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Pertussis: The Disease and New Diagnostic Methods

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INTRODUCTION

The human pathogen *Bordetella pertussis* causes an acute and chronic respiratory disease known as whooping cough or pertussis mainly in infants and young children. Pertussis has been clinically recognized since the 16th century, with the first account of the disease published in 1640 (30). The etiologic agent of the disease, *B. pertussis*, a gram-negative coccobacillus, was later isolated by Bordet and Gengou (13) in 1906 from a patient with the infection. Pertussis was a major cause of childhood morbidity and mortality in the world until the introduction of a whole-cell vaccine in the 1940s which produced a dramatic decrease in the incidence of the disease (28). Yet today, pertussis is still a major health problem in third-world countries, where it is a leading cause of childhood mortality (160). Whooping cough is also re-emerging as a problem in the United States and other developed countries due to fears of reported side effects of the whole-cell vaccine (29, 105). A decrease in vaccination rate has led to an increased incidence of the disease in the United States, United Kingdom, and Sweden (25, 28, 130).

While pertussis is considered to be predominantly a childhood disease, studies suggest that adults may be infected at a much higher rate than previously realized (92, 116, 129). In a recent report by Robertson et al., it was found that 25.7% of adults with persistent cough were infected with *B. pertussis* (129). Thus, these studies demonstrate that pertussis can occur at any age.

The problem of diagnosis of pertussis is that the disease may not always be typical in adults or children (92, 116, 129) and laboratory diagnosis can be difficult (25, 98). As stated in a 1985 Centers for Disease Control editorial, "current methods to diagnose pertussis appear to be inadequate and may impede studies of pertussis transmission" (25). This review will look at presently used diagnostic methods for *B. pertussis*, recent advances, and what future possibilities lay ahead for the development of new rapid diagnostic tests.

PERTUSSIS: CLINICAL DISEASE

B. pertussis is a pathogen only for humans, not naturally causing disease in animals (154). Pertussis is highly contagious, having an attack rate of >90% in unimmunized individuals (82). Humans are the sole reservoir for *B. pertussis*. Infection is spread by exposure of the susceptible population to the organisms by an infected individual via the aerosol route. Pertussis classically is seen in children of <12 years of age (120).

Pertussis can be divided into three symptomatic stages: catarrhal, paroxysmal, and convalescent. After exposure to the infectious agent, the incubation time can vary from 6 to 20 days (84). In the catarrhal stage, the initial symptoms are the same as a mild common cold with rhinorrhea, slight conjunctivitis, coryza, low-grade fever, and a mild cough. It is during the catarrhal stage when one has the greatest chance to recover *B. pertussis* from the patient by culture of nasopharyngeal specimens (162). This is rarely done since the initial clinical signs are so nonspecific.

The catarrhal stage may last for several weeks with increasing episodes of severe and violent coughing, which marks the start of the paroxysmal stage (120). The cough at first occurs at night but in time becomes more frequent during the day, with as many as 15 to 25 paroxysmal coughing episodes occurring in a 24-h period (98). The traumatic coughing attacks lead to the eventual clearance of mucus that has blocked the lung airways. Air is rapidly inspired into the lungs past the swollen glottis, causing the classical whooping associated with the disease (98, 120). Pertussis is generally used in place of the term whooping cough because not all patients whoop, particularly adults

who may have only common cold-like symptoms (167, 174). The coughing episodes can be so severe that vomiting and extreme exhaustion may occur (120). In young children of poor nutritional status, vomiting can lead to weight loss, dehydration, and malnutrition (108).

Many complications can occur during the paroxysmal stage, including hemorrhagic events, hernia, pneumothorax, and pulmonary complications (91, 120). The sequelae of most concern are seizures, encephalopathy, secondary bacterial infections, and death (84, 91, 98, 120). The mortality rate in pertussis is highest for children of <1 year of age, with the major cause of death due to secondary bacterial infections (97, 108).

At this acute stage of the disease, antibiotic therapy has little effect on progression of the illness in the patient (10), but is useful in preventing further spread of the disease to others (92). It is perplexing that during this stage of the infection, when clinical symptoms are so fulminating, it is rarely possible or extremely difficult to isolate *B. pertussis* from the patient (58, 64, 120). Pittman proposes that most symptoms of pertussis at this stage of the disease are caused by a toxin (pertussis toxin [PT]) released by the bacteria (125).

The paroxysmal stage may last for 1 to 4 weeks, with symptoms slowly decreasing. During the convalescent stage, coughing paroxysms can occur sporadically as long as 6 months after the infection (86). Also during this stage, secondary bacterial infections and other complications may arise (44, 98, 120). For a more detailed review of clinical pertussis, readers are referred to papers by Olson (120) and Linnemann (91).

VIRULENCE FACTORS OF *B. PERTUSSIS*

B. pertussis produces a vast array of potential virulence factors which may play a role in the pathogenesis of pertussis. These include PT (125, 138, 149), filamentous hemagglutinin (FHA) (98, 137, 149, 162), hemolysin (161, 164), endotoxin (33, 88, 98, 162), adenylate cyclase (AC) (31, 47, 67, 100), heat-labile toxin (94, 115), tracheal cytotoxin (TCT) (53, 133), and agglutinogens (5, 147, 172). Each of these factors is discussed briefly below.

