through further development and clinical trials.

Antibiotic use without consideration of microbial ecology and evolution rapidly leads to failure. Back in the 1990s, fluoroquinolones were used in poultry. Over the next few years, human *Campylobacter jejuni* isolates from humans acquired a high rate of ciprofloxacin resistance, which had previously been rare[19] and therefore posed a greater risk to human health than previously. Use of modern farming practices, including high stocking densities and use of antibiotics as growth promoters, leads to higher antibiotic resistance rates.[20] Wild birds typically have lower *Salmonella* carriage rates and less antibiotic resistance compared with farmed poultry.[21] The only realistic way to reduce the risk of *Salmonella* in farmed birds over the long term is to alter the ecology that the organism inhabits, including facilities engaged in companion bird breeding. Keeping farmed animals in high population densities increases contact rates, pathogen loads, and stress and lowers barriers to transmission. Increased ease of transmission reduces the selective pressure to keep the host alive and healthy.

Viruses are strictly dependent on host cells for replication. Therefore, living free in the environment as a strategy for dealing with limited host lifespans is not a viable option. There are a number of important properties that impact viral evolution and ecology. Enveloped viruses are surrounded by a lipid envelope. This envelope is usually essential for invading a host cell. Nonenveloped viruses use other mechanisms to invade a cell. The lipid envelope is easily damaged, making disinfection easier when dealing with an enveloped virus.

Segmentation of viral genomes, which allows reassortment, provides a hybrid advantage for crossing host species;

this has been best studied with influenza.[22] Random genetic mutations are much more likely to be deleterious than advantageous. Acquiring functional genes that are from a related organism is significantly more likely to be advantageous. Throughout biology, hybridization is a factor that allows for rapid nondetrimental change and for species to invade novel habitats.[23] New sites of infection or host species are novel virus habitats. Animals and plants invest significant resources into sex; it would be much easier to sit on the couch and bud, rather than having to take a shower and go on a date, but the advantage of more rapid evolution is worth the cost. Viral recombination is the equivalent of sex. Influenza, a negative stranded RNA virus in the family Orthomyxoviridae, is a segmented virus, and it changes so rapidly that a new vaccine is needed every year. Measles and its nearest avian relative, Newcastle disease, are caused by negatively stranded RNA viruses in the family Paramyxoviridae, which are biologically similar but are not segmented. Vaccination for paramyxoviruses typically results in lifelong protection because the virus does not do the viral equivalent of sex and therefore does not change rapidly. Another example of a segmented virus leading to rapid adaptation to divergent hosts is in the genus *Orthoreovirus*; a virus identified in parrots by one institution was nearly identical to one found by another institution in a case of abortion in a Steller sea lion in Alaska, representing an avian–mammal host jump.[24]

Nucleic acid type is another property with a major impact on viral evolution and ecology. Many large deoxyribonucleic acid (DNA) viruses adapt to a balance with their hosts, especially those with intranuclear replication. This is seen with latency or chronic infection, requiring a delicate balance with the host immune system, and a larger number of genes is often needed to maintain this balance. Because they are larger and more complicated, they require more accurate replication to avoid accumulating lethal mutations. DNA viruses usually have much more accurate replication, with either host or viral proofreading mechanisms in place. Many DNA viruses evolve at rates comparable with their hosts, enabling larger viral genomes with greater numbers of genes. Viruses reproducing in the nucleus often utilize the host replication machinery there, unlike viruses replicating in the cytoplasm which must supply their own replication proteins. This results in greater dependence on a given host, and large DNA viruses with intranuclear replication are the most host-specific viruses.[25] Adenoviruses and herpesviruses, both large intranuclear DNA viruses, have co-diverged evolutionarily along with their hosts. In Figure 2-1, a herpesviral phylogenetic tree is shown. The earliest amniote divergence is between mammals and reptiles, as seen in Chapter 1. All known β-herpesviruses and γ-herpesviruses use mammal hosts, and the longer branch lengths in this area indicate that these viruses have diverged over a longer period. In the α-herpesviruses, the first agents to diverge infect squamates; the squamates are the earliest divergence within the reptiles. The next group to diverge are the herpesviruses infecting turtle or tortoise hosts; this is also consistent with host divergence patterns. *Mardivirus* and *Iltovirus* infect avian hosts. However, the mammalian α-herpesviruses nest with the clade infecting avian hosts, closest to *Mardivirus*. The branch lengths within the mammalian α-herpesviruses are relatively short, indicating that these viruses have not diverged from each other to the same extent that mammalian herpesviruses in the other subfamilies have. One plausible explanation for this is that the mammalian α-herpesviruses represent a host jump to mammals from the Dinosauria. Chicken pox, caused by the α-herpesvirus *Human herpesvirus 3*, may be a descendant of an avian virus and more aptly named than had been realized.

The host adaptation of some large DNA viruses provides selective advantage to causing minimal pathology in their hosts. A long-lived host may provide suitable habitat for decades. However, this balance in a definitive host may not apply to other hosts. Hosts that are similar enough for a virus to infect but dissimilar enough for the intricate balance of latency or chronic infection to not work often results in overwhelming and often fatal infection. The most significant pathology associated with herpesviruses is in aberrant hosts. A well-balanced host–virus relationship may actually be beneficial to the host. *Columbid herpesvirus 1*, endemic in rock doves, causes disease in squabs kept in stressful conditions, but the overall pathology is relatively minimal. However, in raptors, which prey on rock doves, *Columbid herpesvirus 1* causes an overwhelming infection that is rapidly fatal.[26] The advantage to the pigeon populations of killing off predators likely outweighs the disadvantage of minor disease in neonates.

RNA viruses reproduce less accurately. They usually lack proofreading and have the highest mutation rates of any organisms on Earth. These mutation rates mean that a large complex genome is not possible because their high error rates would cause offspring requiring a large gene set to be nonfunctional. RNA viruses therefore have small genomes and fewer genes. The advantage of such a high error rate is that RNA viruses are capable of rapidly outmaneuvering the host immune system. The strategy of RNA viruses is typically rapid reproduction and moving on to a new host. Because they have less complex relationships with their hosts, RNA viruses are much more capable of moving to new host species. The ability to move to new hosts reduces the selective pressure to not harm the host, and many RNA viruses are more pathogenic. One meta-analysis found that of the 20 virus families infecting the best-studied vertebrate species, humans, four RNA virus families, Reoviridae, Bunyaviridae, Flaviviridae, and Togaviridae, accounted for more than half of emerging and re-emerging viruses.[27] Most of the high-profile human viral diseases that have recently emerged are RNA viruses, including Ebola (Filoviridae), severe acute respiratory syndrome (SARS) and Middle East respiratory syndrome (MERS) (Coronaviridae), Chikungunya (Togaviridae), West Nile (Flaviviridae), influenza (Orthomyxoviridae), and Hendra (Paramyxoviridae) viruses.

Retroviruses have RNA genomes, and when actively replicating, they have very high mutation rates similar to other RNA viruses. However, retroviruses are unusual in that they reverse transcribe from RNA to DNA, and the DNA copy of their genome is then incorporated into the host genome. This has happened a lot over the course of evolution; approximately 1% of the typical vertebrate genome encodes for vertebrate proteins, whereas 8% to 9% of the typical mammal genome is retroviral in origin. The avian genome is much less burdened with retroviruses, at approximately 1.1% of the genome, but this is still formidable.[28] Numbers of identified endogenous retroviruses range from 132 endogenous retroviruses in the ostrich genome to 1032 in the American crow

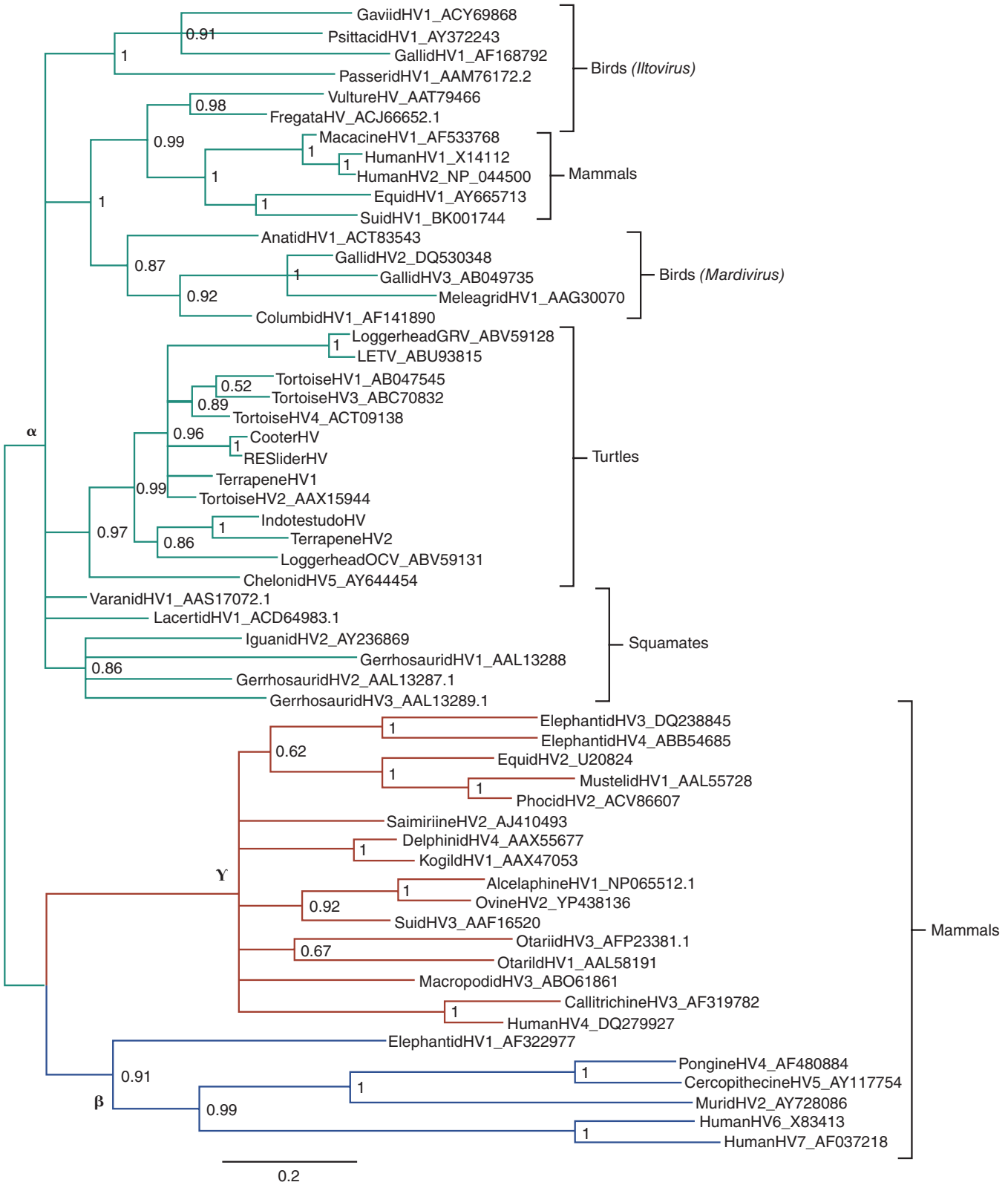

FIGURE 2-1 Phylogenetic tree of the herpesviruses suggestive of coevolution. The β-herpesviruses and γ-herpesviruses are all found in mammal hosts, whereas the α-herpesviruses are mostly reptile viruses and short by host class, with the exception of the α-herpesviruses of mammals, which have shorter branch lengths and cluster with avian herpesviruses, suggesting they may be of avian origin.

genome.[28] This makes retroviral discovery and diagnosis very challenging, not because they are hard to find but because they are widespread and present in such large numbers that it is difficult to sort out disease-associated viruses from clinically irrelevant endogenous viruses. Because of the prevalence of retroviruses in avian genomes, reverse transcriptase, the enzyme that converts viral RNA back to DNA, is commonly expressed in bird cells. This has also resulted in the incorporation of other viruses into host genomes, especially those that replicate in the nucleus, albeit less frequently. Bornaviruses, which have the uncommon trait for RNA viruses of nuclear replication, have been found to be incorporated into the genomes of many vertebrates, including avian species.[28] Incorporation of inactive bornavirus into host genomes complicates the interpretation of nucleic acid–based diagnostics for bornaviruses, some of which have been demonstrated to be causes of proventricular dilation disease in birds.[29,30] Circoviruses, small circular DNA viruses of which the best-studied member in birds is Beak and feather disease virus, are also incorporated into several avian genomes.[28] Interestingly, after retroviruses, the second most common endogenous viruses are the Hepadnaviridae, best known as the cause of hepatitis B in humans; there are 38 copies in the budgerigar genome and 68 in the great cormorant genome.[28] This is not seen in mammals, and this suggests a longer avian–hepadnavirus relationship. Although significant human pathogens, the clinical implications of hepadnaviruses in birds are not yet well understood, and they have only been described relatively recently in companion birds.[31] The chronic nature and lack of pathognomonic histologic lesions in humans make hepadnaviral disease more likely to avoid detection, and these lesions may be a significant unrecognized problem in birds. Endogenous parvoviruses have also been incorporated into avian genomes.[28]

Several routine husbandry practices in the avian pet trade create strong evolutionary selective pressures toward pathogenicity. First, overcrowding is common. The stress of close confinement has significant negative impacts on numerous health parameters.[32] High population densities lower transmission barriers, reducing pressure to keep the hosts alive and selecting toward virulence.[33] It is also common in the bird trade to select for color phases. This usually involves some degree of inbreeding to select for what are often recessive traits. A major driving force for the evolution of sex is the acquisition of genetic diversity for immune function. Inbreeding results in selection for greater disease.[34] Finally, variably stressed birds of species originating from all over the world are brought to breeders or distributors, often in the same facility. This is an ideal situation for pathogens to jump to new host species, which is where the most dramatic disease is seen.[35] The mixing of species by the exotic animal trade has already proved disastrous, with the transfer of monkey pox from Gambian pouched rats to prairie dogs to humans.[36]

Koch's postulates have led to another common erroneous conclusion—that most infectious diseases are caused by a single agent. When infectious disease is more properly considered as ecology, it seems obvious that a more typical scenario is several infectious agents in concert with other environmental factors. Chicken anemia virus and Fowl adenovirus 341 together cause hydropericardium syndrome in chickens, whereas this was not seen with either agent by itself.[37] Avian pneumovirus is much more significant when there is a co-infection with *Escherichia coli*, *Bordetella avium*, or *Ornithobacterium rhinotracheale*.[38] Co-infection does not always result in greater pathogenicity; avian influenza and Newcastle disease interfere with each other, resulting in lower pathogenicity[39].

With the development of next-generation sequencing tools, it has become possible to use nucleotide sequences to truly understand the diversity of flora present, whereas only a small fraction were previously identified by culture methods. Deeper investigation of enteritis in chickens revealed that while no single agent was a sole cause, exposure of specific pathogen-free chickens to flocks with chronic enteritis resulted in colonization with astroviruses, rotaviruses, picobirnaviruses, picornaviruses, and coronaviruses and also had significant shifts in bacterial gut flora.[40] Further work is needed to understand the microbial ecology of pathogen interactions, but the important thing for the clinician to understand at this point is that most infectious diseases involve the interaction of multiple microbes, and a frank single-pathogen disease is atypical.

To reduce the significant selective pressures toward highly pathogenic diseases, major changes in the associated avian industries are indicated. Genetic diversity in populations needs to be valued and monitored through appropriate use of studbooks and cooperative, rather than competitive, interactions with breeders. Breeding for color mutations needs to be discouraged. Housing needs to be entirely revised, with larger enclosures for individual animals or pairs designed such that feeding and cleaning can be done without cross-contamination to other animals. Facilities need to focus on single species and have smaller numbers of animals at lower densities. In conclusion, evolution is central to avian medicine and occurs in a clinically relevant time frame in avian infectious diseases. It is critical for the avian practitioner to take this into account, especially when dealing with the dynamics of interactions with population health (herd health), individual bird health, and infectious diseases.

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AVIAN BORNAVIRUS AND PROVENTRICULAR DILATION DISEASE

Michael Lierz

Proventricular dilation disease (PDD) is a common and fatal disease in birds and affects mainly psittacines. Only anecdotal reports describe the disease in other avian taxa. The disease was first described in the 1970s and named "Macaw wasting disease." The disease was always thought to be transferrable between birds, but the pathogen remained obscured for decades despite various speculations of potential candidates. Only recently, a novel virus, avian bornavirus (ABV) was proven to be the causative agent of this disease. It is now also known that this agent causes digestive tract disorders as well as other clinical signs in birds. Since its discovery, considerable research has been done on the disease and ABV, and knowledge has increased dramatically in the last 5 years. Many questions, however, are still unanswered or not clearly understood, in particular the clinical interpretation of test results in affected birds. This chapter will provide an overview of PDD, focusing on ABV and its clinical significance in the disease and diagnosis.

PROVENTRICULAR DILATION DISEASE

The first reports about PDD originated from the end of the 1970s, where a disease originally named "Macaw wasting disease" was described. Synonyms that have been used to describe the disease since then have included proventricular dilation syndrome (PDS), neuropathic dilation of the proventriculus, and myenteric ganglioneuritis.[1–7] It still remains unclear where the disease originated from, but there are speculations that it was first brought to the United States from Bolivia via imported parrots, followed by a further distribution to Europe.[8]

PDD is typically a disease of psittacines and has been described in more than 60 species.[9] Anecdotally, birds from other taxonomic orders, including Passeriformes, Anseriformes, and Piciformes, have been diagnosed with PDD.[10–12]

PDD is characterized by a nonpurulent inflammation of the peripheral nerves, in particular of the autonomic nervous system of the gastrointestinal tract (GI; esophagus, crop, proventriculus, and ventriculus).[6] As a result, neurologic function is impaired, and the smooth muscles of the GI tract atrophies.[5,13-15] This is followed by a functional impairment of peristaltic function, and food accumulates in the proventriculus and crop.[11] Food is maldigested, and the birds lose weight despite the frequent presence of a normal appetite. Dilation of the proventriculus can be extreme, leaving only a very thin proventricular wall (Figure 2-2), which can even rupture.[16] Nonpurulent inflammation can also be found in the central nervous system (CNS; brain, spinal cord), autonomic nerves of the heart, and adrenal glands.[5,6,17,18]

Clinical signs of PDD are usually nonspecific and detected relatively late in the progression of disease. They usually involve lethargy, weakness, and ruffled feathers but frequently a normal appetite. Owners then detect a loss of weight of the birds. Sudden death, without premonitory signs are described.[14,19] However, maldigestion is typically noted in birds that show a normal appetite, and in the later stages of the disease, undigested seed in feces and vomiting may be seen. Birds often die as a result of cachexia and functional starvation. Cardiac conduction disorders may also be reasons for death in some individuals afflicted with PDD.[20] Parallel to the affected digestive tract, CNS signs are described, mostly ataxia, lameness, tremor, and epileptic convulsions.[19–24] CNS signs and even blindness have also been linked to PDD without digestive disorders.[24,25]

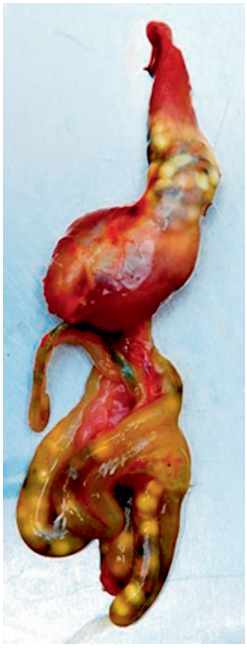

FIGURE 2-2 Gastrointestinal tract of a cockatiel with proventricular dilation disease (PDD). Note the thin proventricular wall, allowing the undigested seed to be seen. The same occurs even in the intestine, which is a rare event.

PDD occurs in all ages, with no recognized age predilection. In a study involving 127 birds, the average age of the affected birds was 3.8 years, with a range of 10 weeks to 17 years;[6] therefore, long latent periods before the appearance of clinical signs were presumed, as single-housed birds also became affected after years of no known other outside exposure.[26]

PDD is often suspected if birds lose weight and have undigested seed in feces, although the differential diagnosis for that particular sign certainly includes other disease concerns. Importantly, a dilated proventriculus may occur because of reasons other than PDD. In particular, mycotic infections with *Candida* spp. and *Macrorhabdus ornithogaster* should be considered. Bacterial or parasitic infections, neoplasia, or foreign bodies are additional potential causes.[8,11] Intoxications, particularly with lead or zinc may be other considerations, especially if CNS symptoms additionally occur.

Conversely, CNS signs often are not part of typically suspected clinical signs, but PDD should almost always be one consideration in the differential diagnosis list for susceptible species and CNS signs. Radiography, including contrast imaging, may demonstrate the presence of a dilated proventriculus in typical cases. This does not have to be the case, however, if the myenteric ganglia of the proventriculus are not affected.

Often, when the proventriculus is enlarged as a result of PDD, the walls of the organ are appreciably much thinner than normal. In cases of a dilated proventriculus caused by other reasons (see above), the organ wall is usually thickened or unaltered. Additionally, the passage time of the contrast medium is prolonged in PDD.[8] Using contrast fluoroscopy, typically there is an absence of normal ventricular contractility that is visible and measurable.[27]

Endoscopic evaluation of a potentially dilated proventriculus has been described,[25] but it is usually difficult to judge the size of the organ and is of less value than radiography. In postmortem examinations, with the classic form of the disease, the proventriculus is highly dilated with a paper thin wall (see Figure 2-2) and filled with undigested food. The diagnosis of PDD is confirmed by demonstration of the typical inflammatory lesions of lymphoplasmacytic infiltrations in the ganglia of the nerves and therefore is often demonstrated post mortem only. In live birds, proventricular biopsies can be difficult to achieve, and may be contraindicated where there is considerable smooth muscle atrophy present. For this reason, crop biopsies have been used.[28] False-negative results of crop biopsy are not uncommon. In a study comparing 29 birds confirmed with PDD, only 22 birds had typical lesions in the crop, whereas 25 birds were positive in the proventriculus and 27 in the ventriculus.[29] These authors concluded that 24% of crop biopsy specimens seemed to have false-negative results, especially as those lesions were not distributed equally through the organ. This and other similar observations led to the recommendations to take proventricular and ventricular biopsy samples in cases where the crop biopsy was negative but the disease was suspected. The possibility of false-positive crop biopsy results should also be considered. Signs of inflammation may be seen in the ganglia but may only be temporary, not necessarily diagnostic for PDD. Evidence for this might be provided by the observation that birds that had positive results in the crop biopsy had repeat biopsy performed, had never been positive again, and did not show clinical signs for years. When these single-biopsy-positive birds ultimately died of unrelated causes, PDD was also not confirmed at postmortem examination. In the past, prior to the discovery of the causal role of ABV, crop biopsy represented the only tool to achieve at least a tentative diagnosis, in a minimally intrusive manner, compared with proventricular or ventricular biopsy. Today, crop biopsy is no longer viewed as a valuable tool, considering its comparative insensitivity and other diagnostic options being available (see below).

In cases where PDD has been diagnosed, therapy is difficult. Although some birds may clinically recover with treatment, many will not, and euthanasia may be appropriate when quality of life is poor. A first component of treatment is the provision of highly digestible, high-energy foodstuffs, preferably a formulated product. Metaclopramide has been symptomatically used to aid in promoting GI motility and cimetidine to reduce gastric acid secretion and for its histamine-blocking effects. Antibacterial medications and antimycotic treatment at the beginning of the therapy can be beneficial to treat secondary infection, if present. The use of cyclo-oxygenase-2 (COX-2) inhibitors and partial inhibitors (e.g., celecoxib (Celebrex, Pfizer; Meloxicam) seems to have the most beneficial effects in treatment, reducing the speed of progression of the disease.[13]

One of the major questions during the last 4 decades in avian medicine was the identification of the causative agent of PDD, although an infectious etiology has always been suspected because the disease seemed to be transferrable between birds. Gough et al[30] isolated cytopathogenic, 83-nm-large, enveloped virus particles from macaws with PDD but were unable to identify them. Gregory et al[31] were the first to prove the transmissible character of PDD, as they were able to reproduce the disease in healthy parrots after subcutaneous and intramuscular inoculation of homogenized tissue from birds with PDD. The inoculates contained 80-nm-large virus particles but could not be further characterized. During the last decades, many other viruses, especially neurotropic viruses, were speculated to be causative agents. This included adenoviruses, herpesviruses, coronaviruses, polyomaviruses, eastern equine encephalomyelitis virus, western equine encephalomyelitisvirus, and the latest avian paramyxovirus serotype 1 and 3.[a] However, none of these potential candidates was regularly demonstrated in birds with PDD, and Henle-Koch's postulates were not fulfilled. Therefore, those candidates always failed to be the proven cause. The latest candidate was identified in 2008 by two independent research groups, both demonstrating the same virus in PDD-affected birds.[35,36] Both groups characterized the virus as part of the bornavirus family and named it "avian bornavirus."

AVIAN BORNAVIRUS

Avian bornavirus (ABV) was first detected in 2008 from PDD-affected birds in Israel and the United States by microarray analysis[36] and pyrosequence analysis.[35] In nonaffected control birds, sequences of this virus were not found. The virus demonstrated a sequence homology to mammalian bornavirus by less than 70% but showed important features of the family Bornaviridae and was therefore named avian bornavirus. More detailed characteristics about the viral structure of ABV can be found in the literature.[37,38]

Until the discovery of ABV, the family Bornaviridae within the order of Mononegavirales contained only one genus (bornavirus) with all strains originating from mammals. The order Mononegavirales also includes Paramyxoviridae, Rhabdoviridae, and Filoviridae. The order is characterized as a relatively large enveloped virus with a monopartile single-stranded RNA genome of negative polarity.[38] In contrast to the other families within this order, bornaviruses use a cellular gene-splicing machinery for protein expression and replicate in the nucleus of the cell.[39–42] Bornaviruses are approximately 90 (70 to 130) nm in size and are neurotropic. In the 1920s, the viral etiology of borna disease in mammals was identified.[3] The virus was described in more detail in the 1970s[44] but since then, only two different genotypes of borna disease virus (BDV) have been described—that is, the genome of BDV is highly conserved.[45,46] BDV was distinguished into the classic BDV-1, where all the isolates shared a genomic nucleotide sequence level of more than 95%,[38] and BDV-2, the only variant so far (No/98) that is 85% similar to the other isolates in the genomic sequence. Only recently a novel mammalian bornavirus was isolated from squirrels that seems to have a

[a]References 2,5,17,23,32-34.

zoonotic potential.[46a] This picture changed dramatically with the discovery of ABV. So far, eight ABV genotypes have been described from psittacines (ABV 1-8)[35,36,47-49] and seven from nonpsittacine birds, including strains isolated from Canada geese and trumpeter swans,[50,51] canaries,[12,52] and estrildid finches.[53] Within the group of ABV, the different strains share a 91% to 100% genomic similarity within their genotype, 68% to 85% between genotypes, and 60% to 69% with BDV.[36,38,51] The obvious difference between ABV and BDV is also supported by the fact that ABV replicates in cells of avian origin and only poorly, if at all, in mammalian cells compared with BDV, which replicates well in both.[38] This diversity within the genus *Bornavirus* required a novel taxonomy. Therefore, Kuhn et al[38] suggested naming at least five different species within the genus *Bornavirus*: Species 1 (mammalian 1 bornavirus), including the classic BDV-1 and BDV-2; species 2 (psittaciform 1 bornavirus), including ABV 1, 2, 3, 4, and 7; species 3 (passeriform 1 bornavirus), including the strains originating from canary birds (C1,C2, and C3) and from a Bengalese finch (LS); species 4 (waterbird 1 bornavirus), including the strains from waterfowl (062_{CG}); and species 5 (passeriform 2 bornavirus), including the isolate from an estrildid finch. ABV 5 and 6, MALL (originating from wild ducks[54]), and reptile bornaviruses described so far have remained unassigned, as the available sequences and the absence of isolates from those genotypes have not allowed classification so far.[38] The authors further suggested that the different bornavirus "variants" be named more descriptively. Therefore, in the future, ABV 1-7 should be named parrot bornavirus 1-7 (PaBV 1-7); C1-3 and LS as canary bornavirus 1-3 (CnBV 1-3) and munia bornavirus 1 (MuBV-1); ABV 062_{CG} as aquatic bird bornavirus 1 (ABBV-1); EF as estrildid finch bornavirus 1 (EsBV-1), and the mammalian BDV 1-2 as borna disease virus 1 and 2 (BoDV 1-2). The variant of the Loveridge's garter snake belongs to a novel species (elapid 1 bornavirus) named Loveridge's garter snake virus 1 (LGSV-1), which is currently placed in the family Bornaviridae but not included in the genus *Bornavirus* so far because of insufficient characterization.[38] Only recently, a distinct ABV has been detected in captive psittacines in Brazil and has been named parrot bornavirus 8 (PaBV-8), forming a separate branch within psittaciform 1 bornavirus species.[49] As these nomenclature changes are not yet internationally accepted, the old nomenclature is used in this chapter. However, in the future, it is fair to assume that the new nomenclature will likely be used.

Interestingly, the avian bornavirus genome was also detected embedded in avian genomes in a low copy number.[55] This may point to the long coexistence of birds and viruses. The author in that report stated that birds obviously seem to be less susceptible to viral genome invasions or prevent them more efficiently compared with other taxonomic groups such as reptiles. So far, it is speculated that ABV represents a rather old virus with the same ancestor as BDV and that BDV evolved later (about 300 years ago).[56] The relationship of the separate lineages from waterfowl, songbirds, and psittacines remains speculative, especially if one evolved from another.[56] Further studies are needed for more detailed conclusions.

ABV as the Cause of PDD

The discovery of ABV in PDD-affected birds was surprising, as up until that discovery, only two mammalian bornavirus strains were known. Borna disease in mammals shows similar lesions to those typically of PDD in birds. BDV is difficult to isolate, however, as it does not show cytopathogenic effects in cell cultures and can easily be overlooked. At that point in time, a high possibility that ABV is the cause of PDD was presumed. However, in part as a result of the remaining large variety of different viruses that were also presumed to potentially have a causative role in the disease, doubts have remained if the cause of PDD was really discovered. First, studies indicated the causative role by demonstrating ABV-antigen in specific PDD lesions.[57,58]. These findings were followed by infection trials, where efforts were undertaken to induce the disease by artificial infection methods.

As a first trial, three cockatiels were inoculated intranasally, orally, and intramuscularly with the homogenized brain of a Grey parrot with PDD that was positive for ABV-4. Two birds demonstrated PDD-like symptoms 21 and 31 days after infection and ABV-RNA was demonstrated in tissue. Postmortem examinations showed histologic lesion typical for PDD.[59] The homogenized tissue, however, contained retrovirus and astroviruses as well, so a conclusive demonstration of ABV as the cause of PDD was not possible. In another trial, two Patagonian conures were inoculated intramuscularly with 8×10^4 international units (IU) of an ABV-4 isolate originating from a PDD-affected macaw. Both birds demonstrated PDD-like symptoms by 66 days after infection and seroconverted; ABV-RNA was found in both birds, and typical PDD lesions were detected at postmortem examination.[60] This experiment supported the hypothesis of ABV as the cause of PDD, but both conures were also known to be infected with a herpesvirus. Mirhosseini et al[61] infected (orally and intramuscularly) two cockatiels with an ABV-2 isolate originating from a cockatiel, PDD-like symptoms occurred 33 and 41 days after infection, and typical histologic lesions were demonstrated at postmortem examination. None of the birds shed ABV-RNA, but ABV-2 was demonstrated in the brain, spinal cord, and intestine. As both birds were known to be infected with ABV-4, the authors concluded that a superinfection with two different strains may cause PDD. Again, this study provided further evidence but still failed to prove ABV as the cause of PDD, particularly because of the low number of birds used and the questioned role of the other viruses found in those previous studies. Piepenbring et al[62] performed a larger infection trial involving 19 healthy cockatiels from a closely monitored research flock, which were known to be free of ABV, paramyxovirus-1, *Salmonella* spp., and *Chlamydia* spp. The birds were divided in two groups of nine birds each and a sentinel bird. One group was infected intracerebrally, the other intravenously with an ABV-4 isolate originating from a macaw. The birds were placed in an incubator, and the sentinel bird was added to the intracerebral group. The birds were closely monitored and sampled every other day for ABV-RNA shedding and weekly for the production of ABV antibodies. The trial ended after 230 days, and all surviving birds were euthanized. During the trial, five birds demonstrated PDD-associated clinical signs. At histopathology, all inoculated birds demonstrated nerve lesions typical for PDD. Immunohistochemistry revealed ABV associated with the lesions, and reisolation of the inoculated ABV strain was successful, proving Henle-Koch's Postulates for the first time. All birds shed ABV-RNA in their feces, starting between day

18 and 71 after infection. All birds seroconverted, with titers constantly rising, up to as high as 1:20480. The first detectable antibodies were noted between day 7 and day 63 after infection. The findings of this study resembled the picture seen in daily practice, where infected birds do not always demonstrate clinical signs. This study clearly demonstrated that ABV is the cause of PDD and that it causes GI symptoms as well as neurologic signs, in combination or individually. Therefore, it has been suggested that PDD should be renamed avian bornavirus disease (ABVD), particularly because many more clinical signs beyond a dilation of the proventriculus can be seen. At present, it is becoming more important to understand ABV infection and its pathogenesis of disease in order to determine the route of transmission and to identify effective prophylactic measures. Knowing the pathogen opens new possibilities in fighting the disease. However, ABV infections are currently not completely understood, which is not surprising, as the virus has only been known for a few years.

Occurrence of Avian Bornavirus

Avian Bornavirus was first discovered in psittacines in single cases of PDD-affected birds in Israel and the United States.[35,36] Additionally, ABV has been demonstrated in Australia, several European countries,[47,63,64] Brazil,[49,65,66] Japan,[67] South Africa,[68] and Canada,[69] indicating a worldwide occurrence. Prevalence studies are rare, as most research has focused on the examination of diseased birds. Within Europe, a prevalence of 22.8% was detected, involving 1442 live and 73 dead parrots from 215 different flocks, including 33 genera of birds.[64] The study demonstrates that in all dead birds with histologically proven PDD, 100% were infected with ABV, whereas only 19% of the birds dying from other causes were ABV positive. In the live birds, 67% of birds showing PDD-like signs were infected compared with 19% of healthy birds investigated during a routine control examination.[64] This study not only supports the link between ABV and PDD, but it also demonstrates that the prevalence of ABV is considerably high in captive parrots and that clinically healthy, ABV-positive birds are common. A similar prevalence in a single flock was detected in 59 birds examined after two birds died from PDD with confirmed ABV infection. In 32.2% of the investigated clinically healthy birds, ABV-RNA was demonstrated in cloacal swabs.[63] In contrast, a study in Japan revealed only 4.3% of 93 investigated psittacines as ABV positive.[70] In the meantime, many breeders and veterinarians began to test and screen psittacine flocks, and it is not surprising that many asymptomatic individuals and flocks have been tested positive. It can realistically be assumed that nearly all larger breeding flocks of psittacines are infected, except those which are specifically making diligent efforts to clear the presence of the pathogen. Within a clinically healthy flock, about 10% to 45% of the birds are ABV positive, but exceptions to this general trend might occur. So far, all reports involved captive psittacines, but Encinas et al[65] detected ABV-4 in free-ranging birds in Brazil for the first time. This provides clear evidence that ABV is a pathogen that is not restricted to captive settings. In canaries, 12 of 30 investigated flocks (40%) were ABV positive, and both clinically healthy birds and diseased birds were seen. In waterbirds, ABV-RNA shedding prevalence in free-ranging asymptomatic birds varied according to species and sampling size between 0% and 13%, whereas antibodies were detected in all groups examined. This also clearly shows a wide distribution of ABV in waterfowl populations.[71] In addition, if waterfowl cases are selected by the presence of PDD-like histologic lesions, the prevalence of ABV-RNA detection in tissue samples of those birds increased up to 88.2%.[69]

In psittacines, predominantly ABV-2 and 4 are detected, with ABV-4 being the most common genotype.[72,73] This seems to be independent of the geographic origin, as those genotypes are reported on the various continents. Despite the reports of ABV-4 in psittacines in Brazil,[65,66] a novel type (PaBV-8) was reported in various birds in one study performed in Brazil.[49] Further studies are necessary to see if this strain is endemic to Brazil or if this will be reported more frequently in future. For now, ABV-2 and ABV-4 need to be considered as the most likely genotypes to be recovered, but seeing the high diversity of different strains, it seems likely that further genotypes will be described. The other psittacine genotypes described so far are only reported in single cases. It seems that other avian taxa do have distinctive specific strains, as waterbirds have consistently other genotypes than songbirds. Also within the family of songbirds distinctive genotypes are reported (canary bornavirus, estrildid bornavirus, munia bornavirus). So far, there is no evidence that the different ABV genotypes are able to cross family borders.

Avian Bornavirus Transmission

Because ABV-RNA is regularly detected in feces and urine as well as in cloacal–crop swabs, a fecal–oral route of transmission has been presumed.[37,72-75] Additionally, it was demonstrated that ABV spreads in a flock after infected birds are introduced into a collection.[76] Contact birds as well as non-contact birds became infected, but as seen in most flocks, not all birds became ABV positive. However, in this case, the prevalence of ABV in the affected flock prior to the first case was unknown, so it remains unclear how many birds were infected after introduction of the PDD case into the flock. By showing the occurrence of more clinically affected birds, the study underlines that transmission within a flock is possible. This is supported by an infection trial using canaries, where five healthy, noninfected birds were placed with 14 experimentally infected birds. Two of those contact birds developed a persistent infection, supporting the obvious conclusion that direct horizontal transmission between birds took place,[52] but three birds remained negative. Interestingly, those findings were questioned by the same researchers when they were able to infect cockatiels and canaries by inoculation and none of the contact birds of either species were shown to seroconvert or shed ABV-RNA.[53] The authors then concluded that horizontal transmission of ABV by direct contact is insufficient in immunocompetent fully fledged birds of the tested species. This finding was already presumed after a sentinel cockatiel, placed together with other experimentally infected birds, tested positive in feather and skin samples after contact, but never in one of the organs at necropsy; nor did they seroconvert. Exposure, therefore, most likely did not achieve persistent infection.[62] Further doubts of an easy transmission of ABV by fecal–oral route were raised when birds remained uninfected after being in contact with positive birds for years.[63] It is also a common finding, when investigating flocks,

that infected and uninfected birds have had direct contact and that even in successful breeding pairs only one partner is ABV positive. The first experimental trials all used more than one infection route (oral, intramuscular, and intravenous)[59–61] or single routes that are artificial and do not represent the natural way of transmission (intracerebral and intravenous).[62] In the first infection trials mimicking more natural routes of transmission, birds could not be infected. In a study, two groups with nine cockatiels each were infected with an ABV-4 isolate orally and intranasally, respectively. The birds were monitored for several months and euthanized at the end of trial. None of the birds seroconverted, and ABV-RNA could not be demonstrated in any of the organs.[77,78] As it was possible to infect cockatiels with the same isolate under the same experimental conditions,[62] it is fair to presume that nasal or oral transmission in healthy cockatiels does not represent the common route of transmission, or at least other co-factors are needed for successful infection. Here the authors discussed the necessity of mucosal or skin lesions for the first time and other factors such as immune deficiency or incompetence in juvenile birds. These assumptions are supported by further trials involving Grey parrots. In a first study, Grey parrots could not be infected by oculonasal ABV gavage but were successfully infected when the same isolate was administered by the subcutaneous route.[79] The difficulties in transmission of the ABV between birds might be related to the viral nature. ABV, similar to BDV, persistently infects cells but those cells only release a very few infectious particles.[37,73,80,81] Potentially only certain cell types might be able to release an efficient amount of virus for transmission, as speculated for kidney cells.[74,81]

Another potential route of transmission that has been intensively discussed is vertical transmission. The first evidence to support the presence of vertical transmission was found in 2011, when the embryos of ABV-positive psittacine parents were tested positive for ABV-RNA in the brain.[82] Similarly, 10 eggs out of 61 eggs obtained from a psittacine flock with PDD-affected birds within it contained ABV-RNA either in the yolk or in the brain of two embryos.[83] This was further supported by a study demonstrating that embryos of ABV-positive sun conures contained not only ABV-RNA but also ABV-specific antibodies.[84] The authors also demonstrated the eggs and embryos of ABV-positive parents to be free of ABV-RNA and concluded that ABV can be vertically transmitted but that it is also possible to get negative offspring from infected parents by hand rearing or foster rearing. Embryonated eggs laid by experimentally infected canaries contained ABV-RNA, but the virus could not be reisolated.[52] However, there is still some doubt regarding vertical transmission of ABV, as all the studies demonstrated ABV-RNA only, and not viable virus. Both Lierz et al[82] and Monaco et al[83] stated that viable virus in a chick hatched from an infected egg needs to be proven before vertical transmission can be assumed. This is supported by findings of Wüst et al[84a] in 2015 investigating the survival of ABV after inoculation in embryonated cockatiel eggs. Of 32 embryos infected at day 3 to day 5 of incubation in the yolk sac, only nine demonstrated ABV-RNA in the brain at day 17 of incubation. All these embryos developed uneventfully, and no inflammation typical for PDD was seen. Reisolation of the virus was still ongoing during the preparation of this text, but these results again demonstrated that vertical transmission is also not common and, if at all, only occurs when certain co-factors that are currently unknown are present. This is additionally supported by a study that failed to demonstrate ABV-RNA in the brain of newly hatched chicks or embryos from Canada geese originating from a known ABV-positive population. Only in the yolk of one unembryonated egg was ABV-RNA detected.[71]

Therefore, from clinical observation, the route of transmission, at least the circumstances of a successful transmission, is currently not fully understood, including vertical and horizontal means. The abilities and means by which ABV can overcome skin or mucosal barriers are unknown, viral factors (different genotypes and pathogenicities) are not fully understood, and host factors such as immunosuppression, incompetency in juvenile birds, or other immunologic variables might still play a role in the development of disease. However, both the irregular horizontal transmission as well as the uncommon vertical transmission of ABV opens large potential opportunities in preventing further spread of the virus and clearing flocks of ABV (see below).

Potential Pathogenesis

Today, there should remain no doubt that ABV is the causative agent of PDD and additional clinical signs, especially CNS abnormalities. However, it is also known that a considerable number of birds are infected but remain clinically healthy for long periods.[63,64,85-87] The circumstances leading an infection to clinical disease are presently not fully understood. ABV demonstrates a clear tissue tropism toward neural tissue,[63] especially in infected but clinically healthy birds. The highest virus load is always found in the brain, retina, or spinal cord.[88] In clinically diseased birds, the virus can be detected in a wider range of tissues, not exclusive to those of neural origin.[37,57,71] On the one hand, this is in part similar to BDV, as the mammalian virus also demonstrates neural tropism, but in contrast, BDV is not detected in various other tissues. Additionally, intravenous inoculation of BDV failed to infect rats,[89] whereas in cockatiels, those infection routes were successful.[62] Therefore, it is fair to assume that the pathogenesis of ABV has certain parallels to BDV but that differences might be present, so further work is needed to clarify the pathogenesis of ABV. However, BDV causes an immune-mediated disease (see also Chapter 11). Immunoincompetent or neonatal rats demonstrate a persistent infection with high virus load in CNS tissue compared with adult rats, which demonstrated an encephalitis 20 to 35 days after infection. Transmission of T lymphocytes from BDV-affected rats to infected but immunoincompetent (symptomless) rats induced clinical signs, clearly indicating a T cell–mediated disease at least in rats.[90] Here a neural invasion of CD8 T lymphocytes seems to cause the cellular damage and not the virus itself.[81,90-93] Obviously, a similar pathogenesis is presumed in ABV, and Payne et al[51]speculated a delayed-type hypersensitivity effect in inducing the clinical disease. It was also shown that ABV uses similar strategies in escaping the host immune system, by removing the 5' termini of the viral genome.[94] The authors further demonstrated that ABV infection of cell cultures is reduced by adding type 1 interferon (IFN) but that quail cells with a high load of viral ABV-RNA did not produce detectable levels of type 1 IFN as a sign of reducing the host response. The same was supported in a study comparing the

type 1 IFN–reducing capacity of the X-protein of BDV and ABV, demonstrating similar capabilities.[95] The authors further detected that the level of depression of IFN-production was dose dependent with the amount of X-protein.

