Glasser's Disease of Swine Produced by the Intracheal Inoculation of Haemophilus suis

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SUMMARY

The intracheal inoculation of pigs with Haemophilus suis led to the production of Glasser's disease at every attempt without significant pulmonary involvement. Isolation of this organism from the experimental animals was possible only in the acute phase of the disease.

The indirect fluorescent antibody technique when applied to frozen sections of tissues obtained from the experimentally infected pigs at autopsy, revealed a few rod forms but mostly "round bodies" of H. suis in animals from which the organism was isolated, and "round bodies" only in the pigs from which the organism was not isolated.

Attention is drawn to the similarities between the lesions caused by H. suis and Mycoplasma hyorhinis, and to the confusion which may result therefrom. It is stressed that the laboratory diagnosis of these two diseases is complicated by the fact that both agents may not be isolated on the media commonly used in diagnostic laboratories. Both organisms necessitate the use of special media where the clinical and autopsy results indicate polyserositis and arthritis.

RESUME

Nous avons produit à plusieurs reprises les lésions de la maladie de Glasser (polysérosite séro-fibrineuse) par l'inoculation intratrachéale de porcs avec Haemophilus suis. L'inoculation par cette voie n'a pas donné de lésions pulmonaires. Il a été possible de réisoler l'agent causal en milieu de culture seulement durant la phase aigue de la maladie.

Nous avons utilisé la méthode de l'immunofluorescence indirecte pour déceler l'agent causal dans des coupes histologiques de tissus congelés. Cette méthode nous a permis de déceler dans les tissus de porcs positifs à la culture bactériologique quelques formes bacillaires mais surtout des formes sphériques de l'agent, tandis que chez les porcs négatifs on ne pouvait déceler que des formes sphériques.

Nous voulons souligner la similitude, donc la confusion possible entre les lésions produites par H. suis et Mycoplasma hyorhinis. Nous notons que la diagnostic de ces deux maladies en laboratoire, est compliqué du fait que ces bactéries ne se reproduisent pas sur les milieux bactériologiques usuels. Des milieux de culture spécifiques doivent être utilisés lorsque l'examen clinique et la nécropsie indiquent la polysérosite avec arthrite.

INTRODUCTION

A specific disease affecting swine mainly from 5-12 weeks of age and characterized by serofibrinous pleuritis, pericarditis, peritonitis and arthritis was described by Glasser (6). He observed bacteria in the serous exudate of affected pigs which stained well with aqueous methylene blue, were Gram-negative and non-acid-fast. Shanks (17) isolated *Haemophilus influenzae suis* (13) from affected pigs, but was unable to reproduce the disease experimentally. Hjarre and Wramby (7) isolated *H. influenzae suis* from cases of Glasser's disease and successfully reproduced it by inoculating pigs subcutaneously with the organism. Bakos *et al* (3) using large doses of *H. influenzae suis* isolated from cases of respiratory disease and Glasser's disease in swine, reproduced the latter by intraperitoneal inoculation. Sutherland and Simmons

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(18) and King (8) in Australia and Braend and Flatla (4) in Sweden reported on the isolation of the haemophilus species from cases of Glasser's disease in swine.

The following is a record of the experimental production of Glasser's disease in sow-reared specific pathogen free (S.P.F.) piglets by the intratracheal inoculation of a strain of *H. suis*[1], and the difficulty encountered in isolating the organism despite its demonstration in the exudate and tissues by the indirect fluorescent antibody technique (FAT).

MATERIALS AND METHODS

The Organism

A capsulated smooth (S) strain of *H. suis* was isolated from an eight week old pig which died in the acute febrile stage of Glasser's disease. The organism was maintained in Levinthal broth and was serially passaged on Levinthal agar plates eight times before lyophilization.

Culture Media

Dependency of the organism for growth on nicotinamide adenine dinucleotide (NAD) or the V factor, and on hemin or the X factor was tested using these two substances as additives to the proteose-peptone medium described by White *et al* (20).

The culture media used to examine the post mortem specimens were 3% sheep blood agar plates, with a staphylococcus streaked diametrically across the plate, Levinthal agar plates, trypticase soy broth (TSB) and Levinthal broth. All media except the blood agar had NAD added.

Preparation of Inocula for Experimental Infection

The bacterial inocula were prepared from 16 hour washed cultures on Levinthal agar plates suspended in a final volume of 4.0 ml of 0.01 M sodium phosphate buffered saline at pH 7.1 (PBS).