PT

PT produces a vast array of biological responses in vivo, including leukocytosis, lymphocytosis, histamine sensitization, lethality, mitogenicity, adjuvant effects, and stimulation of insulin secretion (98, 111, 125, 162). Initially, it was thought that these biological activities were caused by different toxins. Munoz and Bergman were the first to propose that all of these biological activities were due to one exoproduct, which they call pertussigen (111). Various names were used for PT, including lymphocytosis-promoting factor, histamine-sensitizing factor, islet-activating factor, and pertussigen (98, 111, 125, 162). With the eventual isolation and purification of PT, it was found that all of these observed activities were due to one unique exotoxin of *B. pertussis*.

PT has been purified by several groups (137, 140, 150) and found to be an oligomeric protein containing six subunits, five of which are different (140, 150). Tamura et al. have shown that PT is composed of subunits S-1, S-2, S-3, S-4, and S-5 in a molar ratio in the native molecule of 1:1:1:2:1 (150). The molecular weight of PT was found to be 117,000 by equilibrium ultracentrifugation (121), with others reporting similar results (140).

Further studies have shown that PT is a typical A-B toxin. The S-1 subunit is the A (active) portion which catalyzes the adenosine 5′-diphosphate ribosylation of a 41,000-molecular weight membrane protein in a variety of cell types (77). This membrane protein has been shown to be the alpha subunit of the guanine nucleotide regulatory protein (Ni or Gi) which is involved in the inhibitory control of mammalian cellular AC (49). Adenosine 5′-diphosphate ribosylation of the Ni protein inhibits its regulatory function, causing enhanced activity of the mammalian cell AC (49).

The remaining pentamer of PT (S-2, S-3, 2-S-4s, and S-5) makes up the B (binding) portion, which is required for binding PT to specific cell receptors and enables the enzymatic A portion to reach its site of action in the cell (77). It has been found that the B oligomer alone stimulates mitosis of lymphocytes (149). Thus, the multitude of biological effects of PT seems to be due to the mitogenic action of the B subunit and the adenosine 5′-diphosphate ribosylation enzymatic activity of the A subunit.

Studies have demonstrated the critical role that PT plays in immunity to *B. pertussis* infection (35, 135, 138, 139). Sato et al. showed that intraperitoneal injection of anti-PT rabbit hyperimmune sera would passively protect mice against a lethal aerosol challenge with *B. pertussis* (138). Cowell et al. showed that immunization of mice with detoxified PT (treated with glutaraldehyde) protected mice against intracerebral challenge with *B. pertussis* (35). In more recent studies, using both the intracerebral and aerosol mouse models of *B. pertussis* infection, mice were protected by either active immunization with PT-toxoid or passively with anti-PT sera (136).

Using monoclonal antibodies to PT S-1 and S-4 subunits, Sato et al. (135) showed that anti-S-1 monoclonal antibody neutralized leukocytosis-promoting and islet-activating activities of the toxin and also protected mice against lethal *B. pertussis* infection via the aerosol and intracerebral routes. Anti-S-4 monoclonal antibody showed no neutralizing or protective ability.

In contrast to these results, Arciniega et al. found that mice immunized with the B oligomer of PT were protected against PT challenge (4). Immunized mice produced antibodies which neutralized PT toxicity in the CHO cell assay (4). The authors also reported that the A subunit of PT was a poor immunogen in mice.

Similar observations were reported by Nicosia et al. (117) when PT subunits were expressed as fusion proteins in *Escherichia coli*. Mice were immunized with the five subunits separately or as mixtures, with no protection obtained in the intracerebral challenge model. The results suggest that the native conformation of PT is necessary for induction of protection.

Further evidence for the role of antibodies to PT in immunity to whooping cough in humans has been reported recently by Granström et al. (56). They demonstrated that there is a correlation between anti-PT serum titers and long-term immunity to *B. pertussis* infections in patients. Furthermore, in many patients with pertussis, one can observe lymphocytosis, hypoglycemia, and an impaired rise in serum glucose in response to epinephrine (8). These clinical observations are similar to responses observed in experimental animals treated with purified PT.

Recently, the structural gene of PT has been cloned, and intensive work is ongoing to study PT at the genetic level (95, 118). Thus, PT is the most studied and a well-characterized virulence factor of *B. pertussis*.

FHA

Native FHA appears by electron microscopy as a fine filamentlike protein, 2 nm in diameter and 40 to 100 nm in length, which agglutinates various types of erythrocytes (3, 109). FHA has been purified and reported to have a molecular weight ranging from 220,000 to approximately 58,000 (35, 75, 137). Studies by Irons et al. (75) with monoclonal antibodies to FHA suggest that many of the lower-molecular-weight protein bands seen on sodium dodecyl sulfate-polyacrylamide gel electrophoresis are probably breakdown products or monomers of higher-molecular-weight (native) FHA.

While FHA does not have toxic activity, the antigen seems to be important in immunity to *B. pertussis*. In mouse studies, FHA protects against aerosol challenge by active but not passive immunization (119, 136). Immunization with FHA in concert with PT has been shown to have a synergistic effect (136).

Studies by Tuomanen et al. suggest that FHA may be involved in the initial attachment and adherence of *B. pertussis* to ciliated epithelial cells of the upper respiratory tract to initiate infection (155, 156). Studies by Urisu et al., using human WiDr cells in vitro, also indicated a major role of FHA in adherence (157). Thus, antibody to FHA may be protective via inhibition of initial colonization of the upper respiratory tract.