Prior to the detection of ABV, an immune-mediated pathogenicity of PDD was presumed, similar to Guillain-Barré syndrome in humans.[96] The study described antiganglioside antibodies as the cause of the nonpurulent inflammation typical of PDD and provided evidence, as the detection of those antibodies in PDD-confirmed cases were significantly higher than in healthy birds. The authors also concluded later[97] that the trigger for this could be ABV but also any other viral infection. However, this needs to be questioned, as it was not possible to find antiganglioside antibodies in confirmed PDD cases after experimental ABV infection and a very poor connection between ABV positivity and occurrence of antiganglioside antibodies in clinical cases has been shown.[97] A more detailed view of potential immune-mediated pathogenesis of ABV is provided in Chapter 11. It should also be kept in mind that the role of viral factors in the pathogenesis is not yet determined. It is known that different ABV strains act differently within the same host by terms of viral replication and pathogenicity. The first trial comparing the experimental infection of cockatiels with two different ABV genotypes under identical conditions, demonstrated that one strain (ABV-2) was more pathogenic to cockatiels than the other strain (ABV-4) and that viral RNA shedding occurred significantly earlier in ABV-4–infected birds compared with ABV-2–infected birds but that seroconversion occurred significantly earlier in the ABV-2 group.[98] More interestingly, the viral load of ABV-RNA in the different organs was significantly higher in the ABV-4–infected group despite the presence of fewer clinical signs. Additionally, in ABV-4–infected birds the tissue virus load findings were comparable in all birds, independently from the time point of death after infection or the route of inoculation (intracerebral versus intravenous). In the ABV-2–infected birds, the viral load in the different organs after infection depended on the route of infection (intracerebral-infected birds higher compared with intravenous-infected birds) and the time point of death after infection (early death birds had a lower load compared with late death birds). Last but not least, ABV-4 antigen was more often detected in the CNS of infected birds compared with that of birds infected with ABV-2, where antigen was found to be increased in the GI tract.[98] Most interestingly, reisolation was easily possible from ABV-4–infected birds from nearly all tissues within a couple of days, whereas re-isolation of ABV-2 depended on the time point of death or the identifiable disease of the host. Reisolation of virus from the birds that died earlier after experimental infection was successful only after several passages in cell culture compared with what was seen in infected birds that died later, where reisolation was typically possible in the first passage. These results indicated additionally that the amount of virus (and viral replication) is not correlated with the severity and speed of the disease and its progress. In addition, it is apparent that the virus induces the disease through mechanisms (e.g., earlier activation of the immune system) independently of the viral load or even that earlier activation of the immune system causes more severe disease but does not allow the virus to replicate quickly. This seems to be supported by the fact that viral shedding was also noted significantly later compared with the ABV-4 group with a far higher viral replication and less severe symptoms. The infection patterns of ABV-2 demonstrates many parallels to BDV infection in mammals, as it is known that minimal viral replications can trigger the onset of clinical symptoms and that disease progression depends on host immune response.[99]

It needs to be considered, as clearly stated by Lierz et al,[98] that the differences found between ABV-2 and ABV-4 must not only be related to the different genotype, but they could also present strain specific variations and can theoretically also occur in different variants within a genotype. Additionally, the ABV-4 isolate used originated from a macaw compared with the ABV-2 isolate originating from a cockatiel and might therefore be differently adapted to the trial animals (cockatiels). However, they demonstrated varying viral factors influencing the viral kinetics and host–virus interactions. Further studies should focus on the interaction of ABV with the host to better understand the viral and host factors involved in triggering clinical disease after infection.

DIAGNOSIS

The diagnosis of the presence of an ABV infection basically follows the common rules of infectious medicine. It is focused on the demonstration of the pathogen in samples of the birds (direct proof) or the detection of specific antibodies against the pathogen (indirect proof). Both of these basic methods are possible in diagnosis of ABV infection in birds. However, for a straightforward diagnosis, knowledge regarding ABV kinetics in the host and its interaction with the immune system (circumstances of antibody production) must be known, but this knowledge is incomplete. As a result, interpretation of diagnostic test results is challenging. The first problem is when and how to judge a bird to be ABV positive; second, a bird owner or veterinarian will often request a prognosis about the clinical outcome for the bird. The first problem will be discussed below; the second problem has a very clear answer—a clinical prognosis is not possible in infected but clinically healthy birds.

The demonstration of the presence of ABV in samples from birds (e.g., cloacal swabs, tissue samples) is made by the detection of viral-RNA by reverse transcription polymerase chain reaction (RT-PCR). Conservative ABV-consensus PCR-protocols focus on the detection of the M-, N-, P-, or L- Gen,[35–37] with the M- and N- protocols appearing to have a higher sensitivity.[57,100] For an additional quantitative analysis of viral amount real-time RT-Taqman-PCR were initially developed for the detection of ABV-4 (Primer 1034-1322) and ABV-2 (Primer 1367).[35] As with all PCR protocols, the primers are able to detect specific gene sequences. As a result of the high variability of ABV, it should be kept in mind that those primers might fail to detect a specific ABV genome despite its presence because alterations in the specific gene sequence occurred. Therefore, negative PCR results should be interpreted in the context of the kind of PCR that has been used and, balanced with serologic results, the clinical picture in the patient or the potential occurrence of novel genomic variants. Enderlein et al[88] demonstrated that commonly used real-time RT-PCR protocols were not able to detect all known ABV genotypes. Similarly, an ABV-2 variant from

cockatiels was not detected in the previously described real-time RT-PCR protocols[35] but by a conventional consensus RT-PCR,[98] making it necessary to alter the real-time RT-PCR protocol for detection. As ABV-4 and ABV-2 are the most common genotypes in psittacines, it seems fair to use those protocols in an initial diagnostic step. However, as stated earlier, in negative but suggestive cases, or to increase the confidence in the interpretive meaning of the results, further protocols should be applied. If focusing on the detection of ABV from other bird families (e.g., waterbirds, songbirds), specific PCR protocols need to be used.[52,69] Therefore, the laboratory receiving the samples should be able to handle the different PCR protocols that may be required and will need to know the origin of the sample. Additionally, laboratories need to be very experienced in handling samples for ABV or BDV investigation, as it is known that cross-contamination with Bornaviridae-RNA occurs more easily compared with other viruses. It cannot be overstated that ABV detection is a specific task that requires experience and that it is not easy to establish compared with other diagnostic PCR systems as commonly thought by veterinarians or commercial laboratories. Additionally, the details of sample selection, collection, storage, and transport to the laboratory surely can affect the results obtained. Commonly used samples are crop and cloacal swabs, feces, feather calamus, and blood. Feces carry certain disadvantages when used in PCRs, as inhibitors are commonly found in those samples. In a comparison of samples from 55 known ABV-positive psittacines, in 36 birds crop and cloacal swabs were positive for ABV-RNA, whereas in 11 cases only the crop and in 8 cases only the cloacal swab were positive. None of the whole blood samples of those birds were positive by PCR.[88] As a conclusion, a combined sample, including crop and cloacal swabs from one bird, merged in one tube for testing seemed superior for ABV detection in live birds. Interestingly, a recent study identified a high ABV-RNA content in urine,[74] potentially explaining the good results with cloacal swabs. Some authors suggest the feather calamus as a good sample,[101] but this view is not supported by the experience of some laboratories or by experimental studies, demonstrating other tissues more often positive.[62] Additionally, feathers always contain a higher risk of being contaminated by other birds or the environment, leading to false-positive results. In dead birds, brain or retinal tissue is the most superior sample for the detection of ABV-RNA.[62] Additional postmortem samples for viral detection might be the adrenal gland, proventriculus, and ventriculus. After collection, the samples should be stored in a cool environment or ideally placed in a special transport media (RNAlater, Quiagen) to be sent to the laboratory, as RNA within samples are sensitive to degradation, and false-negative results might occur because of poor transport conditions. The samples should reach the laboratory within a few days. In case samples need to be stored longer or are frozen, they should not be thawed and should reach the laboratory in the frozen state. Repeated thawing and freezing cycles degrade the RNA very quickly. The veterinarian should always keep in mind that a negative result of the sample might not automatically mean an ABV-negative bird. Apart from a false-negative result (e.g., sampling issues, loss of detectable viral genome as a result of transport), the sample might just not contain ABV-RNA because at the time of sampling the virus was not present in that location. This is a common problem, especially in live birds, as ABV is shed intermittently in some birds.[56] In tissue samples, the virus might not be in that particular tissue and may be found elsewhere (e.g., the brain). Especially in live birds, repeated testing might be recommended (see below).

ABV antigen can further be demonstrated in tissue by immunohistologic staining. This is commonly used in research settings but is of limited use in daily practice for diagnosis. Viral antigen is stained within the tissue by using polyclonal antiserum against nucleocapsid proteins[102] as well as against phosphoproteins[37,47,103] There seems to be high cross-reactivity between the antigens of the different ABV genotypes as well as between ABV and BDV, but this seems to depend on the target antigen.[104,105] It should also be kept in mind that used primary antibodies might cross-react with tissue antigens, complicating the interpretation of the results.[57]

Last but not least, the isolation of ABV from samples is another method for direct proof of the presence of the pathogen. As this is also not easy, susceptible to false-negative results (as a result of challenges in keeping the virus live during transport and cultivation), costly, and time-consuming, isolation is not a routine method in daily veterinary practice for the diagnosis of ABV infection. However, virus cultivation represents the only method to prove the viability of a virus known to be present, whereas a PCR only demonstrates the presence of a certain RNA sequence. Therefore, virus isolation is essential to answer certain questions and to understand the infection itself. As an example, the proof of vertical transmission requires the cultivation of a viable virus from embryos or newly hatched chicks, similar to the way that detection of means of shedding viable virus and infection trials can only be made if one is working with a live virus in hand. There are difficulties in cultivation of ABV in cell culture. During the search for the causative agent of PDD, several attempts to isolate the potential pathogen failed.[10,106] However, now it is known that ABV does not cause a cytopathic effect and therefore may have been overlooked when using cell cultures. Only Gough et al[30] demonstrated a cytopathic effect when he thought that the pathogen causing PDD was found, but so far it remains unproven if he did find ABV. ABV, independently of its origin (psittacine, canary bird, waterbirds), grows in cell cultures of avian origin such as in duck embryo fibroblasts[48,60,72] or quail cell lines (CEC32, or QM7).[37,48,52,105] ABV does not grow in cells of mammalian origin, and so far, only one study has reported a minimal growth of ABV in VERO cells.[52] The best virus cultivation results were achieved in CEC32 cells.[48,52] It should be kept in mind that different ABV isolates differ in their growth characteristics, especially in speed of replication and ability to infect cells,[98] making several passages necessary in some cases before a negative result can be assumed. As there is no cytopathogenic effect, additional tests such as real-time RT-PCR, immunofluorescence testing, or Western blot testing need to be applied to prove an increase in the amount of viral antigen to confirm a growing virus (Table 2-1).

The detection of ABV-specific antibodies in serologic assays is a very important diagnostic tool. So far, it is not fully understood under which circumstances detectable antibodies are present. In experimental setups, all infected birds developed antibodies independently of the genotype that the birds were infected with[52,62,98] following common rules of infections.

TABLE 2-1

Selected Tests for Detection of Avian Bornavirus Infection in Birds Commonly Offered by Commercial Laboratories*

Test	Use in Practice	Sample	Meaning	Interpretation	Remarks
Direct test to demonstrate the presence of virus particles. Minimum 18 days after infection shedding is detectable according to infection trials[62]; under natural circumstances this might be longer.					
Reverse transcriptase polymerase chain reaction (RT-PCR)	Good, sample easy to take, use transport media, cross contamination possible	Swabs, tissue, secretions	Detection of avian bornavirus ribonucleic acid (ABV-RNA)	Viral RNA demonstrated, does not imply the presence of viable virus	Could be caused by contamination, repeat if serology is negative, excellent for screening (see Figures 2-2 and 2-3)
Virus isolation	Less practical. Takes a longer time. Virus is sensitive to transport issues, and false-negative results can be seen	Swabs, tissue, secretions	Detection of viable virus	Complete viable virus, unlikely result of cross contamination	Takes long, expensive, more for research setups
Immunohistochemistry	Less practical, expensive, takes long, usually not commercially offered	Tissues	Detection of viral antigen in cells	In positive cases infection is clearly demonstrated	Sensitivity questionable, especially in latent cases usually not many cells infected; therefore not applicable in testing live birds
Indirect test (serology) to demonstrate the immunologic reaction of host against the presence of virus particles. Unclear if virus is still present, or even being likely in the case of ABV. Minimum 7 days after infection, seroconversion occurred according to infection trials[62]; under natural circumstances this might be longer.					
Immunofluorescence test – ABV-infected cell culture	Excellent; result takes 3–5 days	Serum, plasma	Detection of anti-ABV-specific antibodies	Bird's immune system had contact with virus. Persistent infection is presumed. Low titers may become negative, demonstrating non-infection of bird. Low titers (up to 1:80) should be rechecked if polymerase chain reaction (PCR) of bird is negative	ABV-infected cell cultures present various ABV antibodies, therefore higher chance of cross-reactivity between different antibodies. Antibodies cannot be distinguished against which protein they are directed

Continued

TABLE 2-1

Selected Tests for Detection of Avian Bornavirus Infection in Birds Commonly Offered by Commercial Laboratories*—cont'd

Test	Use in Practice	Sample	Meaning	Interpretation	Remarks
Multiple protein enzyme-linked immunosorbent assay (ELISA)	Excellent, results may take only 2 days	Serum, plasma	Detection of anti-ABV-specific antibodies	See Immunofluorescence, except that low and high titers should be interpreted by the laboratory using the test	Several proteins act as Antigen. Cross-reactivity likely, but less than as in immunofluorescence test. Depending on ELISA, antibodies, may be distinguishable against which protein they are directed. Low experience at present but may become a very valuable tool for research and clinical prognostication
Single-protein ELISA	Excellent, results may take only 2 days	Serum, plasma	Detection of anti-ABV-specific antibodies	See Immunofluorescence, except that low and high titers should be interpreted by the laboratory using the test	Titers should be rechecked, as huge variations between tests occur. Especially low titers in immunofluorescence test (IFT) sometimes not detected. Comparison trials between the various diagnostic test with large amount of samples necessary
Western blot	Good, result may be quick, only a few laboratories use this test	Serum, plasma	Detection of anti-ABV-specific antibodies	Interpretation of titers difficult	See Single-protein ELISA

*Refer to the text for more details.

In contrast, after natural infection, some birds are detected as shedders without seroconversion.[64,86] Additionally, the titer depends obviously on the time point of infection, the ABV genotype and other unknown factors, making interpretation of serologic test results challenging in spite of the apparent value that serology offers as a tool in flock management (see below). There are also hints that the titer correlates with the potential possibility of developing clinical signs[107] (see clinical interpretations below) and could be used for the interpretation of clinical cases. There are different assays available to detect anti-ABV-specific antibodies. These are indirect enzyme-linked immunosorbent assay (ELISA)[101,108] and Western blot[63] tests, using certain proteins as antigen. Those proteins are recombinant N-,[63,108,109] P-,[63,101] M-,[101] or X-[84] proteins from either BDV or ABV. Usually, single proteins in those tests are used, with expectations of cross-reactivity between BDV, ABV, and different ABV genotypes. However, as this cross-reactivity is not ensured between the different ABV genotypes, the sensitivity of those tests focusing on one protein only needs to be questioned until otherwise proven. At present, this is challenging, since gold standards for those tests for ABV-specific antibodies have not been set, as the disease and definition of ABV positive cases is poorly understood. However, sera from experimentally infected birds are available and should serve as samples for establishing those standards. The first published results of such tests had a

sensitivity of 90% and a specificity of 82% in a Western blot test[72,104] and in an ELISA using the N-protein of 75% sensitivity and 75% specificity,[101] both unsatisfying results for use in clinical setups, especially in flock management and flock pathogen elimination strategies. It is presumed that in case of low titers, those tests might present false-negative results, and this presumption was supported by the first comparison tests performed. Tests based on ABV-infected cell cultures (e.g., indirect immunofluorescence test (IFT),[105] which present a wide range of different ABV antigens, seem to be superior in the detection of anti-ABV-specific antibodies in clinical case and flock management. However, those tests are more complicated to perform compared with ELISA. Additionally, in the indirect IFT, a complete antibody titer is measured, not distinguishing against which specific protein those antibodies are directed. For a clinical situation, this seems to be adequate. For research purposes, especially to obtain a better understanding of pathogenesis and disease development, it might be advantageous to know against which proteins the antibodies are directed in the different phases of infection.[101,110,111] Here, Dorrestein et al[112] made very interesting observations by developing an ELISA using different proteins as antigen. They detected that antibody titers against certain proteins (especially P16 and P24) increased when birds developed disease, whereas in infected but healthy birds only antibodies directed against the recombinant ABV protein P40 were detectable in the beginning.

Clinical Disease of Avian Bornavirus and Disease patterns

Clinically, ABV infections have the largest impact in psittacine birds. There are reports of clinical disease related to ABV infection in canaries, geese, and other species, but those are seen rarely, are usually anecdotal descriptions and the significance is still unclear. In psittacines, infection can lead to a deadly outcome. In psittacine species conservation projects, ABV can have a major impact. As an example, during the early 2000s, about 10% of Spix's macaws in the breeding program, one of the most endangered birds in the world and currently extinct in the wild, died from PDD, the major clinical outcome of an ABV infection.

ABV causes nonpurulent inflammation in nervous tissues with ensuing loss of function. Mainly lymphoplasmacytic infiltrations in ganglia are seen in histopathology. The most known clinical outcome of an ABV infection is PDD, as described above. However, other neurologic disorders should also be considered.[87] Especially, CNS signs seem to be more common than previously thought. CNS signs can range in severity from relatively minor signs, including slight tremor (e.g., of one toe), to epileptic convulsions and incoordination, loss of equilibrium, head shaking, opisthotonus, and so on. Fluck et al[107] examined CNS cases presented to avian practice and could clearly demonstrate a link to ABV infection, with more than half of those cases being ABV positive, by exclusion of other common potential causes. In the past, behavioral problems[70] and feather-damaging behaviors in psittacines had been associated with ABV,[113] but scientific proof for clear causality is still lacking. Fluck et al[107] included birds with feather-damaging behaviors in a study and found that about 50% of the examined birds were positive for ABV, but the amount of antibodies and viral RNA shed was comparable with those of ABV-positive birds in a control group, whereas it was significantly lower compared with a group of CNS-diseased birds. A link between ABV and feather-damaging behaviors cannot be ruled out, but current evidence suggests that it is not likely. This stands out in contrast when compared with what is seen with clinical signs of CNS disease in a considerable number of those birds very likely caused by ABV. This is also supported by experimental infection trials, where cockatiels developed a classic manifestation of PDD after experimental infection (GI tract signs), but some birds also developed clear CNS signs either on their own or in combination with PDD.[62,98] Interestingly, some birds demonstrated only nonspecific clinical signs of ruffled feathers and diarrhea, and a few died suddenly without demonstrating any clinical signs prior to death.

It seems that the quality and quantity of clinical signs depend not only on host factors but also on the ABV variant. In a comparison trial, more birds developed clinical signs after infection with an ABV-2 variant compared with an ABV-4 variant. The incubation time can obviously be relatively short but can also be several months at least. The first signs occurred in experimental trial as soon as 22 days after infection with an ABV-2 variant and 33 days after infection with the ABV-4 variant.[62,98] In this same study, however, some birds developed clinical signs after as much as 20 weeks. It is repeatedly observed that single-housed birds died from PDD (with then-confirmed ABV infection) years after arrival, therefore making it likely that the incubation period of ABV might be as long as several years and the triggering factors might occur any time independent of the duration of existing ABV infection. These types of repeated clinical observations support the hypothesis that there is a delayed pathogenesis after infection in some birds; however, scientific challenge has not yet confirmed these observations to be true. Experimental trials have suggested that the clinical course of the disease in a single bird is unpredictable. After ABV-2 infection, three different types of courses were seen. There are (1) birds with a severe and acute onset of symptoms shortly after infection; (2) birds with a mild course of the disease developing first signs from approximately 80 days after infection, which might then develop to severe signs; and (3) birds with demonstrated signs late after infection (172 days after infection) with a mild progression of clinical disease or even remaining clinically healthy during the complete infection trial.[98] This array of clinical presentations is also seen in daily practice or when observing flocks. After introduction of ABV-positive birds into a collection, some contact birds died quickly with severe clinical signs, whereas others had a slow but progressive onset, and some remained infected but clinically healthy.[101] Additionally, it seems that it is not only the introduction of newly infected birds but other factors such as stress that may also aid in the induction of clinical disease in some birds in a flock. ABV-infected birds were followed up for years after being donated from owners who wished to exclude ABV-positive birds from their flocks. Repeatedly, it was observed that after translocation of those clinically healthy-appearing but ABV-infected birds, an initial die-off period was seen, with some of the translocated birds developing PDD or CNS signs shortly after translocation. Birds that survived this initial period of several months and did not demonstrate clinical signs during this time remained clinically healthy for at least

3.5 years despite being ABV positive. This observation is supported by a study monitoring ABV-infected birds for a year and demonstrating that some birds developed clinical signs, whereas others stayed clinically healthy for long periods.[86] Therefore, the prognosis for ABV-positive birds is nearly impossible to predict, as long as no directly attributable clinical signs of infection are noted.

In bird species other than psittacines, the clinical significance of an ABV infection seems to be low or unknown. The first description of a canary with enteric ganglioneuritis and encephalitis was linked to ABV infection.[47] In naturally ABV-infected canaries, GI and neurologic signs comparable with those observed in psittacines were discovered, but in contrast to the experimental trials in psittacines, these same clinical signs could not be reproduced in canaries experimentally infected with the ABV virus isolated from those diseased birds.[52] Histopathologic lesions, including lymphoplasmacytic perivascular cuffing in nervous tissue, similar to those of PDD in psittacines, were also discovered in Canada geese and trumpeter swans that were positive for ABV infection[69] but could not be linked to clinical signs, as only tissue was examined in that study without clinical history of those affected birds.

Clinical Diagnosis of Avian Bornavirus Infection

The most difficult question at present concerns the clinical interpretation of test results obtained from individual birds. Here, there is a considerable gap between the knowledge gained from experimental infection trials and what is seen in practice.

In two large experimental infection trials involving ABV-negative cockatiels, all inoculated birds seroconverted and started shedding ABV-RNA eventually. There was a difference between those trials in the maximum antibody titer reached, as ABV-4 caused significantly higher titers than an ABV-2 variant infection with lower and more varying titers,[62,98] but in the end, diagnosis of a successful ABV infection was made as all birds seroconverted and shed detectable levels of ABV-RNA. If those birds had been presented in a practice setup, they would easily have been detected as ABV-positive birds. Interestingly, it was also shown that 11 birds first tested positive for RNA shedding before seroconversion occurred, in 6 birds seroconversion was noted prior ABV-RNA shedding, and in 2 birds both occurred at the same time. These observations underline clearly the need for both direct and indirect tests when attempting to diagnose ABV infection. However, in those trials, birds were inoculated by the intravenous or intracerebral route, which might have affected the host response to infection.

In contrast, Heffels-Redmann et al[86] investigated naturally infected psittacines from different flocks repeatedly over a year. In this study, different infection patterns were observed. As in the experimental trials, there were birds with a high anti-ABV-antibody titer and shedding of ABV-RNA during the entire study, some of which developed clinical signs during the observation period, whereas others remained clinically healthy. However, those birds were clearly ABV infected. Another group of birds had a permanent but low anti-ABV-antibody titer and variable detection of ABV-RNA in crop and cloacal swabs. Those birds should also be interpreted as being infected, as shedding of ABV seemed to be intermittent. However, the authors also observed two other groups of birds that are not easy to categorize, and those observations are also regularly seen in daily laboratory practice. There are birds demonstrating an intermittent low anti-ABV-antibody titer, meaning that sometimes they are serologically negative and at other times they have a low but measurable titer. Those birds never were noted to shed viral RNA during the observation period. In comparison, other birds shed viral-RNA in a low amount intermittently but never had a detectable antibody titer.[86] The last group may be explained as persistently infected birds, in which ABV was functionally hidden from the immune system, but the other group is much more difficult to explain. Those individuals may be persistently infected, with irregular contact of the virus to the immune system. However, both these hypotheses are at present very speculative, and the cause of those infection and host response patterns remains obscure. The route of transmission, as well as unclear host factors, might play a role in this. Veterinarians often have difficulties in explaining to bird owners if those birds should be considered ABV positive or not. As long as the details of infection remain incompletely understood, birds that have had positive signs for ABV should be considered potential carriers (see flock management) and tested repeatedly to get a clearer picture. Importantly, those observations very clearly underline the need that the investigation of birds for ABV positivity must include direct and indirect tests, that is, demonstration of viral presence (e.g., through PCR) as well as serology. With the use of only one laboratory test (e.g., PCR), the results are understandably incomplete and often inconclusive. Particularly in daily practice, cross-contamination of one sample with ABV-RNA from the environment cannot be excluded during sampling in places where several birds are kept or pass through.

In clinical settings, the following is suggested as long as the ABV infection is not better understood. Birds positive for the presence of virus (e.g., RT-PCR positive) and that are also positive by serology can be considered clearly ABV positive. Those being shown to be positive by PCR only should be retested to confirm this result and to exclude cross-contamination of the sample as a reason. At best, those tests should be repeated not earlier than 4 to 6 weeks after the first sample to see if seroconversion has occurred. During this time, the tested birds should be kept separate from others. If they seroconvert or are repeatedly positive for viral presence, they should be considered positive. Birds positive by serology only are a more difficult interpretive challenge. Usually, they are presumed to be carriers,[72] but clear evidence for this is lacking. However, birds with a high titer should be considered positive, as it is known that bornavirus in mammals causes persistent infections.[73] As described above, birds with a low titer are seen to be negative in additional tests, and therefore persistent infection in those remains unproven. There seems to be evidence that some birds are able to clear the ABV infection,[108] which could explain the varying titers seen in some birds, usually not seen in birds with a high ABV titer. This, however, needs to be scientifically proven. Additionally, false-positive reactions in serologic tests by cross-reaction with antibodies directed against other antigens cannot fully be excluded, even if unlikely. Therefore, low-titer birds should be kept separate and repeatedly tested by direct virus test

(e.g., PCR) and serology approximately 4 to 6 weeks later. If the titer remains or increases, if virus is detected, or both, the birds should be considered positive. Decreasing titers or seronegativity, together with negative virus detection (PCR negative), should lead to another retest 4 to 6 weeks later. Only if the seronegativity remains for both tests, and there is a repeated failure of virus demonstration by PCR, should the birds be considered ABV negative. Figure 2-3 provides an overview of suggested interpretation of test results to categorize tested birds.

An interesting question is the interpretation of what the meaning of a low or a high antibody titer is. This is not easy to answer, as this depends on the test used. Therefore, the laboratory should be contacted for interpretation. The greatest advantage is with laboratories that are experienced with ABV investigations, as they should have the experience to interpret those questionable results. Ideally, those laboratories have affiliations with clinical practice settings to provide the needed experience to compare with laboratory results obtained. By using the indirect IFT on infected cell cultures (see above) titers ranging up to 1:80 should be retested if viral detection by PCR failed.

As previously stated, PCR and serology laboratory results do not allow an individual prognosis for prediction of the onset of clinical disease in birds. These test results also do not necessarily indicate the cause of the clinical signs seen. Birds with a high titer and a high amount of shed virus remained clinically healthy for at least 3.5 years. It repeatedly is seen that clinically ill birds have a considerably high anti-ABV-antibody titers and do shed a considerable amount of virus or viral RNA. This was also seen by Fluck et al,[107] who detected significantly higher anti-ABV antibody titers and amount of ABV-RNA being shed in a group of neurologically diseased parrots, compared with a control or a feather-damaging group, despite the fact that the total number of ABV-positive birds was comparable between the feather-damaging and the neurologic group. This underlines the fact that laboratory results point to clinical diagnosis. However, there are cases when the antibody titers rise quickly shortly before the onset of clinical disease, but this point in time is not predictable, and birds cannot be monitored permanently in clinical setups. In experimental infection trials, it was also described that some birds developed the clinical signs very quickly after infection without developing high antibody titers or even shedding viral RNA. Therefore it must be summarized that in clinically sick birds, high titers or large amount of shed virus might be used carefully for interpretation of the cause. Conversely, those same results in clinically healthy birds cannot be used for a prognosis of clinical disease.

The question remains if diagnosis of ABV status is helpful for a PDD diagnosis. This question can be answered both No and Yes. In PDD cases, a single test (PCR or serology) for ABV alone is not helpful. An ABV test (PCR, serology, or both) can tell if a bird is infected with ABV, but it does not tell if the clinical signs of concern are caused by this infection. As described earlier, a considerable number of ABV-positive birds remain clinically healthy. If a bird is symptomatic with compatible signs of PDD, the factual cause of those signs can still be unrelated to ABV. Exclusion of other potential differential diagnoses such as toxicoses, gastric foreign bodies, or concurrent infections still is required to aid in the inductive strength of a PDD diagnosis. Therefore, an ABV test—serology, PCR, or a combination—should not be taken as a PDD test. The typical PDD lesions can only be seen in histopathology. However, here comes the "Yes" that ABV investigation is helpful. In the situation where typical clinical signs of PDD or CNS signs are observed, ABV testing (serology and PCR) should be included in the diagnostic workup, and other common causes should be investigated. If those reasons cannot be confirmed, and ABV combined test results are positive, it is very likely that those signs are caused by ABV. This is particularly true if the ABV-titer or amount of shed ABV-RNA is high. For flock management, the combined ABV tests are essential (see below). Therefore it can be summarized that a combined antibody– PCR ABV test may be included in the examination panel in an avian practice but should not be taken as a "PDD test" and that single PCR or serologic assays are considerably less reliable for the purpose of screening and diagnosis of ABV status or the presence of PDD.

THERAPY

While several reports indicate partial success of therapy against PDD (see above), only a few studies have focused on the treatment of ABV infection itself. Antiviral treatment of ABV has not been successful to date.[73] Some authors[100,114] reported that amantadine hydrochloride reduced clinical symptoms in birds, but others[72] could not see an effect in reducing the viral shedding. It remains, therefore, unclear what the mode of action could have been in reducing the symptoms in the described studies. Ribavirin was reported to reduce ABV infection in cells in tissue cultures but also did not demonstrate an effect in reducing viral shedding in birds.[115] As long as no direct antiviral drug is available to reduce ABV infection, a treatment focus might be to interrupt the pathogenesis, thus not allowing the virus to trigger clinical disease. To achieve this, immunosuppressive drugs might be promising, as an increasing survival time of experimentally infected rats with BDV has been described.[91] This therapy focuses on selective T cell suppression, for example, accomplished with cyclosporine, and has been described as beneficial in single case reports in birds treated with cyclosporine-A.[116] Controlled studies in infected and clinically ill birds are necessary to prove the benefits. Additionally, it needs to be discussed how practical it is to immunosuppress a bird and reduce ABV-related symptoms, which would potentially then make the bird more susceptible to other infections. Those birds must then definitely be monitored very closely and may also return to clinical states of disease with PDD when immunosuppressive treatment is stopped. However, studies into this direction will be necessary to evaluate the pros and cons of this type of treatment. Recently, a study suggesting an immunomodulating approach using robenacoxib (anti-COX-2 nonsteroidal antiinflammatory drug) in combination with mycobacterial extracts was reported as being promising. The complete mixture applied remained proprietary, but it was reported that the T cell response was somehow redirected.[117] However, those studies had certain drawbacks, as proper case controls were lacking, and therefore the results should be considered carefully and need further confirmation.

As in the treatment of PDD, antiinflammatory drugs were suggested to reduce the ABV-induced signs of inflammation

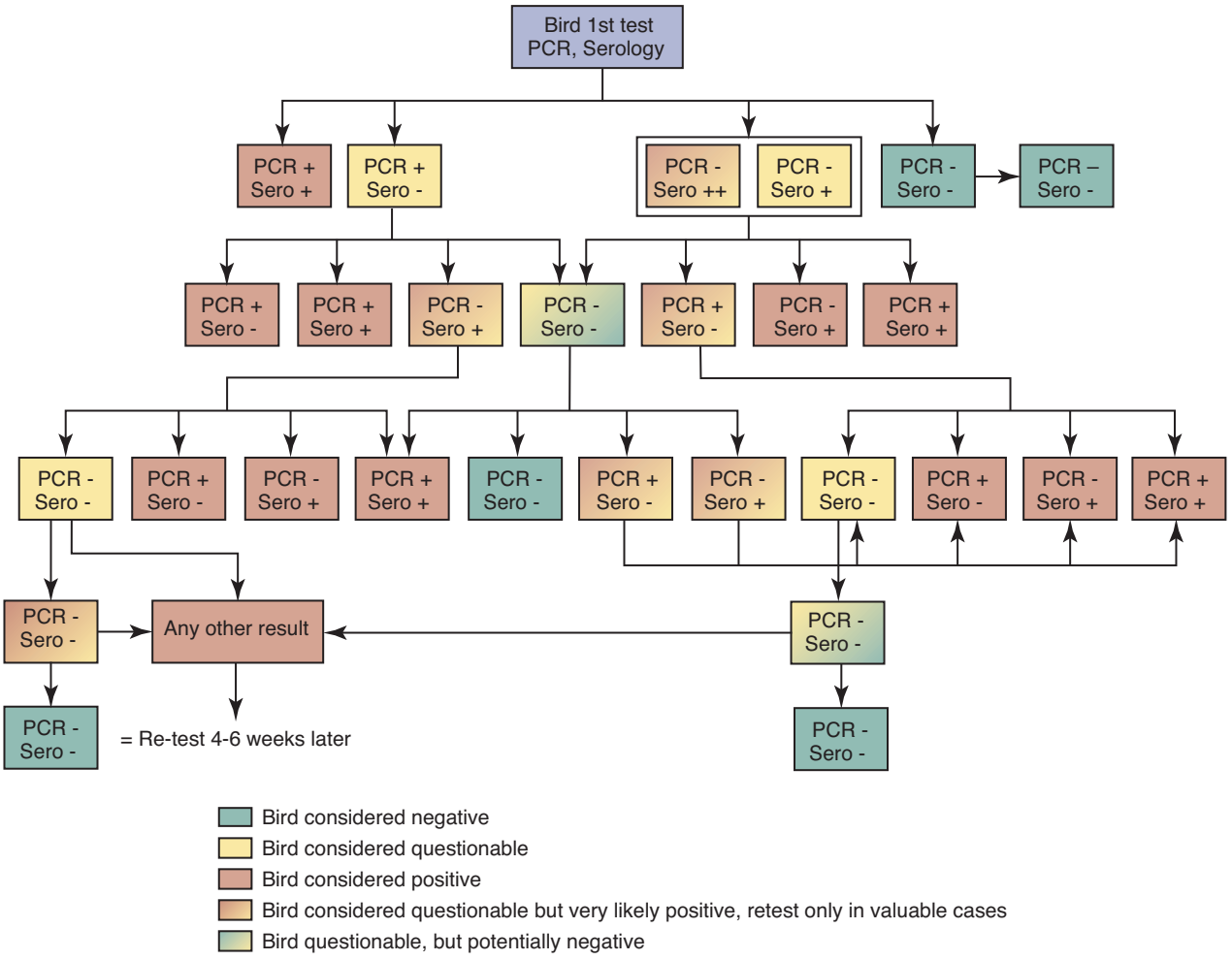

FIGURE 2-3 Suggested interpretation of laboratory test results to categorize birds as positive or negative for avian bornavirus (ABV). Polymerase chain reaction (PCR) should be made from a pooled cloacal and crop swabs of each bird, serology with a multiprotein enzyme-linked immunosorbent assay (ELISA) or preferably Immunofluorescence test based on ABV persistently infected cell culture (Herzog, et al, 2010). – = negative test result, + = positive test result, ++ high anti-ABV-antibody titer. Each box represents an individual bird, and so all the potential test results can be seen by following a box through the complete diagnosing process. Each arrow represents a retest 4 to 6 weeks apart with combined PCR and serology. Usually, most of the birds tested are assigned to a final result in the second row (after two tests) or third row (three tests). Only in very rare cases are more tests necessary, or the birds should be considered positive. First of all, the individual bird is tested and then assigned a positive, negative, or questionable status. Red means that those birds should be taken as positive. Yellow indicates that there is a questionable interpretive status and that from those birds all possibilities may arise; a retest is necessary. Green birds are negative, but it is advised to have at least two negative tests of one individual bird to ensure negativity. In the circumstance that a bird is considered positive, no retest is necessary. Often in clinical circumstances, a negative bird is considered as such in a single test. In the case where the bird is planned to be sold or integrated into a known negative flock, the test should be repeated, and only with a second negative result is the bird considered negative. In case a bird has a high antibody titer with a negative PCR, it should be considered positive. However, in the case of a bird that is deemed very rare or valuable species or individual, the test may be repeated. These birds are depicted as (red/yellow). Questionable birds are retested and usually will be assigned positive or potentially negative status. As described above, only in very rare events birds being positive in one of the test again may still be considered questionable and tested again. The vast majority of these previously positive cases, when retested, will remain positive. Potentially negative birds that were questionable status before are tested a third time and are only considered negative if they are deemed negative again, providing two consecutive negative tests. The odds that from a red/yellow box a result other than a positive can occur are very low, according to the experience of this author, but can theoretically happen.

and thus improve the observed clinical signs. For this, meloxicam was used in experimentally infected cockatiels[115] but demonstrated an adverse effect, with more severe lesions in ABV-infected birds that were treated compared with the control group that was ABV positive and not treated. It is fair to conclude that meloxicam had no observed beneficial effect in reducing clinical signs in experimentally infected cockatiels, but it remains unclear if the treatment had a role in increasing the severity of lesions observed in the treated group.

All in all, immune-modulating or symptomatic treatment might be beneficial in clinical circumstances for individual birds, but it should not be forgotten that those birds will likely remain ABV positive and are a potential risk of infecting other birds and spreading the virus.

FLOCK MANAGEMENT

The loss of a complete flock to ABV (or PDD) is a very rare event, if this has ever occurred. It seems that an initial period of PDD and CNS signs is reported more commonly in an infected flock, followed by longer periods of patency where no or only single birds become diseased. Usually, over years, only single birds die from ABV-related signs in these infected flocks. In some collections, even those single birds might have great value for financial, personal, or genetic reasons, and therefore this loss cannot be tolerated. However, some owners might decide to live with those losses and not to clear the flock from the virus. As ABV is very intensively discussed in the avian community, negative ABV test results are more often requested with a pre-purchase examination, in particular when large parrots are sold. Additionally, more and more boarding facilities request negative ABV test results on record prior to entry. Therefore, there is an increased pressure on breeding flocks to clear their flock from ABV, especially as ABV-positive or untested birds will be more and more impossible to sell. Unfortunately, the specifics of how these birds are being tested may be incomplete, leading to inconclusive findings or erroneous conclusions.

Viewing the common occurrence of ABV-positive birds and the high prevalence in different collections of psittacines, it is rational in some settings to initiate a proper flock health management strategy to exclude the virus from collections, particularly in psittacines. This complex task should only be supervised and planned by a veterinarian familiar with the particular details of the flock's particular management and goals and who understands what is most currently known about ABV and PDD. As with many complex disease processes, effective flock management strategy is far more than a series of test results alone. Informed consent prior to initiating the process to establish an ABV-free population is required.

The clearance of a flock from ABV is a long, expensive, and frustrating task and can only be accomplished with strict compliance of the owners. Many of them are enthusiastic at the start and want their flocks be examined. When they recognize that a considerable amount of birds are ABV positive, conflict often arises, especially if expensive and successfully breeding birds may need to be separated. It is not uncommon for aviculturists to stop further testing and abandon the effort to establish an ABV-free flock. Therefore, owners should be counseled as to exactly what is involved and expected at the onset of the effort to establish an ABV pathogen-free collection. If they accept this, a flock clearance and establishment of an ABV-negative flock is possible. This is particularly true as horizontal transmission of ABV seems to be not that easy (see above).

All birds of the flock need to be tested in a bimodal manner, by direct (virus demonstration, preferably RT-PCR of crop and cloacal swab) *and* indirect (serology) tests. Careful thought should be given to the specific choice of the serologic assay being used. This also applies in all further tests during the clearance process. After the first round of tests, three groups will be established and need to be maintained epidemiologically separate. This implies different logistical setups of compartments away from each other. One group contains the positive birds, the next the questionable birds, and the last the ABV-negative birds according to the diagnostic interpretation mentioned above (see clinical diagnosis and Figure 2-3). The assignment of the birds to one group is purely based on the laboratory results, not on the owners' preference or pairing status of the birds. If an owner does not want to split a pair when one partner is positive and the other negative, both birds must be included in the positive group. The compartments the different groups are located in are treated as separate units. These units are supplied and serviced by different caretakers or by the same caretakers who change their clothes, shoes, and so on, and disinfection is implemented when leaving one compartment. The negative compartment should be supplied (food, water, cleaning) first, followed by the questionable compartment and then the positive compartment. Owners might decide to give the positive birds away, which is a difficult task and might only be reached by rehoming them to private owners to be kept as pets. Euthanasia of positive birds is sometimes also requested but should only be considered in clinically affected birds. Ethically, euthanasia of ABV-positive but clinically healthy birds is very difficult and not recommended. It should also be considered that it seems possible to produce ABV-negative offspring from ABV-positive parents, and therefore those birds, as long as not clinically affected, do have their value as breeding birds (see below). In the end, it is the owners' informed decision, based on the advice of the veterinarian, whether the benefits outweigh the higher workload involved in keeping the ABV-positive birds and the risk of infecting the ABV-negative birds. In many cases of valuable breeding flocks, this is the case, and logistic plans should be made to keep the ABV-positive birds.