Experimental Infection

Sixteen sow reared S.P.F. piglets between six and eight weeks of age were allotted to four groups designated "Groups I, II, III and IV". In Group I the dosage given to pigs P1 and P2 was 3×10^{10} organisms; in Group II pigs P3-P8 received 3×10^{9} organisms. In Group III the five pigs P9-P13 received graded doses of 5×10^{8}, 5×10^{7}, 5×10^{6}, 5×10^{5} and 5×10^{4}, one to each pig. Two of the three control pigs in Group IV received sterile PBS and the other was uninoculated. All inoculations were performed by the intratracheal method described by L'Ecuyer (11).

Following exposure to infection, daily clinical examinations were made. Some pigs were allowed to die as a result of the infection whereas others were killed with intravenous pentobarbitone sodium. All the subjects were examined post mortem and specimens were collected in formal-saline for histological examination. Pleural, pericardial, peritoneal, synovial fluids and portions of viscera were collected aseptically for bacteriological examination. In the third group, frozen sections and impression smears of body fluids and viscera were made, then fixed in acetone at room temperature for 20 minutes for the indirect FAT.

Antiserum Production in Rabbits

Antiserum was prepared after the method of Alexander (1, 2) by harvesting six hour growths of our strain of *H. suis* from Levinthal agar plates in 0.5% formalin in PBS and storing it for 48 hours at 4.0°C before use. The antigen thus prepared was injected intravenously into adult New Zealand white rabbits with the dosage increasing from 0.1 to 2.0 ml for 12 doses at 72 hour intervals. The rabbits were bled 10 days after the last dose, and the serum separated.

Indirect FAT

The globulin fraction of the *H. suis* hyperimmune rabbit serum was precipitated with saturated ammonium sulphate solution, then redissolved in and dialysed against PBS at pH 7.1. The rabbit hyperimmune globulin after absorption with pig liver powder was used as the first stage of the indirect FAT (15), the second stage used fluorescein isothiocyanate conjugated goat anti-rabbit globulin[2]. The specificity of the technique was tested by using it on a wide variety of pathogens isolated from pigs including *Pasteurella spp.*, *Bordetella bronchiseptica* and another strain of *H. suis* isolated from the lungs of a pig with pneumonia.

The indirect FAT preparations were examined at 800 magnifications under oil

[1]We prefer to use Haemophilus suis as classified by Bergey's Manual of Determinative Bacteriology (7th Ed. 1957). See also reference to the taxonomy of this organism by Leidy et al (12).

[2]Difco Laboratories, Detroit, Michigan.

immersion using a Reichert "Zetopan" microscope with "Binolux" illumination apparatus consisting of a 30 watt tungsten filament, and a 200 watt mercury vapour ultra-violet (UV) source with a BG12 exciter filter and GG9 plus OG1 barrier filters. This apparatus allowed examination by anoptral contrast and fluorescence either separately or in combination (20% white plus 80% UV mixtures). A darkfield condenser was also employed for examination using both sources of illumination.

Preparations of Spheroplasts for Comparative Microscopy

Levinthal agar cultures of *H. suis* grown at 37°C for 24 hours were washed off the plates and washed twice with PBS. The organisms were suspended in PBS containing 0.34 m sucrose, Mg^{++} at 1.2 g per ml and 3% glycine, and were incubated for 2 days at 37°C. This treatment of the cells induced the formation of spheroplasts which were observed throughout the 48 hour period by anoptral contrast microscopy and FAT, and by attempts to grow these forms in Levinthal broth simultaneously. The purpose here was to compare these spheroplasts *in vitro* with similar morphological entities seen *in vivo*.

RESULTS

The strain of *H. suis* used in our experiments required NAD for growth, but was not dependent on hemin. All 13 pigs given this organism developed Glasser's disease.

The two pigs in Group I (3×10^{10} organisms) became sick within 24 hours, one died at 55 hours after inoculation and the other, 5 hours later. Both pigs had temperatures of approximately 107°F and were recumbent 48 hours post-inoculation. Autopsy revealed a typical serofibrinous pleuritis, pericarditis, peritonitis, and synovitis of the limb joints.