AC

The presence of a *B. pertussis* AC was first reported by Wolff and Cook (169) in studies with whole-cell vaccine. Hewlett et al. demonstrated that the cyclase accumulates to maximum levels in culture supernatant in exponentially growing cultures by 24 h, but the majority of the cyclase activity was found associated with the bacterial cell surface extracytoplasmically (65). Studies by Utsumi et al. (158) showed that the AC could be recovered from *B. pertussis* cells by urea extraction and that the cyclase activity was quite stable under these conditions. The extract was found to inhibit human polymorphonuclear leukocyte (PMN) functions, including chemotaxis and oxygen consumption. Confer and Eaton (31) reported that dialyzed urea extracts inhibited superoxide production of both human PMN and alveolar macrophage. The urea extract was also found to inhibit PMN chemotaxis and killing of *Staphylococcus aureus*. The extract induced accumulation of cyclic adenosine 3',5'-phosphate (cAMP) to high levels in various mammalian cell types, including PMN, lymphocytes, monocytes, CHO cells, mouse S49 lymphoma cells, and isolated rat pituitary cells (66). cAMP has been shown by others to be an inhibitor of various phagocytic functions (16, 124).

Another unique characteristic of the AC is that it is activated 10- to 1,000-fold by the eucaryotic regulatory protein calmodulin (170). This activation by calmodulin is unusual for a procaryotic cyclase, with the one exception being the edema factor of *Bacillus anthracis*. Leppla (89) has demonstrated that the edema factor of *Bacillus anthracis* is also an AC which is activated by calmodulin.

Confer and Eaton hypothesize that the *B. pertussis* cyclase enters phagocytic cells, is activated by calmodulin, and induces high levels of intracellular cAMP, which impairs PMN and macrophage bactericidal functions (31). The AC could play a significant role in *B. pertussis* pathogenesis by inhibiting host defenses of the tracheobronchial tree, allowing the bacteria to survive, multiply, and cause disease.

The AC is potentially an important virulence factor involved in allowing establishment of the initial pertussis infection by suppressing phagocytic defenses and the usual immunological responses associated with infection. Recently, Weiss et al. (163, 164) attempted to study *B. pertussis* pathogenesis by using a wild-type strain and a series of Tn*5*-induced mutants deficient in production of various virulence factors. They found that avirulent phase mutants, pertussis toxin mutants, and a double mutant deficient in hemolysin and AC were unable to cause disease in an infant mouse model (163). Brezin et al. recently reported that monoclonal antibody to AC was protective in an experimental mouse respiratory infection model (18).

While *B. pertussis* produces PT and AC, two closely related species, *Bordetella bronchiseptica* and *Bordetella parapertussis*, produce only AC. Yet these organisms are still pathogenic for animals (55). These observations strongly suggest that the AC is an important virulence factor in mammalian *Bordetella* pathogenesis.

Several groups have reported the isolation and purification of soluble AC from culture supernatants of *B. pertussis* (79, 83, 100, 142), while others have reported purification of AC from the bacterial cell surface extracts (45–47, 61). These studies report varying molecular weights of AC from 43,000 to 200,000 (46, 61, 83, 100). The differences in molecular weights reported may be due to different methods of isolation used and to isolation of cell-associated versus culture supernatant AC.

The AC has been recovered in two forms, one containing only enzymatic activity and the other having both enzymatic activity and the ability to enter mammalian cells, causing an increase in intracellular cAMP levels (biological activity) (162). Friedman (46), Hanski et al. (45, 61), and Confer and Eaton (31) have demonstrated that the cell-associated AC has biological effects on human cells (PMN, lymphocytes). Whether purified soluble AC has toxic effects on human immunologic cells has not been reported (79, 83, 100, 141–143).

TCT

The TCT, a small glycopeptide, was first discovered by Goldman et al. (53) when they observed that concentrated *B. pertussis* culture supernatant (log phase) caused ciliostasis and specific damage to only ciliated cells in hamster tracheal ring cultures. Exposure of hamster trachea epithelial cells (51) to *B. pertussis* culture supernatant caused a dose-dependent inhibition of deoxyribonucleic acid (DNA) synthesis, but total ribonucleic acid and protein syntheses were not affected (50). Goldman suggests that impairment of DNA synthesis of hamster trachea epithelial cells by TCT reflects impaired cellular regenerative response. These results suggest that TCT may have some toxicity for basal cells in the respiratory epithelium.

The specificity of TCT for ciliated respiratory epithelial cells is of interest because these cells are also the site of initial attachment and initiation of *B. pertussis* infection (50). The bacteria remain localized and multiply to high numbers, causing destruction of the ciliated cells, possibly by TCT. Destruction of the ciliated cells impairs normal lung clearance of mucus, debris, and bacteria, resulting in coughing to clear the airways (50), and may play a role in the development of secondary bacterial infections commonly seen in pertussis (91, 120).

By chemical analysis, the composition of TCT is similar to that of peptidoglycan of gram-negative bacteria containing

diaminopimelic acid, muramic acid, alanine, glutamic acid, and glucosamine residues (50). Muramic acid and diaminopimelic acid are found only in peptidoglycan. The estimated molecular weight of TCT is between 1,235 and 1,400 (52). Recent studies by Rosenthal et al. (133) found that *B. pertussis* releases an almost homogeneous set of soluble peptidoglycan fragments which are identical to TCT. TCT seems to be released by *B. pertussis* via hydrolysis of peptidoglycan fragments from the peptidoglycan matrix of the bacteria and not by release from peptidoglycan precursors (133).