This first step is not the end of the way to clear a flock of ABV. In the second step, both groups (questionable and negative), are retested 4 to 6 weeks later. In the questionable group, some birds will now be treated as positives and transferred into this group, and some now potentially negative will remain here, but separated from other questionable birds (Figure 2-4). From the negative group, some birds will be transferred to one of the other groups and some remain still negative. The negative group can be taken as cleared when two consecutive combined tests performed 4 to 6 weeks apart reveal negative results in *all* birds of this group. Birds from the questionable group should be retested as often as only negative birds remains here, with two consecutive combined tests 4 to 6 weeks apart being negative. Those birds then can be placed back in the negative group. Whenever a bird shows

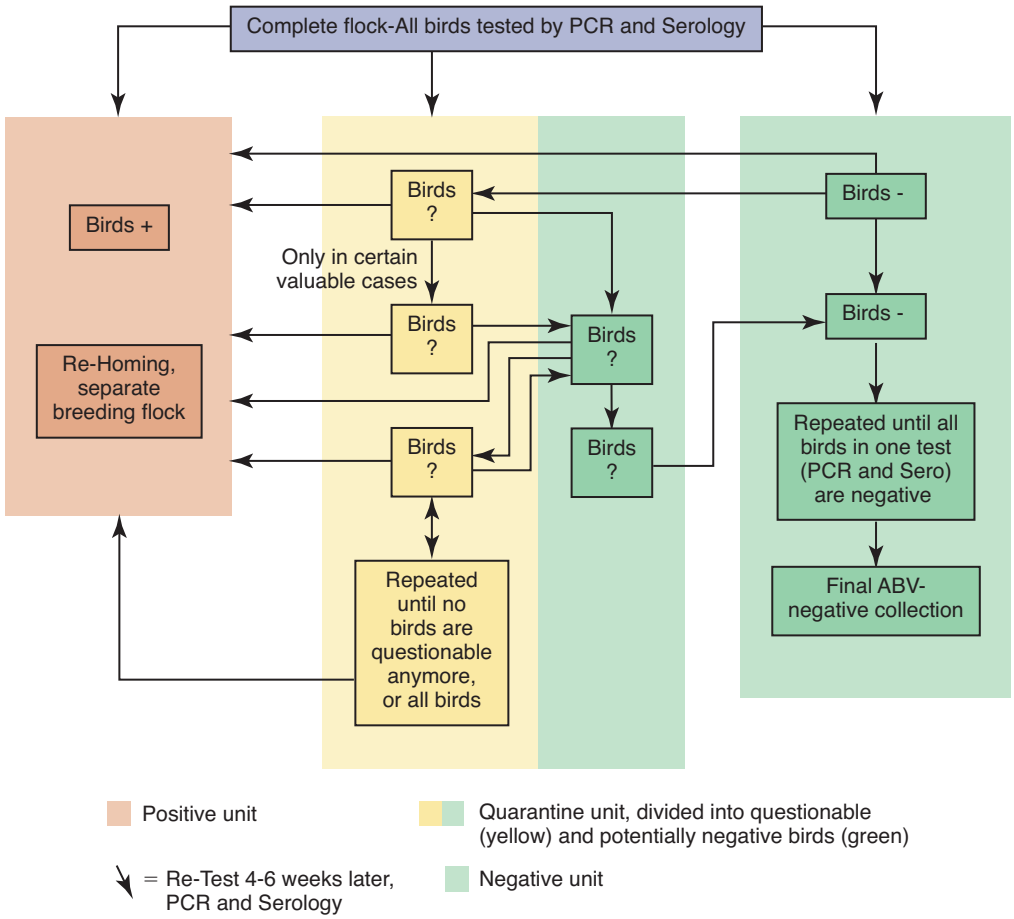

FIGURE 2-4 Suggested diagnostic workup plan to clear a flock from avian bornavirus (ABV) infection. The large box represents the epidemiologic units (populations), which should be clearly separated and treated as independent populations. The smaller boxes represent the group of birds which demonstrated the same results (positive, questionable, negative). Each arrow represents a retest 4 to 6 weeks later of *all* birds in that group at the same time. It is important that all birds within these groups are sampled on the same day and that always polymerase chain reaction (PCR) and serology are performed on an individual bird. First, the complete flock is tested, and three major groups are established (positive, questionable, and negative). A retest 4 to 6 weeks later places additional birds from the negative group into the positive and questionable groups and from the questionable group to the positive group and establishes a potentially negative group within the quarantine section. The negative group is only ultimately considered ABV-negative if ALL birds in that group are tested at the same time and are found to be negative in two consecutive tests. Questionable birds within the questionable groups are only placed in the negative group after two negative tests but are housed separately within the isolation area from the others after their first negative test result. Birds that are retested questionable (very rare; see Figure 2-3) should be considered positive for the purpose of clearing a flock of ABV; however, in very rare or valuable species, the risk might be taken to keep them in quarantine in the questionable group. However, should those birds test questionable again, they should definitely be considered positive. The isolation population is tested as long as all the birds are assigned to one of the other groups (positive or negative) or the owner decides that the remaining birds here should not be retested, and at that time, all remaining birds in isolation will be assigned to the positive group. Once a bird is assigned to the positive group, it will remain here.

up positive in one of those groups, the risk remains that virus was transferred from this bird to another, so the complete group should be retested. As stated above, a "test" means both direct (e.g., PCR) and indirect (serology) investigation (see Figure 2-4). Using this method, flocks were successfully cleared of ABV, with an observation period of approximately 3 years after exclusion of the last ABV-positive bird. This method was also utilized and effective in a flock of Spix's macaws. After separation of ABV-positive birds and strict hygiene measures, no further infections of previously negative birds were recorded over an observation period of 2 years in that flock.[118]

To keep a flock negative, strict measures must be applied to not include new birds in the flock. Only quarantined birds (at least 3 months or, even better, 6 months) that are repeatedly tested, as described above, and are negative should be included. Additionally, the flock should be retested on a regular schedule (yearly, biannually) to ensure the negative status. Also, the acceptance of other bird owners as visitors should be limited and shoe covers and coats should be supplied to them before entering the flock. This also will be beneficial to prevent various other infectious diseases.

As stated above, positive birds might still be used as breeding birds as vertical transmission of ABV seems to be rare, if occurring at all. Examination of eggs from ABV-positive parents has demonstrated that ABV-negative eggs are regularly found. Therefore eggs of positive parents should be taken away and the shell disinfected. It was shown that the shells of cockatiel eggs were effectively disinfected from ABV-RNA after cleaning with 3% hydrogen peroxide.[119] Following disinfection, those eggs should be artificially incubated, and the chicks should hatch separately from each other. Those chicks should then be kept singly housed, hand raised, and tested repeatedly using paired serology and PCR methods. In newly hatched chicks of small species, blood sampling for serology might not be possible, and only in this rare event PCR test for ABV-RNA detection by crop and cloacal swab should be used. It should be kept in mind that an anti-ABV-antibody titer in chicks could represent maternal antibodies transferred through the egg, as previously described.[101] This means that those birds should be retested 4, 6, and 8 weeks after hatch to see if the titer decreases, which is the case with maternal antibodies. If this is not the case and/or ABV-genome is detected, the birds are treated as positive. Chicks repeatedly testing negative can be grouped to avoid human imprints or can be transferred to ABV-negative foster parents. The last seems only to be possible in parrots when the birds are proven negative early enough for this procedure to be accepted by the parents. Transfer of newly hatched chicks to ABV-negative foster parents can be considered, and those chicks can be regularly tested there, although this maneuver poses a risk of the foster parents being infected. This is usually only done in setups where very valuable birds are bred and less valuable pairs are established as negative foster parents.

ABV-positive birds may remain clinically healthy for years. Therefore there may be a demand to rehome those birds as pets when breeders want to exclude them from their flocks. More and more owners also are starting to test their birds for ABV to try to determine their ABV status. This is particularly done if single birds are planned to be paired, even if breeding is not intended. Owners of ABV-negative birds usually request that the potential partner birds also be ABV negative. Conversely, owners of ABV-positive birds might plan to accept ABV-positive partners. Therefore rehoming of ABV-positive birds from flocks that are seeking to become ABV free is possible. Basically, this is a way that may be considered by the supervising veterinarian, if the right information about what is involved is relayed to the owner of the new home to which these birds are being transferred. However, it should be mentioned that ABV-positive birds might potentially carry different ABV genotypes. As a previous study has demonstrated, the infection of ABV-4-positive birds with ABV-2[61] triggered clinical disease; therefore, it might be recommended that the genotype be evaluated first before paring those birds. On the other hand, it remains unclear at present if this observation and the potential different pathogenicity observed is really related to the genotype itself or to the single strain involved.[98] Compared with genotyping, strain differentiation within one genotype is very difficult, and therefore matching of the different ABV strains carried by potential partners is a task that can derail the plan to pair ABV-positive birds. The risk of triggering a clinical disease by pairing two ABV-positive birds remains, for practical extents and purposes, as anyway every ABV-positive bird carries the risk of getting a clinical disease at any time. This recommendation may be abandoned, changed, or supported when the factors triggering clinical disease are better understood.

VACCINATION

Whenever a novel pathogen is discovered as a cause of an important disease, the demand to produce a vaccine as a prophylactic measure and to protect the birds arises. In the case of ABV, this seems to be a very difficult task. As described above, high anti-ABV-antibody titers are detected in clinically diseased birds, and in many cases, there seem to be a correlation between the increase of the titer and onset of clinical symptoms. Therefore it is fair to state that the antibodies that have been detected so far specifically against ABV are not protective.[87] It seems also likely that the pathogenesis of the symptoms caused by ABV are immune mediated and that ABV has developed mechanisms to escape the recognition of the immune system.[94] All these facts do not make steps toward a vaccine production very promising. To the contrary, it might even be that vaccine could in some circumstances induce the clinical disease or at least increase the severity of clinical signs.[72] However, when the pathogenesis and the details of the host immune response and the nature of different anti-ABV-specific antibodies are better understood, the window for the development of specific vaccines might open, especially based on recombinant vaccines.

TAKE-HOME MESSAGE

Avian bornavirus has clearly been identified as the causative agent of PDD and other clinical symptoms such as CNS signs or sudden death. Despite being commonly observed in other avian taxonomic groups, its main clinical importance is seen in psittacines. So far, the triggering factors causing clinical disease out of an ABV infection are unknown, and the pathogenesis of the infection is poorly understood. However, for the clinician, it is important to know that ABV is widely distributed. The means of transmission or the circumstances under which the virus is successfully transmitted between birds are not completely understood, but viral transmission overall does not seem to be easily reproduced. Therefore, psittacine flocks can be managed to clear the virus from the flocks, which is possible by regular testing of birds using direct virus demonstration (e.g., PCR) and indirect (serologic) testing. Positive birds need to be separated from negative birds, and as long as they are clinically healthy, those positive birds should not be euthanized. In summary, ABV is a very important viral infection with a large impact, especially in psittacine management, but it seems to be controllable.

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PSITTACID HERPESVIRUSES AND ASSOCIATED DISEASES

David Phalen

PSITTACID HERPESVIRUS 1

History and Description of the Virus

Psittacid herpesvirus 1 (PsHV-1) belongs to the subfamily *(Alphaherpesvirinae)* and genus *(Iltovirus)*. It has four major genotypes and at least three serotypes (Table 2-2 and Table 2-3).[1] It is the cause of Pacheco disease, which is an acute rapidly fatal disease of parrots and, rarely, passerine species, as well as mucosal papillomas and associated neoplasms of parrots.[1,2]

Outbreaks of Pacheco disease were first recognized in Brazil in the late 1920s in captive parrots and were not seen again until the late 1970s when large numbers of parrots were exported from South America into Europe and North America.[1,2] As of this time, the frequency of outbreaks has diminished to the point where they are relatively rare, but they still occur in mixed collections of parrots originating from multiple sources. The prevalence of mucosal papillomatosis in parrots has also diminished, but to a lesser extent.

Species Affected and Geographic Distribution

Pacheco disease occurs in parrots of either sex and of any age, originating from all of their distributions, so potentially all species are susceptible to infection with PsHV-1 and the development of disease (see Table 2-3).[3] There is no age or sex predilection. Mucosal papillomas have the greatest prevalence in Amazon parrots (*Amazona* spp.), macaws (*Ara* spp.), Hawk-headed parrots *(Deroptyus accipitrinus)*, and conures (*Aratinga* spp.) but have also been infrequently reported in other species. Mucosal papillomas and related neoplasms are found in birds of both sexes. They can also occur in parrots as young as 6 months of age. Pacheco disease outbreaks and or mucosal papillomatosis have been documented in North America, Europe, the Middle East, Japan, New Zealand (in quarantined birds), and Australia.[2,4]

TABLE 2-2

Psittacid Herpesviruses and Their Associated Diseases

Virus	Pacheco Disease	Respiratory Disease	Mucosal Papillomas
Psittacid herpesvirus 1	Yes	No	Yes
Psittacid herpesvirus 2	No	No	Grey parrots only
Psittacid herpesvirus 3	No	Yes	No

Clinical Manifestations

Pacheco disease

Pacheco disease should be suspected when a parrot in a collection dies unexpectedly and when multiple deaths occur over a short period. Signs are rare and generally nonspecific; however, some birds will have biliverdin-stained (yellow or green) urates immediately prior to death.[2]

Mucosal papillomatosis

Mucosal papillomas can occur most frequently in the cloaca and the oral cavity. Signs may be lacking and the lesions only observed on physical examination. When the lesions are advanced, birds may exhibit upper respiratory signs, strain to defecate, and have blood in their droppings, and the papillomatous lesions from the cloaca may protrude. A more generalized form of the disease can also occur where the papillomatous lesions extend into the esophagus, crop, and, rarely, to the level of the proventriculus and ventriculus. These birds may experience a chronic wasting disease. Regurgitation is uncommon but may occur. Mucosal papillomas are typically raised and pink and have a cauliflower-like surface. More diffuse lesions that involve the entire cloaca may have a cobblestone appearance. Oral papillomatous lesions are most commonly found along the margins of the choanae and at the base of the tongue. They can be very subtle, resulting in an asymmetric thickening of the choana or a blunting of the papillae. In many instances, the first indication of oral mucosal papillomas is a loss of pigment at the site of the lesion. Lesions can wax and wane, disappear completely, or become progressive.[2,4,5]

Bile duct and pancreatic duct carcinomas are fairly common sequelae to mucosal papillomatosis. Birds with these lesions do not show evidence of disease until the lesions are severe. When they do show signs, they are typically signs of chronic liver disease, including weight loss, an overgrown beak, and poor feather quality. Bile and pancreatic duct carcinomas develop in the months and years following the onset of mucosal papillomas.[5]

TABLE 2-3

Serotype and Disease Potential for the Four Genotypes of Psittacid Herpesvirus 1

Genotype	Serotype	Pacheco Disease	Mucosal Papillomas	Bile Duct Carcinomas
1	1	Amazons, Australian species	Uncommon	No
2	2	Amazons, Grey parrots	Uncommon	No
3	3	Predominately Amazons, less commonly other species	Very common	Yes
4	1	Most species	No	No

From Tomaszewski EK, Gravendyck M, Kaleta EF, et al: Isolation of psittacid herpesvirus genotype 1 from a superb starling *(Lamprotornis superbus)*, *Avian Dis* 48:212–214, 2004.

Diagnosis

Pacheco disease

It is rare that a bird with Pacheco disease survives long enough to have blood collected and tested, and as a result, the diagnosis of Pacheco disease is usually made at necropsy. If a bird survived long enough to be seen by a veterinarian, experimental infections would indicate that they likely have a leukopenia and marked elevations in their plasma aspartate aminotransferase concentrations.[2] The hematologic picture, however, might change if the birds do not die immediately, which is uncommon.

Gross lesions in birds with Pacheco disease are variable. Some birds will only show very subtle changes in the liver that resemble a diffuse lipidosis. Others will have prominent swelling of the liver and spleen. Multifocal areas of discoloration, representing areas of necrosis, and gross evidence of pancreatitis and enteritis are seen less frequently. Most birds will be in good to excellent body condition. Microscopically, hepatic and splenic necroses, varying from moderate to massive, are characteristic lesions. The pattern of necrosis in the liver may appear to be random, but generally the periportal hepatocytes are spared. Pan-nuclear eosinophilic inclusions are generally present in the liver but, in some instances, can be difficult to find; they are often abundant in the spleen. Pancreatic necrosis and necrosis of the intestinal and crop mucosa with intralesional inclusion bodies occur with infection with certain genotypes. There is a single report of a cockatiel with chronic active pancreatitis secondary to PsHV-1 infection. This bird exhibited both endocrine and exocrine pancreatic insufficiency.[2]

Mucosal papillomatosis and associated neoplasms

Mucosal papillomas are diagnosed through careful examination of the oral cavity and eversion of the cloacal mucosa with a lubricated swab (Figure 2-5). Anesthesia may be required to observe subtle mucosal changes. If there are diffuse lesions, crop thickening may be detected with palpation. The gross papillary changes are characteristic, and biopsy is generally not necessary to make a diagnosis. In the author's experience, PCR assays will reveal all of these birds to be positive for PsHV-1, so testing for PsHV-1 is not necessary.[2]

Despite extensive liver involvement, it is rare that the liver of birds with bile duct carcinomas is sufficiently enlarged that the edge of the liver can be palpated. Radiographically, the liver may have rounded margins. Bile duct carcinomas are readily visualized with ultrasonography as multifocal to coalescing hyperechoic regions of the liver that are replacing the adjacent normal areas of the liver. Increases in the γ-glutamyl transferase (GGT) have been reported in birds with bile duct carcinomas. It has been the author's experience that the GGT will increase in older parrots in a range of species, so changes in the GGT are not specific for bile duct carcinomas. Diagnosis can be confirmed by liver biopsy. Pancreatic duct carcinomas are very difficult to diagnose, but ultrasonography may reveal lesions, and biopsy can also lead to antemortem confirmation of diagnosis.[2]

Grossly, mucosal papillomas can be difficult to recognize in the dead bird, but if the animal is fresh, these mucosal papillomas will retain the same characteristics as those seen in the live bird. Microscopically, mucosal papillomas are made up of multiple fimbriae with a variably wide to narrow base. Each fimbria is composed of a fibrovascular core surrounded by a pseudostratified or stratified cuboidal to columnar epithelium. The lesions may be ulcerated. Lymphoplasmacytic infiltrations of the fibrovascular cores occur intermittently.[4]

Bile duct carcinomas are pale tan to gray colored, confluent to slightly raised, and multifocal to coalescing. Only small amounts of normal liver may remain. Similarly, pancreatic duct carcinomas are gray and nodular, and, in some instances, may be coalescing. Neither the bile duct carcinoma nor the pancreatic duct carcinoma metastasizes.[2]

The author has rarely seen cloacal carcinomas (Figure 2-6) in psittacine birds. However, in the three cases that have been seen, all contained PsHV-1 DNA, and all three metastasized to other organs in the body. Whether these tumors were caused by the virus or not is not known.

FIGURE 2-5 Everted mucosal papilloma of the cloaca of a blue and gold macaw. Persistent soiling of the mucocutaneous junction around the vent has resulted in infection and ulceration.

FIGURE 2-6 Cloacal carcinoma in an Amazon parrot.

Treatment

Acyclovir has been used to treat individual birds and entire aviaries during outbreaks of Pacheco disease. Published reports and anecdotal evidence suggest that treatment is highly effective in preventing mortality in these outbreaks. A range of routes of administration and drug dosages have been used. The author has used acyclovir orally by gavage at 80 to 100 milligrams per kilograms (mg/kg) three times a day for 10 days with apparent success. While treated birds may survive, they are not cured of infection and will become carriers of the virus. Carriers and those with overt mucosal papillomas are not impacted by treatment with acyclovir.[2]

Mucosal papillomas should be left alone unless they are clearly causing the bird discomfort or interfering with breathing or defecation. These lesions are rarely static and may spontaneously regress or may worsen. In many cases, they will shrink only to return again in the weeks or months to follow. If surgical intervention is required, the papillary lesions can be debulked with sharp dissection, laser surgery, radiosurgery, and treatment with topical silver nitrate. It is the author's impression that surgical remove of part of the diseased tissue can result in regression of the surrounding lesion in some instances. Repeated surgical intervention can result in cloacal scarring. To prevent cloacal scarring, the author uses sharp dissection to remove the diseased mucosa and sutures the margins. There is minimal evidence of successful treatments for bile duct carcinomas.[2] There is a case report of treatment of a pancreatic duct carcinoma in a green-winged macaw *(Ara chloroptera)* with carboplatin. In that case, the lesions resolved but ultimately returned.[6]

Epizootiology and Preventive Measures

It has been hypothesized that the four genotypes of PsHV-1 have coevolved with some South American species of parrot.[1] Infected birds shed the virus in oral secretions and droppings. Infection is thought to be the result of ingestion of contaminated material. The incubation period is 5 to 7 days. In the adapted host, infection is unlikely to cause disease, and these birds develop lifelong infections and are potential sources for future outbreaks if housed with other parrots that have not been exposed to the virus. When nonadapted parrots are exposed to certain PsHV-1 genotypes, Pacheco disease occurs. Which parrots in an aviary will develop Pacheco disease will depend on the species of the exposed bird and the genotype of the virus, as well as husbandry and other undefined factors.[1] For example, densely housed indoor collections are more prone to outbreaks of Pacheco disease. Many infections are subclinical, and the affected birds become carriers. Subclinically infected birds and those that have survived Pacheco disease are at high risk for developing mucosal papillomas if they are infected with genotype 3 and have a lower risk if infected with genotypes 1 and 2. To date, all birds that developed bile duct and pancreatic duct carcinomas and were tested were found to be infected with genotype 3.[4]

Keeping the birds infected with PsHV-1 out of a virus-free collection can be achieved by performing routine testing. PCR-based assays that can detect all four genotypes of PsHV-1 have been developed. In one study, the virus was consistently found in birds that were repeatedly tested over the course of a year by a PCR assay of combined oral and cloacal swabs. PsHV-1 DNA could also be detected in heparinized blood, but blood samples proved to be less sensitive than mucosal swabs. Serology may also be a useful tool for detecting subclinically infected birds, but sera would have to be tested against all three serotypes.[7]

Vaccines have been developed from PsHV-1 isolates and may prove to be useful tools in high-risk flocks.[2] It is not known, however, if vaccination with one serotype of PsHV-1 will protect against infection with other serotypes. In at least one instance, two different serotypes have been detected in the same bird, which indicates that a polyvalent vaccine may be required to protect against all three serotypes of PsHV-1.[7]

PSITTACID HERPESVIRUS-2

History and Description of the Virus

Psittacid herpesvirus 2 (PsHV-2) is of the genus *Iltovirus* of the subfamily Alphaherpesvirinae. It is most closely related to PsHV-1 but has never been associated with a Pacheco-like disease.[8]

Species Affected and Geographic Distribution

PsHV-2 has only been detected in Congo African Grey parrots *(Psittacus erithacus erithacus)* in the United States and Germany. Infections have been found in both wild-caught and domestically raised birds. In the only extensive survey done, even in mixed aviaries, infection was confined to Grey parrots and was not even detected in the closely related Timneh parrot *(Psittacus timneh)*.[8,9] The only other bird known to be infected with PsHV-1 was a blue-and-gold macaw in a collection in the United States.[7] Widespread testing for this virus has not been done, so it is expected that it may have a wider geographic range than is currently known.

Clinical Manifestations

PsHV-2 infections can either be subclinical or result in the development of mucosal and, less commonly, mucocutaneous papillomas of the oral cavity and eye. The papillomas are benign but can be fairly extensive. Differential diagnoses for PsHV-2-induced papillomas include cutaneous papillomas caused by parrot papillomavirus-1 (PePV-1) and other neoplastic diseases of the oral cavity. PePV-1 is rare, causes extensive lesions of the skin of the face, and has only been reported in wild-caught birds.[8,9]

Diagnosis

Gross lesions are characteristic but can be confirmed by biopsy where the characteristic fibropapillomatous lesions will be demonstrated. Virus inclusions have not been reported in these lesions. Primers that detect PsHV-1 DNA can be used in PCR assays to detect PsHV-2 DNA in the papillomatous tissue or combined oral and cloacal swabs from clinically and subclinically infected Congo African Grey parrots.[2]

Treatment

There are no reports of treatment attempts. Surgical removal would be indicated if the lesions were interfering with air flow. Given that these lesions are not thought to be

associated with replicating virus, acyclovir will not be effective against them.

Epizootiology and Preventive Measures

Current data suggest that this virus is host-adapted to Grey parrots and entered Europe and the United States by the movement of wild-caught Grey parrots. Twenty percent of aviaries in Germany were found to have infected birds.[9] Individual birds could be screened for infection by using PCR assays of oral and cloacal swabs designed to detect PsHV-2. The sensitivity of this assay is not known.

PSITTACID HERPESVIRUS 3

History and Description of the Virus

For several decades, an uncharacterized herpesvirus that predominately targets the trachea of parrots has been reported to occur sporadically in a range of parrots in North America and Europe. Recent work has shown this virus to belong to the subfamily Alphaherpesvirinae and the genus *Iltovirus*. It has been named psittacid herpesvirus-3 (PsHV-3) and is most closely related to the passerid herpesvirus-1, another respiratory herpesvirus.[10]

Species Affected and Geographic Distribution

PsHV-3 infection has only been confirmed by sequencing of the virus in an outbreak of disease in Bourke's parrots *(Neopsephotus bourkii)* in the United States and in two eclectus parrots *(Eclectus roratus)* in Australia.[10,11] It is likely that PsHV-3 has a more widespread geographic and species range because a similar disease has been described in Amazon parrots (*Amazona* spp.), Indian ring-necked parrots *(Psittacula krameri)*, a cockatiel *(Nymphicus hollandicus)*, and a princess parrot *(Polytelis alexandrae)* in Europe and Japan and other locations in Australia and the United States.[10]

Clinical Manifestations

The outbreak in the two Bourke's parrots lasted several months. Infected birds coughed, had difficulty breathing, and exhibited ocular and nasal discharge. The birds died within 3 to 7 days after signs were first noticed. Other species of parrots in the collection did not develop the disease.[10] Both eclectus parrots were in poor body condition and appeared to have been ill for some time. Respiratory signs were observed in one of the eclectus parrots, but the majority of the signs exhibited by these birds was nonspecific and may have been the result of concurrent infectious diseases.[11]

Diagnosis

Diagnosis in the live bird

No work has been done on the diagnosis of PsHV-3 in the live bird. It should be considered a potential differential diagnosis in any psittacine bird that is exhibiting signs of tracheal or pulmonary disease, especially if these signs are accompanied by ocular and nasal discharge. Cytology of the conjunctiva and trachea has the potential to detect syncytial cells as well as cells with characteristic eosinophilic intranuclear inclusion bodies. The partial sequence of PsHV-3 is known, and virus-specific primers could be developed for use in a PCR assay. It is also possible to detect PsHV-3 by using panherpesvirus primers.[11]

Postmortem diagnosis

Potential gross lesions include conjunctivitis, tracheitis, and changes in the lungs suggestive of diffuse or locally extensive pneumonia and air sacculitis.[10] One of the eclectus parrots exhibited multiple pale foci in the pancreas caused by pancreatic necrosis. Both eclectus parrots also had concurrent aspergillosis.[11] Microscopically, diagnosis of PsHV-3 infection is presumptively made by detecting syncytial cells containing pan-nuclear eosinophilic inclusion bodies in the bronchi and parabronchi and to a lesser extent in the trachea, conjunctiva, air sac, and respiratory epithelium of the turbinates. Similar lesions were also seen in numerous other tissues, including the spleen, pancreas, inner ear, meninges, kidney, thymus, bursa, and gonads. Lymphoplasmacytic inflammation of varying degrees was often present, and hyperplasia of the respiratory epithelium was a common finding. In contrast to the acute form of the disease caused by PsHV-1, the liver is not a primary target.[10,11] Both eclectus parrots also had severe mycotic bronchopneumonia.[11]

Treatment

The outbreak in the Bourke's parrot collection stopped with the onset of acyclovir treatment. However, multiple other management changes were made at the same time, and whether acyclovir treatment was the reason the outbreak was stopped is not known.[10] Given the apparent success and safety of acyclovir treatment in birds with acute PsHV-1 infections, it would seem reasonable to use acyclovir to treat PsHV-3 infections if an antemortem diagnosis were made.

Epizootiology and Preventive Measures

If this virus behaves like many other avian herpesviruses, then it is likely that it is host adapted and does not cause disease in its host or hosts but does cause a persistent infection. If so, persistently infected birds are likely to intermittently or continuously shed the virus and would be the source of outbreaks when they do occur. It is possible that subclinically infected birds could be detected with PCR-based assays of oral swabs, cloacal swabs, or blood samples.

Both eclectus parrots described here were concurrently infected with the psittacine beak and disease virus (PBFDV).[11] It is therefore likely that these birds were immunosuppressed, which resulted in their *Aspergillus* infections. It is possible that immunosuppression may have also resulted in the PsHV-3 infection causing disease, whereas it would not have in an immune competent bird. Testing of birds with confirmed PsHV-3 infection for PBFDV will be necessary to prove this hypothesis.

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PSITTACINE BEAK AND FEATHER DISEASE

Shane Raidal

PSITTACINE BEAK AND FEATHER DISEASE

Psittacine beak and feather disease (PBFD) is a well-recognized disease that clinically presents most often as a chronic and ultimately fatal viral disease of Psittaciformes. While acute forms of the disease can occur in nestling and fledgling birds, the incubation period can be very long, with the slow development of feather dystrophy as molting progresses. All parrots, lorikeets, and cockatoos are considered susceptible to infection,[1,2] and there is evidence of it occurring naturally in wild birds for more than 120 years in Australia, where it is recognized as the main disease threat to many critically endangered birds such as the orange-bellied parrot *(Neophema chrysogaster)*. Increasing evidence suggests that the dispersion of wild-caught Australian parrot species such as the budgerigar *(Melopsittacus undulatus)* since the early 1840s has most likely resulted in the global spread of PBFD because it now affects a wide range of psittacine species in both wild and captive populations worldwide,[1,3-7]

Etiologic Agent

The virus that causes PBFD is beak and feather disease virus (BFDV) and despite many attempts, no method for cultivating BFDV in vitro has been successful, which impeded early research into the disease. The biologic characteristics, pathophysiology, and mode of replication have all been determined by studying the natural virus purified from the tissues of infected birds, by studying recombinant proteins, or by inferring from related circoviruses. The virus is a member of the family of Circoviridae[8] and is perhaps the simplest pathogen known to infect vertebrates. It is highly genetically diverse and prone to mutation[9–11] but relatively antigenically conserved based on serology.[12–14] Unlike other members of the *Circovirus* genus, BFDV is a hemagglutinating virus and has been shown to agglutinate erythrocytes from guinea pigs, geese, and many species of psittacine birds.[15–18] The disease is associated with ongoing massive viral excretion, a feature that can be readily detected with hemagglutination assay (HA) as an antigen detection diagnostic test.

Origins of Beak and Feather Disease Virus

The first recorded description of a feather loss syndrome that almost certainly was PBFD was in South Australia in 1907 in red-rumped grass parakeets *(Psephotus haematonotus)*. Affected birds were described as "quite healthy, except being destitute of feathers,"[19] and this was considered responsible for the decline of the species in the Adelaide Hills, since affected birds were likely to be more susceptible to predation. The use of terms such as "runners," or "hikers"[6] but more commonly "French molt" in historical records, particularly in reference to feather loss syndromes in budgerigars, probably included examples of PBFD as well as avian polyomavirus disease. Nevertheless, the disease has been recognized by aviculturists since the early 1970s.[20]

Until recently, it was thought that different BFDV genotypes or even distinct virus sub-types were responsible for, or at least associated with, disease in certain geographic areas or psittacine bird species. However, emerging consensus indicates that all psittacine birds are susceptible to a diversity of BFDV clades, with no clear association based on host–virus cospeciation. Within the order Psittaciformes as a whole, BFDV exhibits host-generalism with wide species susceptibility.[11]

Phylogenetic analysis of BFDV genomes strongly indicates that no one genotype can be considered more virulent than another; as such, it behaves like a viral quasispecies and host-generalist in the Psittaciformes, with shallow host-based divergence likely reflecting the dynamic ranges of interspecific transmission. Any effort to develop an attenuated strain for vaccination purposes is likely to be confounded by this feature. There is evidence that the Loriinae subfamily, which includes the lorikeets, lories, fig parrots, and budgerigars, may be the most robust or deeply adapted host of BFDV and are potentially super-distributors of this virus, at least throughout Australasia (Figure 2-7).

There is increasing viral genetic evidence that BFDV originated in the Australasian and not African or South American Psittaciformes.[1,21-23] While captive macaws, conures, and Amazon parrots are susceptible to BFDV infection,[9] the conspicuous paucity of unique BFDV genotypes from South American parrots suggests that the disease does not occur naturally in the wild in this region. Given the prominence of neotropical parrots in the North American and European aviculture, it seems likely that if it does occur in the

FIGURE 2-7 Abnormal and discolored plumage in a black-capped lory *(Lorius lory)* on the left with psittacine beak and feather disease. Affected contour feathers are abnormally yellow, while others are missing, including the primary flight feathers. While more than 200 different beak and feather disease virus (BFDV) genotypes have been detected, there are no distinct subtypes or strains, with only weak association with certain geographic areas or psittacine bird species. Emerging consensus indicates that all psittacine birds are susceptible to a diversity of BFDV clades. (Courtesy Dr. Brian Speer.)

wild in South America with an epidemiology similar to that in Australasia, then the disease would have been historically more frequently seen in shipments of wild-caught South American birds. This is because the less-than-ideal disease control in the pet bird trade during the height of exportation in the previous decades would have allowed ample exposure to any neotropical BFDV genotypes admixing from a variety of sources. Such genetic admixing has clearly been documented recently among captive psittacine flocks in Europe.[9] Recent evidence of BFDV infecting wild Cape Parrots in South Africa[7] is likely the result of a recent introduction, given the constrained degree of genetic diversity observed as well as the close relatedness of Cape Parrot isolates to BFDV genotypes from captive birds in Europe.

Features of differential disease expression—seen in Grey parrots at one end of a scale of susceptibility and in lorikeets and cockatiels at the other—are typical of the accentuated virulence seen when a virus switches from its preferred host to another.[24,25] There is evidence for this because BFDV jumps from one host species to another. In quasispecies theory, it is likely that a greater number of genetic variants occur as the most replicatively efficient variants compete within new hosts. This has been recently shown in PBFD-affected orange-bellied parrots[11] and cockatoos,[26] and an overall consequence of this might be the retention or enhancement of virulence across Psittaciformes as BFDV jumps flexibly from one host to another. In other words, the rich range in psittacine hosts probably counteracts any evolutionary trend toward viral attenuation.

Many avian circoviruses have been detected recently, some more pathogenic than others,[27–32] and many more are likely to be discovered serendipitously with the use of next-generation sequencing and metagenomic techniques. Circovirus DNA sequences present in a wide range of invertebrates, protozoans, plants, fungi, algae, and bacteria suggest a likely ancient coevolution of circoviruses with vertebrate hosts,[33,34] and this is likely to be true for the majority of birds. Indeed, the absence of a circovirus lineage in any extant psittacine species is somewhat puzzling, given the recent findings. Increasing evidence supports a post-Gondwanan origin of BFDV in the Australian species,[26] and there is fossil evidence that *Cacatua*[35] and the budgerigar have likely been present in Australia in their present forms for at least five million years.[36] Theoretically it is likely that BFDV has circulated with limited host-based divergence among the Australian Psittaciformes for at least this period. In contrast, there is no strong evidence of BFDV endemicity in native New Zealand parrot species prior to human colonization,[4] and the recent detection of PBFD in wild New Zealand birds is best explained by the introduction of this pathogen with the release of infected feral eastern rosellas *(Platycercus eximius)* from Australia. A similar scenario almost certainly occurred for the Norfolk Island green parrot.[37] The recent characterization of BFDV infection in captive New Caledonian lorikeets and parrots[21] is evidence of contemporary introductions of at least two BFDV lineages in Deplanche's rainbow lorikeets *(Trichoglossus haematodus deplanchii)* and the vulnerable New Caledonian parakeet *(Cyanoramphus saisseti)*. Given the relatedness of the Indonesian, Australian, and Polynesian BFDV genotypes, it seems most likely that continental Australia, or Sahul, has acted as a pathogen reservoir for island seeding in the South Pacific region. Within the Australian parrot species, there is a clinically well-recognized differential host susceptibility to PBFD. Lorikeets have never been reported with the same degree of advanced feather and beak dystrophy as is seen very commonly in sulfur-crested and other white cockatoos,[38] and there is anecdotal evidence that lorikeets frequently make complete clinical recoveries or at least regain relatively normal plumage. The majority of these recovered lorikeets may continue as BFDV carriers, excreting large viral titers in feces for months and possibly years. It is plausible that lorikeets disperse BFDV to the islands. This is supported by the results of a recent survey of captive birds in New Caledonia, which showed a strong infection bias to lorikeets,[21] but in the absence of more widespread sampling of wild birds, iatrogenic reasons, rather than natural expansion, will have to be held responsible in that case.

In the context of BFDV in the Australian landscape, a definitive understanding of disease modeling and population thresholds for a multihost disease such as PBFD may not be possible, given the large number of potential host species and conceivable parameters that could dynamically influence intraspecies and interspecies transmission rates alongside other factors such as abundance of important host reservoir species. Nevertheless, phylogenetic analysis of BFDV genomes strongly indicates that no one genotype can be considered more virulent than another, and as such, BFDV behaves like a viral quasispecies and host-generalist in Psittaciformes, with shallow host-based divergence likely reflecting dynamic ranges of interspecific transmission.

Transmission

The virus is excreted in feather dander and in feces. Consequently, high concentrations of the virus can be detected in

liver tissue, bile, crop secretions, feces, and feathers.[18,39,40] Infection is most likely by oral and/or intracloacal ingestion of the virus, as demonstrated by experimental infection studies.[41] BFDV is suspected to be transmitted vertically[42,43] because BFDV DNA can be found in embryos from infected hens.[43] However, there is no experimental evidence that has conclusively confirmed vertical transmission rather than horizontal transmission to the embryo via cloacal secretions and nesting material. If vertical transmission occurs, it is unlikely to be a significant mechanism for circovirus maintenance in populations, since it is more likely to be a deep force for virus–host coevolution. Recent phylogenetic analyses provide little evidence to support strong host-based divergence. When considered in broader terms of disease ecology, BFDV behaves more as a resource-generalist with flexible host switching. This is much more likely facilitated by horizontal transmission and, at least in Australia, is most likely to occur in tree nest hollows, where there is strong competition between Psittaciformes and other birds for reproductive opportunities.[44–46] The ability of BFDV to persist in the environment,[47] along with the massively high titers excreted by PBFD-affected birds, supports this. As such the role of sequestration of BFDV genotypes within nest hollows, perhaps for many years, may be an important factor in extending the replication strategy of the virus along with re-entry of ancestral BFDV genotypes into host populations.

Clinical Signs

Juvenile or young adult psittacine birds are the most susceptible to PBFD, but birds of all ages can succumb to the disease. Birds kept in isolation for many decades can become infected when exposed to affected psittacine birds or contaminated areas. An acute form of the disease is well recognized in nestling or fledgling birds,[48] particularly in the Grey parrots *(Psittacus erithacus)*,[49,50] which can die within a week of developing signs, and a more commonly encountered chronic form that can occur in all psittacine species. In acute disease, there is rapid development of depression associated with leukopenia, anemia, green diarrhea, biliverdinuria, and death due to hepatic necrosis. Acutely affected birds often become systemically ill and anorexic and/or regurgitate food. There may be pterylodynia with edematous and painful wing tips caused by inflammation, vasculitis, and subcutaneous edema. High viral titers can be detected in the liver and bile of affected birds, and some may die of liver failure without obvious feather lesions. Depending on the age of the nestling and thus the phase of feather development in individual pterylae, affected feathers may be shed all at once, or only the primary flight feathers may be affected, but this is usually seen in a bilaterally symmetric pattern. Fractures of the developing calamus and accompanying intrapulp hemorrhage are the predominant clinical findings. Affected feathers fracture at points of necrosis, usually before the feather has unsheathed.

The more commonly encountered manifestation of PBFD is a chronic disease with a slow subtle development and progression. As the molt progresses, dystrophic feathers replace normal ones, and the affected birds gradually lose plumage, often without other clinical signs of illness. The pattern of ongoing plumage damage is related to the stage of the molt that the bird is in when the disease first begins but is usually bilaterally symmetric and slowly progressive. Dystrophic feathers are usually short and have one or more of the following characteristics: fault lines across the vanes, a thickened or retained feather sheath, blood within the calamus, an annular constriction of the calamus, or curling (Figure 2-8).

While some species such as the cockatiel, *Trichoglossus* lorikeets and New World psittacines appear to have an inherent resistance to BFDV infection or at least to the development of PBFD, if they become infected at all, others such as the gang gang cockatoo and black cockatoos (*Calyptorhynchus* spp.), which occupy specialist ecologic niches, seem more susceptible to succumbing, especially from the acute phase of infection (Figure 2-9).

In all Cacatuidae, the powder down feathers, or pulviplumes, are often the first feathers affected, and the ensuing lack of powder throughout the plumage can result in a glossy or dark pseudodiscoloration of the beak and claws and cause the plumage to become dull. PBFD-affected pulviplumes are fragile or develop an abnormally thickened outer sheath that fails to disintegrate. Powder down feathers may atrophy and create bare patches of affected skin. Claw abnormalities occur occasionally and generally develop well after feather and beak lesions become apparent. The beak can progressively

FIGURE 2-8 Wild Australian king parrot *(Alisterus scapularis)* with early clinical signs of psittacine beak and feather disease, with plumage deficits around the face and head.

FIGURE 2-9 Powder down patch in a gang gang cockatoo *(Callocephalon fimbriatum)* with psittacine beak and feather disease (PBFD) demonstrated atrophic and dysplastic pulviplume feathers, which results in a loss of powder throughout the rest of the plumage.

elongate and/or develop fracture lines, and the affected rhamphotheca may slough off. In severe cases, necrosis of the oral epithelium and osteomyelitis can extend through to the esophagus and crop. On the extremities, PBFD-induced hyperkeratosis can cause the skin to appear excessively scaly, or it may be thickened and moist. Sunlight-exposed skin can become darkly pigmented. Chronic skin ulcers can occur at the elbows and wing tips. Chronically affected birds are predisposed to hypothermia, and secondary infections are common as a result of immunosuppression. These include cryptosporidiosis and bacterial, mycotic, and other viral infections. Most birds with chronic disease eventually have difficulty eating, lose weight, and die. In smaller grass parrots such as the *Psephotus* and *Neophema* species, apparently normal feathers that fall out or are easily plucked may be the only clinical sign. The first clinical sign in birds with green plumage may be the development of yellow feathers which may appear normal in other respects (Figure 2-10).

Clinical Pathology

The acute form of PBFD is associated with severe leukopenia in juvenile birds,[49,51,52] and chronically affected birds may have lower serum protein concentrations, characterized by low prealbumin and gammaglobulin concentrations.[53] The hematologic characteristics of juvenile long-billed corellas *(Cacatua tenuirostris)* were studied following experimental infection with BFDV and compared with vaccinated birds.[54] Significant differences in total and differential leukocyte concentrations, including heteropenia and lymphopenia, were demonstrated in BFDV-infected birds, but packed cell volume (PCV) and total serum protein (TSP) were not significantly affected.

Histopathology

Lesions within the skin and epidermis include multifocal epithelial cell necrosis, necrosis of distal pulp and hemorrhage into the distal shaft of feathers, and epidermal hyperplasia and hyperkeratosis.[20,41,55] There may also be infiltration of heterophils and lymphocytes into the pulp of some feathers. Basophilic intracytoplasmic inclusions can be found within macrophages in the feather pulp (see Figure 2-8), and some epithelial keratinocytes may contain intracytoplasmic or intranuclear inclusions.[20,56] Within the beak, degeneration and necrosis of epithelial cells occur in the basal and intermediate cell layers. Chronic beak lesions are also associated with inflammation as a result of the presence of bacteria within the exudate and the keratinized layers of epithelium.[20]

The liver may be congested, with multifocal areas of necrosis of varying severity.[56] Characteristic basophilic inclusions may be present in Kupffer cells within the liver, and occasionally erythrophagocytosis may be seen in the liver and spleen. The thymus and bursa may show varying degrees of atrophy and necrosis. Focal aggregates of necrotic lymphocytes often contain macrophages with typical inclusions, and necrotic lymphocytes with intranuclear inclusions may also be visible.[20,56] Intracytoplasmic inclusion bodies within macrophages are variable in size and shape, and electron microscopic examination shows that they are composed of particles 17 to 20 nm in diameter arranged in a paracrystalline array.[20,41,55]

Immunohistochemistry and in situ hybridization[57] can be used to demonstrate BFDV antigens in a wide range of tissues,[58] but the best organs to assess are the bursa of Fabricius, feather follicles, spleen, esophagus, and crop. In some species, there appears to be differential expression in these tissues (Figure 2-11).

Diagnosis

The chronic form of PBFD can be diagnosed clinically with a high degree of certainty by careful physical clinical examination. Very few other diseases can mimic the bilaterally symmetric feather dysplasia seen in this disease, but endocrine conditions such as hypothyroidism should be considered in rare cases. Cases of feather plucking may be present, resulting in widespread iatrogenic plumage damage, so it is important to examine the feathers around the head and face area, where single birds cannot easily inflict self-trauma. In pairs or groups of birds, occasionally excessive allopreening might result in physical trauma to facial and head feathers that can mimic the lesions seen in PBFD. Surgical biopsy of skin and developing feather follicles has been used to detect histopathologic evidence of infection, but the sensitivity of detection is low unless chronic fulminate disease is present. In some species such as the grass parrots, *Neophema*, and *Psephotus* parrots, viral inclusions can be rare or difficult to confirm without immunohistochemistry (Figure 2-12).