None of the six pigs in Group II (3×10^9 organisms) died. However, all were lame with swelling of the carpal and tarsal joints and adjacent tendon sheaths. All had temperatures of around 106°F from Day 5 to Day 8 after inoculation. One pig became recumbent and was killed on Day 6. At autopsy this pig was found to have lesions similar to those found in Group I, but joint involvement was confined to the carpi and tarsi only. These joints had swollen and hyperaemic synovial membranes and were filled with an excessive quantity of cloudy fluid. The five remaining pigs were lame for the two weeks when recovery took place. Two of these pigs became pyretic again on Day 13 with temperatures of 106.0° and 106.6°F respectively returning to normal in 24 hours. They were killed on Day 21, and all appeared normal at autopsy.

The graduated doses (5×10^4 to 5 to 10^8) administered in Group III gave rise to Glasser's-like disease in all five pigs. The pig receiving 5×10^6 organisms never had a temperature above the normal range, but became lame on Day 5 after inoculation and remained so until Day 13 when it was killed. At autopsy it was found to have a mild pericarditis and cloudy fluid in the carpal and tarsal joints. The pig receiving 5×10^7 organisms was visibly lame on Day 5 but did not have a febrile response until Day 14. This animal when killed on Day 18 was found to have an excessive quantity of synovial fluid in the carpi and tarsi. The remaining three pigs were killed in the early acute febrile stage of the disease; the two receiving 5×10^8 and 5×10^5 organisms respectively being killed on Day 4, and the pig receiving 5×10^4 organisms on Day 5. The lesions were mainly confined to the pericardium and joints with small areas of fibrinous pleuritis and peritonitis.

No clinical signs were observed in the control pigs. Nothing abnormal was seen in these pigs at autopsy and swabs from the appropriate sites when used to inoculate our media yielded no growth of organisms.

Histopathological studies of macroscopic serosal lesions in the infected pigs revealed an acute sero-fibrinous inflammatory reaction. Sometimes however the reaction was composed primarily of mononuclear cells. Small areas of collapse were evident in the lungs as a result of bronchiolar plugging by fibrin and leucocytes; the bronchiolar epithelium appeared normal in these areas. Nothing abnormal was detected histologically in the tissues of the control animals.

Haemophilus suis was isolated from the lungs, limb joints, pericardium and meninges of the pigs in Group I and the pig killed on Day 6 in Group II. It was not isolated from the remaining five pigs in Group II, although they had passed through an acute phase of the disease followed by slow recovery.

The bacterial isolation and direct microscopy results of the material from pigs in Group III are correlated in Table I. The mode of isolation is also indicated in this table, showing whether the organism was

TABLE I. Results of Clinical, Bacteriological and Fluorescent Antibody Examination of the H. suis Infected Pigs in Group III.

		Bacteriological Results				Indirect FAT	
Pig	Tissue	Plates*	Broths** 24 hr	Broths** 48 hr	48 hr broth to plates	Presence of round bodies	Presence of rods
P9. killed day 4	Brain	−	−	+	+	+	−
with temp. 105.4° F	Pericardium	−	+	+	+	+	+
and lame.	Lung	+	+	+	+	+	−
Highest temp.	Liver	−	−	+	+	+	−
105.6° F on day 3.	Spleen	+	−	+	+	+	−
Dose: 5 x 10^8	Joints	+	+	+	+	+	+
P10 killed day 18	Brain	−	−	−	−	+	−
with temp. 102.6° F	Pericardium	−	−	−	−	+	−
and lame.	Lung	−	−	−	−	+	−
Highest temp.	Liver	−	−	−	−	+	−
106.2° F on day 14.	Spleen	−	−	−	−	+	−
Dose: 5 x 10^7	Joints	−	−	−	−	+	−
P11 killed day 13	Brain	−	−	−	−	−	−
with temp. 100.4° F	Pericardium	−	−	−	−	−	−
and lame.	Lung	−	−	−	−	−	−
Highest temp.	Liver	−	−	−	−	−	−
104.4° F on day 1.	Spleen	−	−	−	−	+	−
Dose: 5 x 10^6	Joints	−	−	−	−	+	−
P12 killed day 4	Brain	−	+	+	+	+	−
with temp. 106.4° F	Pericardium	+	+	+	+	+	−
and lame.	Lung	+	+	+	+	+	−
Highest temp.	Spleen	+	+	+	+	+	−
106.4° F on day 4.	Joints	+	+	+	+	+	−
Dose: 5 x 10^5							
P13 killed on day 5	Meninges	−	−	+	+	NE	NE
with temp. 106.2° F	Brain	−	−	−	−	−	−
and lame.	Pericardium	−	−	−	−	+	−
Highest temp.	Lung	−	−	−	−	+	−
106.2° F on day 5	Liver	−	−	−	−	−	−
Dose: 5 x 10^4	Spleen	−	−	−	−	+	−
	Joints	+	+	+	+	+	+