Parallels can be made to *Neisseria gonorrhoeae*, a gram-negative pathogen that colonizes the mucosal surfaces of the urogenital tract. Rosenthal and co-workers found that *N. gonorrhoeae* releases large amounts of low-molecular-weight peptidoglycan fragments that are actually peptidoglycan monomers produced by a gonococcal transglycosylase (132, 144). Studies by Melly et al. demonstrated that these peptidoglycan monomers have specific toxicity for ciliated cells of human fallopian tube epithelium in vitro, causing loss of ciliary activity and cell extraction (102). Goldman suggests that *B. pertussis* TCT and the peptidoglycan monomers of *N. gonorrhoeae* may be examples of a general virulence mechanism that other gram-negative bacteria may use (50).

Hemolysin

When virulent *B. pertussis* are grown on Bordet-Gengou (BG) agar plates (containing 15% blood), zones of hemolysis are produced around isolated colonies. Over the years it has been suggested that the hemolysin could be an important virulence factor of *B. pertussis* (111, 161), but this has not been extensively studied. Yet studies on other bacterial pathogens have shown the importance of hemolysins as virulence factors (24).

Genetic studies by Weiss et al., using Tn*5*-mediated mutagenesis in *B. pertussis*, provided the first evidence that the hemolysin and AC may be important in pertussis pathogenesis (164). Kawai et al. (78) reported that a modified ornithine-containing lipid isolated from *B. pertussis* has hemolytic activity. Whether this is the same compound that causes hemolysis on BG plates has not been determined. To date, it has not been demonstrated whether *B. pertussis* produces a true hemolysin. Extensive studies on the potential role of the *B. pertussis* hemolysin in pertussis pathogenesis are critically needed.

LPS

The lipopolysaccharide (LPS) of *B. pertussis* has the same biological activities as endotoxin from other gram-negative bacteria. This includes pyrogenicity, toxicity, induction of general and local Shwartzman phenomena, induction of nonspecific immunity in mice, induction of interferon production, and adjuvant properties (7, 98, 162). Pertussis LPS is reactive in the *Limulus* lysate assay, as are other endotoxins (112). An unusual biological activity of *B. pertussis* LPS is its ability to induce nonspecific antiviral activity (7, 168).

Studies have found that the LPS of *B. pertussis* has a different chemical structure from that of LPS from enterobacteria (7). Endotoxin from virulent *B. pertussis* is composed of two major types of LPS: LPS-I, which contains lipid X; and LPS-II, which contains lipid A and two different polysaccharides (PS1 and PS2). Lipid X is different in that it contains 2-methyl-3-hydroxydecanoic and -tetradecanoic acids while lipid A does not (59). PS1 is bound to lipid A via a molecule of nonphosphorylated 3-deoxy-2-octulosonic acid, while PS2 is bound to lipid A via a molecule of phosphorylated 3-deoxy-2-octulosonic acid. The point of attachment to lipid X is unknown (88). Recently, the structure of the tetrasaccharide contained in PS1 has been described (107).

Studies by Ayme et al. (7) found that lipid X exhibits all of the pyrogenic, sensitizing, and toxic properties of LPS while pertussis lipid A does not. Pertussis lipid A had reduced pyrogenicity and toxicity while having normal adjuvant and antiviral activity (7). What role *B. pertussis* LPS plays in the pathogenesis and immunity to the disease is presently unknown.

Heat-Labile Toxin (Dermonecrotic Toxin)

Heat-labile toxin or dermonecrotic toxin was the first reported toxin of *B. pertussis* (14). As its name suggests, the toxin causes inflammation and necrotic skin lesions after subcutaneous injection at low doses in mice but is lethal at high doses (34, 94, 115). The toxin is heat labile, with toxic activity lost upon heating at 56°C for 10 min (98).

Studies by Cowell et al. found that heat-labile toxin was localized intracellularly in the cytoplasm and was not actively secreted by *B. pertussis* (34). Munoz et al. had previously reported that after cell lysis heat-labile toxin activity was associated with the soluble lysate fraction (113). Recent studies by Livey and Wardlaw suggested that the heat-labile toxin may be partially exposed on the bacterial surface, with the majority of toxin activity localized in the cytoplasm (94).

Heat-labile toxin partially purified by gel filtration was reported to have a molecular weight of 89,000 (94). Nakase and Endoh recently reported purification of the toxin with a molecular weight of 102,000 (115). They reported that the toxin is composed of four polypeptides, two each of 30,000 and 24,000 molecular weight (115).

Kume and Nakai have reported the purification of a dermonecrotic toxin from *B. bronchiseptica* (80). The *B. bronchiseptica* toxin was found to consist of a single polypeptide chain of 190,000 molecular weight which dissociated, via dithiothreitol and urea treatment, into two polypeptides of 75,000 and 118,000 molecular weight, respectively. Both polypeptides were required for toxic activity (80). These studies suggest that *B. pertussis* heat-labile toxin has a similar structural composition (80, 115).

In studies with the purified *B. pertussis* heat-labile toxin in vitro, the toxin was found to inhibit rat kidney Na^+-K^+ adenosine triphosphatase activity. In vivo, when the toxin was instilled onto surgically exposed peripheral blood vessels of guinea pigs or mice, vasoconstriction occurred within 15 min (115). This resulted in decreased blood flow leading to ischemia, hemorrhaging, and leukocyte influx which occurred over a 5-h period (115). Studies by Roop et al. implicate the role of dermonecrotic toxin in atrophic rhinitis caused by *B. bronchiseptica* in young pigs (131). Further studies are needed to definitively determine the mode of action of heat-labile toxin and its role in the human disease.