In Australia, serology and antigen detection have proven to be valuable diagnostic tests for detecting and quantitating BFDV excretion and antibody responses, and when used in combination, different tests have proven extremely useful for understanding the impact of viral infection in individual birds and to identify potential false-positive and false-negative results.[13] While a number of antibody-detecting enzyme-linked immunosorbent assay (ELISA)–based tests[59,60] have been developed, they are not used extensively in diagnostic testing, primarily because the cross-reactivity between the immunoglubulin Y (IgY) of different psittacine birds is not known, and it is impossible to guarantee the validity of the assay when used with sera from other species. Hemagglutination inhibition (HI) avoids such issues and remains the gold standard for antibody detection.[14] HI assays tend to be technically simple and rapid and do not require anti-species-specific secondary antibodies or highly purified antigen. Antibody

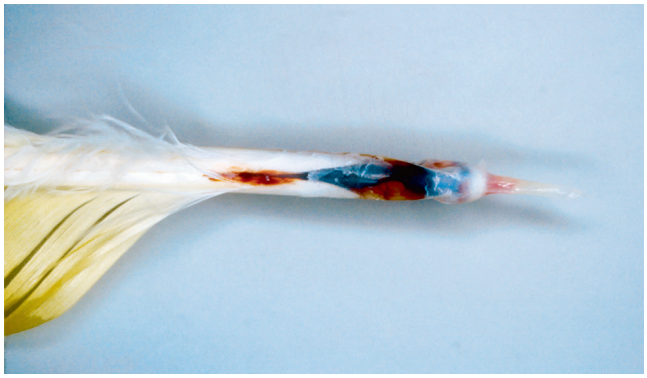

FIGURE 2-10 A dystrophic feather, showing blood within the calamus and annular constrictions of the calamus.

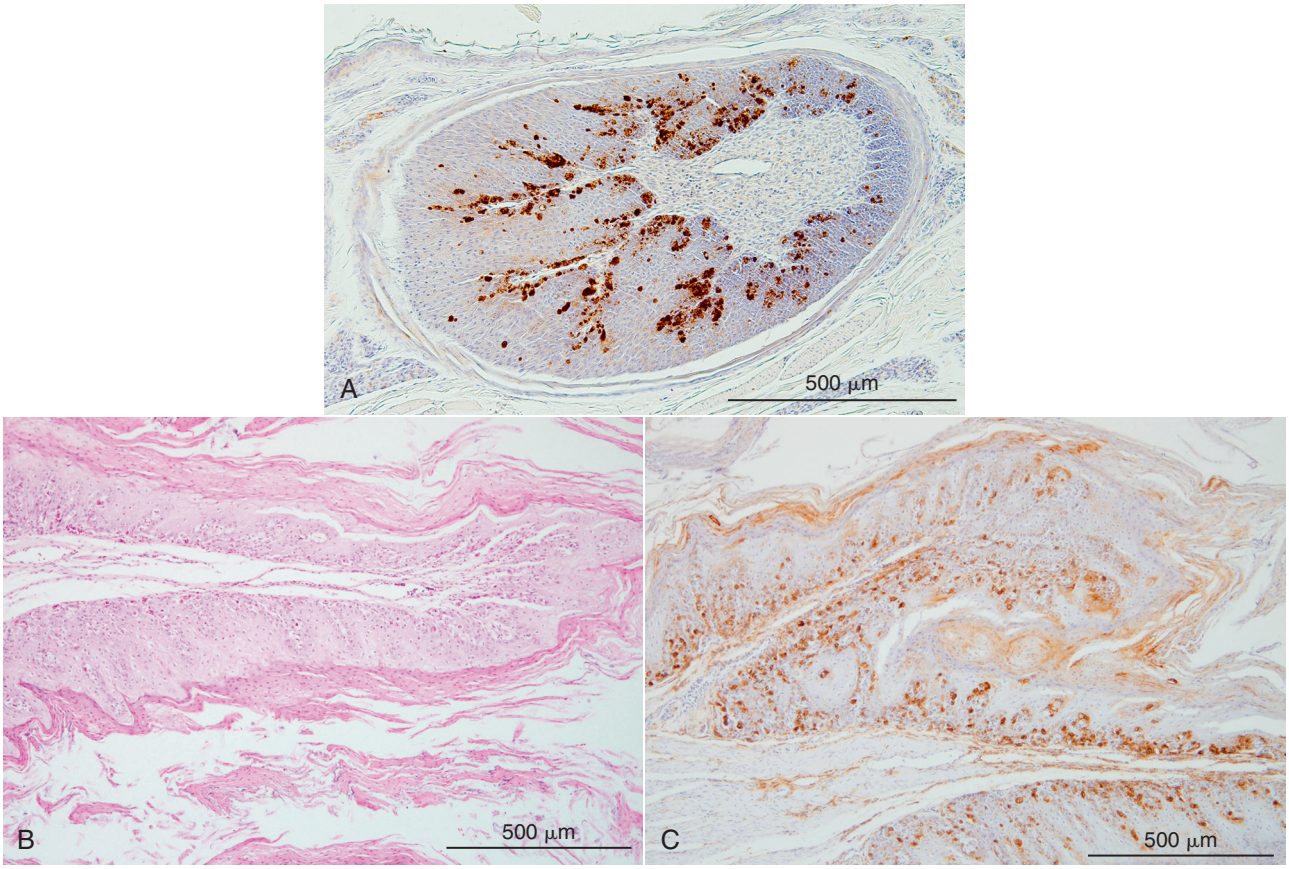

FIGURE 2-11 Typical strong positive immunohistochemistry reaction shown in the developing feather of a cockatoo with chronic psittacine beak and feather disease (PBFD) **(A)**. Beak and feather disease virus (BFDV) antigen can be detected in other organs such as the esophagus shown in a hematoxylin and eosin–stained section **(B)** from a gang gang cockatoo *(Callocephalon fimbriatum)*, which had areas of epithelial cell apoptosis and spongiosis. In the absence of characteristic botryoid amphophilic intracytoplasmic inclusions, immunohistochemistry has shown a positive reaction to antigen **(C)**.

FIGURE 2-12 Loss of normal feather coverage around the head and face in a female eclectus parrot with abortive attempts at follicular regeneration, resulting in small atrophic and dysplastic feather stumps.

measurements using HI have reasonable precision if attention is paid to minimizing interassay variation by titrating standard virus and antibody activity against each other and against the erythrocytes from multiple birds prior to testing. Even so, HI assays are still prone to an appreciable amount of intertest variations, especially if performed infrequently or without standard reference antigen and sera.[61]

Along with HA and HI, polymerase chain reaction (PCR) testing has been used extensively for managing PBFD,[13] and in most countries, PCR testing has become the main method for detecting BFDV infection. As mentioned above, there is a wide variation in BFDV genetics, which has the potential to confound the PCR test design. Nevertheless, the BFDV *Rep* gene is relatively conserved,[1,62] and the PCR primer set P2-P4,[63] which targets this gene, has proven to be reliable for detecting BFDV DNA; even so, there are some rare genotypes that do not match perfectly with this primer set, and at least one study has revealed wide variations in diagnostic laboratory accuracy.[64] The PCR product from this diagnostic test covers a 700-nucleotide segment of the *Rep* gene, providing an ideal length for routine DNA sequencing,[65] which is useful for tracing the origin and establishment of infection in

a flock.[66] This can be an important legal aspect of a diagnostic investigation. Within the diagnostic laboratory, it is also a useful step to monitor or identify potential sources of DNA contamination. Clinicians need to be aware that different laboratories might target slightly different parts of the BFDV genome or the capsid gene that has a higher degree of genetic diversity. Even for the same primer sequence set, they may use different PCR amplification conditions in their diagnostic assay, and the potential number of different variables that goes into designing a test protocol means that laboratory results from different laboratories should not be considered as equal.

In real-time PCR assays, primer dimers and other artefacts can result in false-positive interpretations. Other sources of false-positive PCR results include contamination of samples from the environment, especially if feathers are being collected, as well as amplicon contamination in the laboratory during DNA extraction. Copious shedding of BFDV occurs in the environment of PBFD-affected birds, and the risk of contamination of samples precludes the use of any material such as feathers exposed to the environment for meaningful PCR diagnosis in individuals that are not isolated for a prolonged period. Collection of bodily tissues such as blood is ideal for PCR testing. So it is important for clinicians to use and change gloves when handling and collecting samples from multiple birds. In the laboratory, a number of steps can be taken to investigate suspected false-positive reactions. First, the tests can be repeated using a second round of DNA extraction from the original submitted sample. Second, a separate PCR test that targets a different part of the viral genome, such as the capsid gene, can be used. Third, DNA sequencing can be done on the amplicons and compared with reference or positive controls. If the clinicians are suspicious of the results, they should contact the laboratory and ask for further validation of results.

Appropriate sample collection is important for determining the infection status of suspect birds. One study showed that in a flock of 56 peach-faced lovebirds *(Agapornis roseicollis)*, of the 47 birds that were PCR positive on blood samples, only 10 were also positive on feather samples (Figure 2-13).[13]

While rarer than false-positive results, false-negative PCR results do occur. There are various reasons for this, but most importantly, the clinician needs to consider whether the sample collected was appropriate for the question being asked and if it has been appropriately stored. False-negative PCR results can occur due to a number of intralaboratory errors in technique, but more importantly, the degree of genetic variation that occurs in BFDV can lead to errors in primer annealing.

More recent studies in clinical research and diagnostics have used high-resolution DNA melt (HRM) curve analysis for routinely identifying differences in genetic sequences.[67] Newer-generation PCR machines can do this automatically as part of the diagnostic analysis. The melting profile of a PCR product is dependent on length, sequence divergence, guanine-cytosine (GC) content, and heterozygosity and is an accurate, robust, and cost-effective alternative to existing methods for genotypic differentiation of BFDV. Compared with sequencing, the technique is faster, and results can be obtained within 5 hours from receipt of blood or feather specimens.[68]

FIGURE 2-13 A peach-faced love bird *(Agapornis roseicollis)* with advanced clinical signs of plumage deficits due to PBFD. (Courtesy Dr Brian Speer.)

Management of Disease and Treatment

Individuals within many species may make full recoveries from clinical PBFD. For example, lorikeets (*Trichoglossus* sp.) and Eclectus parrots (*Eclectus* sp.) often develop protective HI titers alongside cessation of virus excretion. The immunologic mechanisms that control whether or not a bird succumbs to full-blown disease or recovers from subclinical infection is not well understood. Successful therapeutic regimes are likely to be developed but almost certainly will have a higher rate of success in subclinically infected birds rather than those with chronic disease. Current therapeutic options for PBFD are mainly supportive. Birds with chronic BFDV can live for many years, even after the development of significant beak lesions.

There have been few studies on possible therapeutic interventions for PBFD. Interferon alpha (IFN-α)–modulatory CpG sequences have been described in other circoviruses and likely also exist within BFDV. These oligodeoxyribonucleotides (ODNs) have been shown to have both inhibitory and stimulatory effects on the induction of IFN-α and an inhibitory effect on the production of tumor necrosis factor alpha (TNF-α) in natural interferon-producing cells,[69–71] independent of viral replication or the presence of capsid proteins. Cytokines show promise for the treatment of many viral diseases, and the use of chicken IFN-γ has been promoted anecdotally. Interferon of avian origin is not yet commercially available, and its efficacy for the treatment of chronic cases is yet to be investigated, especially in light of findings that IFN-γ may enhance circovirus replication in cell culture.[72]

In one case, treatment with b-(1,3/1,6)-D-glucan from oyster mushroom was claimed to have cleared BFDV DNA from four out of the six BFDV-infected horned parakeets *(Eunymphicus cornutus)*, and four subclinically affected Major Mitchell cockatoos *(Lophochroa leadbeateri)* some 9 months after the treatment commenced.[73] However, the absence of BFDV DNA in blood should not lead to the conclusion that an effective clearance because an insufficient number of birds were treated, no control group was included, and no evidence that

absence of BFDV DNA was not simply a result of the development of an appropriate antibody response was provided.

Prevention and Control

It is presumed based on its physicochemical characteristics that BFDV is resistant to extremes of temperature and various chemical disinfectants.[18] However, disinfection using peroxide compounds (Virkon S) has been recommended for use in captive breeding programs of endangered psittacine species.[74] Strict quarantine and diagnostic screening of new additions to the flock, using a combination of assays to detect potentially infected birds, is recommended. In countries where free-flying PBFD-infected birds may exist, prevention of access to the flock by wild birds is important, as is prevention of contamination of the flock by feces from wild birds. Stringent hygiene protocols should be in place, including regular cleaning with an appropriate disinfectant such as Virkon S in a 1% solution, which has been shown to inactivate nonenveloped viruses and bacterial spores.

Currently, there is no commercially available vaccine for BFDV. An experimental inactivated vaccine using inactivated virus or recombinant proteins has been shown to be effective.[75,76] As no cell culture system has been developed to grow the virus successfully in vitro, recombinant techniques show the most promise for the development of effective vaccines that may be produced on a large scale. Recombinant capsid proteins, expressed in bacterial and insect cell–based systems have been proposed for use in diagnostic tests and vaccines for BFDV.[77,78] It should be noted, however, that vaccination does not prevent viral replication,[79,80] so effective control of PBFD will always depend on a combination of diagnostic testing, hygiene measures, and the maintenance of high levels of flock immunity.

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AN OVERVIEW OF AVIAN INFLUENZA IN DOMESTIC AND NONDOMESTIC AVIAN SPECIES

Darrel K. Styles

Influenza poses significant disease risk to both animal and public health, causing extensive morbidity and sometimes high mortality. Influenza may cause global pandemics such as the recent 2009 H1N1 pandemic, and avian influenza results in the loss of billions of dollars of poultry yearly, hence the older term for its disease "fowl plague." While biologics are useful in limiting the spread of influenza, the rapid rate of viral evolution and its ability to elude the immune response make it a challenging disease to control. This chapter is intended to provide a cursory overview of influenza virology and disease dynamics and discuss the disease risk and sequelae in both domestic and wild avian species.

Influenza viruses belong to the family Orthomyxoviridae and are enveloped, single-stranded negative-sense ribonucleic acid (RNA) viruses with segmented genomes consisting of eight gene segments coding for 11 known proteins. These viruses are broadly subdivided into types A, B, and C. Type A influenza viruses are further classified into subtypes based on the major antigenic proteins that festoon the viral capsid, namely, hemagglutinin (H or HA) and neuraminidase (N or NA). These two proteins provide the basis for the subtype nomenclature (e.g., H5N1, H3N2, and H7N9), because there are 16 recognized HA and 9 NA confirmations that occur in different combinations and comprise a range of avian influenza subtypes. Avian influenzas are widely distributed in waterfowl and shorebirds, which are considered the natural hosts for influenza viruses.[1,2] However, recent reports have described bat species as being hosts for two novel strains, H17N10 and H18N11, showing that influenza virus host diversity extends beyond avian species.[3,4] Type A influenza viruses are antigenically diverse and express a cosmopolitan host preference. These viruses may affect many avian species and a broad range of mammals, including, but not limited to, humans, swine, horses, dogs, ferrets, bats, and marine mammals. Clinically, influenza A viruses are responsible for outbreaks, epidemics, and pandemics.

Type B influenza viruses are also further classified into subtypes and express a more restricted host range, which includes humans, seals, and, experimentally, ferrets. Influenza B viruses typically cause outbreaks and epidemics but not pandemics. Type C influenza viruses are the least antigenically diverse and are largely confined to humans, although both canine and swine infections and experimental infections in ferrets have been reported. Therefore influenza C viruses cause outbreaks and highly localized epidemics but are not involved in pandemics.

BIOLOGY OF TYPE A INFLUENZA VIRUSES

Avian influenza viruses (AIVs) are Type A influenza viruses and are thought to be the progenitor of all influenza A viruses regardless of their host species. AIVs are found in waterfowl and shorebirds globally, and these species are the natural reservoirs for the virus. AIVs may adapt to mammalian species and become established in those populations; however, how this occurs has not been well elucidated, although there may be select evolutionary mechanisms by which this transition occurs.

Type A influenza viruses are highly subject to mutation and evolution, and primarily change by two mechanisms, antigenic drift and antigenic shift. Antigenic drift occurs because the virus' RNA polymerase has no proofreading function; therefore substitutions are introduced resulting in a somewhat predictable error rate between 1×10^{-3} and 8×10^{-3} substitutions per site per year.[8] Antigenic drift may contribute to the agent's ability to elude the host immune response, but generally it does not result in significant virulence changes in the virus. By contrast, antigenic shift can radically change the virus' pathogenic potential. Antigenic shift generally occurs by reassortment of heterologous influenza virus gene segments when the host is co-infected with two different influenza subtypes. The influenza genome is segmented and the gene segments of different influenza viruses can reassort to create unique viruses.[8] For example, if a host is co-infected with H5N1 and H3N2, then new reassortant viruses such as H5N2 and H3N1 can result. Antigenic shift can greatly increase virulence or host adaptation in a single viral generation. This shift may potentially advance zoonotic potential and certainly enhances the ability to elude the host immune response.

VIROLOGY, PATHOBIOLOGY, AND ECOLOGY OF AVIAN INFLUENZA VIRUSES

Type A influenza viruses attach to a host's respiratory or gastrointestinal (GI) epithelial cell's sialic acid receptor by means of their hemagglutinin protein. Mammalian adapted influenza viruses and AIVs demonstrate different receptor preferences for the confirmation of the terminal galactose on the polysaccharide chain of the sialic acid. AIVs prefer this terminal sugar to be in an α-2,3 orientation, whereas mammalian adapted influenza viruses prefer an α-2,6 orientation. This

specificity helps to partially explain host preferences for these viruses. Avian species have a greater density of α-2,3 receptors on their epithelial surfaces, whereas mammals have a greater density of α-2,6. However, mammals do possess α-2,3 receptors of variable concentration, and in humans, these are typically found in the lower respiratory tract. This is one of the possible pathways for avian influenza viruses to infect mammalian hosts. Swine have demonstrated the potential for being readily infected with both avian and mammalian strains, and this has, in part, been attributed to receptor sialobiology.[8] Therefore, swine have been postulated to be the "mixing vessels" for avian and mammalian strains and capable of adapting avian strains to mammals. However, other findings suggest that the distribution of receptor type in swine is not dissimilar to humans; hence the mechanism(s) for avian strain adaptation to mammals is more complex than receptor biology alone. Quail have also been postulated to play a similar role in this type of adaptation scheme; however, whether this might occur has not been established.[9]

Once the influenza virus hemagglutinin protein has been bound to the sialic acid receptor with the carbohydrate moiety in the appropriate confirmation for that virus and species, an essential enzymatic cleavage of the hemagglutinin protein must occur in order for the virus to enter the host cell. This cleavage has important implications for AIV virulence, which will be discussed later.

AIVs are further classified by their pathogenic potential for poultry, namely, highly pathogenic avian influenza (HPAI) and low pathogenicity avian influenza (LPAI). Therefore, when designating an avian influenza virus, the HA/NA subtype designation is preceded by its pathogenic potential (e.g., HPAI H5N1, LPAI H7N9). HPAI infection typically causes severe illness and death in avian species, but the clinical signs of LPAI range from subclinical to mild, depending on the species infected and the strain of virus. Clinical signs described in susceptible chickens infected with HPAI include ocular and nasal discharges, coughing, snicking and dyspnea, swelling of the sinuses and/or head, apathy, reduced vocalization, marked reduction in feed and water intake, cyanosis of the unfeathered skin, wattles and comb, incoordination, nervous signs, diarrhea, and acute death. In laying birds, additional clinical features include a marked drop in egg production, usually accompanied by an increase in numbers of poor-quality eggs. None of these signs is considered to be pathognomonic for HPAI infection. LPAI viruses that normally cause only mild or no clinical disease in poultry can result in more severe disease if concurrent infections or adverse environmental factors are present. LPAI infections in poultry are often detected serologically with hemagglutination inhibition (HI) assays, agar gel immunodiffusion tests (AGIT), and enzyme-linked immunosorbent assays (ELISA). Virus isolation with LPAI viruses can be challenging, but often partial sequencing for characterization can be accomplished by molecular methods such as reverse transcriptase polymerase chain reaction (RT-PCR). By contrast, birds with HPAI may succumb to disease before seroconversion and molecular testing (e.g., RT-PCR) and sequencing and/or virus isolation is used to characterize the virus.[10,11]

Domestic poultry and other birds are usually infected by LPAI from spillover from infected wild migratory waterfowl. While such events typically result in a mild or asymptomatic infection, LPAIs of the H5 and H7 subtype are subject to mutation in land-based poultry (e.g., chickens, turkeys) and can evolve into highly pathogenic strains. All currently known HPAI viruses are restricted to subtypes H5 and H7 (although not all H5 and H7 are highly pathogenic; in fact, most are LPAI viruses). HPAI virus infections may cause high flock mortality up to 100%. Some subtypes of HPAI viruses have become adapted to wild migratory waterfowl and may crossover directly into poultry from that compartment.

Commercial poultry in the United States is subjected to rigorous surveillance for LPAI and HPAI; infection in commercial poultry is usually detected through this routine serologic or molecular surveillance for subclinical infections or by clinical illness and production losses. Commercial flocks showing clinical signs consistent with avian influenza are subjected to extensive diagnostics and may be depopulated if an H5 or H7 subtype is detected. LPAI infection of land-based poultry is largely confined to the respiratory tract, unless the infection is exacerbated, whereas in waterfowl it is largely subclinical and confined to the gastrointestinal tract.[5] This tissue tropism is a result of the necessary enzymes being present in the target cells to cleave the hemagglutinin protein after attachment, typically confined to the avian intestinal or respiratory tract. LPAI viruses have a single cleavage point within their HA protein that helps to convey this cell-type specificity where only the target cells (e.g., GI tract) possess the necessary enzymes to cleave the HA protein and allow ingress. By contrast, HPAI viruses have multiple basic amino acid cleavage points within their HA protein, which permits an array of cell types to actively cleave the HA protein. Therefore, HPAI viruses are systemic in nature and infect multiple organ systems.

DIAGNOSIS

Identification of the Agent

Samples of oropharyngeal and cloacal swabs, feces, or specimens from dead birds can be submitted for virus isolation. RT-PCR, targeting one or more segments of the virus genome (usually the matrix protein, HA, and NA) offers accurate and rapid results.[12,13] The matrix or M protein is a highly conserved protein across all subtypes of influenza, and a PCR-positive result suggests that there is an influenza A virus in the sample. Subtyping the virus may be accomplished by HI and neuraminidase inhibition tests against a battery of polyclonal or monospecific antisera to each of the 16 hemagglutinin (H1–16) and 9 neuraminidase (N1–9) subtypes of influenza A virus. However, sequencing is more frequently used in determining virus subtype. Pathogenicity is determined by inoculation of live susceptible chickens in a virus-secure biocontainment laboratory to determine the intravenous pathogenicity index (IVPI), which defines the threshold for HPAI designation when the mortality rate is 75% or greater, and/or by sequencing the H5 or H7 gene and determining whether the genes possess the multiple basic amino acid cleavage sites common to all HPAI viruses.[14–16]

Serology

Serological diagnostics have been validated for poultry species, but may not be fully applicable across the range of avian

species that may be examined. Some pen-side antigen capture tests have demonstrated effectiveness for detection of avian influenza virus both in terms of sensitivity and specificity.[17] ELISA assays that have been validated for veterinary use are preferred for veterinary diagnostic laboratories. AGITs are used to detect antibodies to the conserved nucleocapsid and matrix antigens of influenza A viruses, and are therefore used as general screening tools for domestic poultry monitoring.[18,19] AGITs may be less reliable for detection of antibodies to influenza A in species of birds other than domestic poultry, so results from nondomestic species should be carefully interpreted.[20] HI tests can be used in diagnostic or screening serology; however, these tests also may lack sensitivity because of the subtype specificity of the hemagglutinin used. ELISA is used to detect antibodies to influenza type A–specific antigens in either species-dependent (indirect) or species-independent (competitive) test formats.

REPORTABLE (NOTIFIABLE) AVIAN INFLUENZA

Infection of poultry by H5 or H7 strains is considered to be a reportable disease to State and Federal animal health authorities. Older regulatory language refers to infection of poultry by H5 or H7 subtypes as "notifiable," but this language is considered obsolete by the World Organization for Animal Health (OIE), which provides regulatory guidance for the international trade of animal commodities. OIE defines avian influenza for its purposes in the Terrestrial Code Chapter 10.4, which is paraphrased as "infection of poultry caused by any influenza A virus of subtype H5 or H7; HPAI viruses demonstrate an IVPI of 1.2 (75% mortality) or greater or possesses multiple basic amino acid cleavage sites within their HA protein." LPAI viruses are considered to be all other H5 or H7 viruses in poultry that cannot be classifed as HPAI viruses by the aforementioned criteria. Infection of commercial poultry by non-H5 or non-H7 LPAI subtypes (e.g., H1, H3) is not immediately reportable, but these LPAI viruses may cause mild disease or production loss. Detection of any HPAI strain in commercial poultry is immediately reportable to regulatory authorities and has a swift and direct impact on the interstate and international movement of poultry commodities. All poultry infected with or exposed to HPAI, and some classes of poultry infected with LPAI H5 or LPAI H7, are quarantined and depopulated to control the spread of the disease or prevent evolution into a more dangerous strain.

ZOONOTIC POTENTIAL OF AVIAN INFLUENZA VIRUSES

While influenza infects a broad range of avian species, outbreaks in domestic poultry remain the primary concern due to the potential for the H5 and H7 subtypes to evolve into highly pathogenic strains. Zoonotic transmission is also a possibility with some subtypes of avian influenza, which—if they could achieve sustained lateral transmission in people—might result in a pandemic.[6] Both HPAI H5 and HPAI H7 subtypes have demonstrated the potential for zoonotic transmission. This is exemplified by the emergence of the virulent genotypes of the Asian strains of HPAI H5N1 in 1996, which have caused an epizootic extending from a pan-Asian distribution to parts of Africa and resulted in scores of human cases and fatalities. However, LPAI viruses such as LPAI H7N9 and LPAI H9N2 have also exhibited zoonotic behavior. LPAI H7N9 emerged in China in 2013 and is sustaining ongoing infections, which are characterized by land-based poultry subclinically infected by LPAI H7N9 and are being transmitted to people, resulting in human illness and fatalities.[7] How avian influenza viruses adapt to mammals is not well understood, but the potential for emergence of zoonotic or pandemic strains exists within the avian population.[21] It has been speculated that virulence changes occur when the viruses attempt to adapt to novel hosts (e.g., land-based poultry, mammals).

AVIAN INFLUENZA IN BIRDS OTHER THAN WATERFOWL AND POULTRY

For purposes of this discussion, the nondomestic avian species that will be addressed are psittacines, passerines, Columbiformes, Accipitriformes, and ratites. Avian influenza has been reported in a number of different bird species.[22] However, these were likely coincidental infections resulting from spillover from either infected waterfowl (the natural hosts) or infected domestic poultry.[23] Epidemiological data suggest that LPAI and some HPAI viruses may spread from wild waterfowl along their migratory route to domestic birds such as chickens, turkeys, or even ostriches. However, HPAI had rarely been detected in any wild bird species until the appearance of the current pathogenic clades of Eurasian HPAI H5N1 that emerged in 1996 and then re-emerged with the more modern pathogenic clades in 2003.[24] Since 2003, HPAI H5N1 has been detected in a number of avian species other than wild waterfowl and domestic poultry, which is likely due to the magnitude of the epizootic across Asia and extending into Africa.

Occasionally, raptor and passerine species are incidentally infected by spillover from the infected domestic poultry compartment or infected wild waterfowl. This transmission can occur indirectly through the contaminated environment (passerines) or directly through consumption of infected poultry or waterfowl (raptors). Columbiforms appear to be highly resistant to avian influenza virus infection and are not considered to be a high-risk species.

Pet Bird Species

Because psittacines figure prominently in pet bird culture, they will be the focus of this discussion, but the findings are largely applicable to other conventional pet bird species. Parrots have been reported to have been infected with HPAI H5N1, HPAI H5N2, and LPAI H9N2, as well as other LPAI subtypes.[25–28] However, these infections have likely occurred because of housing the birds in close proximity to infected poultry, waterfowl, or other avian species that are shedding virus. Situations that are associated with pet bird species being infected with avian influenza include trapping of wild caught birds that may be exposed to infected poultry or other infected species in places like the live-bird markets that are common in the developing world.

Psittacines would not normally encounter high-risk influenza species in the wild, and experimental studies have shown that avian influenza virus infection and transmission is not efficient in parrots. However, avian influenza infection can cause serious disease or death in pet bird species and can mimic the clinical signs of other viral diseases (e.g., Newcastle, avian polyomavirus).[25] Neotropical parrots were infected when being co-housed in a quarantine station with infected bulbuls and vireos from Asia (which may have originated from live-bird market environments), resulting in the depopulation of the station. The infection of these passerines likely occurred in the exporting country through exposure to infected poultry or waterfowl prior to export and the virus was then transmitted to the psittacines.

Psittacines can be treated palliatively for avian influenza (including HPAI infections), and there is a case where an infected pet parrot was isolated and supported until it cleared the infection.[25] While some subtypes of avian influenza have been shown to be zoonotic, parrots have been experimentally infected with avian influenza, including HPAI H5N1 and the zoonotic LPAI N7N9; but it is questionable how efficient these birds could be as vectors of avian influenza to other birds or animals.[22]

CURRENT HPAI DYNAMICS IN THE WORLD

Eurasian HPAI H5N1 continues to cause disease in both domestic poultry and wild birds across Asia and parts of Africa. It also continues to cause infections in humans, but to date has not increased in virulence or transmission potential in regard to its zoonotic capacity. However in Asia, HPAI H5N1 over the past decade or so has generated a number of reassortants including H5N2, H5N3, H5N5, H5N6, and H5N8. Of these reassortants, Eurasian HPAI H5N6 and H5N8 have proven to be particularly robust. This clade of Eurasian H5 viruses appears to be uniquely adapted to select species of dabbling ducks (genus *Anas* spp.) where the birds manifest infection asymptomatically and serve as reservoirs of the viruses without consequence. Eurasian HPAI H5N8 (EA H5N8) has spread to much of Asia and even made incursions into Europe in late 2014. EA H5N8 reached North America sometime in late 2014 and was detected in the United States in December 2014. Presumably, EA H5N8 arrived in wild migratory waterfowl from Asia during the migration season in 2014 via the Pacific flyway along the Aleutian chain. Shortly after EA H5N8's arrival, it reassorted with an endemic LPAI N2 virus to create Eurasian/North American HPAI H5N2 (EA/NA H5N2). Both EA H5N8 and EA/NA H5N2 have been detected in wild birds and domestic poultry in the Pacific, Central, and Mississippi flyways of the United States. Only the Atlantic or Eastern flyway has no reported detections to date (May 2015). EA H5N8 has also generated another reassortant, EA/NA HPAI H5N1, but this virus has only been found in a single wild bird and has not been detected since. These viruses appear to be moving in subclinically infected dabbling duck (mallards and their relatives) species and precipitating outbreaks in both commercial and backyard poultry, which intensified during the spring migration period of 2015. Disease in the wild bird population is rare (the viruses have only been detected in a few species other than dabbling ducks) but the dynamics of these viruses are still being studied in the many different avian species that are being exposed. However, both captive and wild raptors feeding on infected waterfowl have died from the disease in the United States, thus generating extreme concern within the falconry community. These Eurasian H5 viruses will likely persist in the wild migratory waterfowl compartment and may spread throughout all U.S. flyways at an increased prevalence in the fall of 2015, exposing both domestic poultry and other avian species to infection. What the ultimate fate of these Eurasian H5 viruses will be in North America is uncertain. They could potentially attenuate over time or be subsumed into the larger endemic LPAI community. Nevertheless, all holders of birds should be implementing good biosecurity practices and should notify their state animal health officials if diseased or dead birds are observed.

VACCINATION

Vaccination for avian influenza is strictly controlled in the United States by state and federal authorities because of the impact that seropositive birds may have on interstate and international commerce. Vaccination for LPAI (e.g., H1, H3) does occur in the United States for some species such as turkeys. However, vaccination for any H5 or H7 subtype is highly restricted to a case-by-case basis.

Nondomestic birds, including parrots, were vaccinated in zoos in Europe during the HPAI H5N1 crisis in the mid-2000s.[29] However, the likelihood of any nondomestic pet bird housed outdoors in a secure enclosure (excluding waterfowl and galliforms) being infected by a wild migratory waterfowl is low. Therefore, vaccination may provide no more additional protection of outdoor caged birds than does adequate biosecurity. Moreover, the performance of many vaccines and the vaccination schedule in nonpoultry species is not well defined. Therefore, vaccination of nondomestic avian species is done only under extreme situations.

There are many platforms of avian influenza vaccine available, ranging from inactivated (killed) to modified-live vectored vaccines. However, only inactivated or nonreplicating vectored vaccines would likely be recommended for nondomestic species should they become eligible for vaccination.

SUMMARY

Avian influenza is a complex virus that will continue to be a challenge to poultry production and other avicultural operations for the foreseeable future. The high mutability of the virus complicates vaccination efforts, but biosecurity practices coupled with surveillance and depopulation of infected birds helps control any outbreaks. While avian influenza has been detected in pet bird species, it is usually the result from spillover from the infected waterfowl or poultry compartments and does not naturally circulate in pet bird species in the wild. The risk that avian influenza poses to pet birds is largely a function of the potential exposure to infected high-risk species and disruptions in commerce caused by the presence of the disease.

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ASPERGILLOSIS

An Martel

Aspergillosis is one of the most frequently occurring mycotic diseases in birds and is caused by infection by the genus *Aspergillus*. Although other *Aspergillus* species such as *A. flavus*, *A. niger*, *A. glaucus*, *A. nidulans*, *A. terreus*, *A. clavatus*, *A. oryzae*, *A. ustus*, and *A. versicolor* have been isolated from patients with aspergillosis, *A. fumigatus* is the predominant species of this airborne infection.[1] The pathogenesis of this respiratory disease is still poorly understood. Acute or chronic disease can occur, varying in spectrum from local involvement to systemic dissemination. Although epizootics as flock diseases with severe mortality from brooder-borne or litter-sourced infection can occur, in most cases, only an individual is infected.

ETIOLOGY

A. fumigatus is a ubiquitous saprophytic ascomycetous fungus, which is identified on the basis of its macromorphology and micromorphology. The macromorphology comprises the features that can be observed with the naked eye or the stereomicroscope. Colonies are dark blue-green in color (Figure 2-14) and consist of a dense felt of conidiophores, intermingled with aerial hyphae.[2] Hyphae are the main mode of vegetative growth and are collectively called a *mycelium*.[3] To examine the micromorphology, a smear is stained with lactophenol blue or new methylene blue staining (Figure 2-15). The conidial heads (conidiophores or fruiting bodies) are columnar, resembling a "holy water sprinkler."[4] Conidiophores are specialized hyphae with a swollen end, known as *vesicle* (15–30 μm in diameter), from which the green phialides (5 to 9 μm length) directly arise. A chain of green smooth-walled conidia (2 to 3 μm in diameter) emerges from each phialide.[3]

A. fumigatus is a rapidly growing fungus and is thermophilic, with growth occurring at temperatures as high as 55° C, and survival at temperatures up to 70° C.[3,5] It grows rapidly on

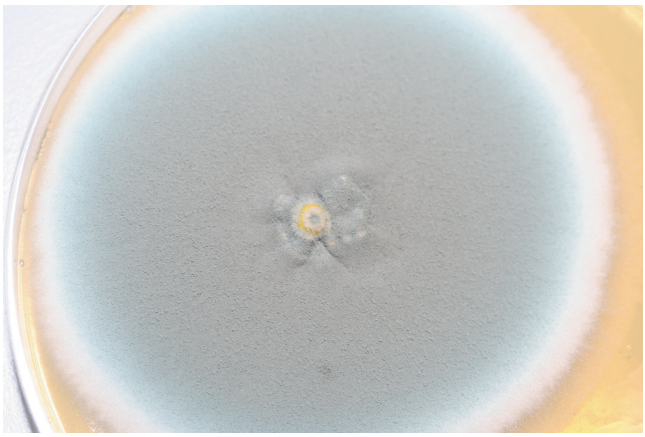

FIGURE 2-14 *Aspergillus fumigatus* culture grown on Sabouraud dextrose agar plate.

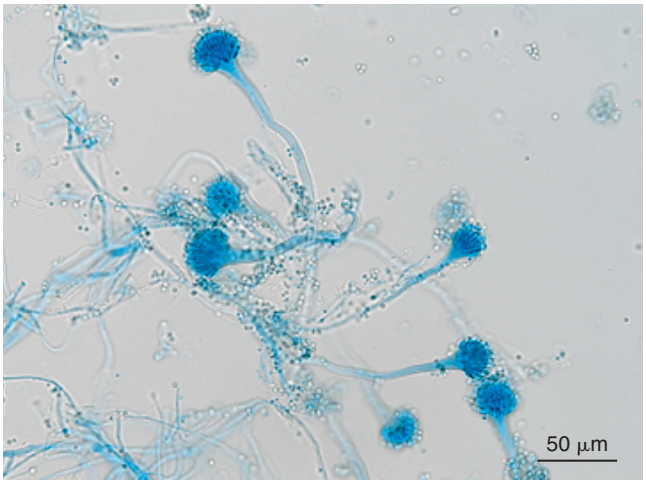

FIGURE 2-15 Lactophenol blue stain of a smear.

Sabouraud dextrose agar (see Figure 2-14), Czapek yeast agar or potato dextrose agar at 25° C to 37° C. Colonies develop a diameter of approximately 3 to 4 cm in 7 days. A young *A. fumigatus* colony is white but turns green to dark blue-green after a few days of growth due to sporulation. As the colony matures, conidial masses become gray-green, but the colony edge remains white (see Figure 2-14).

DISEASE PREDISPOSITION

The major risk factors for an *Aspergillus* infection are exposure to an overwhelming number of conidia and/or immunosuppression of the host. An overwhelming amount of spores can rapidly develop in a warm humid environment with poor ventilation and poor sanitation.[4,6,7] Besides, improperly stored feeds can be a source of fungal pathogens *(A. fumigatus*, *A. flavus*, *A. glaucus*, and *A. niger)*.[8,9] In those feeds, not only the fungi but also immunosuppressive mycotoxins such as zearalenone, trichothecenes, aflatoxins, and/or fumonisins can be present.[10,11] Intensive production strategies, severe genetic manipulation, and inadequate management and husbandry practices of domestic birds may also weaken the immunologic defense.[12,13] Other immunosuppressive factors that can predispose birds to aspergillosis include administration of tetracyclines, vaccination (e.g., against infectious bursal disease, infectious bronchitis, or Newcastle disease), overcrowding, shipping, quarantine or capture of wild birds, starvation, thermal discomfort, migration, inbreeding, *Psittacine circovirus* infection, lymphoproliferative disorders, toxicosis (e.g., heavy metals, being oil soaked), traumatic injuries, and reproductive activity.[1]

All bird species are considered particularly susceptible to aspergillosis, probably because of the anatomic and physiologic characteristics of the avian respiratory system compared with those of mammals and humans. These characteristics include the high average body temperature (38° to 45° C), which is favorable for the growth of thermophilic fungus; the absence of an epiglottis, which otherwise prevents particles from reaching the lower respiratory tract; the lack of a diaphragm, which disables a strong cough reflex; the limited distribution of ciliated epithelium through the respiratory tract; a greater respiratory surface area and a thinner air-blood capillary barrier; and the presence of an air sac system, which widely extends throughout most of the body.[7,12] The warm and oxygenated air sacs provide a favorable condition for the vegetative growth and even sporulation of *Aspergillus*.[7] In addition, the unidirectional air flow in the lungs and the bidirectional air flow in the air sacs hinder the elimination of inhaled particles.[14] The paucity of free respiratory macrophages in the avian respiratory system is also assumed to obstruct the respiratory immunity against respiratory pathogens, but this might be compensated by the phagocytic epithelial cells in the atria and infundibula, the pulmonary intravascular macrophages, and subepithelial macrophages, which can be efficiently translocated to the epithelial surface.[12,13,15-19]

A. fumigatus infections have been observed in a wide number of taxonomic orders, including Accipitriformes, Anseriformes, Charadriiformes, Ciconiiformes, Columbiformes, Falconiformes, Galliformes, Gruiformes, Gaviiformes, Passeriformes, Psittaciformes, Rheiformes, Sphenisciformes, Strigiformes, Struthioniformes, and Tinamiformes.[1] Although every bird species is intrinsically susceptible to the disease, some authors report some species to be more susceptible than others. Based on empirical data, several authors claim that birds of prey, especially gyrfalcon *(Falco rusticollis)* and hybrids, rough-legged hawk *(Buteo lagopus)*, and red-tailed hawk *(Buteo jamaicensis)* are highly susceptible to aspergillosis.[7,21,22] In psittacines, the Grey parrot *(Psittacus erithacus)*, the blue-fronted amazon *(Amazona aestiva)*, and pionus parrots (*Pionus* spp.) seem highly susceptible.[4] Another group of birds considered extremely susceptible to infection are seabirds. Although there are a few sporadic cases reported for these birds in the wild, the incidence increases substantially in birds coming into captivity to be rehabilitated.[23,24] There have been a number of documented cases of aspergillosis in penguins, either during rehabilitation or in zoologic settings.[25–27] Most of these data are coming from observations of the prevalence of aspergillosis in avian clinics and zoos. Limited experimental studies concerning the true susceptibility of these presumptively highly susceptible species have been conducted. Because of the lack of scientific research in this area, it is not clear if these highly susceptible birds are

intrinsically more susceptible to aspergillosis or if other factors such as stress renders them more vulnerable to develop the disease. Van Waeyenberghe[28] demonstrated that there was no difference in species susceptibility between 8-month-old Gyr-Saker hybrid falcons and pigeons to a single-dose exposure of 10^7 *A. fumigatus* conidia, supporting the latter hypothesis.

PATHOGENESIS OF ASPERGILLOSIS

Because of continuous inhalation of the ubiquitously present and small-sized *A. fumigatus* conidia, the respiratory system is primarily affected, although other body sites such as the eye or skin can be infected as well.[4,7,29-33] Some inhaled *A. fumigatus* conidia are not trapped in the nasal cavity and trachea and are therefore able to colonize the lungs and air sacs.[34] The tracheal bifurcation can also be infected due to conidia deposition in the narrow lumen. The consequences of colonization of *A. fumigatus* conidia ultimately depend on the interaction between the host immune system and the fungus.[35]

The *A. fumigatus* conidia that colonize the lung get embedded in the atria and in parts of the infundibula in the parabronchus and are first attacked by the phagocytic epithelial cells, subepithelial macrophages, and intravascular macrophages.[12,13,18,34] If the conidia overwhelm the immune defense, they break dormancy and start germinating by mitotic divisions.[4,36] Germination switches the fungal morphotype from unicellular conidia to multicellular hyphae, which extend and enable tissue invasion.[35] As the hyphae invade, tissues necrotize, and plaques are formed in the lung and respiratory tract and obstruct the trachea or bronchi or fill an air sac.[4] Occasionally, sporulation occurs in the aerated spaces of lungs and air sacs (Figure 2-16).[37,38]

Because hyphae are tissue invasive, extension of the infection can occur through the air sac wall to adjacent tissues and organ systems. In addition, hematogenous spread can occur.[39] In this circumstance, hyphae as well as host cells play a role in the hematogenous spread of infection. Conidia can become attached to erythrocytes or be ingested by respiratory macrophages and then carried by the bloodstream and the lymph stream to other organs.[40,41]

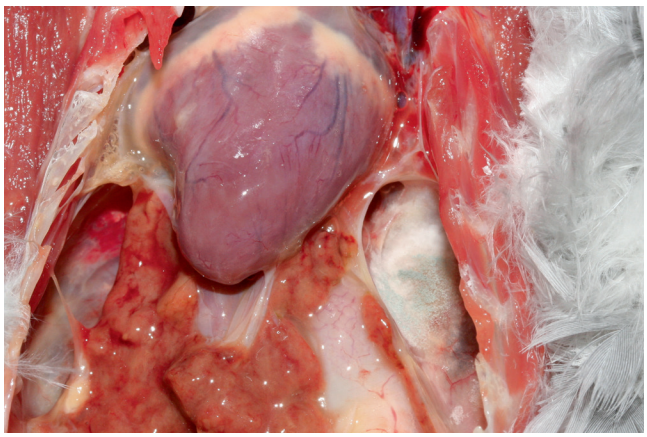

FIGURE 2-16 *Aspergillus fumigatus* granuloma in the air sacs of a Grey parrot.