* "Plates" = were blood agar staph. streaked, and Levinthal agar.
** "Broths" = refers to TSB and Levinthal broth.
NE = Not examined.

isolated directly on plate media, or after 24 or 48 hours in a broth medium. The organism was recovered from the pigs killed in the acute febrile stage of the disease, (P9, P12 and P13). In these pigs *H. suis* was isolated in broth from 13 of 18 potentially positive sites. The tissues of the two pigs killed at a later stage in the disease (P10 and P11) were negative on bacteriological culture despite the fact that they were still lame.

The indirect FAT was found to be specific for both our strains of *H. suis* and when applied to the other bacterial species isolated from swine did not show cross reactions. The technique applied to preparations from the negative control pigs showed fluorescence of some of the cytoplasmic granules in some of the eosinophils. The indirect FAT examination of preparations from pigs in Group III (graded doses) revealed the presence of a few bacterial rod forms of *H. suis* in the joints of the pig receiving 5 x 10^4 organisms (Fig. 3) although one rod only was seen in the smear of the pericardial fluid of this animal. Fluorescing round bodies were seen in all five pigs in the spleens and joints (Figs. 3 and 4) and in the brains, pericardial fluid and lungs of the others.

The spheroplasts induced *in vitro* were evident several hours after the treatment commenced and after 18 hours the transition from bacilliary to spheroplast forms appeared complete. The spheroplasts were identified by anoptral contrast microscopy of wet mounts and were specifically fluorescent by the FAT (Fig. 2). The spheroplasts thus formed grew as the rod form when inoculated into the Levinthal broth

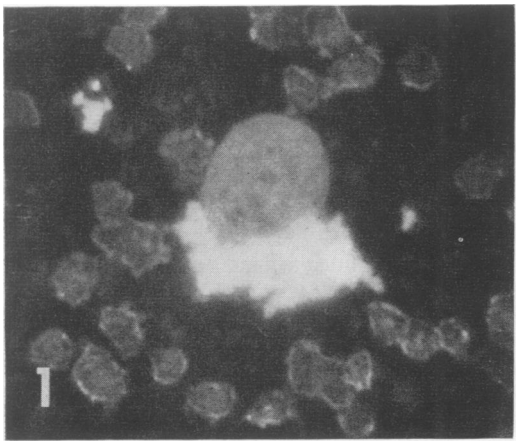

Fig. 1. Impression smear of lung of pig taken in the acute febrile stage of Glasser's disease produced by intratracheal inoculation of H. suis. Clusters of organisms are seen in the cytoplasm of a large mononuclear cell. FAT technique. X1000.

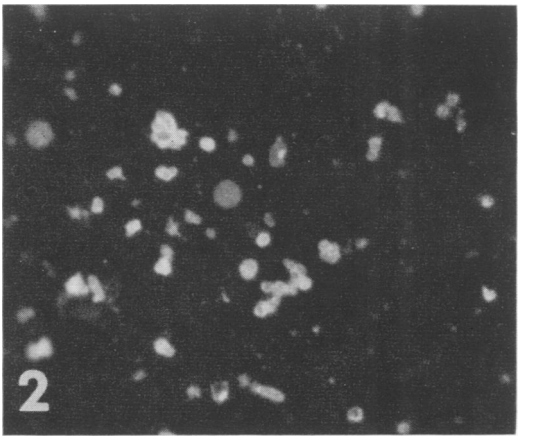

Fig. 2. Spheroplasts produced with glycine in liquid medium at 24 hours which revert to the rod form on solid medium. FAT. X 1000.