Agglutinogens (Fimbriae)

B. pertussis, like many other gram-negative bacteria, have on their surface fimbriae or pili. Antibodies to fimbriae cross-link bacteria (due to high surface antigen concentration) and cause agglutination (98, 162). Bacterial agglutination caused by use of type-specific sera raised against

various strains of *B. pertussis* has been used for many years as a serotyping system, with its initial development by Eldering et al. (41, 42). The surface antigens that caused the agglutination were called agglutinogens, with six serotypes proposed by Eldering (41, 42). More recently, Preston et al. proposed that three (1, 2, and 3) were major agglutinogens and three (4, 5, and 6) were minor agglutinogens (126).

Studies by Ashworth et al. were the first to suggest strongly that agglutinogens and fimbriae of *B. pertussis* were one and the same (5). Fimbriae of agglutinogen types 2 and 6 have been purified (36, 147, 172). The fimbriae have a helical structure formed by repeating units of monomeric fimbrial protein of approximately 22,000 molecular weight (147). Type 2 fimbriae are distinct from FHA, which is also a filamentous antigen on the surface of *B. pertussis*. The purified fimbriae do not hemagglutinate erythrocytes and are structurally, antigenically, and chemically different from FHA (172). Whether other agglutinogens are fimbrial in nature has not been determined.

B. pertussis fimbriae may be involved in the initial attachment and colonization process in whooping cough. Thus, antibody to fimbriae may be protective by blocking initiation of the disease process. Previous studies have reported that pertussis does not occur in patients with high agglutinogen antibody titers (98). Zhang et al. found that mice immunized with purified type 2 fimbriae were protected from a lethal aerosol challenge of *B. pertussis* (173).

LABORATORY DIAGNOSIS OF PERTUSSIS

Collection, Transport, and Culture

Sensitivity of *B. pertussis* culturing is affected by time between disease onset and initial culturing and whether or not the patient is on antibiotic therapy (9, 20). Also, age and immunization status of the patient may affect culture results (20). Other factors that contribute to problems of *B. pertussis* isolation include incorrect collection procedures, delay in transit of specimens, overgrowth by other microorganisms, and lack of experience in recognizing the bacteria in culture (20).

Reported results vary on recovery rates of *B. pertussis* by culture of clinical specimens. A Scottish study reported that the isolation rate was 42% from infants of <6 months of age clinically diagnosed with pertussis. In children over 1 year old, the percentage of isolation dropped to 20% (38). An Australian study reported an isolation rate of only 50% (90), while Broome et al. reported 83% of cases in an outbreak of pertussis in Atlanta, Ga., identified by culture techniques at the Centers for Disease Control (21). Thus, isolation from clinical specimens is highly variable and difficult, especially for hospitals that do not routinely culture for *B. pertussis* (93).

The specimen most commonly collected for culture of *B. pertussis* is the pernasal nasopharyngeal swab (two are usually taken), which uses a swab on a fine flexible wire (123). The swab is inserted via the nostril to the nasopharynx for up to 1 min. Various types of swabs have been reported to be used, including cotton, dacron, and calcium alginate (123, 124). Calcium alginate and dacron swabs seem to be the best because fatty acids present in cotton swabs may inhibit *B. pertussis* growth (123, 134). Nasopharyngeal secretion aspirates may also be used for culturing when available.

Specimens need to be cultured as quickly as possible. Optimally, direct plating of the specimen should be done at bedside. This is not possible for most pertussis patients because they are usually seen by private-practice pediatricians, who do not routinely culture for *B. pertussis*. In these situations a transport medium is utilized to send the specimen to a diagnostic laboratory for culture.

Several studies have been done with various transport media, including Amies, Stuart, Jones-Kendrick, and Regan-Lowe (71, 73, 127, 134), to determine which is best suited for recovery of viable *B. pertussis*. While the reported results in some cases were contradictive (73, 134), the overall results demonstrated that Regan-Lowe and Jones-Hendrick transport media are superior for recovery of *B. pertussis*. There are several reasons for these results. Stuart and Amies media are non-nutritive basal salts medium which are not optimal for *B. pertussis* survival. Regan-Lowe and Jones-Kendrick media are based on charcoal, starch, nutrients, and antibiotics (cephalexin). Normal nasopharyngeal flora is suppressed by cephalexin and cannot overgrow the specimen in transit. *B. pertussis* remains viable and can increase in numbers in the transport medium, increasing its chance of survival and isolation upon plating.

The temperature at which the inoculated transport media is stored or transported may also have an effect on recovery of *B. pertussis*. Ross and Cumming (134) compared storage at 4°C and room temperature and observed better isolation after storage at 4°C. Hoppe et al. reported that preincubation of Regan-Lowe transport medium at 36°C for 1 to 2 days led to better isolation on plates than storage at room temperature or 4°C (71).