Tissue reactions to *A. fumigatus* infections in birds can be granulomatous and/or infiltrative, depending on the immune status of the bird. The granulomatous form is characterized by a necrotic center containing hyphae and/or heterophils surrounded by abundant inflammatory cells, including giant cells, macrophages, and lymphocytes, and encapsulated by an outer layer of fibrous connective tissue. Neither exudative inflammation nor vascular lesions are seen in the neighboring tissues.[1] The infiltrative types of tissue reaction include exudative cellular inflammation with giant cells, macrophages, heterophils, and lymphocytes. In this type, the fungus frequently invades blood vessels and forms aggregates of radiating hyphae containing a large number of conidiophores and conidia without forming structured granuloma due to T-cell suppression.[1]

Host Immune Response to Aspergillosis

Both cellular and humoral immunity are involved in the bird's immune response to infection. Macrophages and heterophils play the primary role in phagocytizing the invading *A. fumigatus* conidia and hyphae, followed by antibody reactions for adaptive immunity.[42,43]

The respiratory macrophages form the early immune defense against *A. fumigatus* infection in birds.[42] Birds lack free respiratory macrophages in the respiratory system.[13,15,16] Instead, respiratory macrophages are present in the epithelia and the subepithelial interatrial septa of the atria and infundibula and can be reinforced by the pulmonary intravascular macrophages.[12,15,18,44] These macrophages can transmigrate from the epithelia and the interatrial areas or the vascular system into the air surface and play an important role in the removal of particles or pathogens from the air.[12,13,18-20] In vitro studies with *Aspergillus* conidia and avian macrophages demonstrate that they may prevent early establishment of infection unless the number of *A. fumigatus* conidia exceeds the macrophage killing capacity, leading to intracellular germination and lysis of the phagocytic cells, which may contribute to colonization of the respiratory tract.[45]

Immunosuppressive agents such as mycotoxins, frequently present in parrot feeds,[11] can alter the macrophage functions. It has been demonstrated that the mycotoxin T-2, on the one hand, impairs the antifungal activities of chicken macrophages against *A. fumigatus* conidial infection; on the other hand, it stimulates a proinflammatory response in infected macrophages, which might compensate for the observed macrophage functional impairment.[46] However, fungal growth in the presence of T-2 induces a stress response in *A. fumigatus*. The net outcome of decreased macrophage defense, increased proinflammatory response, and induction of fungal stress in birds exposed to T-2 is an overall exacerbation of aspergillosis.[47]

The avian immune response is regulated by cytokines, which can be produced by virtually every cell type, and chemokines are a group of cytokines that regulate leukocyte traffic. Recruitment of leukocytes (e.g., macrophages, heterophils, and dendritic cells) to the infection cite is primarily mediated by the interaction between the circulating leukocytes and the chemokines released from the infection cite.[48] Instead of the oxidative mechanisms used in neutrophils, heterophils use cationic proteins, hydrolases, and lysozymes to kill fungal hyphae, but more research is needed to elucidate the fungal killing mechanisms in avian heterophils.[7]

Also, the avian adaptive immune response against aspergillosis is poorly known.[49] A study of the humoral response of pigeons to *A. fumigatus* antigens showed an early rise of immunoglobulin M (IgM) and a later rise of IgG following injection of *A. fumigatus* culture filtrate.[21,50-53]

Aspergillus fumigatus Virulence Factors

Most airborne fungi rarely cause disease. This suggests that *A. fumigatus* produces specific virulence factors that are important for the fungus to colonize avian tissues. In humans, several factors, including phospholipase, protease, elastase, and gliotoxin, play a role in the pathogenesis of aspergillosis.[54–56] The relevance of these factors in avian aspergillosis is not well known because research concerning this subject in birds is minimal. One study conducted in turkeys revealed marked variability in pathogenicity between several *A. fumigatus* isolates.[57] Other studies in turkeys have considered that gliotoxin may be involved in the pathogenesis of aspergillosis.[58,59]

CLINICAL SIGNS AND LESIONS

Clinical manifestations of aspergillosis depend on the infection dose, the pathogen distribution, pre-existing disease, and the immune response of the bird. The disease may be either localized or diffuse but often causes a progressive illness leading to mortality if untreated. Although aspergillosis is predominantly a disease of the respiratory tract, any organ can be infected.[60] Avian aspergillosis is distinguished into two forms: acute and chronic.[1]

The *acute form* is thought to be caused by exposure to an overwhelming number of *Aspergillus* conidia.[61] Onset of clinical disease is rapid. The acute signs include dyspnea, anorexia, tail bobbing, open mouth breathing, and gasping. Potential general signs are acute depression, inappetence, vomiting, crop stasis, ascites, polydipsia, polyuria, and cyanosis. Death usually occurs within 7 days.[4,61,62] At necropsy, a white mucoid exudate and marked congestion of the lungs and air sacs can be noted. Although multiple foci of pneumonic nodules may be present, because of the rapid progress of the disease, large pulmonary granulomas are frequently absent. The *chronic form* is generally associated with immune suppression as a localized or disseminated disease.[62] The chronic signs include decreased appetite, lethargy, weight loss, change or loss of voice, cough, open beak breathing, cyanosis, polyuria, depression, and vomiting.[61,62] With the exception of *mycotic tracheitis*, little, if any, respiratory sign is seen at the beginning of the disease.[61,63] In case of tracheitis, a milky white tracheal discharge, loss of voice, and the occasional cough can be observed.[61,63] *Airsacculitis* with extension to the lungs, is the most frequently encountered form of the disease.[6] Aspergillomas may be found throughout the entire respiratory tract (see Figure 2-16; Figure 2-17).

Localized aspergillosis involving the upper respiratory tract often presents as chronic rhinitis and sinusitis (Figure 2-18), possibly accompanied by malformation of the nostrils, beak, and cere and a purulent nasal discharge.[33] Wheezing respiratory sounds may be caused by the formation of rhinoliths or oronasal granulomas obstructing the upper airways.

Mycotic keratitis can cause blepharospasm, photophobia, periorbital swelling, turbid discharge, swollen and adhered eyelids, cloudy cornea, and cheesy yellow exudates within the conjunctival sac.[30,31] Fungal infections of the eye are rare in birds, and most reported cases result from the extension of pre-existing upper respiratory infections, although ocular trauma and corticosteroid therapy are other predisposing factors.[30,31,64]

Encephalitic and meningoencephalitic lesions may occur with *disseminated aspergillosis*. Depression, unilateral wing drooping, paralysis, ataxia, weakness or general disinclination to move, unsteady gait, falling on the side or back, torticollis, and tremors are potential neurologic signs caused by *Aspergillus* infection.[65–67]

Epidermal cysts associated with *A. fumigatus* have been described in the comb of a silky bantam chicken.[32] Although necrotic granulomatous dermatitis from which *A. fumigatus*

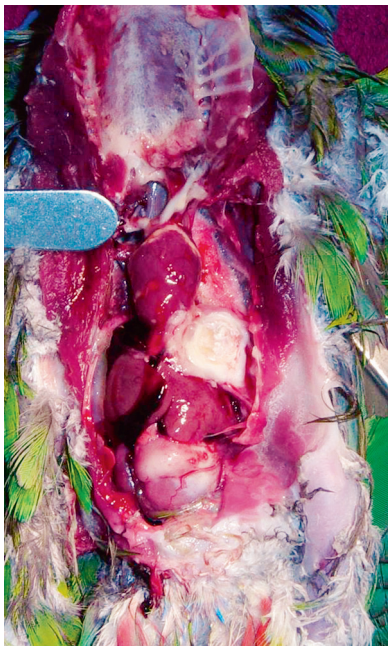

FIGURE 2-17 Air sac aspergilloma. (Courtesy Dr. Brian Speer.)

FIGURE 2-18 Chronic sinusitis in a citron crested cockatoo. (Courtesy Dr. Scott Ford.)

was isolated has been described in chickens, *cutaneous lesions* caused by *Aspergillus* occur rarely in avian species.[68]

DIAGNOSIS

Because the clinical signs of aspergillosis are nonspecific, the diagnosis of the disease is difficult.[39] Moreover, there is no single test that provides certainty. Most of the time, the diagnosis relies on an accumulation of evidence from history, clinical presentation, hematology and biochemistry, serologic tests, radiographic changes, endoscopy, and culture of the fungus.[53] Anamnesis can reveal a stressful event , some underlying environmental factors, and/or an immunosuppressive condition or treatment.[63] It may also reveal chronic debilitation, weight loss, voice change, and exercise intolerance.[4] Since clinical signs are nonspecific and depend on which aspergillosis form a bird develops and which organs are involved, aspergillosis should be included in the differential diagnosis of most respiratory tract diseases as well as systemic diseases.[39,53]

Unfortunately, aspergillosis is frequently diagnosed at postmortem examination, often based on identifying characteristic caseous nodules in the lungs or plaques in the air sacs, followed by cytologic and histologic examinations of the lesions and culturing of the fungus.

Hematology and Serum Chemistry

Hematology and serum chemistry in birds is considered rather indicative than diagnostic of any particular disease.[53] Leukocytosis of 20,000 to more than 100,000 white blood cells per microliter, heterophilia with a left shift (degenerative shift), monocytosis and lymphopenia, nonregenerative anemia, and increased serum total proteins are described in birds with aspergillosis.[b] An increase in β-globulins and an increase of β-globulins and/or γ-globulins can be noticed in acute and chronic infections, respectively. Multiple studies in birds stated a decreased concentration of albumin and a decreased A/G ratio as the most marked electrophoretic changes with aspergillosis.[70–72] In falcons with aspergillosis, lower prealbumin values were noted compared with healthy falcons.[73,74] However, immune-suppressed birds may have hypoproteinemia, and white cells may be in the normal range.[71,72,75]

Serum biochemical changes in specific organ parameters will vary, depending on the organ system affected, and are not specific to aspergillosis.

Antibody and Antigen Detection

Although humoral immunity is generally considered to have less importance than cellular immunity in fungal infections, it is possible to use the antibody response as a diagnostic aid.[57] However, in the acute stage, the antibody production trails behind antigen exposure by 10 to 14 days, and in case the bird is immunosuppressed, the low antibody production results in false-negative results.[21,51] In these cases, detection of circulating *Aspergillus* antigen in serum may be more helpful.[71] Also, high antibody titers against *Aspergillus* antigen have been demonstrated in healthy as well as in *A. fumigatus*-infected raptors.[74,76] Overall, negative serologic test results do not rule out aspergillosis, and positive test results are only considered diagnostic by accumulation of evidence from other diagnostic aids.

Counter-immunoelectrophoresis is a technique that detects precipitating antibodies against *Aspergillus* spp. with the use of metabolic or somatic *A. fumigatus* antigens. The number and intensity of the precipitation vary in function of the precipitant antibody concentration.[77] Precipitating antibodies against *Aspergillus* spp. can also be measured by agar gel immunodiffusion.[57] Both tests, however, result in a poor sensitivity, possibly because of the requirement of a higher antibody concentration than commonly found in patients.[76]

An indirect enzyme-linked immunosorbent assay (ELISA) has been developed to detect antibodies against *Aspergillus* spp. (The Raptor Center at the University of Minnesota, Minneapolis, MN). Although false-negative results can occur, this assay appears to be a useful clinical tool, especially in the detection of subclinical cases of aspergillosis.[69,78] In a report of 23 falconiform birds with confirmed aspergillosis, 43% of the birds had moderate to marked antibody titers, whereas 22% had negative titers. In contrast, in the same study, the owls with confirmed aspergillosis had negative antibody titers.[78] In a study of captive penguins with confirmed aspergillosis, many birds had increased titers, and only 20% had negative antibody titers.[69] The indirect ELISA is limited by the inability of the conjugated antibodies to cross-react with all avian orders.[21,51]

A commercial direct ELISA Platelia *Aspergillus* kit (Bio-Rad, France), which was developed for humans, detects the fungal antigen galactomannan, a major cell wall constituent of *Aspergillus* species, in serum using rat monoclonal antibodies. Galactomannan levels have been 2.6-fold elevated in psittacines with aspergillosis.[79] False-positive results do occur[77,80] and may be explained by the cross-reaction with antigens of other organisms, feeding soybeans, and the use of beta-lactam antibiotics.[81–83] In addition, the sensitivity of this serologic test appears to be low to moderate.[71,77,84] Possible reasons for false-negative results are not well known. Cray[71] hypothesized that necrotic areas that are not nutrient or oxygen rich may decrease the amount of released galactomannan. Also, since galactomannan antigens are large, a degree of angioinvasion may be necessary for the antigen to reach the circulation. Finally, the report suggested that antibodies may bind to galactomannan reducing the test sensitivity.

Concentrations of (1→3)-ß-D-glucan in plasma samples have been shown to be significantly higher in aspergillosis-positive birds than in aspergillosis-negative birds, with the highest averaged values in infected sea birds, followed by companion birds, and raptors.[85]

In birds with aspergillosis confirmed by necropsy, the *Aspergillus* toxin fumigaclavine A (FuA) has been detected in air sac samples with the use of an enzyme immunoassay (EIA).[86,87] Little is known whether this EIA can be used in serum or plasma samples of birds. One study on experimentally infected falcons was not able to demonstrate FuA in blood samples.[74]

Radiology

Lateral radiography and ventrodorsal radiography are part of the routine clinical examination of a sick bird. Radiographic changes noticed in aspergillosis patients can be bronchopneumonia with a prominent parabronchial pattern; thickening of

[b]References 4,53,61,63,66,69.

the air sac walls, reducing the detail in the coelomic cavity; distinct nodular lesions; and/or air sac hyperinflation as a result of airway obstruction or loss of air sac compliance.[62] Although intraluminal granulomas of the syrinx, trachea, and main stem bronchi are fairly common, they can be seldom visualized radiographically.[6] Organ enlargement can be noticed in systemic disease. A disadvantage is that radiographic features indicating an *Aspergillus* spp. infection are only obvious in the late phase of the disease. In addition, the changes of pneumonia and consolidating airsacculitis are nonspecific, leading to a broad differential diagnosis that includes bacterial pneumonia, hypovitaminosis A, pulmonary hemorrhage or infarction, and neoplasia.[62]

Other imaging techniques such as computed tomography (CT) or magnetic resonance imaging (MRI) avoid the superposition of overlying structures and can be useful for demonstrating small lesions that are not visible on radiographs. However, the definitive diagnosis of aspergillosis still requires identification by biopsy, histopathology and/or cytology, or culture.[6]

Polymerase Chain Reaction

Polymerase chain reaction (PCR) assays have been developed for the diagnosis of human aspergillosis. Few reports of different PCR assays (including real-time PCR) tested on heparinized whole blood, tracheal washings, air sac fluids, respiratory tract granulomas, and (biopsy) tissue samples from birds support the value of this assay for the diagnosis of avian aspergillosis.[39,71]

Endoscopy

By using endoscopy, the respiratory tract, including choanal opening, glottis, trachea, syrinx, lung, and air sacs, and the coelomic cavity, can be evaluated. With this invasive technique, the lesions can be visualized and the extent as well as the progress of infection during treatment can be followed up (Figure 2-19).[21,53] Tracheal endoscopy is useful to visualize a lesion (e.g., a plaque or white discharge) occluding the trachea or syrinx (Figures 2-20 and 2-21). The use of bronchoscopy is limited by the size of the bird. Samples for culture, cytology, or histology should be taken directly with biopsy forceps or via air sac lavage.[4]

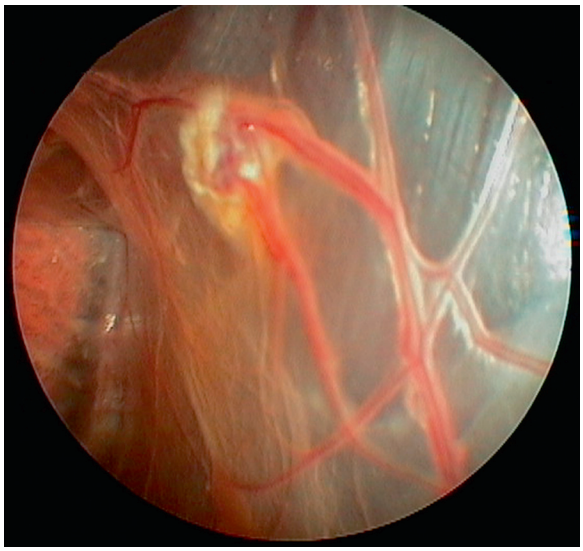

FIGURE 2-19 Endoscopic view of an aspergilloma in the cranial thoracic air sac of a hybrid falcon *(Falco rusticolis x F. cherrug)*.

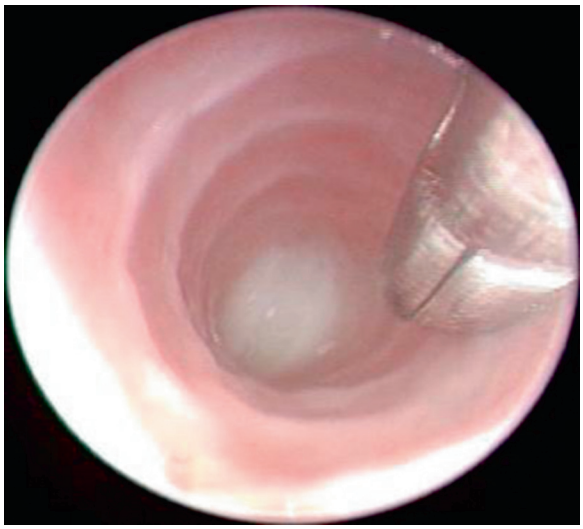

FIGURE 2-20 White discharge occluding the trachea as visualized by tracheal endoscopy. (Courtesy Dr. Brian Speer.)

FIGURE 2-21 Aspergilloma of the syrinx in a mallard duck *(Anas platyrhynchos)*.

Cytology

Cytologic evaluation of clinical samples can aid the diagnosis of aspergillosis. Squash preparations are prepared and stained with lactophenol cotton blue of methylene blue stain. Conidiophores and hyphae can be identified.

Histopathology

Histologic characteristics can be indicative for aspergillosis (Figure 2-22), but because in-vivo hyphae of hyaline filamentous fungi are very similar and their in situ manifestations are not pathognomonic, this technique does not allow fungal species identification.[71,88] To identify *Aspergillus* spp. PCR or immunohistochemistry could be used using monoclonal or polyclonal antibodies.[67,88-91]

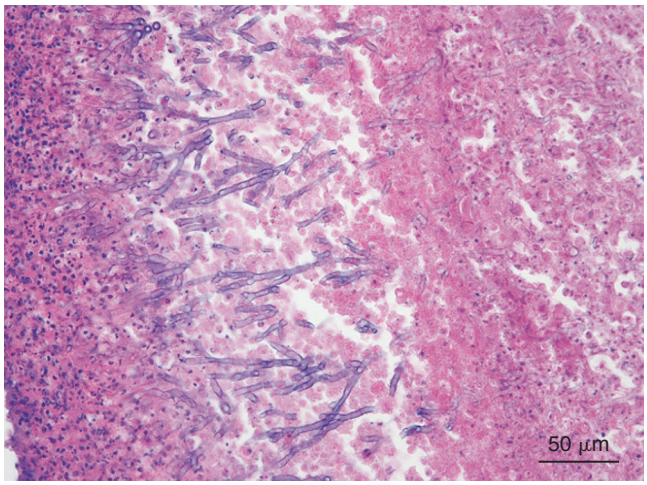

FIGURE 2-22 Hematoxylin and eosin staining from aspergilloma in a peacock.

Confirming the Diagnosis: Fungal Culture

Isolation of the fungus is considered the gold standard of an etiologic diagnosis of aspergillosis. However, it is important to mention that isolating the fungus alone is not confirming the infection because *Aspergillus* fungi are ubiquitous and can be a contaminant. An abundant culture from any organ is considered diagnostic. However, depending on the sample place (e.g., trachea swab), a negative culture does not rule out aspergillosis.[21]

TREATMENT

Treating avian aspergillosis is a challenge because of the limited knowledge regarding the pharmacokinetics of antifungal agents in different bird species; the presence of granulomatous inflammation, which makes it difficult for the drug to reach the fungus; the presence of concurrent disease and/or immunosuppression; and the late stage at which birds mostly are presented.[1] Prolonged antifungal therapy for periods up to 4 to 6 months or even greater is often necessary for treatment success.

Topical therapy after debulking the granulomatous lesions, in combination with an early, systemic antifungal therapy is recommended when the lesions can be easily removed. In most patients, however, granulomatous lesions are difficult to remove because of their location and/or extent. In these cases, only systemic antifungal therapy can be applied. Topical therapy can be administered through nebulization, nasal or air sac flushing, and endoscopic or surgical irrigation of abdominal cavities or lesions, while systemic therapy can be administered intravenously or orally.[4,6] A summary of the administration routes and doses of antifungal agents for birds is presented in Table 2-4.

Polyenes

Amphotericin B is a polyene macrolide that acts by binding to ergosterol, which is the principal sterol in the cell membrane of the fungus. This binding alters membrane permeability, causing leakage of sodium, potassium, and hydrogen ions and leads eventually to cell death.[92–94] In mammals, amphotericin B is nephrotoxic because of the binding to mammalian sterols (e.g., cholesterol) in the cell membrane. Pharmacokinetic studies with amphotericin B conducted in domestic turkeys and three raptor species reported that the half-life is much shorter than in mammals.[95] This finding may be responsible for the lack of nephrotoxicity in avian species in contrast to mammals.[80] Despite this fact, clinicians are still advised to monitor the renal function of their avian patients. Amphotericin B is fungicidal to a variety of organisms, including *Aspergillus* spp.[94] Native reduced susceptibility of *A. terreus* and *A. flavus* and acquired resistance in *A. fumigatus* to amphotericin B is documented.[96,97]

TABLE 2-4

Summary of the Administration Routes and Doses of Antifungal Agents Used for Treatment of Avian Aspergillosis

Antifungal Agent	Administration Route	Dose
Amphotericin B	Intravenous	1.5 mg/kg q8h 3–7 days (most species)[93,99]
	Intratracheal/nasal flush	1 mg/kg q8-12h, dilute to 1 mL with sterile water (psittacines, raptors)[114,115]
	Nebulized	7 mg/mL 15 min q12h (most species)[116]
Clotrimazole	Nasal flush	1% solution[93]
	Nebulized	1% solution, 30–60 min[116]
Enilconazole	Nebulized	0.1 mL/kg in 5 mL sterile water, 30 minutes q24h 5 days on/2 days off (raptors)[117]
Itraconazole	Oral	5–10 mg/kg q12-24h (toxicity is reported in Grey parrots: recommended dose for this species 2.5–5 mg/kg PO 24h)[114,118]
Ketoconazole	Oral	10–30 mg/kg q12h 21 days[99]
Terbinafine	Oral	15–30 mg/kg q12h[105]
	Nebulized	1 mg/mL solution (can be combined with itraconazole)[119]
Voriconazole	Oral	10 mg/kg q12h (chickens, pigeons, Grey parrots)[100,107,108] 12–18 mg/kg q12h (Grey parrots)[120] 12.5 mg/kg q12h (falcons)[121]

mg/kg, Milligrams per kilogram; mg/mL, milligrams per milliliter; min, minutes; q8h/q12h/q24h, every 8/12/24 hours; PO, orally; q8-12h, every 8 to 12 hours.

Amphotericin B has been used to treat both systemic and topical fungal infections in birds. It can be administered intratracheally, intravenously, in sinus flushes, and through nebulization. Topically, the drug can be very irritating to tissue, and to reduce the risk of iatrogenic sinusitis or tracheitis, the drug must be diluted in water (saline inactivates amphotericin B).

Azoles

Azoles inhibit the enzyme cytochrome P450-dependent 14-α-sterol demethylase, required for the conversion of lanosterol to ergosterol.[75,98] Exposed fungi become depleted of ergosterol and accumulate 14-α-methylated sterols. This causes disruption of membrane structure and function, thereby inhibiting fungal growth.[98] Vertebrates have slightly different cytochrome P450 enzymes compared with fungi. The relative toxicity of the drug depends on the specificity for binding the fungal enzyme instead of the vertebrate one.[93] The side effects of the azole family of drugs in general are anorexia, vomiting, and liver alterations.[99] With the exception of voriconazole, azoles are known to be fungistatic at the doses used in birds and need several days to reach steady-state concentrations.[61,80,93,100] Hence, months of therapy are often required to cure patients. Itraconazole (first-generation triazole) and voriconazole (second-generation triazole) are the azoles most thoroughly studied in birds. Pharmacokinetic studies with itraconazole were conducted in pigeons, amazon parrots, red-tailed hawks, and ducks.[7,94,101-104] These studies documented species-dependent variability, suggesting that different dosage regimens of itraconazole may be required for various species of birds. Grey parrots are reportedly more sensitive to itraconazole and may exhibit adverse drug effects (anorexia, depression, and death) at normal dosage levels. For voriconazole, a high interindividual variability, dose-dependent pharmacokinetics, and a possible induction of liver enzymes were found in raptors and Grey parrots.[100,105,106] In chickens, the bioavailability of orally administered voriconazole was found to be poor.[107] Compared with itraconazole, voriconazole shows good distribution to the tissues in which *A. fumigatus* is mostly located: the respiratory tract and the brain. Moreover, voriconazole may be a valuable alternative in Grey parrots that do not tolerate itraconazole.[105,108] Compared with amphotericin B, voriconazole has the advantage that it can be administered orally in addition to intravenously, which makes this drug suitable for long-term use in birds. However, since liver toxicity was observed in studies in racing pigeons, blood biochemistry should be closely monitored for potential side effects.[100] To maximize drug concentrations in the lungs, the upper respiratory system (nose and sinuses), and skin (in case of dermal aspergillosis) and to minimize adverse effects, topical treatment for localized aspergillosis is preferable to oral or intravenous treatment. However, it has been shown that nebulizing the intravenous formulation of voriconazole does not provide good plasma or lung concentrations in racing pigeons.[100] Nebulization of a nanosuspension of itraconazole is well tolerated by pigeons.[109] In poultry, enilconazole fumigation is frequently used to treat infected chicks and the litter. Acquired resistance of *A. fumigatus* strains to azoles is increasingly reported.[97,110]

Allylamines

Allylamines (terbinafine) act by inhibiting the ergosterol synthesis by interfering with squalene epioxidase, another key enzyme in the biosynthesis of ergosterol. In common with other antifungal agents that interfere with the biosynthesis of ergosterol, allylamines result in ergosterol depletion and accumulation of toxic sterols.[105] In pharmacokinetic studies in Grey parrots (15 and 30 milligrams per kilogram [mg/kg]) and raptors, no therapeutic concentrations were achieved in the plasma.[105] Despite these findings, some reports document successful treatment in birds using terbinafine or terbinafine combined with itraconazole, at a dosage of 15 milligrams per kilogram (mg/kg) orally every 12 hours.[105]

PREVENTION

In order to prevent aspergillosis, it is important to minimize the risk factors, which are an overload of spores and immunosuppression. Birds at high risk for aspergillosis can be treated prophylactically with antifungals during the risk period. In the presence of live animals, nebulization of facilities with commercial disinfectants is frequently used to lower the environmental load of *Aspergillus* spp. and the risk of infection.

A number of vaccination strategies have been attempted in birds, with the use of different preparations of vaccines, but the results have been inconsistent.[99,111,112] The use of the immunostimulant levamisole did not decrease aspergillosis associated lesions in turkeys.[102] In humans, clinical improvement of aspergillosis is documented after adding interferon-gamma and granulocyte-macrophage colony stimulating factor to the antifungal treatment.[113] Whether the favorable effect of these products could have value in future treatment protocols for avian aspergillosis is not known.

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COCCIDIAL DISEASES OF BIRDS

James F. X. Wellehan Jr.

BIOLOGY OF COCCIDIA

Coccidia are one of the groups of single-celled parasitic eukaryotes in the phylum Apicomplexa. The Apicomplexa may be distinguished by the presence of an apicoplast, an organelle derived from an endosymbiont much like a mitochondrion or chloroplast. This organelle is essential for the organism's survival and serves as a useful drug target. Related noncoccidial Apicomplexa include the hemosporidians (e.g., *Plasmodium*, *Leucocytozoon*) and the piroplasms (e.g., *Babesia*, *Theileria*). Within the Apicomplexa, the taxa known as coccidia include the families Cryptosporidiidae, Eimeriidae, and Sarcocystidae. Initial morphologic identification has resulted in a number of errors in coccidian taxonomy that have been revealed with deoxyribonucleic acid (DNA) sequence-based phylogeny. Evidence has emerged that the Cryptosporidiidae are more closely related to other apicomplexan taxa than to the Eimeriidae and Sarcocystidae,[1] and this is reflected in their biology and medical treatment. However, for the purposes of this discussion, these three families and their members affecting the Dinosauria will be discussed.

An important consideration when dealing with coccidia is the life cycle of the organism. Some taxa have direct life cycles, involving only one definitive host, whereas others have indirect life cycles, utilizing an intermediate host for asexual reproduction and a definitive host for sexual reproduction. Although some pathology such as causing diarrhea may help with transmission, killing the definitive host is typically disadvantageous for a parasite; it loses its breeding habitat. Coccidian evolution therefore involves significant selective pressure against killing definitive hosts. However, when the parasite is in the intermediate host, the life cycle is most typically completed by the definitive host eating the intermediate host. A bird with encephalitis caused by *Toxoplasma gondii* is more likely to be eaten by a cat. Causing significant disease in the intermediate host may therefore be advantageous, and the most significant pathology is seen in intermediate hosts. Management of coccidial disease in an avian collection requires knowledge of the life cycle; management of coccidia with indirect life cycles is centered on separating intermediate and definitive hosts, whereas management of coccidia with direct life cycles centers on hygiene. It is therefore crucial to properly identify coccidian species for effective management.

CRYPTOSPORIDIIDAE OF BIRDS

The family Cryptosporidiidae contains one genus, *Cryptosporidium*. Cryptosporidiidae are more closely related to other apicomplexan taxa than to the Eimeriidae and Sarcocystidae,[1] and this is reflected in their biology and response to pharmacologic therapy. Cryptosporidia develop on the apical surface of the epithelium in the gastrointestinal, respiratory, and urinary tracts. All cryptosporidia have direct life cycles. Management centers on exclusion of *Cryptosporidium* spp. from collections through quarantine surveillance and by disinfection of contaminated areas. *Cryptosporidium* spores are exceptionally stable in the environment; they are not very susceptible to

ultraviolet disinfection, and even 6% bleach with a 2-hour contact time was shown to only result in 92.7% reduction. Chloro-m-cresol was more effective.[2] Cleaning should involve as much mechanical removal of feces as possible.

There are many species of *Cryptosporidium* with significantly different clinical implications. The vast majority of *Cryptosporidium* spp. seen in birds are still unnamed. Morphologic species identification is not reliable, and sequence-based techniques are needed to differentiate species.[3] The known species of *Cryptosporidium* form two clades, one with gastric tropism and one with primarily intestinal tropism (and sometimes respiratory or urinary tropism), indicating that the site of infection has had greater long-term fidelity than host species.[4] This is useful for the prediction of the site of infection of unknown species lacking histologic data; *Cryptosporidium* avian "genotype IV" has only been identified through fecal surveillance but would be expected to be gastrotropic.[5] Knowledge of the site of infection of a given *Cryptosporidium* spp. is also important for diagnostic sampling. Gastric lavage was found to be a better sample for detection of *C. serpentis*, a gastrotropic species found in snakes compared with cloacal swabs; this would not be expected for a species tropic for the cloaca.[4] Gastrotropic *Cryptosporidium* spp. tend to be associated with decreased appetite, weight loss, and chronic vomiting.[5] Enterotropic *Cryptosporidium* spp. tend to be primarily associated with weight loss and diarrhea. *Cryptosporidium* spp. tropic for the urinary tract have been associated with gout and renal failure.[5] *Cryptosporidium* spp. tropic for the respiratory tract have been associated with respiratory distress, otitis, and ocular disease.[6]

Cryptosporidium spp. vary significantly in their host specificity; some are highly tropic for one host taxon, whereas others have a broad host range. Within species infecting bird hosts, *C. meleagridis*, an enterotropic species first described in turkeys, has the broadest known host range.[5] Initially discovered in turkeys, it affects a wide range of avian species, including parrots, chickens, partridges, and columbiform birds, and has also been reported in dogs, cattle, pigs, rabbits, and rodents, and is zoonotic.[5–8] In companion psittacines, most infections are caused by species that have yet to be named. Specifics of select species may be seen in Table 2-5.

TABLE 2-5

Select Cryptosporidiidae Infecting Birds

Species	Site	Known Affected Species
C. galli	Gastric	Diverse avian species
C. "avian genotype III"	Gastric	Psittacines, passerines, gulls
C. meleagridis	Intestinal	Diverse vertebrates, zoonotic
C. baileyi	Respiratory, ocular	Diverse avian species
C. "avian genotype V"	Urinary tract, cloaca	Psittacines, green iguana

EIMERIIDAE OF BIRDS

The family Eimeriidae contains several different genera, not all of which have proven to be valid when further examined with sequence data. The clinically relevant genera in avian hosts include *Caryospora*, *Eimeria*, and *Isospora*. Eimeriidae typically have direct life cycles but may facultatively have indirect life cycles as well. There are diverse species with significantly different clinical implications. Specifics of select taxa may be seen in Table 2-6.

Caryospora are an Eimeriid genus with both direct and indirect life cycles, containing more than 25 species. Oocysts have a single sporocyst with eight sporozoites. Carnivorous birds and other carnivorous reptiles serve as definitive hosts, in which *Caryospora* spp. replicate in the intestines. Known intermediate hosts are typically prey mammals, in which extraintestinal tissue cysts may be in the skin. However, birds may also be directly infected with oocysts shed by another bird.[9] Clinical signs in carnivorous birds center around enteritis, with weight loss and diarrhea being the most commonly seen signs. Management is focused on the exclusion of *Caryospora* spp. from collections through quarantine surveillance, disinfection of contaminated areas, and breaking the life cycle by obtaining food animals that are free of *Caryospora*. Mammals beyond those normally preyed upon by birds may be intermediate or aberrant hosts; dogs and pigs are susceptible to *Caryospora* dermatitis, and the concern of possible zoonotic infection exists.[10]

Eimeria is a diverse eimeriid genus with direct life cycles, containing more than 200 species infecting birds. Oocysts have four sporocysts, each of which contains two sporozoites. Most species replicate in intestinal epithelium, but some species replicate in renal tubular epithelium, and disseminated visceral infections are a significant problem in cranes. Management focuses on the exclusion of *Eimeria* spp. from collections through quarantine surveillance and disinfection of contaminated areas.

Eimeria spp. tend to be very host specific, and host jumping between distantly related avian taxa is rare. However, there are potential concerns for transmission between more closely related taxa; *E. dunsingi*, first identified in budgerigars with intestinal coccidiosis, has been found to be capable of infecting the enterocytes of musk lorikeets.[5,10] In the case of enterotropic species, disease tends to be most significant in young or otherwise compromised birds; co-infections with agents such as adenoviruses may play significant roles in disease manifestation. Infections in otherwise healthy adult animals are often subclinical. Nephrotropic *Eimeria* infection may result in signs such as weakness, depression, and wasting and has been associated with mortality events, but many infections may be subclinical.[11]

The most significant eimerian pathology is seen with disseminated visceral coccidiosis in cranes, with *E. reichenowi* and *E. gruis* being the most common etiologies. These agents appear to infect all crane species. Cranes may display weakness, lethargy, diarrhea, and oral granulomas. Again, subclinical infections are also common. Necropsy may reveal additional disseminated granulomas, most commonly in the liver. Infection is disseminated via the peripheral blood monocytes and can be seen on a blood smear, bearing significant resemblance to extraintestinal *Isospora* infection in passerines. Oocysts are then produced in both the lungs and the intestine.

Isopora is a diverse eimeriid genus with direct life cycles. Oocysts have two sporocysts, each of which contains four sporozoites. However, molecular sequence data have shown

TABLE 2-6

Select Eimeriidae Infecting Birds

Species	Site in Avian Host	Known Affected Species	Life Cycle	Intermediate Host	Notes
Caryospora sp.	Intestinal	Carnivorous birds	Indirect or Direct	Typically small mammals	Potentially zoonotic
Eimeria dunsingi	Intestinal	Budgerigar, Musk lorikeet, Musschenbroek's lorikeet	Direct	None	No sequence data
E. psittacina	Unknown, likely intestinal	Budgerigar	Direct	None	No sequence data
E. haematodi	Unknown, likely intestinal	Rainbow lorikeet	Direct	None	No sequence data
E. aestivae	Unknown, likely intestinal	Blue-fronted Amazon	Direct	None	No sequence data, morphologically similar to *E. aratinga*
E. amazonae	Unknown, likely intestinal	Yellow-crowned amazon	Direct	None	No sequence data, morphologically similar coccidian seen in blue-fronted Amazon
E. ochrocephalae	Unknown, likely intestinal	Yellow-crowned amazon	Direct	None	No sequence data
E. aratinga	Unknown, likely intestinal	Orange-fronted conure	Direct	None	No sequence data, morphologically similar to *E. aestivae*
E. ararae	Unknown, likely intestinal	Blue-and-gold macaw	Direct	None	No sequence data
E. auritusi	Renal	Double-crested cormorant	Direct	None	
E. truncata	Renal	Canada goose, Lesser snow goose, gray-lag goose	Direct	None	No sequence data
E. reichenowi	Disseminated	Cranes	Direct	None	
E. gruis	Disseminated	Cranes	Direct	None	
Isospora greineri	Disseminated	Superb starlings	Direct	None	
I. superbusi	Disseminated	Superb starlings	Direct	None	

conclusively that what was formerly considered *Isospora*, based on sporulation patterns, consisted of two evolutionarily distinct groups. The presence or absence of Stieda bodies, the use of paratenic hosts, and reptilian or mammalian host specificity are more phylogenetically informative than the number of sporocysts or sporozoites for these genera.[12] All former *Isospora* spp. of mammals are members of the Sarcocystinae, not the Eimeriidae, and were moved into the new genus *Cystoisospora*. All true *Isospora* spp. utilize reptilian hosts, including the Dinosauria (birds). The diversity of *Isospora* has not been well defined; although there are over 140 species in birds referred to in the literature, much of this is based on the likely incorrect assumption of host specificity; experimental data have shown that *Isospora* infection in evening grosbeaks could be transmitted to other passerine species but not to ducks.[13] There are only 10 named true *Isospora* spp. for which sequence data are currently available; the majority of publically available sequence data is not associated with named organisms. Most species replicate in intestinal epithelium, but some species have extraintestinal stages that may be associated with significant pathology. Species with extraintestinal stages are found in passerine birds and were formerly known as *Atoxoplasma* but have now been shown not to be distinct from *Isospora*.[12] Similar to *E. reichenowi* and *E. gruis* in cranes, after initial infection in the small intestine, the peripheral blood mononuclear leukocytes are infected, and the infection is disseminated to other tissues, especially the liver and the lungs. Transmission is primarily by the fecal–oral route, but it has been hypothesized that hematophagous arthropods may also serve as vectors. Systemic isosporosis has been recognized as important in passerines, having caused significant mortality in captive populations of the endangered Bali mynah.

SARCOCYSTIDAE OF BIRDS

The family Sarcocystidae contains several different genera, not all of which have proven to be valid when further examined. The clinically relevant genera in avian hosts include *Sarcocystis*, *Toxoplasma*, and *Neospora*. Sarcocystidae typically have indirect life cycles, although some species may be facultatively direct in their definitive hosts. There are diverse species with significantly different clinical implications. Specifics of select taxa may be seen in Table 2-7.

Sarcocystis are a sarcocystid genus with obligate indirect life cycles, containing more than 120 known species. Some species

TABLE 2-7
Select Sarcocystidae Infecting Birds

Species	Site in Avian Host	Known Affected Avian Species	Definitive Host	Intermediate Host	Notes
Sarcocystis falcatula	Disseminated	Diverse avian hosts	Virginia opossum	Diverse birds	
Sarcocystis calchasi	Intestinal in hawks, disseminated in other species	Pigeons, parrots	Hawks	Diverse birds	Likely further avian hosts susceptible
Toxoplasma gondii	Disseminated	Diverse avian hosts	Cats	Diverse animals	Zoonotic
Neospora caninum	Disseminated	Passerines, parrots, pigeons	Dogs	Diverse animals	Galliform birds not susceptible

utilizing raptors as definitive hosts were formerly known as *Frenkelia* but have now been shown not to be distinct from *Sarcocystis*.[14,15] Oocysts have two sporocysts with four sporozoites, similar to *Isospora* in the Eimeriidae. Carnivorous or omnivorous vertebrates serve as definitive hosts, in which *Sarcocystis* spp. replicate in the intestines. Clinical disease in definitive hosts includes enteritis, with weight loss and diarrhea being the most common signs. Definitive host ranges tend to be more limited than intermediate host ranges. Intermediate hosts are prey animals, in which initial stages replicate in blood vessels, followed by the development of sarcocysts in tissues, commonly muscle. Sarcocysts are often grossly visible and may appear as white streaks in muscle. The most significant disease is seen in intermediate hosts, in which signs may include depression and sudden death. The most clinically significant species of *Sarcocystis* seen in birds are *S. falcatula*, which uses Virginia opossums as definitive hosts, and *S. calchasi*, which uses hawks in the genus *Accipiter* and possibly *Buteo* as definitive hosts.[16] Management focuses on excluding *Sarcocystis* spp. from collections through separation of definitive hosts and their feces from intermediate hosts. It is not uncommon for Virginia opossums or hawks to perch on top of outdoor avian enclosures, and thus their feces fall inside.

Toxoplasma is a sarcocystid genus with a facultatively indirect life cycle, containing one species, *T. gondii*. Cats are the definitive hosts. *T. gondii* has very little specificity for intermediate hosts and is a zoonotic disease. All avian species should be considered susceptible intermediate hosts; those that ingest other intermediate hosts or cat feces are at greater risk, and pigeons and canaries are most likely to present with clinical disease.[16a] Clinically, toxoplasmosis may mimic systemic isosporosis in passerines. Management focuses on excluding *T. gondii* from collections through exclusion of cats and cat feces and, in the case of carnivorous and omnivorous birds, exclusion of potential other intermediate hosts that may be ingested.

Neospora is a sarcocystid genus with a facultatively indirect life cycle, containing two species, *N. caninum* and *N. hughesii*. Dogs are the definitive hosts. Although ruminants are the best-studied intermediate hosts, diverse species may be infected. It is not known to be zoonotic. One of four experimentally infected pigeons died with these organisms found in the lungs, heart, central nervous system, liver, spleen, and kidney.[17] *Neospora* has also been identified in wild parrots and passerines; however, quail are resistant to infection.[18]. Management centers around excluding *Neospora* from collections through the exclusion of dogs and dog feces and, in the case of carnivorous and omnivorous birds, exclusion of other potential intermediate hosts that may be ingested.

DIAGNOSIS OF COCCIDIAL DISEASES

When dealing with coccidian species that utilize birds as definitive hosts, fecal flotation is often the best screening test, especially for strictly enteric species. There are circadian differences in the shedding of at least some coccidia; *I. lesouefi* in regent honeyeaters was identified in 21% of fecal samples shed in the morning and 91% of samples shed in the afternoon.[19] This timing has been observed in other *Isospora* spp., and afternoon fecal samples should be chosen. *Cryptosporidium* spp. have relatively few distinguishing features, but do stain acid-fast positive unless they have been formalin fixed, so an acid-fast stain may improve detection. When dealing with gastrotropic *Cryptosporidium* spp., a gastric wash is preferable to a fecal sample; this is reversed for intestinal species. For species that are found in peripheral blood, such as *Toxoplasma*, *Isospora* spp. in passerines, or *Eimeria* spp. in cranes, examination of blood smears is helpful for detection. Organisms may be seen in mononuclear leukocytes. After identification in blood, gastric wash, or feces, species identification is indicated to determine the life cycle for disease management.

Diagnosis of coccidiosis in indirect hosts is significantly more challenging. The first sign seen in indirect hosts is often death, and a necropsy may be the first feasible diagnostic test. With *Sarcocystis* spp., light-colored sarcocysts may or may not be visible in the muscle on gross examination, but typically nothing specific is seen without histopathology. It is important to collect a set of tissues in formalin for histopathology as well as a set of tissues frozen without formalin for additional diagnostics, as indicated by histopathology. Once a coccidian agent has been identified histopathologically, species identification is indicated to determine the life cycle for disease management.

Historically, morphologic identification was used for coccidian identification, but this resulted in numerous errors. Organisms now known to be in two different families had been classed as *Isospora*, and *Cryptosporidium* spp. cannot be reliably differentiated morphologically.[3] With carnivorous birds, it is not uncommon to see pass-through in the feces of coccidia from prey that are clinically irrelevant.