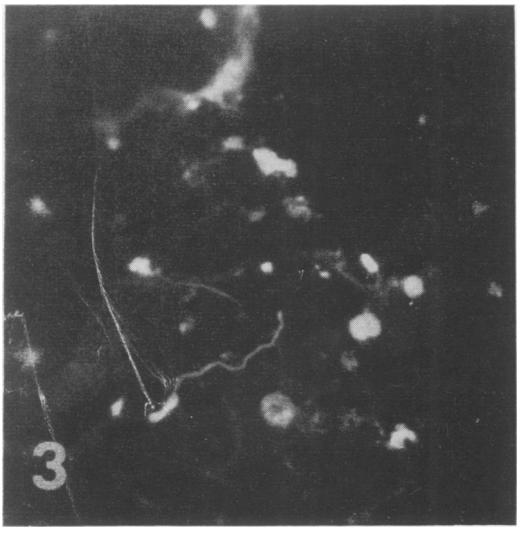

Fig. 3. Two rods, transition forms and spheroplasts observed in synovial fluid of joint and from which the rod form of H. suis was cultured. FAT X 1000.

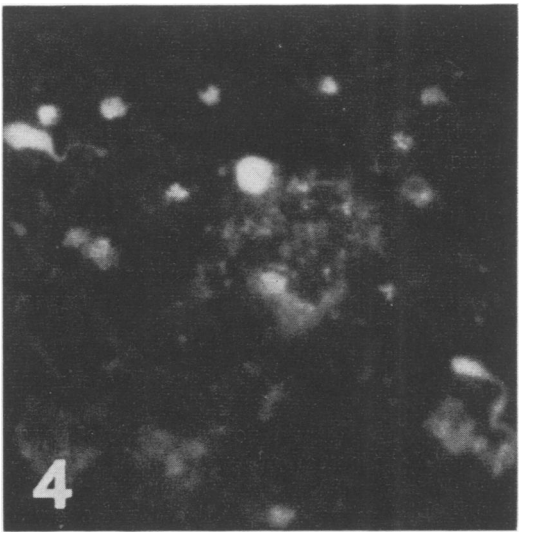

Fig. 4. Transition forms and round bodies observed in synovial fluid of pig from which the organism was not cultured. FAT. X 1000.

up to 30 hours after the commencement of glycine treatment. Subsequently the spheroplasts did not revert or demonstrate growth of any kind. These spheroplasts remained identifiable by FAT.

DISCUSSION

The disease produced by the intratracheal administration of *H. suis* to young pigs appeared similar to that reported independently by Hjarre and Wramby (7) and Bakos *et al* (3). Lecce (10) distinguished two similar diseases in swine, one caused by *H. influenzae suis* and the other by *M. hyorhinis* (19) (16). He was able to produce a clinical haemophilus infection only in colostrum-deprived, S.P.F. pigs. Cordy *et al* (5) produced a similar disease by the intravenous and intraperitoneal inoculation of swine with a caprine mycoplasma. They suggested that the inoculum used by Hjarre and Wramby (7) may have been carrying a mycoplasma, and that this agent may have been responsible for the disease production and not *H. influenzae suis*. Without referring to the work of Bakos *et al* (3), they regarded the work of Lewis and Shope (13) as indicating that *H. influenzae suis* was not pathogenic alone, but only in concert with a virus. It appears

that the inconsistent isolation of *H. influenzae suis*, the pathological similarity of two diseases, and the lack of attention given to the experimental evidence in which *H. influenzae suis* had satisfied all of Koch's postulates as the etiological agent of the disease as described by Glasser, has led to unwarranted confusion. Recently King (8) reporting on studies of porcine polyserositis and arthritis in Tasmania, pays particular reference to mycoplasma and *H. suis* as distinct aetiological entities in the production of the condition.

Bronchiolar plugging with no other histopathological abnormalities in the lungs of experimentally infected pigs suggests that the residues of the inoculum were successfully cleared in these organs, without having any severe effect, and yet the lungs received the direct insult at the time of, and following intratracheal inoculation. Nevertheless the non-fatal *H. suis* infection was clinically manifest as pyrexia and subsequent lameness, indicating the high degree of specificity of the organism as a potential primary pathogen in porcine arthritis.

The severity of the disease may be related to the size of the infective dose. The high dosage used in Group I produced a rapidly fatal disease with no specific clinical signs. Groups II and III received fewer organisms and a non-fatal protracted form of the disease developed following an initial febrile response in most cases. The clinical signs seen in the non-fatal form were characterized by pyrexia for about two days, followed by a period of lameness and lethargy lasting about one week. Pig P10 (Table I) is an exception in that it did not show a febrile response until Day 14, and P11 never exhibited pyrexia. Recovery appeared to be complete in the five surviving animals in Group II. Recovery of the organism appeared to be related to the form of the disease and was dependent on the use of plate and broth media containing an adequate source of NAD.