Several different media have been developed over the years for the isolation of *B. pertussis*. The first to be developed in 1906 was BG agar (13), which is composed of starch, NaCl, and 15 to 20% defibrinated sheep or horse blood. BG agar, while still used by many diagnostic laboratories, has several disadvantages. The medium has a short shelf life due to the high percentage of blood, and it is usually recommended that freshly poured BG plates be used for isolation (70, 123). This makes it an inconvenient medium to use for many clinical laboratories. The addition of antibiotics such as methicillin or cephalexin has helped to partially alleviate the problem of overgrowth by nasopharyngeal normal flora (123, 146). It should be noted that some strains of *B. pertussis* can be inhibited by penicillin or cephalexin; therefore, both selective and nonselective media should be used in attempts to isolate the organism.

Because of these problems, other media were developed for growth and isolation of *B. pertussis* (76, 106, 127, 148). Mishulow et al. developed a blood-free beef heart charcoal agar which contained yeast extract and starch (106). This agar was further developed and modified by Jones and Kendrick (76) by the addition of penicillin. Sutcliff and Abbott developed a medium similar to Jones-Kendrick agar which contained 10% horse blood and 40 μg of cephalexin per ml (148). Cephalexin seems more selective for *B. pertussis* and has a wider spectrum of action than penicillin (146, 148). Regan and Lowe modified this medium and used it as a transport medium (127). Today, Regan-Lowe or charcoal horse blood agar is recommended for use in diagnostic laboratories.

A study by Hoppe and Vogl compared charcoal horse blood, BG, and Jones-Kendrick agar for isolation of *B. pertussis*. They reported that charcoal horse blood agar was superior in rapidity of *B. pertussis* growth, numbers of strains forming colonies, and number of developing colonies (70). Jones-Kendrick agar was slightly inferior but has the advantage of a longer shelf life of 2 to 3 months (70, 146) as compared with 8 weeks for Regan-Lowe agar (48).

In another study, commercially prepared BG agar was compared with Regan-Lowe agar for the ability to recover *B. pertussis* from 25 patients with the disease. Bacteria were isolated from only four patients when BG agar was used, while *B. pertussis* was recovered from all 25 patients with Regan-Lowe agar (F. Chan, E. Rossier, A. M. R. MacKenzie, and A. Comos, Abstr. Annu. Meet. Am. Soc. Microbiol. 1983, C260, p. 355). With the addition of the antibiotic cephalexin to Regan-Lowe medium, less overgrowth by other organisms occurred as seen with BG agar (46). These studies strongly suggest that, in particular, Regan-Lowe agar has the advantages of sensitivity, selectivity, and extended shelf life compared with BG agar.

Recently, a completely synthetic medium, Stainer-Scholte agar with added cyclodextrin and cephalexin, was developed (6). Cyclodextrin is a compound that stimulates growth of *B. pertussis* (74). The cyclodextrin solid medium suppressed normal nasopharyngeal flora growth and provided better isolation rates than BG agar with or without cyclodextrin. This medium was also reported to be stable after 3 months of storage (6). While these results are impressive, one major disadvantage of this new medium is the limited availability and great expense of cyclodextrin.

DFA Test

The direct fluorescent-antibody (DFA) test utilizes polyclonal fluorescein-labeled antibodies against *B. pertussis* to directly detect the organism in nasopharyngeal specimens (123). A DFA test is positive for *B. pertussis* when short rods with a rim of bright-green fluorescence are visualized in smears stained with the antisera and not with control sera (123).

Several early studies reported that the DFA test was more sensitive than culture (26, 120, 165). Whitaker et al. found that 78% of specimens from 128 patients with clinical pertussis were DFA test positive (165). In patients not receiving antibiotics and having symptoms for <3 weeks, DFA test results were 94% positive. In patients with the disease for longer than 3 weeks or those treated with antibiotics, DFA test results were 57 and 11% positive, respectively (165). These studies did not compare DFA results with culturing results. Chalvardjian reported that, of 100 specimens, 29 were positive by culture on BG plates while 46 were positive by DFA test (26). Whether any specimens were both DFA and culture positive was not reported.

More recent studies report that culture is more sensitive than the DFA test (21, 48, 127). In the studies of Regan and Lowe, using their charcoal horse blood agar, culturing was reported to be more sensitive than the DFA test for detection of *B. pertussis* (127). Broome et al. compared use of the DFA test and culture on BG agar of the same specimens during a pertussis outbreak in Atlanta, Ga., in 1977 (21). Of 58 cases, 83% were positive by culture while only 61% were positive by DFA test. Recently, Gilligan and Fisher reported that culturing on Regan-Lowe agar was more sensitive than the DFA test for detection of *B. pertussis* (48).

Direct immunofluorescence provides more rapid results but has limited sensitivity due to the number of organisms necessary for microscopic visualization. DFA also suffers from difficulties in interpretation and variability (25). One major problem with DFA is the high percentage of false-positives, ranging from 6.7 to 40% (21, 25, 69). Much of this problem is due to variability between technologists in interpreting DFA test results, as well as to poor-quality slides and nonspecific reagents (21). As an example, two recent studies have reported cross-reactivity of DFA reagents for identification of *Legionella* species and serogroups with several different strains of *B. pertussis* (12, 81). Cross-reactivity problems could be alleviated by use of specific affinity-purified antibody or monoclonal antibody for detection of *B. pertussis*. Because of these problems with the DFA test, culturing must still be used to confirm cases of pertussis (121, 123).