Immunodiagnostics are also possible in some cases. However, especially in avian species whose coccidial diversity has not been well studied, there is concern of nonspecific cross-reactivity of antibodies against as-yet unstudied, but antigenically related, species. Although antibody cross-reactivity does correlate with genetic distance, small genetic distances may be highly clinically significant.

DNA-based methods are the diagnostic modality of choice. The methods most commonly used are based on polymerase chain reaction (PCR). Primers may be designed to be very specific for a given species or even strain or may be designed for regions conserved across a wider taxonomic group. Pan-coccidial primers have been used to discover diverse novel taxa.[20,21] Validation of primers is critical, and laboratories should provide a peer-reviewed publication on the validation of a given primer set for diagnostic use to clinicians for evaluation. Once a PCR product has been amplified, it is then essential to validate that product. Older methods included gel electrophoresis, restriction digestion, and SYBR-green real-time PCR. None of these methods sufficiently identifies the sequence of the product, and they should not be considered acceptable. Acceptable methods of product identification for diagnostic use include DNA sequencing and probe hybridization quantitative PCR (qPCR, or TaqMan real-time PCR).

DNA sequencing provides not only the possibility of identifying known organisms but also the characterization of novel organisms by comparison to reference sequences and subsequent phylogenetic analysis. Although capable of identifying novel organisms, it is slower and more labor intensive than probe hybridization qPCR.

Probe hybridization qPCR involves a probe with a dye that matches the expected sequence between the two primers. With proper temperature and salt conditions, the probe will not bind unless it matches perfectly. This allows the product to be validated while the PCR is running. The more of the target DNA in the sample, the fewer rounds of PCR are required to release a threshold amount of dye. This can be measured against a standard curve of known amounts of target DNA, enabling measurement of the amount of target DNA in a sample. Knowing the amount of target pathogen in a lesion may be important for assessing clinical significance. When well designed and properly validated to ensure the assay specifically identifies only the target DNA, probe hybridization qPCR can be a sensitive, specific, rapid, quantitative, and relatively inexpensive test. Proper validation is critical, and laboratories should provide a peer-reviewed publication on the validation of a given probe hybridization qPCR assay for diagnostic use to clinicians for evaluation.

TREATMENT OF COCCIDIAL DISEASES

Central to any coccidial treatment and control plan is an understanding of the life cycle of the species and appropriate management changes. Without appropriate management changes, pharmacologic therapy will fail. For species with indirect life cycles, removing access to other hosts in the life cycle is critical. Coccidial species utilizing the avian species of concern as a definitive host require strict hygiene to prevent reinfection; moving birds to a simpler, more easily cleaned enclosure may be necessary. *Cryptosporidium* spp. are especially stable in the environment and resistant to many disinfectants.[22] Peroxide-based disinfectants are the most effective without resorting to toxic agents such as gluteraldehyde. Disinfection should focus on mechanical removal of all feces. Drying will reduce coccidial persistence, and ultraviolet irradiation is also helpful for disinfection.[23]

As eukaryotic parasites, coccidia have diverged more recently from animals compared with bacteria. Antimicrobial drugs target biochemical differences between a pathogen and a host, and there are fewer differences between a coccidian and an animal than between a bacterium and an animal. There are therefore fewer options for classes of anticoccidial drugs than for antibacterial drugs, and available drugs are often more toxic. Pharmacologic therapy of coccidia needs to be done with discretion; overuse of anticoccidial drugs in poultry has resulted in extensive resistance,[24] and indiscriminate use will rapidly lead to resistant coccidia in a collection. Empirically, a good choice for pharmacologic therapy of Eimeriidae or Sarcocystidae is toltrazuril or toltrazuril sulfone (ponazuril). Toltrazuril is a triazine anticoccidial drug.[25] It is thought to act on the apicoplast, an apicomplexan-specific organelle, and affected organisms develop vacuoles and degenerate. It appears to be a relatively safe drug, and significant adverse effects have not been observed in pharmacokinetic studies in mammals.[26] Pharmacokinetic studies in chickens have shown the half-life of toltrazuril sulfone, the active and longest-lasting metabolite, to be approximately 15 hours,[27] and doses of 5 to 20 mg/kg daily have been suggested.

Pharmacologic therapy of *Cryptosporidium* is more problematic; there is no available drug with very good efficacy and safety data. Monensin, salinomycin, alborixin, lasalocid, trifluralin, and nicarbazin have some activity in vitro.[28] Toltrazuril, spiramycin, and halofuginone have been used in stone curlews with *C. parvum*,[29] and azithromycin has been used in scops owls with *C. baileyi*,[30] but there were no control animals to evaluate efficacy or safety.

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MACRORHABDOSIS

*David Phalen**

HISTORY AND DESCRIPTION OF *MACRORHABDUS ORNITHOGASTER*

Macrorhabdus ornithogaster is an anamorphic ascomycetes yeast that has only been found to grow at the junction of the proventriculus and ventriculus in birds.[1] It was first recognized in the early 1980s in the United States in budgerigars and was thought to be a yeast.[2] Concurrent investigations in the Netherlands described it in canaries and incorrectly concluded it was a bacterium and gave it the name *Megabacterium*, which continues to be used improperly to the present.[3] A subsequent study claimed to be able to isolate the organism from budgerigar stomachs by using traditional bacterial isolation methods; however, the authors of that study did not characterize their isolate sufficiently, and subsequently it was shown that the isolate that was described in this study was a bacterium and not *M. ornithogaster*.[4]

The true nature of *M. ornithogaster* was only conclusively demonstrated recently. Studies in Australia demonstrated that it was not sensitive to antibiotics but was sensitive to amphotericin, suggesting that it was, in fact, a fungus.[5] It was shown to stain for chitin, a protein that is only produced by eukaryote organisms, thus proving that it was not a bacterium. Investigators were then able to purify the organism and sequence portions of the deoxyribonucleic acid (DNA) that code for ribosomal ribonucleic acid (RNA). Comparing this sequence to other known yeast it was then shown that *M. ornithogaster* was not only a novel species of yeast but, in fact, was the only known representative of an entirely new genus of yeasts.[1]

It can infect many species of birds.[4] There is convincing evidence *M. ornithogaster* can cause disease in its host, but it is also clear that many birds live with this organism without obvious signs. The only effective treatments for *M. ornithogaster* are a few antifungal drugs and these drugs do not always lend themselves to large-scale flock treatment. Because *M. ornithogaster* was thought to be a bacterium (*Megabacterium*) for more than 20 years, many assumptions about this organism's biology have subsequently proven to be untrue. Continued referencing

*This chapter is a modified version of Phalen DN: Update: diagnosis and management of *Macrorhabdus ornithogaster* (formally Megabacteria). *Vet Clin North Am Exot Anim* 17(2):203–210, 2013.

of some of these flawed studies and anecdotal reports often creates confusion for veterinarians and bird owners alike.[4]

HOST RANGE

The reported host range of *M. ornithogaster* includes a wide range of psittacine birds, passerine birds, poultry, and other species. It has a worldwide distribution and is found in both wild and captive birds.[4,6]

The species of psittacine birds most commonly infected with *M. ornithogaster* are the budgerigar *(Melopsittacus undulates)*, lovebirds (*Agapornis* sp.), and to a lesser extent cockatiels *(Nymphicus hollandicus)*. Infection has also been reported to be common in parrotlets (*Forpus* sp.). In wild Australian birds, the organism is commonly found in recently fledged Galahs *(Eolophus roseicapilla)* and Corellas (*Cacatua* sp.) with chronic diarrheal disease and weight loss. These birds have other intestinal parasites and at least some have concurrent infections with the psittacine beak and feather disease virus. The full host range of *M. ornithogaster* in psittacine birds is unknown, and infection should be considered in any species of psittacine birds presenting with gastrointestinal signs.[4]

Passerine species infected with *M. ornithogaster* include pet canaries *(Serinus canaria)*, zebra finches *(Taeniopygia guttata)*, and Gouldian finches *(Erythrura gouldiae)*. It has also been found in a range of wild European finches and the sisken *(Carduelis spinus)*, and in feral European goldfinches *(Carduelis carduelis)*, and wild-caught feral European goldfinches and green finches *(Carduelis chloris)* captured for the pet trade in Australia.[4]

M. ornithogaster infections have now been reported in chickens *(Gallus gallus)* on four continents—Europe, North and South Americas, and Australia. Other gallinaceous birds reported to be infected with *M. ornithogaster* include the gray partridge *(Perdix perdix)*, the Japanese quail (Coturnix japonica), domestic turkey *(Meleagris gallopavo)*, chukar partridge *(Alectoris chukar)*, and guinea fowl (genus and species not reported). Infection has also been reported in ducks, geese, and ibis, although no supporting evidence on how the diagnosis was made in ibis was provided. Recently, *M. ornithogaster* has been reported in captive raised greater rheas *(Rhea americana)*. Morphologically, these organisms are consistent with those that have been reported in other species. However, they still remain to be characterized by molecular techniques.[4,6]

There are two reports of an organism resembling *M. ornithogaster* infecting the upper respiratory tract of a dog and a cat. These organisms were never described, and given that *M. ornithogaster* is microaerophilic, its growth on a respiratory epithelium does not seem plausible. Recent infection attempts in mice provide additional evidence that *M. ornithogaster* cannot grow in mammals.[7]

Isolation attempts from stomach contents of greater rheas by using growth conditions that are inconsistent with the metabolic requirements of *M. ornithogaster* have resulted in the isolation of a small motile organism, which the investigators suggest is *M. ornithogaster*. This uncharacterized organism has been shown to be able to colonize the stomach of mice. Given that this organism grows in conditions that are incompatible for *M. ornithogaster* growth, that it has morphologic characteristics that have never been seen in *M. ornithogaster* either in vivo or in vitro, and that it has never been characterized genetically, it is the author's opinion that the conclusion that this organism is *M. ornithogaster* is premature and is likely to be incorrect.[6]

CLINICAL MANIFESTATIONS

The signs of *M. ornithogaster* in birds include vomiting, regurgitation, diarrhea, and chronic weight (Figure 2-23). Disease has been seen in young and adult birds. Disease in budgerigars occurs most commonly in middle-aged birds. An acute hemorrhagic disease has been reported in parrotlets. Weight loss, anorexia, melena, and anemia are commonly seen in cockatiels and occasionally in other species that have gastric ulceration secondary to *M. ornithogaster* infection. Canaries and other finches with *M. ornithogaster* infections are often found dead with no premonitory signs but are generally emaciated, which suggests that they had been ill for at least a few days prior to death.[4]

DIAGNOSIS IN THE LIVE BIRD

Detection of *M. ornithogaster* infection in the live bird is most commonly done by microscopic examination of feces. Feces made into a slurry with water or saline can be scanned for *M. ornithogaster* using 40× magnification. Alternatively, fecal smears can be stained with a quick stain or Gram stain. A rapid way of concentrating *M. ornithogaster* and separating it from other solid matter in feces is to homogenize a dropping with approximately 20 times its volume of physiologic saline in a small tube, let it sit for 10 seconds, and then examine a small drop of the suspension collected from the meniscus. As *M. ornithogaster* takes longer to settle than most other material in feces, it is more easily seen in wet preparations after this treatment.[4] A polymerase chain reaction (PCR) assay to detect *M. ornithogaster* in feces is also available in North America (Veterinary Molecular Diagnostics, Milford, OH).

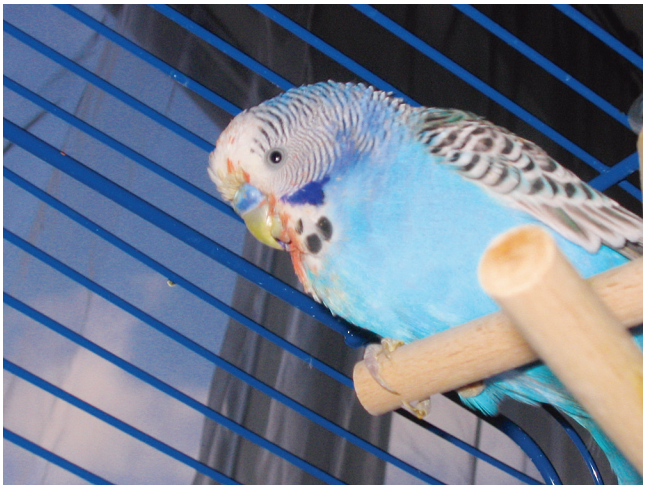

FIGURE 2-23 Budgerigar with *Macrorhabdus* infection and a history of vomiting. The pink stain on the feathers is from an antibiotic that had been vomited after the owner had attempted home treatment.

M. ornithogaster is a long, slender, straight stiff rod with rounded ends when it is found in feces (Figures 2-24 and 2-25). In some circumstances, the long rod may bend slightly in a gentle curve. Y-shaped organisms can be seen (see Figure 2-25), but extremely rarely. Viewed directly in a wet mount, small, oblong, refractile structures found at regular intervals are readily seen. These structures are the nuclei. The nuclei stain with Giemsa stains. *M. ornithogaster* ranges in length from 20 to 80 µm and is consistently 2 to 3 µm in width. The organisms often stain poorly with quick stains and the Gram stain instead of staining uniformly with only pickup small droplets of the stain. When they do stain well, they are gram positive and stain dark blue with quick stains (Figure 2-26). Unlike bacteria and other yeasts, the contents of the cell stain, but not the cell wall. It is the author's impression that they do not stick well to glass slides unless the slide has been heat fixed. It is also the author's impression that heat fixing makes them more likely to stain uniformly.

Birds infected with *M. ornithogaster* may shed the organism in low numbers, in large numbers, or not at all. It has been the author's experience that the majority of birds that exhibit disease as the result of *M. ornithogaster* infection will be shedding large numbers of organisms. However, this may not always be the case, and the absence of *M. ornithogaster* in feces does not completely rule out infection.[4]

There can be other things in feces that resemble *M. ornithogaster* (Figure 2-27). An unknown structure commonly seen by the author in the droppings of many birds is approximately the size of *Macrorhabdus* but has a straight not rounded

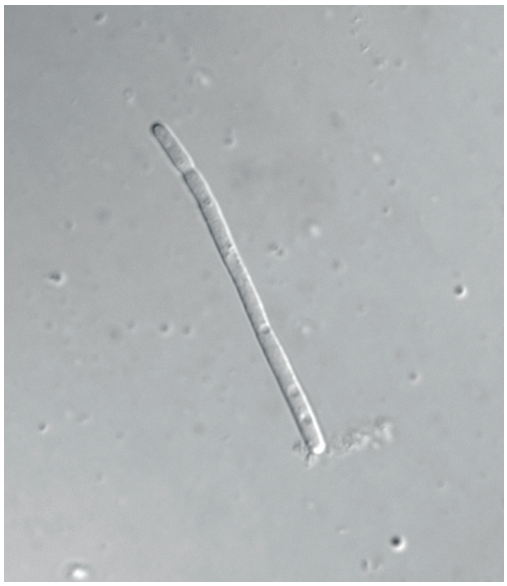

FIGURE 2-24 Unstained *Macrorhabdus ornithogaster*. Original magnification 100×.(With permission from Phalen DN: Update: diagnosis and management of *Macrorhabdus ornithogaster* (formally Megabacteria), *Vet Clin North Am Exot Anim* 17:(2):203–210, 2013.)

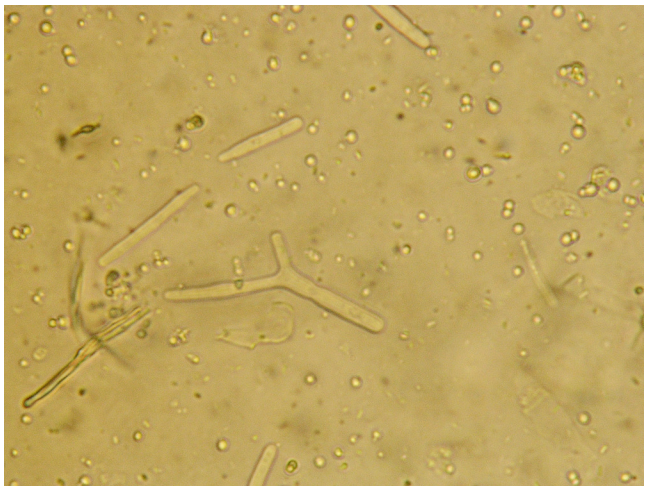

FIGURE 2-25 Unstained wet mount of *Macrorhabdus ornithogaster* showing typical rod-shaped organisms and an unusual Y-shaped organism. Original magnification 100×.

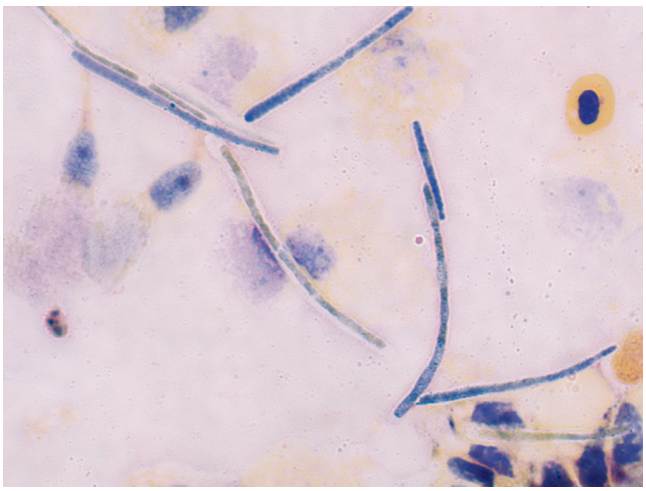

FIGURE 2-26 *Macrorhabdus ornithogaster* stained with Gram stain. Original magnification 100×. (With permission from Phalen DN: Update: diagnosis and management of *Macrorhabdus ornithogaster* (formally Megabacteria), *Vet Clin North Am Exot Anim* 17:(2):203–210, 2013.)

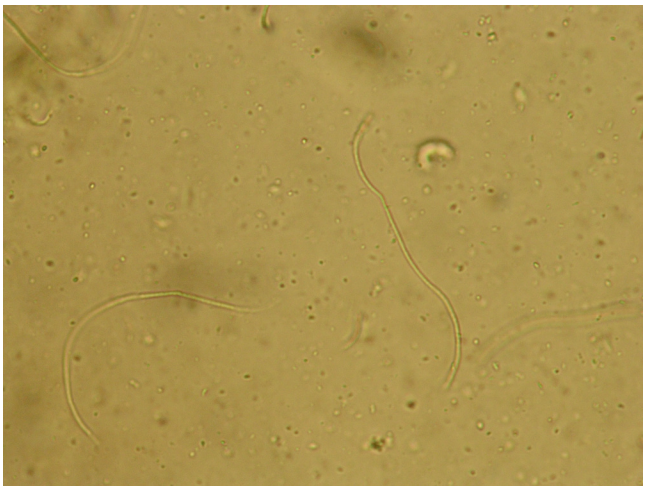

FIGURE 2-27 Unstained wetmount photograph of a finding that may be misinterpreted as *M. ornithogaster*. Other things can be found in the feces that may resemble *M. ornithogaster,* including unknown organisms or structures and filamentous bacteria.

terminal end that appears to be the result of the structure breaking off of something larger. *M. ornithogaster* always has rounded ends. Filamentous gram-positive bacteria can also approach the size of *M. ornithogaster*. These bacteria, however, are often segmented, are thinner than *M. ornithogaster*, generally curve back and forth, and thus are readily distinguished from *M. ornithogaster*.

POSTMORTEM DIAGNOSIS

M. ornithogaster infection is readily made at postmortem examination. A saline preparation of a scraping of junction (isthmus) of the proventriculus and the ventriculus will demonstrate the organisms and they will generally be abundant. *M. ornithogaster* is also readily demonstrated in hematoxylin and eosin–stained sections of the isthmus. It is eosinophilic and is found forming the characteristic log-jam pattern on the surface of and between the mucosal glands. Because it is a fungus, it stains with silver stains and the periodic acid–Schiff stain.[1]

Showing that *M. ornithogaster* infection has contributed to a bird's death, however, requires more proof than just finding the organism. Budgerigars and passerines with disease caused by *M. ornithogaster* will grossly have a thickened mucosa of the proventriculus, and there will be increased mucus in the lumen. Some birds may have one or more bleeding ulcers of the proventriculus. In birds with clinical signs caused by *M. ornithogaster* infection, growth extends beyond the isthmus into the proventriculus and the koilin of the ventriculus and may disrupt the structure of the koilin. Lymphoplasmacytic inflammation is common in birds with heavy *M. ornithogaster* growth but is less likely in birds with minimal superficial colonization by the organism.[4]

GROWTH IN VITRO

M. ornithogaster is readily grown in vitro given the correct substrate and conditions. It must be provided with a microaerophilic environment and grown in a medium with a pH between 3 and 4. Traditional cell culture media containing up to 20% fetal bovine serum and 1% to 5% glucose or sucrose has been shown to support its growth. Its optimal growth temperature is 42° C. Addition of antibiotics to the growth media is recommended to prevent the overgrowth of bacteria. It can be cultured from the isthmus scrapings or from feces.[8]

TREATMENT

A few treatment trials have been performed in birds with *M. ornithogaster* infection.[4] In many of these trials, the measure of successful treatment was the cessation of *M. ornithogaster* shedding in feces, as opposed to the less common trial in which treated birds were killed and the stomach examined directly.[4,5,9] Although it is likely that the cessation of shedding may be the result of a cure, it is also possible that some of these treated birds may have remained infected at low levels.

Amphotericin B is used widely to treat *M. ornithogaster* and appears to be effective and safe when administered orally by gavage and, in some circumstances, in water. Various dosages have been recommended. The author has used 100 milligrams per kilogram (mg/kg), twice a day for 14 days, with direct oral administration but has been gradually reducing the amount and is now using 25 mg/kg, twice a day for 14 days, with apparent success. Success of treatment has been judged by the rapid cessation of *M. ornithogaster* shedding and resolution of signs. Amphotericin B can be purchased as a powder (Gallipot, St. Paul, MN) and compounded into a formula that can be given orally. The 2.5% water-soluble powder from Vetafam (3 Bye Street Wagga Wagga, NSW, Australia) that has been used extensively in the past is no longer available at the time of this writing. There is one report of resistance of *M. ornithogaster* to amphotericin B. It is not known how widespread the resistance may be.[4]

The ability of nystatin to kill *M. ornithogaster* may vary from strain to strain. In vitro trials by Bradely et al showed that *M. ornithogaster* is sensitive to nystatin at concentrations of 0.1 units per milliliter (units/mL).[10] In one clinical trial, the authors also saw a cessation of *M. ornithogaster* shedding after treatment with nystatin. In a recent study, a flock of budgerigars was treated with nystatin at 3,500,000 international units per liter (IU/L) of drinking water for 2 days, followed by 2,000,000 IU/L for 28 days.[9] Some birds in this study were euthanized at the end of treatment and were found to be free of infection. Resistance of some strains of *M. ornithogaster* to nystatin has been reported following clinical trials performed by others.[5]

Research by Bradley et al[10] has shown that *M. ornithogaster* cultured in vitro is highly sensitive to sodium and potassium benzoate and sodium sorbate. Treatment attempts with sodium benzoate in drinking water in live birds have been studied by the author and others.[4] The author's experiences have not been uniformly successful, and in many cases, shedding and clinical signs have not resolved.[4] The reason for the failure of treatment in these instances is not known, but inadequate consumption of the treated water may be to blame. In another trial in which a flock of breeding budgerigars were treated, *M. ornithogaster* shedding stopped, but some of the treated birds died. The cause of the deaths was not determined but could have been the result of sodium toxicity. Water consumption in the treated budgerigars was very high because they were feeding young and because it was the middle of the summer and day time temperatures were very high.[4] The use of potassium benzoate has not been studied, but it may be safer than sodium benzoate because it is more difficult for potassium toxicity to result from ingested potassium than it is for sodium toxicity to result from ingested sodium. The use of any of these chemicals requires additional research before they can be recommended for routine use. There are many potential sources of sodium and potassium benzoate. The product used by the author is purchased as a 99% pure product (Sigma-Aldrich, St. Louis, MO).

Fluconazole has been used to effectively treat *M. ornithogaster* in experimentally infected chickens at dosage of 100 mg/kg. In trials in budgerigars, this dosage rate was found to be toxic and a lower dosage was not effective. Gentian violet was found to prevent *M. ornithogaster* growth in vitro. Gentian violet at moderate concentrations, however, was found to be toxic to budgerigars (Phalen, unpublished information, 2005).

CONCLUSION

M. ornithogaster is found in many species of birds around the world. It can be a significant cause of both morbidity and mortality. Detecting the infection in the live bird requires the direct observation of the organism in feces or its detection by PCR; however, these assays are not so sensitive that a negative result rules out infection. Diagnosis is readily made at postmortem examination of scrapings of the isthmus and histopathology of the proventriculus and ventriculus. The only consistently proven treatment for infected birds is direct oral administration of amphotericin B, although nystatin and sodium benzoate may also be effective under some circumstances.

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CHLAMYDIOSIS (PSITTACOSIS)

Lorenzo Crosta, Alessandro Melillo, Petra Schnitzer

Chlamydiosis is an infectious disease of birds and mammals, including human beings. The disease is so well known in psittacine birds that it has been named after them (psittacosis).

There are several ways to diagnose psittacosis, but many diagnostic approaches that are commonly used only provide degrees of inductive strength to the argument for the diagnosis rather than confirmatory diagnosis. In fact, a generalized disease with clinical signs related to the respiratory, digestive, and nervous systems; leukocytosis with heterophilia; and elevated liver leakage enzymes is rather common in several diseases of psittacines and not specific to chlamydiosis.

DEFINITION

Avian chlamydiosis is an infectious disease; it is contagious, most often systemic, and sometimes deadly. The causative organism is the intracellular, gram-negative bacterium *Chlamydia psittaci* (until recently controversially named *Chlamydophila psittaci*). Depending on the particular strain involved and the host species, chlamydiosis can present with different clinical features that may range from lethargy and anorexia, to ocular and nasal discharge, diarrhea, and green to yellow-green feces.[1]

HISTORICAL DATA

The disease which was already named psittacosis in the 1940s was differentiated into "ornithosis," used to describe the disease when nonpsittacine birds were infected and "psittacosis" when psittacines were affected or when another nonavian species was infected by a parrot.[2] This subdefinition was made on the presumption that the human form of the disease acquired from infected chickens or pigeons is less serious than the clinical form deriving from infected psittacines. Currently, we know this is not true, and often the human infections originating from turkey strains of *C. psittaci* may be even more serious.[3]

ETIOLOGY

The systematic of the order *Chlamydiales* was revised in the late 1990s, and the old name *Chlamydia psittaci* was reclassified as *Chlamydophila psittaci*.[4] However, this reclassification has always been debated, and in 2009, a subcommittee of the International Committee on Systematics of Prokaryotes re-examined the taxonomy and nomenclature issues, and a decision was made to again merge species in the genus *Chlamydophila* into *Chlamydia*.[5]

To date, the family *Chlamydiaceae* comprises nine species in the genus *Chlamydia*: (1) *C. trachomatis*, a causative agent of sexually transmitted and ocular diseases in humans; (2) *C. pneumoniae*, which causes atypical pneumonia in humans and is associated with diseases in reptiles, amphibians, and marsupials; (3) *C. suis*, found only in pigs; (4) *C. muridarum*, found in mice; (5) *C. felis*, the causative agent of keratoconjunctivitis in cats; (6) *C. caviae*, whose natural host is the guinea pig; (7) *C. pecorum*, the etiologic agent of a range of clinical disease manifestations in cattle, small ruminants, and marsupials; (8) *C. psittaci*, comprising the avian subtype and etiologic agent of psittacosis in birds and humans; and (9) *C. abortus*, the causative agent of ovine enzootic abortion.[6]

To summarize, *C. psittaci* is a gram-negative bacterium, is an intracellular obligate parasite, has specific energy needs, and cannot move. It differs from more conventional bacteria for several reasons. However, as deeper studies are carried on and new techniques come into the hands of dedicated researchers, it is becoming clear that some facts, which were considered unequivocal in the past such as the inability of *C. psittaci* to form compounds rich in energy, for example, adenosine triphosphate (ATP), are no longer valid.

C. PSITTACI REPLICATION CYCLE

The replication cycle starts with the attachment to, and penetration of, a target cell (mainly columnar epithelial cells of mucous membranes and mononuclear macrophages),[7] by the infecting and cytotoxic elementary bodies (EBs); they contain deoxyribonucleic acid (DNA) and measure about 300 nanometers (nm) (0.3 micrometer [μm]). The EBs represent the form that chlamydiae, as a group, use to survive outside the host cell. Since they cannot move by themselves, they attach to the microvilli of the epithelial cells and enter by endocytosis (Figure 2-28). The receptor site on the membrane is very specific and is identified in the major outer membrane protein (MOMP) of the EBs; in fact, the MOMP is found only on the EB. Once endocytosis has occurred, the EB is enveloped by a membrane produced by the host cell to form a vesicle. Through a mechanism not yet known, *Chlamydia* inhibits the fusion between this new vesicle (or vacuole) and lysosomes, thereby preventing the formation of a phagolysosome and then the digestion of the agent, particularly in macrophages.

At this point, protected by the vesicle (Figure 2-29), the EB of *C. psittaci* starts a reorganization process, during which the EB, which was metabolically inert, goes through some intermediate forms, to be transformed in the larger (0.5–1.5 μm) and metabolically active reticulated body (RB) (Figure 2-30).

It must be remembered that growth and propagation of RB requires a high-energy source, and for this purpose, the RB sprouts special projections. These are able to cross the membrane of the intracytoplasmic vacuole (the protection of the EB), allowing the uptake of substances from the host cell: most important, the mitochondrial ATP is transferred with the help of an ATP–ADP (adenosine diphosphate) translocase produced by *C. psittaci*.[7] However, it has recently been discovered that *C. psittaci* has more metabolic capabilities than previously believed. Principally among these was the discovery that *Chlamydia* has a nearly complete peptidoglycan synthesis pathway. This discovery confirmed previous work showing that chlamydial development is highly sensitive to beta-lactam antibiotic treatment.[6] Furthermore, other analyses have also revealed that *Chlamydia* can also produce its own ATP, which contradicts previous thoughts that *Chlamydia* was entirely dependent on the host cell's energy reserves. The RB also contains a genus-specific antigen (lipopolysaccharide), which is also located on the surface of the host cell and, probably by increasing the viscosity of the cell wall, appears to protect the infected cell from the cytotoxic effects of T lymphocytes. The growth by binary fission of the RB leads to the formation of microcolonies of 100 to 500 *C. psittaci* cells, called also *inclusions* or *Levinthal-Cole-Lillie bodies* (LCL bodies). The duration of the propagation cycle depends on the *C. psittaci* strain and the type of host cell, but it generally takes from 20 to 40 hours.

During the last stages of the replication process, proteases produced by *C. psittaci* lyse the host cell (these enzymes are sensitive to antibiotics). Simultaneously, the enzyme system of the host is activated, with endotoxicosis of the host cell. This contributes to the release of a new generation of EBs. However, before cell lysis and exocytosis happen, the RB "matures" in its condensed form by concentrating the DNA and stabilizing the cell membrane, until the new EBs are completed and ready to leave the host cell. The whole cycle takes up to 48 hours (Figure 2-31). The enzyme systems mentioned previously destroy the host cell, but the EBs may be shed continuously. With this system, the host cell is permanently infected, maintaining its functions and replicating capabilities. Besides being very interesting, this mechanism is also strategically advanced because the EBs can infect various types of macrophages, after the initial entry through the epithelial cells. Furthermore, since *C. psittaci* can survive in the host cells during mitosis, it is able to infect the next generation of macrophages.

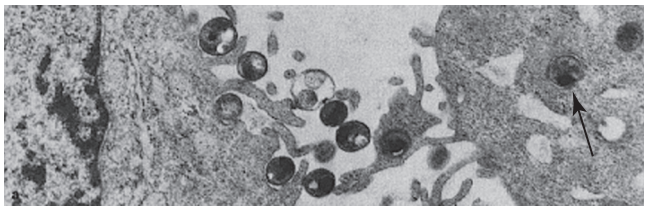

FIGURE 2-28 Chlamydial elementary bodies are shown attaching to the microvilli of epithelial cells on this electron microscopic image. (From Harrison GJ, Ritchie BW, Harrison LR: *Avian Medicine: Principles and Application,* Lake Worth, FL, 1994, Wingers Publishing.)

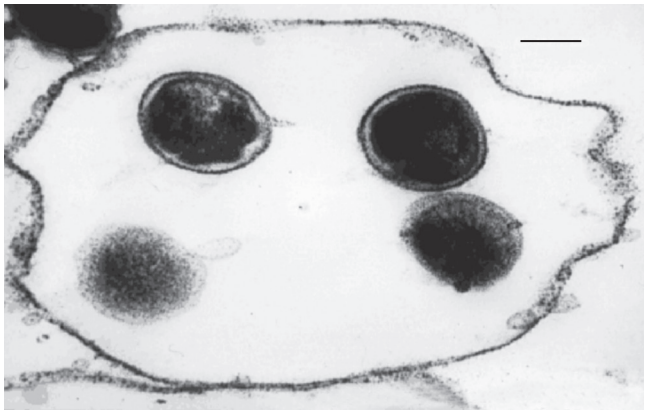

FIGURE 2-29 Elementary body of *C. psittaci* beginning reorganization. (Source: http://chlamydiae.com/twiki/bin/view/Cell_Biology/GrowthCycle. Electronic micrograph by Michael Ward. From Ward ME: The chlamydial developmental cycle. In Baron AL: *Microbiology of Chlamydia,* 1988, CRC Press.)

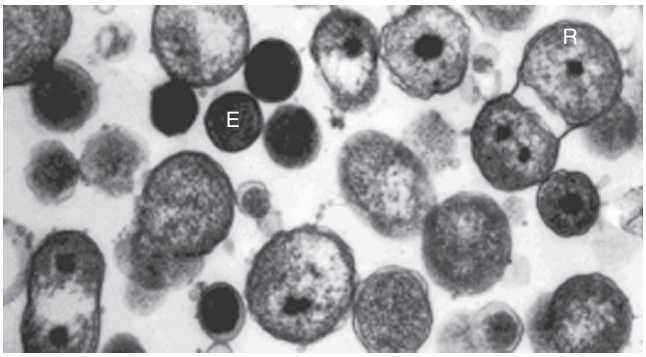

FIGURE 2-30 Elementary bodies (E) being transformed into the larger, metabolically active reticulated bodies (R). (Source: http://chlamydiae.com/twiki/bin/view/Cell_Biology/GrowthCycle. Electronic micrograph by Michael Ward.)

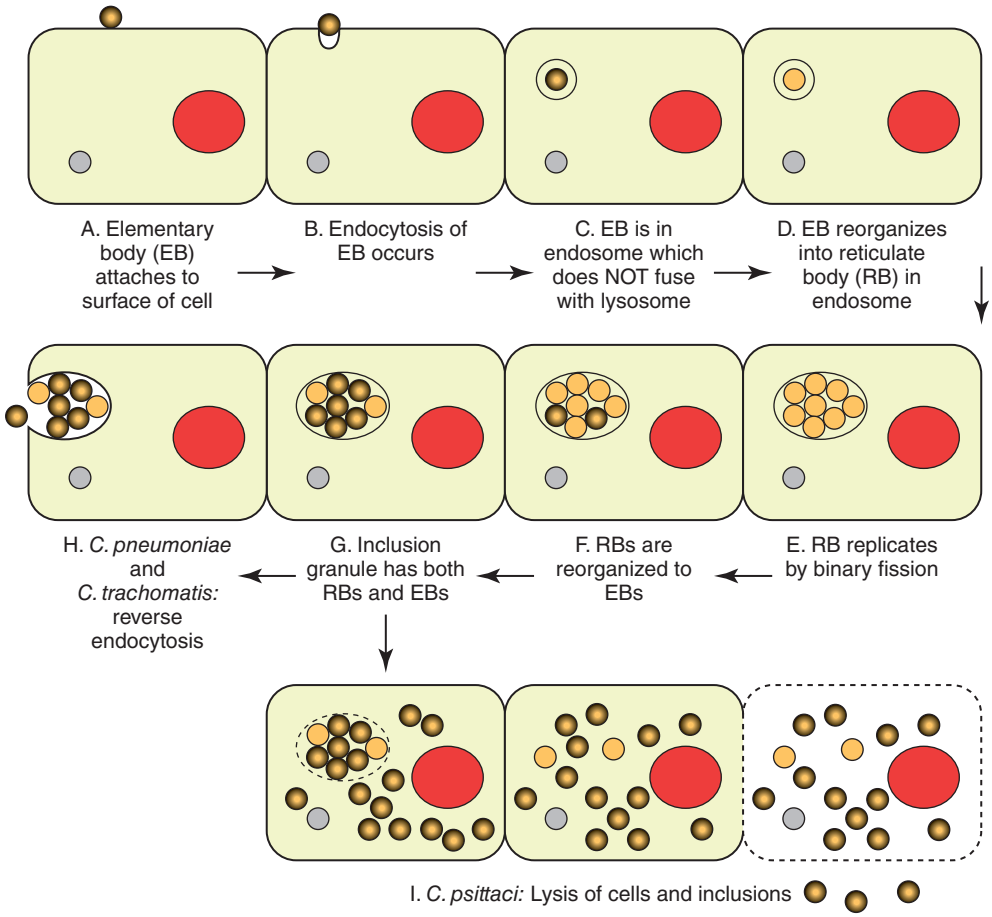

FIGURE 2-31 Replication life cycle of *Chlamydia psittaci*. (From Microbiology and Immunology on-line. University of South Carolina – School of Medicine.)

Under stress conditions, triggered by a range of factors, including the presence of antibiotics, the effects of cytokines such as γ-interferon, and the depletion of glucose and essential amino acids, chlamydial RBs may enter an alternative developmental stage, which involves the formation of large, pleomorphic cellular forms known as the aberrant bodies (ABs). The ABs will persist inside host cells, which led to the term "persistence," until the developmental trigger is removed or the nutrients are replaced. Importantly, in terms of treatment, in vitro studies have indicated that persistent chlamydial infections are refractile to standard antibiotic treatment.[6] This would explain why the idea that treatment of psittacosis with enough time to have some subsequent generations of macrophages replaced is no longer valid. The inhibition of the defense mechanisms of the infected animal, such as phagolysosomes and cytotoxic T lymphocytes, allow for the survival of *C. psittaci* inside the host cell. Even if specific lipoglycoproteins are stimulating the production of antibodies, this does not correlate with an immunologic protection. The hepatotoxic and nephrotoxic toxin, which disappears after *C. psittaci* enters into the host cell and which is linked to the presence of the MOMP, is able to stimulate the production of small quantities of antitoxin antibodies. Unfortunately, these toxins are not useful for the production of vaccines, as the antibodies are not protective and only indicate a previous exposure to *C. psittaci*. Thus, they can eventually be used for diagnostic purposes.

We may make the following conclusions from *Chlamydia psittaci* biology and replication strategy:

1. *C. psittaci* is able to inhibit the defense mechanisms of the host. This enables the infectious agent to survive inside the host cells during its propagation and may cause chronic, long-lasting (sometimes lifelong) infections. Some of those chronic infections cannot be treated adequately inside the host cell.
2. Inhibition of the phagolysosomes in the macrophages compromises the immune response. In birds that overcome the clinical disease, antibodies can be detected, but they are not able to protect the host from the infection.
3. The high variability of the different *C. psittaci* strains is determined by the MOMP, which defines receptor ability and toxic properties of a given strain and thus virulence of the strain and susceptibility of the host.
4. The persistence of *C. psittaci* in the macrophages of a given avian species is naturally selecting strains with low virulence for that species, which may still be much more virulent for other species.
5. Although the detection and discrimination between multiple repeat infections and a single chronic infection remain difficult, persistent infections have been associated with a range of chronic infections.

PATHOGENICITY

The pathogenicity of *C. psittaci* cannot be fully explained by the simple, direct damage to the host cell. In fact, the most important virulence factor is a toxin, intimately bonded to the outer membrane of the elementary bodies. This toxin is present in different amounts in the different *C. psittaci* strains. During the growth and replication of *C. psittaci* in a specific avian host, the bacterium can be modified, either in its structure or in its metabolism, and this will alter its antigenic profile and pathogenicity. The modification rate depends on the number of subsequent replication in a given species, and the surface of the new EBs contains "new" heterologous antibodies, which are supposed to be host-specific.[8] *Chlamydia* interspecific jumps, or spillovers—as may happen in quarantine stations, breeding facilities, or pet shops—can modify its physical–chemical characteristics, toxic components, and, virulence, also the host range.[9] However, these newly acquired characteristics are not necessarily permanent.

The clinical outcome of an infection depends largely on the relationship between the EBs and macrophages. A lytic and lethal reaction takes hold in phagocytic cells infected with a large number of virulent chlamydial particles. Low concentrations of virulent strains are rapidly inactivated by polymorphonuclear and mononuclear phagocytes. If the macrophage is damaged, the chances of chlamydial survival are reduced. Low concentrations of a nonvirulent strain will not stimulate an adequate lytic reaction, producing macrophages that are transformed into epithelioid cells, which remain infected chronically. The average lifespan of these epithelioid cells should be the index to determine the duration of treatment, but little or nothing is known about the lifespan of these cells in birds.[10] Further, during mitosis in the bone marrow, it is very likely that the infected macrophages transfer the chlamydial inclusion bodies in the next generation of macrophages.[11] The partial removal and phagocytosis in new macrophages promotes the selection of strains with low virulence for the species in question. However, these chronic infections facilitate the diffusion of a large number of chlamydiae that can potentially be very virulent for other avian species.[10]

RESISTENCE OF *C. PSITTACI*

The infectious elementary bodies, which can be stained with the Giemsa, Macchiavello, Gimenez, Stamp, or Castaneda stain, can survive out of the host (protected by organic material), and inside the host cells, for several weeks.[7] The tissue destruction induced by bacteria and the presence of feces inactivate the microorganism rapidly. On the other hand, the "free" EBs are relatively unstable in the environment and will be inactivated in a few days. *C. psittaci* is particularly sensitive to heat and is inactivated by relatively low concentrations of formaldehyde (1%), if the room temperature is above 20° C. Quaternary ammonium salts and lipid solvents are not a good choice for the elimination of *C. psittaci*. Its infectivity may, however, be eliminated in just a few minutes by using benzalkonium chloride.[12] Also, hydrogen peroxide has shown some efficacy against *C. psittaci*.

TRANSMISSION

C. psittaci can be detected in feces 10 days before the onset of clinical signs. A large number of chlamydial elementary bodies can be found, continuously or intermittently, in feces (up to 10^5 infectious units per gram of feces), urine, tears, nasal discharge, oropharyngeal mucus, and crop-milk (limited to Columbiformes) of infected birds. Unfortunately, not enough information is available about the period during which the clinically symptomatic birds or asymptomatic carriers can transmit the infection. In this way, a high concentration of bacterial particles can be aerosolized, and the wing flapping of a large number of birds in an enclosed collection can further facilitate this overhead suspension. Therefore, the infection can occur either by inhalation or by ingestion of these infectious particles. The following must always be kept in mind:

- Infection takes place very quickly.
- *C. psittaci* will replicate in the lungs, air sacs, and pericardium of infected birds, as soon as 24 hours after infection.
- Within 48 hours, *C. psittaci* is present in the bloodstream.
- After 72 hours, the infected birds are able to shed *C. psittaci* in the environment.

These facts by themselves can explain how the disease can spread rapidly within a closed group of animals.

It appears that when the infection takes place via the respiratory route, it spreads through the lungs and air sacs to other organs, resulting in a symptomatic disease. However, oral–intestinal infections are less likely to cause symptomatic chlamydiosis and more often lead to chronic, nonsymptomatic forms of the disease.