Recovery of the organism from pigs in the acute stage was readily accomplished. In contrast, lame afebrile pigs yielded the organism irregularly. Bacteria were recovered principally from the joint fluid, in contrast to the results obtained by Lecce (10) who reported the brain to be the best site for isolation.

In Group III (Table I) where the FAT was used, numerous round bodies and a few rods were seen in tissue and fluids from those pigs killed in the acute phase of the disease (Figs. 1, 3) and the organism was recovered from them (P9 and P13). The round bodies (Fig. 4) were easily demonstrable in P10 but the organism could not be recovered. In P12 these bodies were numerous and rods were not demonstrated, yet the organism was recovered from all tissues.

These specimens where only round bodies and transition forms were identified by FAT, but where the typical bacterium was recovered in the culture media inoculated with the specimens, are similar to the spheroplasts we produced *in vitro* after the 30 hour mark. Lapinski and Flakas (9) reported on the presence of transition forms and round bodies in the bronchial secretions of patients under antibiotic therapy for bronchitis involving *H. influenzae*. Although our pigs were not treated, the *H. suis* present in the non-fatal infections appeared to be affected by adverse circumstances as yet unspecified to the point where we were no longer able to isolate it, but still able to demonstrate the presence of round bodies possessing competent antigen. Whether these forms are protoplasts of *H. suis*, or non-viable spheroplasts remains unanswered. There may be a progressive loss of bacterial cell wall leading to the production of spheroplasts and progressing to protoplasts with virtually no remaining wall material. Bacterial spheroplasts are capable of reversion on media suitable for the entire organism, but protoplasts will not revert. It may be possible in the future however to isolate the protoplasts as the stable L-form. Such a phenomenon would explain the variable culture results obtained in the isolation of *H. suis* from Glasser's disease. Negative culture results for *H. suis* from cases of Glasser's disease may mean very little, especially if the outbreak is of several days duration when specimens are taken for culture. Furthermore, if the tissues are negative on culture for *M. hyorhinis* the possibility of covert haemophilus infection must be considered. Whether the round bodies of *H. suis* persist in the tissues as L-forms and cause latent infection as suggested by McKay *et al* (14), or are degenerate forms of no consequence, we do not know. The application of such labelling techniques as the FAT makes the prospect of further studies in this direction possible.

The disease syndrome produced by *H. suis* is quite similar to the polyserositis

and arthritis caused by *M. hyorhinis* and this undoubtedly is the basis of the confusion outlined in this discussion. There can be no reasonable doubt that Glasser's disease is caused by *H. suis*, and the disease in pigs caused by it and *M. hyorhinis* are pathologically similar but etiologically distinct.

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Book Review

A CLASSIFICATION OF THE MAJOR GROUPS OF HUMAN AND OTHER ANIMAL VIRUSES, 4TH EDITION. *Burton I. Wilner. Published by Burgess Publishing Company, Minneapolis, Minn. 1969. 250 pages.*

This is the 4th edition of a book which, since its introduction in 1964, has become a recognized manual of prime necessity for students of virology, and a valuable reference in the laboratory. In comparison to other books of this nature, this one appears more oriented to the student.

It commences with a well written section on the present criteria of viral taxonomy and ends with an index list of viruses complete to this date. In between are chapters dealing with the individual families of viruses based on the system of classification. In addition, there is a summary of the properties of the major groups of viruses, a chapter listing the viruses under their host species and a bibliography of 882 carefully selected references.

The chapters on the different virus families are presented in a two column, notebook style with the individual viruses listed in the left hand column and a short written description on the right. This style is perhaps suitable for quick reference, but it may not be the easiest system for locating specific items of information relating to individual viruses.

The usefulness of such a book presupposes a knowledge of the fundamentals of virology. It serves as a useful companion to volumes dealing with virology and viruses in more general terms. As its title implies, it deals with viruses of mammals, birds, amphibians, reptiles and fish, but not with plant or insect viruses.

It is not difficult for this reviewer to recommend this book to all those interested in virus classification. — *A. S. Greig.*