NEW DIAGNOSTIC TECHNIQUES

The difficulty and questionable sensitivity of culture and the DFA test contribute to substantial underdiagnosis of pertussis. Culture of *B. pertussis* is time-consuming (3 to 7 days) and not always successful. The DFA test is highly variable and difficult to interpret and requires special equipment (25). The failure to diagnose pertussis early can cause problems for infection control. Undiagnosed patients can spread the disease to other family members and other close contacts, increasing the risk of epidemic outbreaks. Presently, whooping cough is diagnosed mainly on the basis of the clinical picture and epidemiologic evidence (98). Diagnosis of whooping cough in a patient via clinical picture, positive culture, and positive DFA test from nasopharyngeal specimens is rare (98).

Thus, there is a great need for new, more sensitive and specific laboratory diagnostic tests for detection of *B. pertussis* in clinical specimens. The availability of such diagnostic tools would help define more fully the epidemiology of whooping cough and significantly aid in the control and spread of the disease during outbreaks (25). This section reviews recently developed diagnostic techniques for detection of *B. pertussis* and future prospects.

Serologic Techniques

Various serologic tests have been used to demonstrate pertussis infection or immunization. Techniques to detect antibody reactive with *B. pertussis* include agglutination (39, 42, 43, 104, 166), complement fixation (17, 96), bactericidal assays (1), and indirect hemagglutination (96). These serological tests are insensitive and cannot determine whether an antibody response is the result of immunization or disease (121).

More sensitive assays to detect specific antibodies to *B. pertussis* have been developed, using enzyme-linked immunoassays (ELISAs) (23, 121, 129). This simple methodology has been applied to serologic diagnosis of numerous infectious diseases (68).

Various ELISAs for detection of pertussis antibodies use different antigen preparations, including purified FHA and PT (23), partially purified FHA (57, 60), whole cells (54, 114), and whole-cell sonic extracts (84, 103, 104, 129, 159). Onorato and Wassilak suggest that, while use of whole-cell or bacterial sonic extracts may increase the sensitivity of the ELISA, it may also increase the risk of cross-reactivity with other microorganisms (121). Use of purified antigens produced only by *B. pertussis* (PT) or *Bordetella* species (FHA) in an ELISA would make the assay highly specific (121).

One major problem with serologic ELISAs is the usual difficulty in obtaining paired specimens of acute- and convalescence-phase sera from suspected cases of pertussis for antibody titer comparison. This usually limits specimens to single convalescence-phase samples with no way to determine the titer rise of the individual patient. In these cases antipertussis antibody titers must be compared with a nor-

mal control population, which as Onorato and Wassilak discuss, can cause difficulties of interpretation (121).

Recent studies have found that natural infection with *B. pertussis* causes a rise in pertussis-specific immunoglobulin A (IgA) antibodies while immunization induces mainly an IgM and IgG antibody response (23, 114). In infants with pertussis, initially IgA antibody is not detectable (23), but it reaches detectable levels 6 to 7 weeks after infection (114). Robertson et al., using an ELISA for *B. pertussis*-specific IgA in serum, demonstrated that 25.7% of adults with persistent cough gave positive results in the assay, suggesting infection with *B. pertussis* (129). Recently, Lawrence and Paton reported that 67% of 1,240 acute-phase sera from patients with suspected pertussis were positive by ELISA for class-specific antibodies to *B. pertussis*. This increased to 77% when convalescence-phase sera were tested (85).

Goodman et al. developed an ELISA for detection of pertussis-specific IgA antibody in nasopharyngeal secretions as an indicator of recent infection (54). They observed that IgA to *B. pertussis* appeared during week 2 or 3 of the disease and persisted for 3 months or longer. They suggested that the assay could be useful as a diagnostic tool in culture-negative cases of pertussis (54).

ELISAs which require antibody production by the patient can be used only in retrospect to confirm clinical diagnosis of pertussis (54, 114, 121). They would be most useful in epidemiologic studies or at the stage of pertussis at which cultures are unlikely to be positive. Presently, they have limited use for rapid diagnosis of pertussis.

Detection of *B. pertussis* Antigens

Another potential approach for rapid diagnosis of pertussis is the use of antibodies to detect *B. pertussis* antigens directly in clinical specimens. A variety of tests have been developed for detection of soluble bacterial antigens in patient body fluids. These include counterimmunoelectrophoresis (CIE), latex agglutination, and coagglutination (11). Sensitive ELISAs have been developed for detection of *Haemophilus influenzae* (11, 37, 145), *Streptococcus pneumoniae* (62, 145), and *Neisseria meningitidis* (145) antigens in clinical specimens, using polyclonal or monoclonal antibodies. Various enzyme immunoassays have also been developed for detection of viral and bacterial antigens in respiratory secretions (2, 27, 101, 171), cerebrospinal fluid (152), and endocervical and urethral specimens (122, 152). Rapid diagnosis of respiratory syncytial virus in nasal secretions from infants and young children by ELISA has been carried out by several laboratories (19, 63, 72).

These observations suggest that viral or bacterial antigens may be stable in secretions and detectable by antibodies much longer than viruses or bacteria could remain viable for culturing. Also, antigen detection rather than isolation of viable organisms would be easier to do when antibiotic therapy has begun on a patient.