Vertical transmission, through the egg, has been demonstrated in the domestic duck,[13] in the black-headed gull *(Chroicocephalus (Larus) ridibundus)*,[13] and in the budgerigar,[15] and it is suspected to occur in the turkey. Cockatiels *(N. hollandicus)*, are frequent carriers of *C. psittaci* and can shed the agent through their feces for more than 1 year after an active infection. Ducks can shed *C. psittaci* in feces for 100 days and can harbor the bacterium for up to 170 days.[7] In any case, carriers may start eliminating the organism after a stressful event; the hypothesis that in a group of birds, the organism may be eliminated within 4 to 5 months has never been confirmed. Finally, it should be noted that while transmission through invertebrate vectors (mites and bloodsucking insects) is possible, direct transmission of *C. psittaci* from dogs, cats, horses, pigs, and humans to members of the same species does not seem to occur.[7]

CLINICAL FEATURES

Psittacosis is a highly heterogeneous disease. The clinical course has been described in detail in chickens and pet birds; however, well-documented cases in wild birds are less common, and usually it is assumed that the latter have simple respiratory signs.[16] Generally, acute, subacute, and chronic forms are described.[1] However, in these forms, overt clinical signs are often evident and include various combinations of respiratory signs, dyspnea, oculonasal discharge, anorexia, regurgitation, vomiting, and greenish diarrhea. On some occasions, psittacosis manifests in a more subtle way. In these situations, clinical signs may range from a simple conjunctivitis to neurologic symptoms such as tremors or torticollis;

sometimes nonspecific signs such as loss of productivity of the flock are present, or sudden deaths occur, with no prodromal signs.

To help overcome the difficulties that may be encountered in the diagnosis, given also the legal implications of the disease, the National Association of State Health Veterinarians (NASPHV), the Centers for Disease Control and Prevention (CDC), and the Council of State and Territorial Epidemiologists (CSTE) have established national case definitions for epidemiologic surveillance of psittacosis in the United States. The most up-to-date case definitions were published in 2010.

AVIAN CHLAMYDIOSIS

The usual incubation period of *C. psittaci* ranges from 3 days to several weeks. However, active disease can appear with no identifiable exposure or risk factor. Clinical signs of chlamydiosis in birds are often nonspecific and include lethargy, anorexia, and ruffled feathers. Other signs include serous or mucopurulent ocular or nasal discharge, conjunctivitis, diarrhea, and excretion of green to yellow-green urates. Severely affected birds may become anorectic and produce sparse, dark green droppings, followed by emaciation, dehydration, and death. Whether the bird has acute or chronic signs of illness or dies depends on the species of bird, virulence of the strain, infectious dose, stress factors, age, and extent of treatment or prophylaxis.

Case Definitions in Birds

Clinical signs may be subtle or not always evident in infected birds.

A confirmed case of avian chlamydial infection is defined on the basis of one of the following:

- Isolation of *C. psittaci* from a clinical specimen
- Identification of chlamydial antigen in the bird's tissues by use of immunofluorescence (fluorescent antibody)
- A fourfold or greater change in serologic titer in two specimens from the bird obtained at least 2 weeks apart and assayed simultaneously at the same laboratory
- Identification of Chlamydiaceae within macrophages in smears or tissues (e.g., liver, conjunctival, spleen, respiratory secretions) stained with Gimenez or Macchiavello stain

A probable case of avian chlamydial infection is defined as compatible illness and one of the following:

- A single high serologic titer in a specimen obtained after onset of clinical signs
- Chlamydiaceae antigen (identified by use of enzyme-linked immunosorbent assay [ELISA], PCR or fluorescent antibody) in feces, a cloacal swab specimen, or respiratory tract or ocular exudates

A suspected case of avian chlamydial infection is defined as one of the following:

- A compatible illness that is not laboratory confirmed but is epidemiologically linked to a confirmed case in a human or bird
- A bird with no clinical signs and a single high serologic titer or detection of chlamydial antigen
- Compatible illness with positive results from a nonstandardized test or a new investigational test
- Compatible illness that is responsive to appropriate therapy

PSITTACOSIS IN HUMANS

As mentioned earlier, psittacosis is a zoonosis, and the level of attention to this disease may vary in different countries or states. In humans, the first symptoms of disease tend to appear after an incubation period of 5 to 14 days, but on occasion, periods of more prolonged incubation have been observed (up to a month).

The first systemic manifestations can be nonspecific: fever, chills, headache, muscle aches, dry cough, and symptoms related to the upper respiratory tract. Usually, involvement of the lungs is detected radiographically, with evidence of pulmonary consolidation. However, the disease can affect organs that do not belong to the respiratory system, such as the liver, myocardium, skin, and brain. Coughing, when present, is usually a late symptom, generally with little mucopurulent expectorate. Sometimes, slowed heartbeat, chest pain, and splenomegaly can be observed, and myocarditis, encephalitis, and thrombophlebitis may occur as complications, or recurrences.

Psittacosis can be suspected in people who, in addition to presenting symptoms compatible with the disease, have had contact with birds. The diagnosis is confirmed with the isolation of the infectious agent from sputum, blood, or tissues. The analysis must be carried out in laboratories with appropriate protective measures. The etiologic diagnosis may be difficult in patients who have been treated with broad-spectrum antibiotics. People in direct contact with wild, domestic, or captive birds, such as owners and breeders of exotic birds, workers in poultry farms or poultry meat processing plants, avian veterinarians and veterinary support staff, and so on are considered to be at risk. Case definitions have been published for humans, as well:

A (human) patient is considered to have a confirmed case of psittacosis if clinical illness is compatible with psittacosis and the case is confirmed by a laboratory with the use of one of two methods:

- Isolation of *C. psittaci* from respiratory specimens (e.g., sputum, pleural fluid, or tissue) or blood
- Fourfold or greater increase in antibody (immunoglobulin G [IgG]) against *C. psittaci* by use of complement fixation (CF) or microimmunofluorescence (MIF) between paired acute-phase and convalescent-phase serum specimens obtained at least 2 to 4 weeks apart.

A (human) patient is considered to have a probable case of psittacosis if the clinical illness is compatible with psittacosis and one of the two following laboratory results is present:

- Supportive serology (e.g., *C. psittaci* antibody titer [immunoglobulin M {IgM}] of ≥32 in at least one serum specimen obtained after onset of symptoms)
- Detection of *C. psittaci* DNA in a respiratory specimen (e.g., sputum, pleural fluid, or tissue) via amplification of a specific target by polymerase chain reaction (PCR) assay.

DIAGNOSIS

The diagnosis of psittacosis in the living animal cannot be made only on the basis of clinical suspicion but must be confirmed by a series of laboratory tests. This is very important given the zoonotic nature of the disease, which carries legal implications, especially in the case of human infection.

Clinical Diagnosis

Following clinical suspicion (clinical signs), the veterinary practitioner may use supportive diagnostic tools such as hematology, blood chemistry, radiology, and endoscopy. Further, the discovery of circulating antibodies, antigen, or both plays an important role in the diagnostic process. Even the response to specific therapy (ex juvantibus diagnosis) proves to be a fundamental diagnostic factor for the clinician.

From the perspective of hematology and blood chemistry, birds with psittacosis often show anemia (hematocrit <30%), marked leukocytosis (white blood cells [WBCs] >30,000) and heterophilia (>70% to 80%). Often there is an increase in the levels of aspartate aminotransferase (AST), lactate dehydrogenase (LDH), creatine phosphokinase (CPK), and bile acids. Radiographically (Figure 2-32) and endoscopically, there are often signs of pneumonia (Figure 2-33), airsacculitis (Figure 2-34), and splenomegaly, the last being often marked in macaws.

Isolation of *C. psitacci*

Theoretically, the preferred method for diagnosis is through isolation and identification of the living organism. However, because of the time needed, the need of flawless sampling methods, and the possible risks to laboratory personnel, often other techniques are preferred. These include the immunohistochemical staining of cytologic and histologic samples, as well as enzyme-linked immunosorbent assay (ELISA) and PCR, with their many variants.

However, the *OIE Manual of Diagnostic Tests and Vaccines Standards* outlines the various steps and methods to follow and apply for the correct diagnosis of psittacosis. Samples should be collected aseptically, avoiding contamination by other bacteria. In live birds, the best sites for the collection of samples are the throat and the choana, but fecal, cloacal, and conjunctival swabs can also be used, alone or in combination, as well as the peritoneal or air sac exudate.[17] Furthermore, to optimize the chances of isolating and cultivating *C. psittaci* from a living patient, choanal and cloacal samples should be collected for 3 to 5 consecutive days. The samples should be pooled and sent to the laboratory.[18,19]

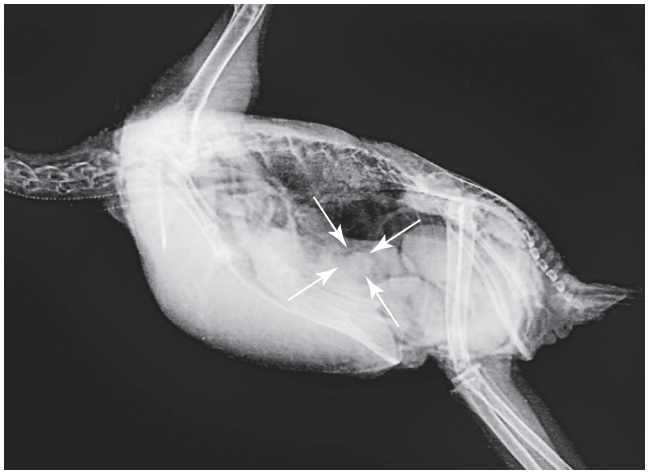

FIGURE 2-32 Mild splenomegaly in a red-tailed Amazon *(Amazona brasiliensis) (white arrows)* with chlamydiosis.

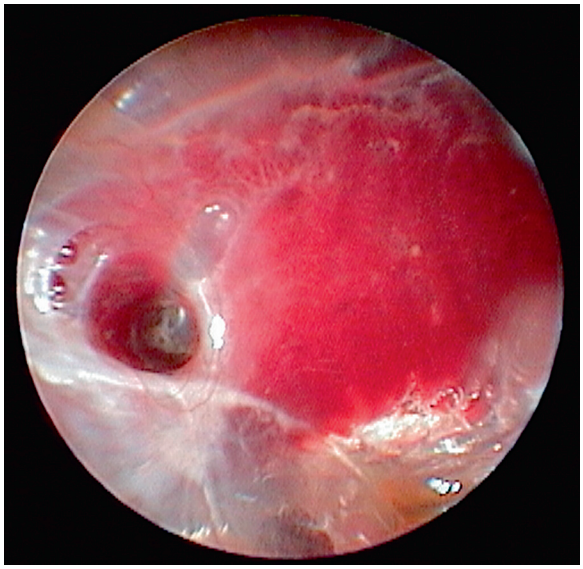

FIGURE 2-33 Pneumonia with congestion in a lovebird (*Agapornis* sp.).

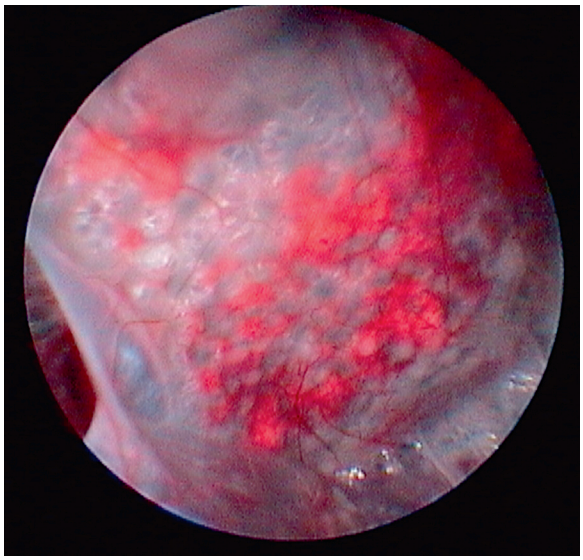

FIGURE 2-34 Airsacculitis with hemorrhage, in a blue-and-gold macaw *(Ara aurana)* with chlamydiosis.

There are specific transport media for Chlamydiae, for example, the GSP transport medium for Rickettsiae or the UTM (Coban). These media work well and carry added vancomycin, streptomycin, nystatin, and gentamicin to reduce contamination by other microorganisms. Moreover, these media can be used as diluents in the laboratory and to freeze chlamydia.[20]

Isolation of Chlamydiae in Eukaryotic Cell Cultures

Cell cultures are one of the best methods for the isolation of *C. psittaci*,[21] and there are different cell lines suitable for the purpose, such as BGM, McCoy, He-La, Vero, and others.[22] Normally, the cell lines contain antibiotics that do not inhibit the growth of *C. psittaci*. However, isolation is laborious and

costly and has largely been replaced by other techniques such as PCR.

In the selection of equipment and materials for cell cultures, it is important to remember the following:

1. Chlamydiae can be identified by using direct immunofluorescence or other appropriate staining techniques.
2. The inoculum is usually centrifuged to increase its infectivity.
3. The sample may have to undergo a blind passage at 5 to 6 days to increase sensitivity of isolation.
4. The sample should be tested two or three times during each step.
5. *C. psittaci* may be infectious to humans.

Chlamydiae can be isolated on cells that replicate normally. Cells that are not able to replicate are preferred for isolation, since they can provide a greater amount of nutrients for the growth of organisms. Moreover, these cells can be monitored and observed for longer periods.

Cell cultures should be checked for the presence of Chlamydiae at regular intervals. This is generally done at day 2 or 3, as well as on day 5 or 6. Cultures that are negative on day 6 are harvested and replanted.

Several staining methods may be used to stain *C. psittaci* inclusions, but the preferred one is direct immunofluorescence, in which inclusions appear fluorescent green.[22–24] *C. psittaci* inclusions may also be demonstrated by using indirect immunofluorescence and immunoperoxidase.[22,24,25] Direct staining can be done with the Gimenez, Giemsa, Ziehl-Neelsen, or Macchiavello stain. With the exception of immunofluorescence, all the other techniques have the advantage of being suitable for a normal optical microscope.

Isolation on Embryonated Eggs

Chicken embryos can still be used for the isolation of *Chlamydia*. The standard procedure is to inject up to 0.5 milliliters (mL) of inoculum in the yolk sac of embryos of 6 or 7 days. The eggs are then incubated at 39° C rather than 37° C. In fact, the multiplication of Chlamydiae increases at high temperatures. The replication of the organism causes the death of the embryo in 3 to 10 days. If it does not happen, two other blind steps are done, at the end of which, if the embryo is still alive, the sample is declared negative. Infection with *Chlamydia* is determined by typical vascular congestion of the yolk membrane. These are collected and homogenized with a 20% suspension of SPG buffer, and then they are frozen or inoculated on eggs or cell cultures.[26] The organism can be identified by preparing an antigen from an infected yolk sac and then testing a smear with an appropriate staining method or with a serological test.

Histochemical Stains

Giemsa, Gimenez, Ziehl-Neelsen, and Macchiavello stains are normally used to stain the Chlamydiae on liver or spleen direct smears. Alternatively, a modified Gimenez (or Pierce-van der Kamp) stain can be used.

Immunohistochemical Staining

These techniques can be used for the identification of *C. psittaci* in cytologic or histologic preparations. The technique is more sensitive than standard histochemical staining, but some experience is required, since some cross-reactions with certain bacteria and fungi are to be expected. For these reasons, specific morphologic aspects have to be evaluated as well. Most of the common immunohistochemical staining can be adapted to obtain satisfactory results; however, the selection of the primary antibody is very important. Both monoclonal and polyclonal antibodies can be used. When having to work with Chlamydiae that have been inactivated by formalin, since formalin damages the antigen, it is better to use polyclonal antibodies. The *Chlamydia* strain that is used does not really matter, since the antibodies will react with antigens that are common to the whole group.

Enzyme-Linked Immunosorbent Assay

The ELISA technique has been widely advertised in the form of simple kits for the diagnosis of human chlamydiosis. These kits seek a lipopolysaccharide antigen (LPS), which is common to all the *Chlamydiae* and therefore will be able to identify all species of the group. Many commercial tests of this type were tested in birds, but no test has been officially approved.[27] One of the problems with these tests is that the chlamydial LPS has epitopes in common with other gram-negative bacteria and the frequent cross-reactions give many false-positive results. This problem seems to be overcome, or at least reduced, with the newest kits that use selected monoclonal antibodies (MAbs). However, these kits do not have high sensitivity, and they still need hundreds of antigen units to give a positive reaction. For this reason, many clinicians believe that the diagnosis of psittacosis in birds with antigen ELISA should be considered valid only if the reaction is very positive in symptomatic birds. Part of the reason is that a high number of false positives can be obtained; therefore, a positive ELISA antigen result in an individual nonsymptomatic bird should be considered not significant.

Polymerase Chain Reaction

The PCR technique is based on the identification of DNA (or RNA) sequences that are specific to a given organism[28,29] and has long been used for the diagnosis of *C. trachomatis* in humans, in whom it is considered highly sensitive and specific. Unfortunately, the test is not directly applicable to other Chlamydiae because it searches a plasmid that is present only on *C. trachomatis* and, therefore, cannot be used for the diagnosis of psittacosis in birds. A further problem lies in the variety of different samples used for the extraction of DNA in the veterinary field, such as oral, conjunctival, and cloacal swabs, as well as tissue samples.[30]

In recent times, PCR has replaced traditional methods as the preferred method for detecting chlamydial infections in humans and animals.[31,32] This method is reliable, rapid, and highly sensitive and can be used on nonviable specimens. Additional advantages include opportunities for subsequent speciation and fine-detailed molecular typing of isolates.

PCR assays targeting a variety of chlamydial genes are available, and a range of specificities and sensitivities has been reported. The most common amplification targets are genes encoding chlamydial 16S rRNA, 23S rRNA, and ompA (encoding MOMP). PCRs that target the 16S rRNA-23S rRNA region are generally able to amplify all members of the order Chlamydiales but make subsequent speciation difficult because of the conservation between species.[4] ompA has also been widely used but is highly variable; this makes selection

of conserved PCR primers difficult, but the variability can be used for fine-detailed epidemiologic analysis of chlamydial outbreaks in animal populations.[6]

Serology Tests

Serologic tests are a great aid in the diagnosis of psittacosis, but they have a greater significance when used in association with other analyses and observations. Serologic assays are particularly valuable in cases with symptomatic populations and individual animals and to monitor response to treatment.[19,33]

Complement fixation test

Serology, particularly in the form of the complement fixation test (CFT), is still a convenient and commonly used technique for detecting present and past chlamydial infections in humans, cats, birds, cattle, and small ruminants. Although the sensitivity is significantly less than 100%, the ease of collection of blood samples and the availability of technology to perform these tests make these ideal for screening large numbers of samples (particularly suited to screen livestock).[6] The standard serologic test for chlamydial antibodies is the CFT. The modified direct CFT can be used with most sera. The antigen is a group-reactive lipopolysaccharide antigen present in all strains. The occurrence of high complement fixation titers in the majority of individuals in a flock with clinical signs is presumptive evidence of active infection. The demonstration of a fourfold increase in titer in an individual bird is considered to be diagnostic of a current infection.[20]

Other tests

Other serologic tests such as ELISA, latex agglutination, EB agglutination, microimmunofluorescence, and agar gel immunodiffusion tests can be used. These tests are of value in specific cases and may replace the CFT; however, comparisons of reliability and reproducibility are not yet available.[20]

Some trials of ELISA for the detection of antibodies against both *C. trachomatis* and *C. psittaci* indicate that in many cases these can replace the CFT.[34–37] However, the tests must be standardized and are not available for all avian species. However, some modified ELISA kit tests for research of anti–*C. psittaci* in parrots are already available commercially.[38,39]

Other possible diagnostic tests are agar gel immunodiffusion (AGID),[40] latex agglutination (LA), EB agglutination (EBA),[41,42] and micro-immunofluorescence (MIF) tests. AGID is less sensitive than complement fixation, although it is more easy and quicker to perform.[43] There is a correlation of 72.5% between latex agglutination and direct complement fixation. LA has a sensitivity of 39.1% and a specificity of 98.8%, compared with direct complement fixation.[43] This test searches both IgM and IgG (IgY), but it is more suitable for the detection of IgM. Therefore, it is recommended to be used for active or recent infections. The EBA can only identify IgM and is therefore indicated only for active infections. The MIF is easy and quick to perform, but unfortunately species-specific fluorescent sera are not always available.

THERAPY

The "traditional" treatment for avian chlamydiosis is based on the administration of tetracyclines for 45 days. The duration of treatment depends on the fact that the antibiotic cannot penetrate the macrophages and therefore cannot reach *C. psittaci* and neutralize it. During the 45-day treatment, at least two complete replicative cycles of avian macrophages take place, and during the division phase, the microorganism should be released from the cells and, if the plasma concentration of antibiotic is sufficiently high to inhibit *C. psittaci*, the organism should be eliminated.

Doxycycline is the accepted first-choice drug for treatment. This is probably because whatever the route of administration, doxycycline shows good bioavailability; furthermore, its absorption is not influenced by the intake of dietary calcium compared with other tetracyclines.[19] There are some established protocols, the most used being intramuscular injections of high doses of doxycycline (75 to 100 mg/kg) on days 0, 7, 7, 7, 7, 6, 5, and 5. The shortening of the interval between injections depends on the development of enzymatic mechanisms that facilitate the metabolism of the drug, which thus would not be able to maintain a therapeutically effective concentration in the last stages.

Also, the formulation of the finished drug, including the doxycycline salt and the vehicle used, affects its absorption. Thus, after various studies and clinical trials, the most used preparation is a product for human use (Vibravenös, Pfizer). Although it is a preparation for intravenous use, it has been demonstrated that it can be used intramuscularly in birds. Among other things, particularly in the case of untamed birds, it may be easier to bring the patient to the veterinarian (or administer the injection) every 5 to 7 days than to catch the bird(s) on a daily basis for a month and a half. The dose of doxycycline that is injected varies somewhat, and readers are encouraged to seek species-specific recommendations in the formulary of this text or others. Alternatively, doxycycline and other tetracyclines can be given orally via drinking water or medicated food or through direct administration.

Administration via drinking water is carried out at a concentration of 400 mg doxycycline hyclate per liter of water for cockatiels *(Nymphicus hollandicus);* for other species, the doxycycline concentration can be increased up to 600 mg/L.[19,20] Unfortunately, no data on the possible toxicity of this treatment regimen are available; however, the reported symptoms are depression, anorexia, biliverdinuria, and alteration of liver enzymes.[19]

Therapy with medicated food can give good results, especially because it is easier to control the intake of food compared with water. For this purpose, in the past, corn was soaked in a solution of chlortetracycline, but currently extruded and pelleted diets, with 1% chlortetracycline, are available. The drawback to this method is that the birds should already be accustomed to eating pelleted or extruded diets before the onset of treatment. For this reason, medicated food is often used for the prophylaxis of groups that are first adapted to the diet and for individual patients that already eat extruded or pelleted diets.[19] Direct, oral treatment is practical and easy to do, but it can cause minor gastrointestinal symptoms. The recommended dosage in parrots varies among studied species, with a generic recommendation from 25 to 50 mg/kg, every day.[19] Species-specific dosage recommendations are as follows: 25 to 35 mg/kg every 24 hours for cockatiels; 25 to 50 mg/kg for Senegal parrots and blue-fronted and orange-winged Amazon parrots; and 25 mg/kg every 24 hours for Grey parrots, Goffin's cockatoos, blue and

gold macaws, and green-winged macaws. Precise dosages cannot be extrapolated for other species; however, 25 to 30 mg/kg every 24 hours is the recommended starting dosage for cockatoos and macaws. If the bird regurgitates or refuses orally administered doxycycline, another treatment method should be used.

In the past, it was hypothesized that a 3-week treatment can be as effective as a 45-day course of antibiotics. In addition, it was supposed that most treatment failures were not due to the drug or to the duration of therapy but to the microorganism.[44] Recent publications have demonstrated the possibility of shortening the treatment time to less than half (21 days), compared with the classic duration of 45 days, by administering oral doxycycline at lower doses (35 mg/kg) and with shorter intervals (24 hours).

Alternatively, fluoroquinolones (enrofloxacin) or macrolides (azithromycin) have been proposed for the treatment of avian chlamydiosis. Although results of early experimental studies suggested enrofloxacin as a potential alternative treatment, anecdotal use of enrofloxacin in clinical practice showed that many birds fail to eliminate infection.[45]

Macrolide antibiotics such as azithromycin and clarithromycin have been used for years to treat humans with *C. trachomatis* or *C. pneumoniae* infections.[45] The main advantage of the newest macrolides is their ability to enter the macrophages in an active form. For this reason, it has been theorized that azythromicin is a better drug for the treatment of long-term, chronic, or "persistent" infections, while the classic intramuscular doxycycline is a better choice for the acute clinical forms, with the classic psittacosis symptoms.

THE AVIAN CHLAMYDIOSIS CONNECTION: AN INFORMAL WORLD TOUR AS IT IS SEEN BY SURVEYED AVIAN VETERINARIANS

One of the most sensitive points about chlamydiosis is its zoonotic nature. The capability of this organism to infect humans has a great impact on the perception of the general public about the disease. Furthermore, depending on the geographic location in the world, several clinical aspects of psittacosis may vary. For example, these may include the prevalence of the agent in a given wild population of birds or the incidence of disease; the variety of captively maintained or domestic species that are presented for diagnosis and treatment; the treatments used by veterinarians; and the legal implications in a given country or region. All of these factors and more will influence many aspects of disease recognition, diagnosis, and treatment options put into play.

To get an idea about how often the disease is seen in practice, how it is managed, and how often it is reported, a small, informal Internet survey of several veterinarians around the world was conducted. The outcome is interesting, and the author (LC) is grateful to those participating colleagues who took the time to answer the questions. The questions included the following: (1) Do you see chlamydiosis cases? (2) If yes, which species are most commonly seen with the disease? (3) What are the predominant clinical signs that you see? (4) How is diagnosis made most often? (5) What is your most common therapeutic plan? (6) Have you seen or heard of cases in humans? (7) Is avian chlamydiosis (psittacosis) a notifiable (reportable) disease where you practice? The survey results are presented in Table 2-8.

The most common species recognized with the disease seemed to be the cockatiel *(Nymphicus hollandicus)*, followed by the budgerigar *(Melopsittacus undulatus)*. Larger parrots or other species were mentioned less often, and the frequency of these other species presenting with this disease may be dependent, at least in part, on the geographic location or the nature of practice exposure. The most commonly described clinical presentation included the "classic" upper respiratory form, with conjunctivitis and oculonasal discharge; this may or may not be combined with the other set of clinical signs—those associated with hepatic disease. In addition to clinical signs compatible with the disease, leading to a heightened index of suspicion, other diagnostics that play an important role in diagnosis included supportive clinical pathology and diagnostic imaging. PCR, performed on conjunctival, oral, and cloacal swabs, appears to be a commonly employed diagnostic tool. Serologically, Immunocomb, a commercially available modified ELISA antibody test, appears to be a fairly common test utilized in diagnosis (but this brand is not available everywhere in the world). A 45-day course of parenteral doxycycline seems to be widely used as the "standard treatment" for psittacosis, although orally administered azythromicin in some regions appears to be a common option. Differences of opinion and treatment options seem to exist with regard to the treatment method for small or larger psittacines and also for tame pet birds and breeding or avicultural specimens. Most of the surveyed practitioners reported having seen cases of human chlamydiosis. However, these appear to be infrequent, overall. Interestingly, two of the interviewed colleagues had suffered from psittacosis themselves. Other colleagues noted that their diagnoses in humans had been confirmed following their recommendations to see their physicians. There was concern expressed about lack of familiarity in some physicians with the disease, its diagnosis in humans, and treatment. The medicolegal aspects of notifiability, that is, the level and manner of notifiable diagnosis, with regard to chlamydiosis seem to vary . Each country seems to treat the problem differently, and even where there is a federation of different states (e.g., Australia, United States), those different states may apply different laws and regulations. In some countries, even if avian chlamydiosis is a notifiable disease, case definitions and the circumstances under which the disease should be reported vary. It appears advisable for avian veterinarians to obtain the relevant recommendations from the appropriate authorities in their area in order to most optimally pursue diagnosis (suspect, probable, or confirmed) and properly address human health concerns, where applicable. An example of case definitions (human and birds), with diagnosis and treatment options, can be found and downloaded in PDF format from the Centers for Disease Control (CDC) website: http://www.nasphv.org/Documents/Psittacosis.pdf.

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TABLE 2-8

Summary of Findings from Informal Survey of Avian Chlamydiosis As It Is Seen by Avian Veterinarians in the World

1	Do you see Chlamydiosis cases?	Yes	Occasionally	Not as much as in years past	Yes	Yes, but not commonly	Yes	Yes
2	If yes, which species are most commonly seen with the disease?	Cockatiels	Small psittacines	Cockatiels, budgerigars	Red-Tailed hawks	Pigeons, budgerigars	Sun conures, cockatoos	Cockatiels, budgerigars, Grey parrots
		Occasionally large and wild parrots	Rarely larger parrots, particularly macaws	Less common large parrots		Parrot nurseries	Feral pigeons and wild sparrows	Amazons, macaws
3	What are the predominant clinical signs that you see?	Conjunctivitis, sinusitis	Generalized (systemic) disease, conjunctivitis	Oculonasal discharge, depression	Poor body condition, lethargy	Upper respiratory signs	Chronic weight loss, oculonasal discharge	Nonspecific signs of illness, hepatopathy
		Systemic disease, airsacculitis, hepatitis		Hepatopathy	Poor feather condition, oculonasal and respiratory signs	Hepatitis, sudden death	Chronic respiratory disease, hepatopathy	Respiratory signs, feather damaging behaviors
4	How is diagnosis made most often?	Clinical signs, serology (Immunocomb)	Clinical signs, serology and polymerase chain reaction (PCR); splenic biopsy	Clinical signs, serology (Immunocomb)	Clinical signs, PCR, necropsy	Clinical signs, PCR, necropsy	Clinical signs, PCR	Clinical signs, complete blood count (CBC), blood chemistries, serology (Immunocomb), PCR
5	What is your most common therapeutic plan?	Parenteral doxycycline (intramuscular [IM])	Large birds: Parenteral doxycycline (IM); small birds: enrofloxacin	Doxycycline IM or orally (PO)	Euthanasia	Doxycycline	Doxycycline, azythromicin	Parenteral doxycycline (IM) 45 days; azythromicin 3 weeks
6	Have you seen or heard of cases in humans?	Yes: coworkers	Yes: myself	Yes: but few	Yes: but many years ago	Yes: several	Yes: veterinarians	Yes: but unconfirmed ones
7	Is Avian chlamydiosis (psittacosis) a notifiable (reportable) disease where you practice?	Yes	No: there are no formal rules	No	Yes	No	Yes: there is a specific government manual	Yes

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Yes	Yes	Yes	Yes	Yes	Yes	Yes, approximately twice each year	Yes, but less often than in in the past	Yes
Quaker parakeets, blue fronted Amazons, Patagonian conures	Gray parrots, cockatiels	Cockatiels	Quaker parakeets, budgerigars	Blue-fronted Amazons, Quaker parakeets	Cockatiels, budgerigars	Macaws and Amazons	Amazons, conures, macaws	Eclectus parrots, budgerigars, sulfur-crested cockatoos
	Less common lovebirds		Amazona aestiva, *Cyanolyseus patagonicus*	Chickens, pigeons	Larger parrots	Less Greys and cockatiels	Less frequent lovebirds	Cockatiels, chickens
Upper respiratory signs	Conjunctivitis	Nonspecific signs of illness	Conjunctivitis, sinusitis	Conjunctivitis, nasal discharge, diarrhea	Conjunctivitis, rhinitis, acute or chronic hepatopathies	Conjunctivitis, nasal discharge	Weight loss, chronic hepatopathies, poor plumage quality, biliverdinuria	Lethargy, anorexia, conjunctivitis, respiratory signs, generalized ill thrift
Conjunctivitis, oculonasal discharge	Hepatopathies less often	Conjunctivitis, weight loss, diarrhea	General depression		Generalized ill-thrift, weight loss	Chronic weight loss, poor feathering		
Clinical signs, direct stain from swabs, serology (enzyme-linked immunosorbent assay [ELISA]), immunofluorescence	Clinical signs, PCR	Clinical signs, PCR	Clinical signs, direct stain from swabs, PCR	Clinical signs, PCR	Clinical signs, history, clinical pathology findings, diagnostic imaging, response to treatment, high antibody titer, PCR, immunofluorescence assay (IFA) or special stains of tissue samples	Clinical signs, clinical pathology findings, serology (Immunocomb), PCR	Clinical signs, CBC, protein electrophoresis, serology (Immunocomb), PCR	Clinical signs, CBC, blood chemistry, diagnostic imaging, serology (Immunocomb), ELISA antigen capture or PCR from choanal/cloacal swab, necropsy
Doxycycline	Doxycycline, enrofloxacin	Doxycycline, enrofloxacin, azythromicin	Doxycycline, enrofloxacin, azithromycin	Doxycycline, azithromycin	Parenteral doxycycline (IM) predominantly	Parenteral doxycycline (IM), 45 days	Pet birds: azithromycin PO; wild or aviary birds: doxycycline in water	Parenteral doxycycline (IM) for 6 Weeks; oral doxycycline as an alternative
Yes: several	Yes: few	Yes	Yes: particularly bird breeders	Yes: several	Yes: some	No	Yes: but not recently	Yes
No	Yes	Yes		Reportable	Reportable	Reportable	Reportable	Not notifiable

MYCOBACTERIOSIS

Angela Lennox

ETIOLOGY AND PREVALENCE

Mycobacterial infections have been described in companion, zoo, and free-ranging birds for many years. Strict eradication programs have nearly eliminated mycobacteriosis in commercial poultry flocks.[1] Mycobacterial infections consist of tuberculous and atypical nontuberculous species. Most infections in birds are atypical nontuberculous and involve species such as *Mycobacterium genavense*, and *M. avium*. A recent 2013 report described *M. genavense* as the most commonly identified species in psittacines.[2] Other atypical organisms include *M. marinum* and others.[3] Tuberculous mycobacteriosis, which is a major health concern in humans, is rarely reported in bird species.

Many different avian species have been affected with mycobacteriosis and include psittacines, passerines, waterfowl, and wild and zoo species. An earlier survey of pet psittacines birds suggested an overall infection prevalence of 0.5% to 14%, with the following species most commonly affected: brotogerid parakeets (*Brotogeris* spp.), Amazon parrots (*Amazona* spp.), budgerigars *(Melopsittacus undulates)*, and Pionus parrots (*Pionus* spp.).[4] Similarly, a more recent updated review of 123 cases in psittacines indicated the most commonly affected psittacines were Amazon parrots (*Amazona* sp.), and gray-cheeked parakeets *(Brotogeris pyrrophterus)*.[2] As these organisms are slow growing and disease is chronic, this tends to be a disease of older birds. There is no reported gender predilection.

EXPOSURE

Atypical mycobacterial organisms are commonly found in the environment, especially in water and soil. Organisms can survive in the environment for long periods. It is assumed that birds acquire atypical mycobacteriosis via ingestion of organisms in food or water or through contact with infected soil.[1,2] Although there are no confirmed cases of bird-to-bird transmission, outbreaks have occurred in free-ranging nonpsittacine birds, including an epizootic in free-ranging flamingos that resulted in 18,500 deaths within a short period. This outbreak was associated with malnutrition and other conditions resulting in immunodeficiency, in combination with dense populations and overwhelming exposure to organisms shed in feces.[5] In humans and some other species, immunocompromise or exposure to large numbers of organisms are a prerequisite for infection with atypical mycobacteriosis.[6] It is uncertain if immunocompromise is required for disease in individual captive birds.

Sporadic cases of tuberculous mycobacteriosis (*M. tuberculosis*) have been reported in birds, including a macaw, an Amazon parrot, and a canary.[7,8] In the case of the blue-and-gold macaw, the source was assumed to be an infected owner.[8] There are no current confirmed cases of transmission of any mycobacterial organism from birds to humans.

Routes of Infection and Pathogenesis

Many body systems can be infected, including the gastrointestinal (GI) tract, liver, respiratory tract, bone, dermis, and others, and this is likely related to the route of infection. A recent review of avian submissions indicated the liver was most commonly affected, followed by the spleen and then the GI tract.[2] Fatal *M. genavense* infection in the central nervous system (CNS) of a spectacled Amazon parrot *(Amazona albifrons)* has been described.[9]

After ingestion of organisms, the organisms infect the small intestine and the liver. Hematogenous spread leads to infection of the bone marrow, lungs, air sacs, spleen, gonads, and, rarely, kidney and pancreas.[2]

Inhalation of organisms may lead to pulmonary infections. Organisms may also enter wounds. The author noted a case of atypical mycobacteriosis associated with a wing web tattoo site used to indicate gender after surgical sexing.

Atypical nontuberculous infections tend to cause diffuse enlargement of the affected organ(s) secondary to macrophage accumulation within the organ parenchyma. The liver may appear enlarged and tan-colored, without visible granulomas, and the intestinal loops may be thickened. In contrast, *M. tuberculosis* infections tend to produce visible nodules containing epithelioid cells, giant cells, and heterophils. The cytoplasm of affected cells is filled with acid-fast organisms.[4]

CLINICAL PRESENTATION

Symptoms of mycobacteriosis are generally nonspecific and vary widely, depending on the length and severity of infection and the organ system affected. Birds can present with weight loss, poor feathering, polyuria, diarrhea, and abdominal distention. Birds with respiratory infections may present with abnormal respirations (rate or effort) and audible respiratory sounds. Some birds die acutely without recognized signs of illness. Less common physical examination findings can include lameness, cutaneous masses, and ocular lesions (Figures 2-35 through 2-37). Weight loss appears to be the most consistent finding in birds with mycobacteriosis. In many cases, birds fail to respond or only temporarily respond to routine antibiotic therapeutic choices.[4,10,11]

Because of the wide range of body systems affected and the chronic nature of the disease, the differential diagnosis list can be extensive and include numerous other infectious, neoplastic, or metabolic diseases. In cases of dermal or conjunctival masses, differential diagnoses include inflammation, infection, cysts, and neoplasia.

Supportive Laboratory Data and Diagnostic Findings

General diagnostic testing results are often nonspecific. Hemogram abnormalities vary; however, typical "textbook" disseminated mycobacteriosis in psittacine birds tends to produce moderate to marked increases in white blood cell numbers characterized by heterophilia and monocytosis. Reactive lymphocytosis can be present. Birds with impaired immunologic function or only localized disease may not exhibit leukocyte abnormalities. Packed cell volume (PCV) is often decreased as a result of chronic infection or inflammation; however, in some cases of primary respiratory mycobacteriosis, PCV can be greatly increased. These hematologic abnormalities can be present in many inflammatory and chronic disease conditions other than mycobacteriosis.[12,13]

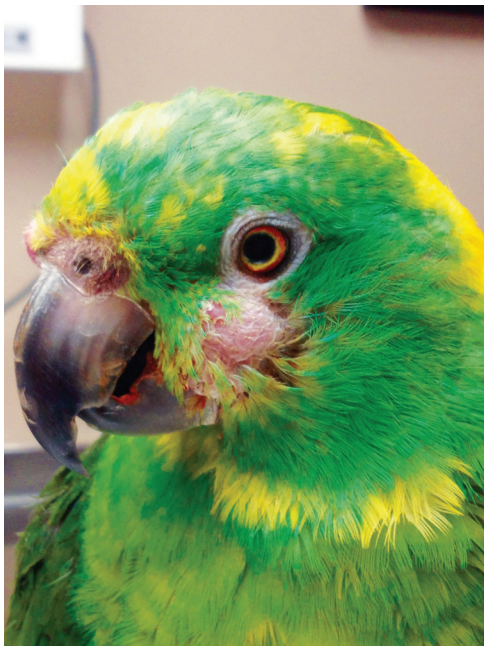

FIGURE 2-35 Left-sided view of a yellow-naped Amazon parrot *(Amazona auropalliata)* with facial and oral lesions confirmed as *Mycobacterium genavense*. (Courtesy Dr. Brian Speer.)

FIGURE 2-36 Right-sided view of the same patient as shown in Figure 2-35, yellow-naped Amazon parrot *(Amazona auropalliata)* with facial and oral lesions confirmed as *Mycobacterium genavense*. Note the asymmetry of the appearance of the lesions. (Courtesy Dr. Brian Speer.)

FIGURE 2-37 Necropsy images of a cockatiel *(Nymphicus hollandicus)* with *Mycobacterium genavense*–related conjunctivitis. (Courtesy Dr. Geoff Olsen.)

Hepatic mycobacteriosis can produce increases in concentrations of enzymes such as alanine aminotransferase (ALT), aspartate aminotransferase, (AST), and lactate dehydrogenase (LDH). However, these enzymes are also present in varying amounts in a variety of other tissues such as muscle, kidney, and heart. Therefore, increases may not truly reflect hepatocellular damage. Conversely, enzyme concentrations can be normal in the face of severe hepatic disease. If liver function has been compromised, hepatic mycobacteriosis may produce increased concentrations of serum bile acids. Plasma protein electrophoresis results are variable.[14–16]

Radiographic findings are also variable and can include evidence of enlarged liver and spleen, thickened intestinal loops, and pulmonary lesions. Infections of bone have produced lesions described as increased opacity of endosteal bone.[4,10]

In birds with GI infections, nonstaining bacterial rods may appear in fresh fecal cytologic samples. These samples can be submitted for acid-fast staining. It should be noted that since organisms can be present in food and water, presence of mycobacterial organisms in feces may not be proof of actual infection and disease, and the amount of fecal shedding can vary, resulting in impaired sensitivity and specificity of the fecal acid-fast test as a screening tool. Acid-fast positive organisms may be identified in other tissue samples as well, including cytologic preparations of liver or cutaneous masses.

CONFIRMATION OF DIAGNOSIS

Diagnosis of cutaneous forms of mycobacteriosis is often straightforward and is achieved by biopsy, cytology, histopathology, or all of these, with additional testing for organism identification. Diagnosis of internal forms of the disease (liver, kidney, spleen, or pulmonary) is significantly more difficult and, in the author's experience, is achieved after extensive workup, including biopsy of abnormal organs, followed by additional testing for organism identification (Figure 2-38). Many infections are identified at postmortem examination.[1]

Histopathology can reveal lesions consistent with mycobacteriosis but cannot confirm nor identify the species in question. Confirmatory diagnostic methods include polymerase

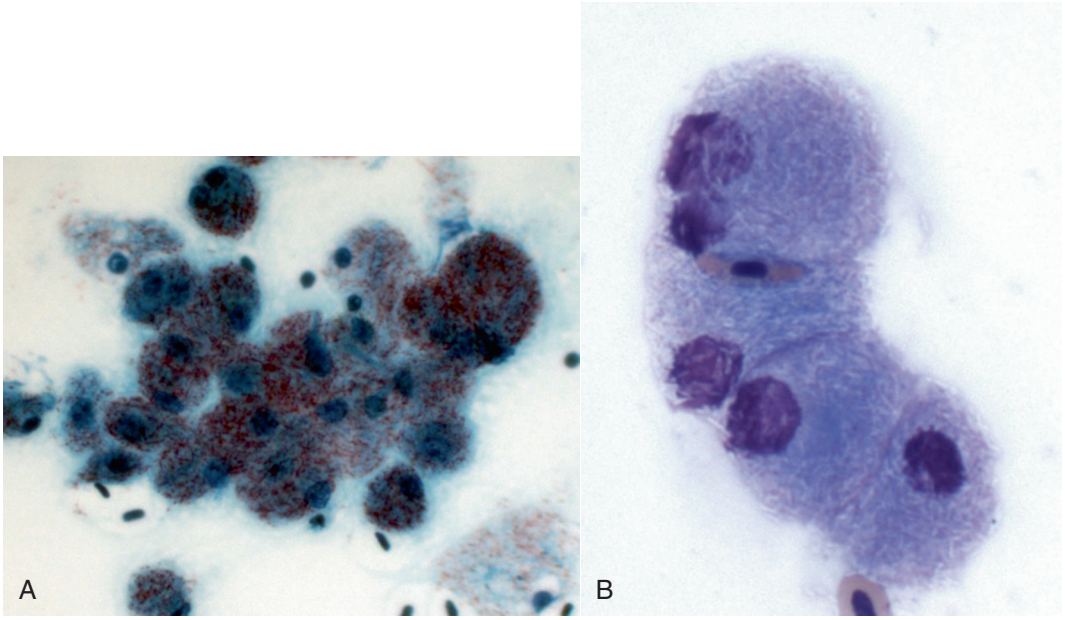

FIGURE 2-38 Specimens from a red-lored Amazon *(Amazona autumnalis)*. **A,** Cytologic preparations of an intestinal aspirate demonstrating macrophages with acid-fast positive bacteria in the cytoplasm. Stain: Acid-fast blue. Objective 40×. **B,** Cytologic preparations of intestinal aspirate demonstrating macrophages with nonstaining rod-shaped bacteria in the cytoplasm. Stain May-Grunwald Giemsa. Oil Objective 100×. (Courtesy Dr. Drury Reavill.)

chain reaction (PCR) and culture and sensitivity of feces and target tissues. It should be noted that intradermal tuberculosis testing, as used in humans, correlates poorly with the presence of disease in psittacines.[1] PCR testing of specimens is the most useful diagnostic test both for confirmation of infection and for speciation of the organism in question, which, again, is a critical consideration when considering treatment. Samples include feces and fresh tissue specimens. PCR can detect very low numbers of organisms and provide rapid results. Many diagnostic laboratories now offer PCR for mycobacterial organisms in exotic species (Table 2-9). Some laboratories may have the ability to perform PCR on paraffin block tissues prepared from histopathology submissions, but many cannot. Therefore, practitioners should develop the habit of collecting, saving, and freezing additional biopsy tissue samples.

Culture of avian tissue and fecal samples for mycobacterial organisms is difficult. *M. avium* may require 1 to 6 months to grow, and *M. genavense* does not grow on conventional mycobacterial media.[1] Some human diagnostic laboratories are willing to provide culture and sensitivity of animal samples, which may guide treatment decisions (see Table 2-9). A study of Japanese quail experimentally inoculated with *M. avium* demonstrated that culture and PCR of target tissue samples were much more sensitive than either method used to detect organisms in fecal samples, respectively.[17]

TABLE 2-9

Specific Diagnostic Testing for Mycobacteriosis

Mycobacterium sp.: PCR	Research Associates Laboratory www.vetdna.com
Mycobacterium sp.: PCR	Veterinary Molecular Diagnostics www.vmdl.com
Mycobacterium sp.: PCR	Washington State University Animal Disease Diagnostic Laboratory www.waddl.vetmed.wsu.edu
Mycobacterium sp.: PCR *Mycobacterium* sp.: Culture and sensitivity	National Jewish Medical and Research Center, Denver, CO www.testmenu.com/NationalJewish Contact laboratory for submission instructions and availability

PCR, Polymerase chain reaction.

ZOONOTIC CONSIDERATIONS

Human infection with *M. tuberculosis* is of serious concern, and there are concerns worldwide about organisms resistant to some of the drugs used for treatment of this disease in humans. For these reasons, most experts recommend euthanasia of birds that are confirmed as being actively infected with *M. tuberculosis*. Despite this caution, a single case of successful treatment of a pet bird with *M. tuberculosis* has been described.[18] Atypical mycobacteriosis, however, is extremely rare in humans with normal, competent immune systems. In these cases, infection is often traced to overwhelming exposure, such as inhalation of organisms in water vapor from an improperly sanitized hot tub or spa. One recent report described 36 cases of "hot tub lung" and a single case of hypersensitivity pneumonitis reaction to *M. avium* in household water, likely acquired during routine showering.[6,19] Treatment in these cases was successful.

In contrast, atypical mycobacteriosis is common in immunocompromised human patients. Prior to the acquired

immune deficiency syndrome (AIDS) epidemic (pre-1981), infections with *M. avium* complex were actually rare, with an estimated 3000 cases occurring worldwide per year. Most cases involved patients having undergone organ transplantation or suffering from hairy cell leukemia. Current recommendations for patients with AIDS include preventive treatment for atypical mycobacteriosis. Without preventive treatment, approximately 40% of patients with AIDS will eventually develop *M. avium* complex infections. In the early 1990s, *M. genavense* was also recognized as a cause of mycobacteriosis in patients with AIDS, although with much less frequency than *M. avium* complex.[20]

In humans, the source of atypical mycobacterial infection is most likely environmental, as organisms are common in food, water, and soil.[21] Humans with disseminated disease caused by *M. avium* complex and *M. genavense* typically have heavy infection of the GI tract, suggesting ingestion of the organisms as the primary route of exposure. Results of one study demonstrated DNA of *M. genavense* present in 25% of intestinal biopsy samples collected from patients without human immunodeficiency virus (HIV) infection.[22]

For these reasons, mycobacteriosis in pet birds caused by *M. avium* complex or *M. genavense* are unlikely to be a health risk to humans with normal immune systems. However, persons with HIV infection or other diseases impacting the immune system are likely at increased risk, especially when CD4 T-lymphocyte counts drop below normal.[21,23,24]

It is interesting to note that U.S. Government-sponsored publications, including the *Guidelines for the Prevention of Opportunistic Infections in Persons Infected with Human Immunodeficiency Virus*, published by the U.S. Public Health Service/Infectious Diseases Society of America (USPHS/IDSA) and similar publications from the CDC do not recommend avoidance of birds for prevention of mycobacteriosis to normal or immunocompromised persons. [25]

TREATMENT OF MYCOBACTERIOSIS

Both treatment successes and failures have been reported.[3,26,27] Prior to considering treatment, the organisms should be positively identified in order to rule out cases of *M. tuberculosis* and to help guide treatment decisions for birds with atypical mycobacteriosis. Birds with confirmed atypical mycobacteriosis should be treated on a case-by-case basis, keeping in mind overall condition and likelihood of treatment success, with input from the owner's physician. It should be kept in mind that treatment requires daily administration of a combination of medications for a year or longer, which may be difficult for many owners. Treatment with a single agent and incomplete or sporadic treatment of mycobacterial infections are both linked to development of resistant organisms and should be discouraged.[1]

Reviews of outcomes of large numbers of birds treated for mycobacteriosis with specific drug combinations at specific dosages are unavailable. Therefore, treatment is based on drug combinations used in humans and other animals for similar species. Without the benefit of culture and sensitivity (which is available but difficult and expensive), the practitioner is advised to research medications currently used for similar organisms in human patients. Drugs reported used for mycobacteriosis in birds include enrofloxacin, rifampin, ethambutol, clarithromycin, and others. Drug dosages are entirely extrapolated from other species (Table 2-10).

Publications in human medicine report that clarithromycin and ethambutol (with or without rifampicin) are commonly used to treat pulmonary *M. avium* complex in humans.[28] No known toxicities have been reported with the use of these drugs in psittacine birds.

The author has found improved owner compliance when selected antimycobacterial drugs are prepared by a compounding pharmacy in the smallest volume dose possible and mixed with powdered sugar. Food is removed the night before and the dose offered to the bird sprinkled on a small amount of a favorite moist table food. No other food is offered until the entire dose is consumed. Many birds require several weeks of "practice" before learning to eat the entire dose. However, birds accustomed to a single food item (e.g., seeds only) may be difficult to reliably medicate. One budgerigar refused to eat the powdered sugar. For this patient, medications were compounded in liquid form, and the bird was toweled and medicated daily. The owner admitted compliance was irregular with this method. Operant conditioning, where these patients undergoing long-term treatment can be trained to participate in their medication process can greatly facilitate treatment.

FOLLOW-UP

The diagnosis of disease can be challenging and so is judging response to therapy, especially when diagnosis was based on biopsy of an organ such as the liver. Clinical abnormalities may appear to resolve, and supportive clinical evidence such as changes in the hemogram may show marked improvement. Birds with GI forms of the disease may be screened for the presence of acid-fast bacteria; however, it should be kept in mind that shedding of mycobacterial organisms is sporadic.

In human medicine, patients are generally treated for a year or more, and in the case of humans with pulmonary disease, patients are released from treatment after being determined to be "culture negative" for 1 year. In the author's experience,

TABLE 2-10

Published Dosages of Antimycobacterial Drugs Used in the Treatment of Mycobacteriosis in Humans and Birds

	Dosage (q24h)	
Drug	**Human Pediatric**	**Psittacine Bird**
Clarithromycin	7.5–15 mg/kg	60 mg/kg
Clofazamine	1–2 mg/kg	6 mg/kg
Ciprofloxacin	10–15 mg/kg	80 mg/kg
Ethambutol	10–15 mg/kg	15–30 mg/kg
Isoniazid	10–20 mg/kg	30 mg/kg
Rifabutin		15–45 mg/kg
Rifampin	10 mg/kg	10–45 mg/kg
Streptomycin	20–40 mg/kg	20–40 mg/kg

Mg/kg, Milligrams per kilogram; q24h, every 24 hours.

treatment is continued for 1 year, and discontinued when the result of a follow-up biopsy of the target organ is negative; however, it should be kept in mind that a single biopsy can miss lesions. All patients finishing treatment for mycobacteriosis should be monitored carefully for any evidence of return of clinical signs.

CONCLUSION

Mycobacteriosis is an uncommon but well-recognized disease in companion psittacine birds. Molecular diagnostic techniques have improved the ability to confirm this disease in pet birds. Studies on efficacious therapeutic protocols in humans, and case reports of successful treatment in psittacine birds provide the avian practitioner with realistic treatment options. Although current research indicates that mycobacteriosis in psittacine birds is unlikely to represent a significant zoonotic risk, the potential risk cannot be ignored, particularly in the case of pet owners who may be immunocompromised.

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USUTU VIRUS

Johannes Thomas Lumeij

Usutu virus (USUV) is a mosquito-borne Flavivirus of African origin and belongs to the Japanese virus encephalitis group. Since it was identified as a cause of significant mortality in European blackbirds *(Turdus merula)* in Vienna in 2001, and because of the similarity to other members of the Japanese encephalitis antigenic complex, such as West Nile virus (WNV), it has received increased attention as an emerging epornitic with a zoonotic potential. The emergence and spread of USUV from Central Europe is well documented. So far, the risk for wild avian populations

seems mainly limited to USUV-naïve European blackbirds, while of the captive populations, some species of USUV-naïve Strigiformes seem to be predisposed. The risk to humans seems limited to immunocompromised individuals. In healthy individuals, USUV may lead to a benign skin rash or seroconversion. The potential to spread to other than the African or Eurasian continents is unknown, but considering the similarity to WNV, this seems plausible.

THE VIRUS

Taxonomy and Description

The species USUV belongs to the family Flaviviridae and the genus *Flavivirus*. Other genera within the family Flaviviridae include *Hepacivirus* (hepatitis C virus) and *Pestivirus* (bovine viral diarrhea virus, border disease virus, and classic swine fever virus). The genus name *Flavivirus* is derived from the Latin word *flavus* (yellow) and refers to the jaundice seen in humans infected with one of the representatives of this group: the Yellow fever virus (YFV). Flaviviruses are single-stranded, enveloped ribonucleic acid (RNA) viruses of 40 to 65 nanometers (nm), with an icosahedral nucleocapsid. The positive-sense, single-stranded RNA of USUV contains approximately 11,000 bases. The entire *Flavivirus* genome is translated into a single polyprotein of 3400 amino acids. The USUV polyprotein contains three structural proteins, the capsid protein (C), the precursor membrane protein/membrane protein (prM/M) and the envelope protein (E), and seven nonstructural (NS) proteins with a regulatory function. The entire polyprotein is later cleaved into its components. In contrast to the alphaviruses, this process starts with the structural proteins in flaviviruses.[1,2] More than 70 different species of *Flavivirus* have been reported, some vectorborne and others without known vectors. Classification is primarily based on the type (or absence) of vector, and further classification is based on antigenic cross-reactivity.[3] Phylogenetic relationships have proven to match this antigenic based classification.[4,5] Those flaviviruses that are transmitted by bite from tick or mosquito had historically been classified together with alphaviruses and some other virus families as arboviruses (arthropod-borne). Well-known serocomplexes are the tick-borne encephalitis group, including tickborne encephalitis virus (TBEV or FSMEV from German: Frühsommer Meningoenzephalitis Virus) and louping ill virus (LIV), the Yellow fever virus (YFV) group, the Dengue virus (DENV) group, and the Japanese encephalitis virus (JEV) serocomplex. Human infections with arboviruses are mostly incidental; humans are dead-end hosts because the virus replication is insufficient to reinfect the arthropods and continue the infectious cycle. Examples of exceptions are DENV and YFV. These viruses are so well adapted to the human host that an urban human–mosquito cycle without an animal host is possible, in contrast to the sylvatic cycle, in which monkeys are the reservoir host. The JEV serocomplex of flaviviruses has 11 representatives, of which JEV, Murray valley encephalitis virus (MVEV), St. Louis encephalitis virus (SLEV), and WNV are currently of most concern to humans. All members of this group have a bird–mosquito transmission cycle, and mammals are dead-end hosts. An exception to this is JEV, for which domestic pigs also serve as amplifying hosts. The subject of this section, USUV, is also a member of the JEV serocomplex but has hitherto only caused disease in a limited number of humans, whereas large-scale bird mortality seems associated with infections of virus-naïve avian populations. Alphaviruses are slightly larger (40 to 75 nm) than flaviviruses and differ in the organization of their genomes and protein synthesis. Examples of alphaviruses include Sindbis virus and Western-, Eastern-, and Venezuelan equine encephalitis viruses (SV, WEEV, EEEV, and VEEV).

Physical and Chemical Properties

Flaviviruses are inactivated by drying, organic solvents, low pH, and proteases. Procedures such as those applied to the production of plasma products that inactivate other flaviviruses (e.g., the use of solvents and detergents) are likely to be effective against USUV as well.[2] There are no specific studies on the stability of USUV.

Biologic Properties

Susceptible species

Apart from a wide range of avian species that can be affected by USUV, clinical infections have also been reported in humans. In some bird populations that have not been exposed to USUV infections previously, infections can lead to significant mortality. In humans, there have only been a limited number of documented cases of infections leading to clinical signs. The common (European) blackbird *(Turdus merula)* is the most widely reported affected species in areas where USUV has been recently introduced. Captive great gray owls *(Strix nebulosa)* were also commonly affected.[6,7] In Switzerland, the house sparrow *(Passer domesticus)* was commonly affected.[8] So far, clinical disease in birds seems mainly limited to representatives of the orders Passeriformes and (captive) Strigiformes. Apart from clinical infections in the aforementioned avian species, a number of avian species have shown seroconversion without clinical signs, notably rock pigeons *(Columba livia)*, mallards *(Anas platyrhynchos)*, and magpies *(Pica pica)*.[9]

The prevalence of USUV infection in specific avian species can be dependent not only on the virulence of the virus strain and host susceptibility but also on the innate preference by the mosquito vector for certain host species. Preference trials with adult female *Culex pipiens*, relating to the epidemiology of WNV in the United States, have shown that host-seeking *Cx. pipiens* were three times more likely to enter the American robin *(Turdus migratorius)*–baited traps compared with traps baited with the sympatric European starling *(Sturnus vulgaris)*.[10]

The mosquito–avian transmission cycle is dependent on the presence of an ornithophilic mosquito vector, in which virus replication and dissemination occurs to such a degree that virus transmission through infected saliva is possible. USUV has been isolated from a large variety of African mosquito species, including *Cx. perfuscus*, *Cx. quinquefasciatus*, *Aedes (Aedimorphus) minutus*, *Mansonia africana*, and *Coquillettidia aurites*,[11] but their vector role is unknown. Results from studies on *Cx. neavei* from Senegal strongly suggest this species acts as a vector for USUV.[12] The low abundance of *Cx. neavei* in inhabited areas in combination with the low anthropophily has been mentioned as a possible explanation for the rarity of reported USUV infections in humans in Africa. In Europe, USUV isolations have been reported mainly from

Cx. pipiens but also from *A. albopictus*.[13,14] For human infections to occur, the feeding pattern of the mosquitoes must be more opportunistic to enable the mosquitoes to act as bridge vectors between the avian populations, in which the infection is enzootic, and the human dead-end host. In Europe, *Cx. pipiens* is considered the most important vector. The *Cx. pipiens* taxonomic complex contains two distinct biotypes: (1) *pipiens* and (2) *molestus*. Although they are morphologically indistinguishable, they differ in physiology and behavior. *Cx. pipiens* requires a blood meal for each batch of eggs (anautogenous), is seasonally active and mainly ornithophilic, whereas *Cx. molestus* is autogenous, active throughout the year and mammophilic, especially anthrophilic.[15] In the northeastern United States, a large proportion of *Cx. pipiens* complex has been found to be a hybrid between both biotypes. These hybrids have lost their host specificity and have become opportunistic feeders, feeding both on birds and on humans. In Europe, the biotype *pipiens* is considered the most widespread, whereas the biotype *molestus* is only known from underground breeding sites. Hybridization between the biotypes has so far been reported only in a restricted number of places. It has been claimed that these opportunistic *Cx. pipiens* hybrids may have served as an important bridge between the avian and human populations and may have had a major contribution to the emergence and spread of WNV in the United States.[16] Likewise, *Cx. pipiens* hybrids might play a role in the transmission of USUV in Europe from birds to humans.

Cell cultures

Flaviviruses can be grown in both vertebrate and mosquito cell lines, but the susceptibility of cell cultures from different species varies.[17] Even cell lines from the same organ of a particular species may show differences in susceptibility. Often, multiplication of flaviviruses in cell lines does not result in alteration of the macromolecular structure, resulting in a persistent infection without a cytopathogenic effect (CPE). From a study conducted on vertebrate cell lines, it was concluded that the most appropriate cell lines for isolation and plaque reduction test for USUV are from the green monkey (Vero), the porcine kidney (PK15), and the goose embryo fibroblast (GEF). Although one would expect that avian cell lines are more susceptible to USUV than mammalian cell lines, it was found that chicken embryo fibroblast monolayers and chicken embryos seem resistant to USUV infection.[17] USUV from pooled trapped mosquitoes was successfully propagated with accompanying CPE in an *A. albopictus* C6/36 mosquito cell line.[18] Three human cell lines (human long adenocarcinoma epithelial A549, human epidermoid larynx carcinoma Hep-2, and human epidermoid oral carcinoma KB) were susceptible to USUV infection and developed a clear-cut CPE, comparable with that produced in Vero cells.[19]

Antigenic properties

The lipid bilayered viral envelope of Flaviviridae is composed of two proteins, of which protein E is the primary target of the immune response of the host. Protein E is the aspecific viral hemagglutinin responsible for seroconversion of infected individuals. The strong cross-reactivity of hemagglutinating antibodies to the various species within the Flavivirus family is a characteristic on which the various flaviviruses have been classified and it should come to no surprise therefore that it is impossible to make a species-specific diagnosis. A clinical diagnosis of acute *Flavivirus* infection, however, can be helpful to a clinician. Apart from the envelope glycoprotein, flaviviruses have other antigenic components and antibodies against the prM and NS1 proteins have also been reported.[20-23] Maternal antibodies from adult birds are transferred to their offspring through the egg yolk, where the antibody is absorbed and enters the circulatory system. USUV maternal antibodies were detectable up to 2 months in Ural owls *(Strix uralensis)*.[24] Cross-reacting antibodies play an important role for both serologic diagnosis and the protection of individuals or populations, but our limited understanding of the inconsistent relationships within and between the various serocomplexes complicates serologic diagnosis and the prediction of protection.[25]

OCCURRENCE

History

USUV was first isolated in 1959 in South Africa by B.R. McIntosh from *Cx. neavei* (originally classified as *Cx. univittatus*, but renamed in 1971[26]) during a study on the prevalence of viruses in mosquito species. It was named after the Usutu river in Swaziland.[27] This South African reference strain is identified as SAAr 1776. Additional isolates were found in the following years in a variety of mosquitoes from a variety of African countries. Mosquito species involved include *Cx. perfusus*, *Mansonia africanus*, *M. autites*, and *A. minutus*. Apart from a report of USUV from a human patient in 1981 in the Central African Republic, followed by a second case in 2004 from Burkina Faso, USUV has been largely ignored by the scientific community, until it emerged in 2001 in central Europe as a cause of avian mortality, especially of Eurasian blackbirds, *Turdus merula*, and great gray owls, *Strix nebulosa*.[28] Later studies have shown the presence of USUV in a variety of other avian species in neighboring countries. Retrospective analysis of archived tissue samples from bird deaths in the Tuscany region of Italy in 1996[29] identified USUV. Partial sequencing confirmed identity with the 2001 Vienna strain and provided evidence for at least a 5-year earlier introduction of USUV into Europe than previously assumed.[7] Currently, USUV is considered enzootic in central Europe, with a mosquito–bird transmission cycle and the potential to spread to other geographic areas. The zoonotic potential in healthy human subjects seems to be limited to a transient skin rash, with possibly a more severe clinical course in immunocompromised patients. The first human cases outside Africa were seen in immunocompromised patients in Italy. These patients showed meningoencephalitis.[30,31] Seroepidemiologic studies in humans indicate that USUV can be endemic in certain areas with no signs of disease in infected people.

Geographic Range

USUV has been reported in a number of African countries, including Burkina Faso, Cote d'Ivoire, Uganda, Nigeria, Senegal, Morocco, and the Central African Republic. After its first documented European appearance in Italy in 1996,[7]

USUV-associated clinical disease in birds has been reported from Austria,[6] Hungary,[32] Switzerland,[8] Czech Republic,[33] Spain,[34-36] Germany,[37,38] and Belgium.[39] Neutralizing antibodies to USUV have also been found in birds from the United Kingdom,[40,41] Poland,[42] and Greece (Figure 2-39).[43] Considering the erratic results seen with *Flavivirus* serology, final conclusions about the presence or absence of USUV in these latter countries can be drawn after more specific confirmatory tests. There is also insufficient information to evaluate the USUV seropositive samples in the period from 2001 to 2005 from Germany. Seroconversion in horses[44] and neuroinvasive disease in humans have been reported from Croatia.[45] Phylogenetic analysis of the currently known complete USUV genome sequences from Africa, central Europe, and Spain has revealed that at least three distinct genetic clusters circulate in Europe.[46] USUV strains isolated from Africa showed an even greater genetic diversity.[47] The two distinct clusters circulating in Spain, which seem to differ in their virulence for avian hosts, are most likely independently introduced by migratory birds from Africa, whereas the central European cluster seems to be an independent introduction.[46] Seroconversion without clinical disease in birds from the British Isles, Poland, and Greece might indicate that the geographic distribution of USUV in Europe is wider than currently realized. However, the limitations of only serologic diagnosis should be kept in mind. A more definitive proof from these countries, in the form of virus isolation and identification, would be more convincing.

CLINICAL FINDINGS

In contrast to a number of avian species that seem highly susceptible to USUV infections and may show clinical signs, a wide range of avian species seems to show only seroconversion after infection with USUV without showing any clinical signs. Clinical signs may vary from nonspecific (immobility, ruffled plumage, half-closed eyes, and anorexia) to neurologic signs such as depression, ataxia, jerky movements, torticollis, and nystagmus, followed by mortality. A poor nutritional status has been reported in wild birds, whereas the nutritional status of captive birds was more variable.[8] This might be related to the more protected captive environment, where food is freely available to the affected individuals.

In humans, infections often go unnoticed, as can be concluded from finding neutralizing antibodies against USUV in clinically healthy blood donors from an endemic region in Italy[48,49] and from a blood donor in southwestern Germany.[50] In Austria, 52 out of 203 individuals from an endemic area who had developed a skin rash of unknown cause were seropositive against USUV, as concluded after finding neutralizing antibodies. In one of these patients, the USUV genome was detected by using PCR.[51] A more serious neuroinvasive form of USUV infection has been seen in two immunocompromised humans.

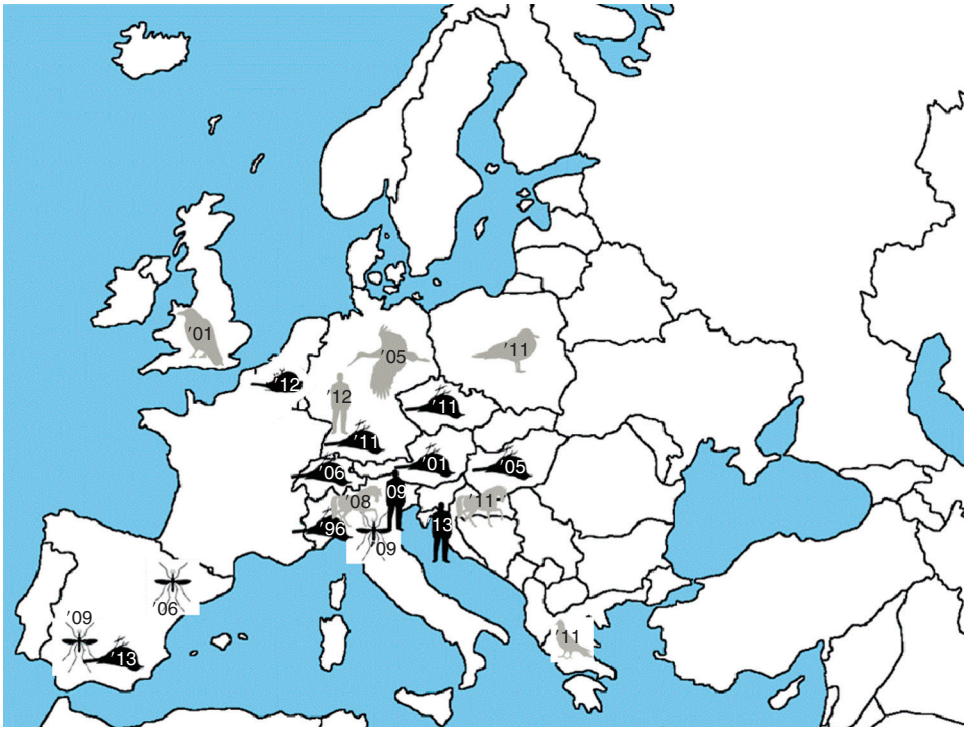

FIGURE 2-39 Usutu virus in Europe. Solid black silhouettes indicate clinical disease (in immunocompromised humans), USUV confirmed mortality (birds), or virus isolation (mosquitoes). The two USUV strains circulating in Spain are different from the Central European strain, which seems to have spread from Italy since 1996. Gray silhouettes indicate seroconversion only (birds, horses, or humans) and possible USUV circulation. Flavivirus serologic results should be interpreted with caution. The numbers indicate the year in which first occurrence was demonstrated.

PATHOLOGIC FINDINGS

Birds that have died from USUV infection may be in good body condition without obvious gross pathologic lesions, indicating peracute mortality. Splenomegaly, hepatomegaly, and pulmonary hyperemia may be seen in affected animals. Histopathologic examination of the cortex and brainstem may reveal multifocal neuronal degeneration and perineuronal clustering of glial cells. Cerebellar lesions may include degeneration of Purkinje cells, formation of glial shrubberies, lymphoplasmacytic perivascular cuffs, and mild degeneration and necrosis at the molecular–granular layer interface (Figure 2-40). A miliary pattern of liver necrosis and a scattered cellular necrosis may be seen in the myocardium (Figures 2-41 and 2-42).[52] Paraffin-embedded tissue can be processed for immunohistochemical staining by using rabbit USUV-specific antibody (Figure 2-43). However, with immunohistochemistry (IHC), there is still the potential for cross-reactivity with other flaviviruses. In situ hybridization (ISH) is based on the complementary pairing of labeled deoxyribonucleic acid (DNA) or RNA probes with USUV-specific nucleic acid sequences in tissue sections (Figure 2-44).

DIAGNOSTIC ASPECTS

Flavivirus infections cause a short-lived viremia of maximum 2 days. A serologic response follows after the viremic stage. Circulating immunoglobulin M (IgM) is produced within 6 days, followed by IgG. Identification of specific immunoglobulin M and seroconversion to IgG or a fourfold rise in titer between acute and convalescent sera taken 10 days apart indicate an acute infection.[1,2] The possibility of false-positive results should be borne in mind, as was demonstrated with IgM antibodies against WNV.[53,54] Individuals that have shown acute mortality are poor candidates for serologic diagnosis because they did not have enough time to seroconvert. Less vulnerable species are likely to show no clinical signs of disease and to still seroconvert, thereby providing an opportunity for serologic screening of populations.

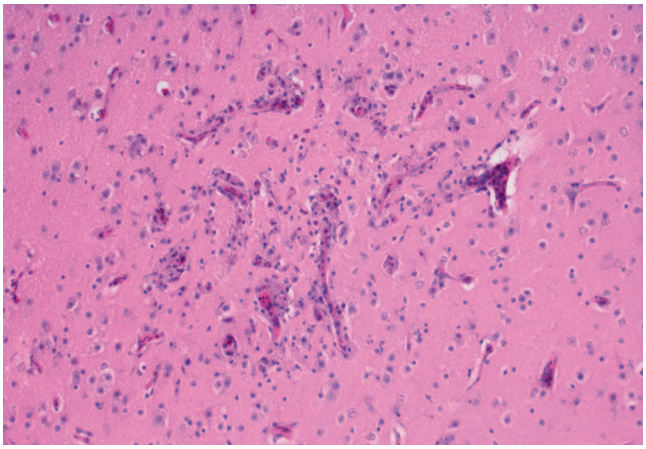

FIGURE 2-40 Nonsuppurative encephalitis in a case of Usutu virus infection, blackbird *(Turdus merula)*. Hematoxylin and eosin stain. Original magnification 203. (Courtesy Herbert Weissenböck, DVM, University of Veterinary Medicine, Vienna.)

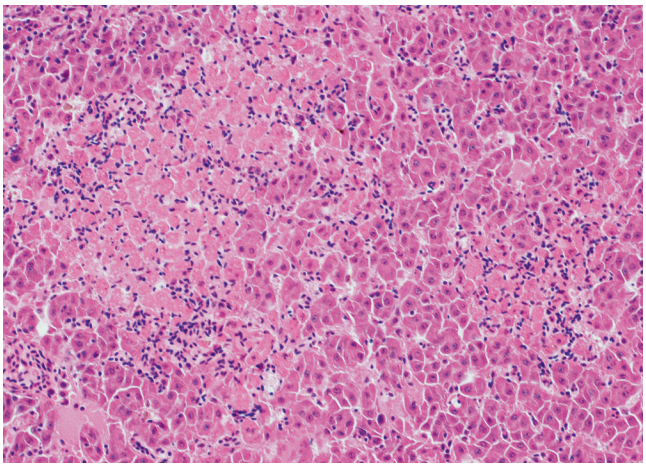

FIGURE 2-41 Irregularly demarcated liver necrosis in a case of Usutu virus infection, blackbird *(Turdus merula)*. Hematoxylin and eosin stain. Original magnification 20×. (Courtesy Herbert Weissenböck, DVM, University of Veterinary Medicine, Vienna.)

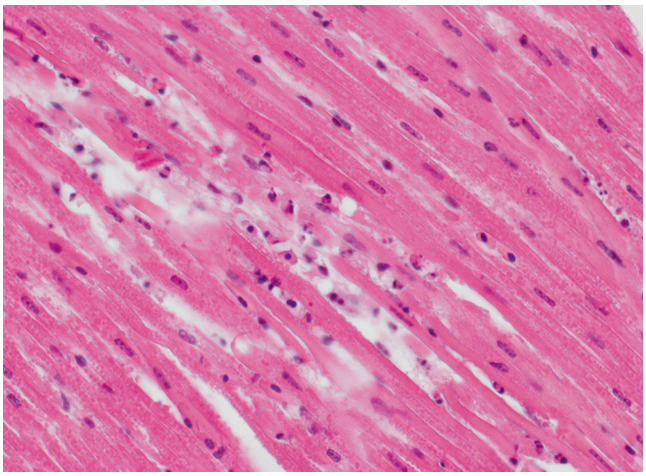

FIGURE 2-42 Focal necrosis of myocardial fibers in a case of Usutu virus infection, blackbird *(Turdus merula)*. Hematoxylin and eosin stain. Original magnification 40×. (Courtesy Herbert Weissenböck, DVM, University of Veterinary Medicine, Vienna.)

For initial serologic screening of populations for USUV, the hemagglutination inhibition test (HIT) can be used.[55,56] This test is not considered specific. Cross-reactions with other flaviviruses such as TBEV and WNV do occur, and distinction between USUV infections and other flavivirus infections are difficult. For confirmation of suspected cases, the more specific plaque reduction neutralization test (PRNT) can be used.[57] However, although the PRNT is considered the gold standard within the Flaviviridae family, cross-reactivity also occurs with the PRNT. All sera from a human cohort vaccinated with JEV and TBEV caused neutralization of LIV, and some sera also neutralized WNV, which was enhanced by YFV vaccination.[58] The specificity of the PRNT using WNV and USUV test sera has been investigated recently by using the USUV strain Blackbird Vienna 2001 and WNV topotype strain Eg-101. Cross-reactivity only occurred in sera with high titers to one of the viruses, to a titer of at least four dilutions steps less than the homolog

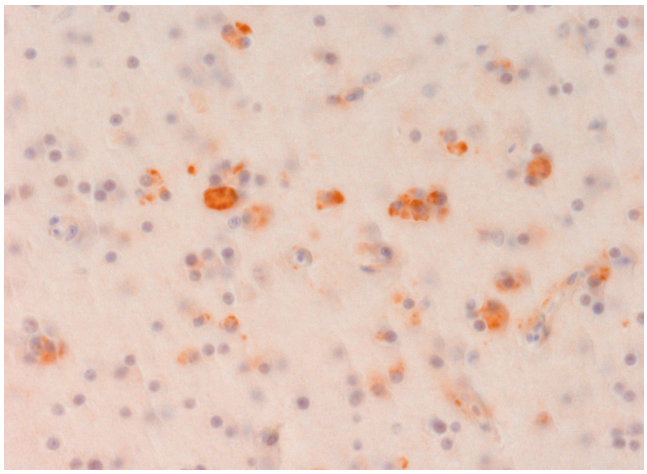

FIGURE 2-43 Usutu virus antigen demonstrated by immunohistochemistry in the cytoplasm of neurons and glial cells in the brain of a blackbird *(Turdus merula)*. Original magnification 40×. (Courtesy Herbert Weissenböck, DVM, University of Veterinary Medicine, Vienna.)

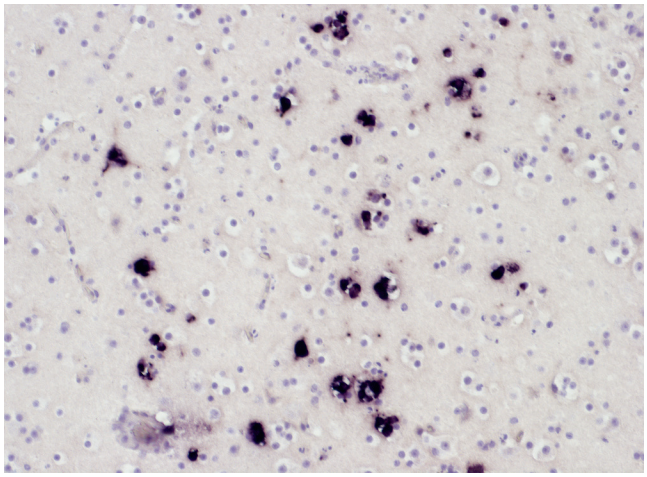

FIGURE 2-44 Usutu virus nucleic acid demonstrated by in situ hybridization within neurons and glial cells in the brain of a blackbird *(Turdus merula)*. Original magnification 20×. (Courtesy Herbert Weissenböck, DVM, University of Veterinary Medicine, Vienna.)

virus. Considering the broad antigenic cross-reactivity between different flaviviruses and therefore the erratic and unpredictable results from *Flavivirus* serology, other strains might yield different findings. Diagnostic experience with WNV has shown that a PRNT must be evaluated by testing neutralizing antibodies against a panel of related viruses. Based on experiences with WNV and considering the diversity and constant evolution of circulating strains, the choice of reference strains should include recent isolates that are known to circulate in the region, and the use of old strains should be avoided. Technical variations between different laboratories might also contribute to variations observed.[59]

The most reliable method to establish a causative diagnosis is reverse transcriptase (RT)-PCR. USUV-specific amplicons can be amplified by RT-PCR on unfixed or formalin-fixed paraffin-embedded brain tissue, and RT-PCR amplification products can be sequenced and compared with already known USUV sequences available in Genbank.[7,60]

DIFFERENTIAL DIAGNOSIS

The most important differential diagnosis, both clinically and serologically, is an infection with another virus of the Japanese encephalitis complex. Apart from cross-reactions with other flaviviruses, nonspecific reactions may also occur.[53,54] Because flaviviruses might share the same vector, simultaneous infections with various viruses might occur, as has been shown for USUV and WNV. For this reason, it is important to rule out exposure to other flaviviruses through natural contact (travel history to *Flavivirus* endemic areas in humans; migratory patterns in birds) or vaccination status (YFV, TBEV, JEV) and to always use specific confirmatory tests.

EPIZOOTIOLOGY

Like WNV, USUV spreads in cycles between ornithophilic mosquito vectors and an avian reservoir host. Once the appropriate vector becomes infected, other susceptible hosts such as humans can be infected. They act as dead-end hosts, since viral replication is insufficient to cause reinfection of another vector. Humans, however, may transmit arboviruses through blood transfusions. Documentation of two cases of blood transfusion–induced WNV infection in humans has led to screening of blood donors for WNV and rejecting donors who have fever and headache during the week of blood donation. For USUV, blood transfusion–induced infection in humans has not been proven. Despite high arboviral titers in reservoir hosts, infections may remain subclinical.[61] For *Flavivirus* enzootics to occur, there must be a sufficient number of specific vectors and susceptible reservoir hosts.[62] Seasonal differences in prevalence are associated with climatic conditions that influence overwintering of vectors and migration patterns of reservoir hosts. Migration of birds may be a factor in reintroduction of new viruses in a specific region or spreading to new regions. Longitudinal studies with USUV in Austria in the period 2003–2006 have shown a year-to-year decrease in USUV-associated wild bird mortality since the initial outbreak in 2001, which was associated with an increasing proportion of seropositive wild birds. It has been hypothesized that the apparent disappearance of USUV-associated avian mortality in Austria can be explained by the rapid establishment of resident flock immunity. Other possible factors are climatic factors and decreased virulence. It has been shown, for example, that in American crows, the virulence of WNV is strain dependent.[63] The high percentage of seropositive birds (>50%) to a circulating arbovirus with an avian–mosquito transmission cycle, as observed by Meister et al,[24] has not been seen in other enzootic transmission cycles of flaviviruses so far and awaits further explanation.

Most of the European USUV epornitics have occurred in urban areas. It has been speculated that the presence of supervised avian collections or bird sanctuaries in urban areas would enhance the detection of new cases, whereas the abundance of predators in the wild would explain the absence of detection of outbreaks in rural areas. However, when the spatial distribution of blackbird mortality in Austria was plotted against the average number of hot days per year, a correlation between

local environmental temperature and blackbird mortality became clear.[64] The emergence of USUV infections was mainly related to those regions around Vienna, where the number of hot days exceeds 10 days per year. This region of the district Lower Austria has the densest human population. Apart from the heat island effect of urban areas, it has been shown that urbanization favors the propagation of *Cx. pipiens* by the proliferation of artificial container habitats.[65] Through mathematic modelling, Rubel et al explained the multiseasonal dynamics of USUV infections in Austria.[66] In this model, the seasonal dynamics of mosquitoes and birds and the density-dependent avian–mosquito infection cycle was considered. USUV dynamics were mainly determined by the interaction between bird immunity and environmental temperature. Higher temperatures increase the reproduction and biting rate and decrease the extrinsic incubation period of mosquitoes. On the basis of this mathematic USUV avian–mosquito model, historical temperature recordings, as well as temperature predictions from global climate models from the Intergovernmental Panel on Climate Change (IPCC), both historical and future scenarios for R_0 were calculated. When R_0 is less than 1, the infection will die out in the long run, but if R_0 is greater than 1, the infection will be able to spread in a population. From 1900 until 2100, R_0 increased for the worst-case IPCC scenario with 0.61 per 100 years and for the best-case scenario with 0.51 per 100 years. For both scenarios R_0 would be consistently larger than one from 2040 onward, whereas it never reached values greater than 1 before 2000. This means that on the basis of this model, it is unlikely that undetected outbreaks would have occurred before 2000, whereas from 2040 onward, the disease will be endemic in the area. For the future, it can be predicted that the disease will become endemic in Austria from 2040 onward, and new outbreaks will occur in immunologically naive avian populations in other European countries, mainly in Passeriformes (blackbirds and house sparrows) and Strigiformes. Outbreaks can be expected during the peak of the *Cx. pipiens* reproductive season between mid-July and mid-September. Urban areas are predisposed because they cause the heat island effect and offer a wide variety of breeding sites for *Cx. pipiens* in the form of stagnant water pools, varying from birds baths to disposed car tires. Urban areas are probably more likely to show benign human infections because these areas are more likely to harbor the aforementioned opportunistic feeding *Cx. pipiens* hybrids. USUV epornitics that do occur in rural areas might go unnoticed because bird carcasses disappear relatively quickly as a result of the abundance of avian or mammalian scavengers.[67]

PROPHYLAXIS

Surveillance

To monitor USUV circulation in a specific region, several approaches have been used, including the use of sentinel animals (horses and chickens), virologic examination of dead wild birds, serologic examination of backyard poultry flocks and mosquito collection, and identification and testing for USUV in pooled mosquito samples. Serologic examination of sentinel animals should be done twice a year, that is, before and after the mosquito season. Considering the host specificity of USUV within the order Passeriformes, a targeted surveillance with special attention for dead blackbirds and house sparrows might prove to be more cost effective than a random search for USUV among all avian species. Although rock pigeons *(Columba livia)* and mallards *(Anas platyrhynchos)* have not been reported to be clinically affected, they have proven to be useful species for serologic examination.[9] It is important to be aware of the close antigenic relationship between various flaviviruses and take appropriate precautions to enable differentiation between the *Flavivirus* species. With regard to the low susceptibility of chicken embryos to USUV infections, and the limited pathogenicity of USUV for chickens,[68] it is questionable whether backyard chickens are the most appropriate species to act as sentinel animals. Considering the findings of Savini et al, feral pigeons *(Columba livia)* might be a better choice.

Vaccination

There is currently no specific USUV vaccine available, and the need is questionable. Although there are human vaccines available for a number of flaviviruses, including JEV, TBEV, and YFV, and an equine vaccine for WNV and sera raised against one of these agents may cross-react in serologic tests with other flaviviruses, cross-protection against USUV has not yet been reported. The cross-reactive properties of flaviviruses are potentially useful for the development of a broad-spectrum vaccine for emerging flaviviruses. However, a worsening of disease symptoms through a possible antibody-dependent enhancement effect should be carefully evaluated.

Mosquito Prevention

Since outbreaks are related to the avian–mosquito transmission cycle, captive susceptible avian collections can be protected against USUV (and other arbovirus) infections by mosquito control. Prevention of stagnant pools of water (in flower pots, gutters, buckets, pool covers, water dishes, discarded tires, birdbaths) has been advocated as a valuable method to reduce the number of breeding sites for the mosquito vector of WNV. *Cx. pipiens* is a lazy flier, and the lack of breeding sites in the vicinity may reduce the number of mosquitoes considerably. Cyclopoid crustaceans[69] or aquatic vertebrates such as fish[70] or terrapines[71] may be used to reduce larvae in pools or holding tanks that cannot be drained. Indoor housing during the mosquito season of immunologically naive Passeriformes and Strigiformes, including young birds and new additions, has been suggested as a method to protect valuable avian collections.[8] Mosquito screens on windows and doors can help keep mosquitoes outside. Air conditioning during the critical months may be an effective alternative to natural ventilation for climate control. Ectoparasites might also contribute to the spread of the disease. Although it is unknown whether the virus can replicate outside the specific *Culex* mosquito vector, it seems a wise precaution to treat susceptible avian collections on a regular basis against ectoparasites.

In addition to the above, humans can protect themselves from *Cx. pipiens* bites by dressing in loose-fitting, long-sleeved clothes, staying indoors at dusk and dawn, and using a 50% solution of N,N-Diethyl-meta-toluamide (DEET) on the bare skin. DEET is considered the most effective repellent against *Cx. pipiens* outdoors during these feeding periods.

Although dying black birds are inherent to nature and in general giving a symbolic meaning to finding deceased black birds can be categorized under superstition, the USUV-induced blackbird mortality can be considered a bad omen to what the future will bring with regard to climate change–related vectorborne diseases. Since USUV epornitics outside its former tropical range are linked to climate change and global warming, the most important consideration in combatting this and other emerging arbovirus epidemics is a global reduction of greenhouse gases. In an attempt to reduce the heat island effect of urban areas, a long-term heat island reduction strategy of planting shade trees and increasing urban albedo by using light-colored, highly reflective roof and paving materials should be included in the plans of city planners, environmental managers, and other decision makers.[72,73]

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