Whether *B. pertussis* antigens, such as PT, FHA, or AC, are produced and secreted into the nasopharyngeal environment at levels detectable by using specific antibodies has not been reported in the literature. Evidence that PT and FHA are produced and secreted during infection in vivo comes mainly from serological studies which demonstrate the presence and increase of antibody titers to the two antigens following infection or vaccination in humans (23, 56) and in experimental animal studies (136). Clinical observations of lymphocytosis, hypoglycemia, and an impaired rise in serum glucose in response to epinephrine in many patients with pertussis also suggest the presence of PT during an infection (98). These observations suggest, theoretically, that *B. pertussis* antigens may be present in respiratory secretions of patients with pertussis. Assays that use specific monoclonal antibodies could be used to directly screen nasopharyngeal aspirates or swab specimens by ELISA or a dot-blot-type assay for *B. pertussis* antigens. For such assays, monoclonal antibodies to PT would be optimal, since only *B. pertussis* is known to produce this exoproduct. Extensive studies are needed to determine the feasibility and potential of such a rapid diagnostic test for pertussis.

Confer and Eaton have proposed and have developed an assay for detection of *B. pertussis* via its AC enzyme (32). This assay uses the unique ability of the *B. pertussis* AC in the presence of calmodulin to convert adenosine 5′-triphosphate to cAMP. The conversion is monitored with a cAMP radioimmunoassay. Studies found that calcium alginate swabs with as few as 100 *B. pertussis* organisms induced detectable levels of cAMP in the assay (32). When screened in the assay, nasopharyngeal secretions from healthy donors gave negative results, as did addition of various concentrations of common bacterial pathogens isolated from the upper respiratory tract (32). One disadvantage of the assay is the use of radionuclides for determination of cAMP concentration. These initial studies are very promising and could lead to the use of such an assay as a rapid diagnostic test for *B. pertussis* infections in humans. Further clinical studies are needed to determine the sensitivity and specificity of the assay.

Boreland and Gillespie have reported the use of CIE as another method for detection of *B. pertussis* antigens in clinical specimens (15). Serum and urine samples from clinically diagnosed cases of pertussis were tested in CIE, using rabbit antiserum against pertussis vaccine. Cultures of pernasal swabs were also done on BG agar for a comparison of the two detection methods. The CIE method was 53% positive with serum and only 33% positive with urine from 17 clinically suspected pertussis cases. By culture, 47% of pernasal specimens were positive. Whether positive culture and CIE results were obtained from the same cases was not reported. Further studies are needed to evaluate CIE to determine its potential as a rapid diagnostic test for pertussis.

DNA Probes

The development of recombinant DNA technology over the last several years has lead to the use of DNA probes for diagnosis of infectious diseases (40, 151). The probe is usually a small piece of DNA cloned from the microorganism of interest in either a plasmid or other type of vector. The DNA probe is usually labeled with a radionucleotide (^{32}P), made single stranded, and allowed to interact with the clinical specimen present on a nitrocellulose filter. If there is nucleic acid homology between the probe and DNA in the specimen, hybridization will occur and can be detected by autoradiography.

DNA probes have been used in various clinical studies. Moseley et al. used cloned genes for heat-labile and heat-stable *E. coli* enterotoxins to screen stool specimens directly for the presence of enterotoxigenic *E. coli* (110). The gonococcal cryptic plasmid was used by Totten et al. to screen urethral exudate or swabs for infection by *N. gonorrhoeae* in 71 cases of symptomatic urethritis. Sixty-three of 71 cases were positive with the DNA probe and also by culture (153). DNA probes for detection of other bacterial pathogens have

also been developed (40). Techniques have also been developed to label DNA with biotin so that bound probe may be detected colorimetrically and not by use of radioisotopes, making DNA probes more practical for the clinical laboratory (87).

Recently, the genes for PT (95, 146) and FHA (22, 128) have been cloned. These genes have potential use as DNA probes for detection of *B. pertussis*. These probes could be used for direct screening of sputum specimens or screening of swab cultures after 1 to 2 days of growth on Regan-Lowe agar plates. These probes would be specific for *Bordetella* species since recent studies have found nucleotide sequence homology to PT in *B. bronchiseptica* and *B. parapertussis* (99). Similar observations have not been reported but are expected for FHA.

McLafferty and co-workers have reported the isolation and use of a sequence of DNA highly repeated within the *B. pertussis* genome (M. A. McLafferty, K. Bromberg, D. R. Harcus, and E. L. Hewlett, Abst. Annu. Meet. Am. Soc. Microbiol. 1987, C322, p. 377). This DNA sequence was used as a DNA probe to screen nasopharyngeal aspirates from children clinically diagnosed with pertussis, and results were compared with DFA and culturing done on the same specimen. The DNA probe was positive in 89% of the specimens, while 44% were culture positive and 67% were positive by DFA. The authors found that the DNA probe could detect as few as 10^3 *B. pertussis* and showed slight cross-reactivity with *Pseudomonas aeruginosa* and other *Bordetella* species. These initial results suggest that DNA probes may have great potential for rapid diagnosis of pertussis. Further field trials are in progress to verify these initially reported results.

CONCLUSION

While progress has been made in the development of rapid diagnostic tests for *B. pertussis*, presently culture and the DFA test are still the methods of choice for confirmatory diagnosis of pertussis. Serologic ELISAs for detection of anti-pertussis antibody in sera or nasal secretions hold promise as important tools in epidemiologic studies of pertussis but not as rapid diagnostic tests. The most promising diagnostic methods for the future appear to be the use of DNA probes and methods to directly detect *B. pertussis* antigens in nasopharyngeal specimens. This includes use of specific monoclonal antibodies or assaying for the AC enzyme of *B. pertussis*. Further extensive laboratory studies, research, and field trial evaluations will need to be done to determine the sensitivity, specificity, and value of these methods as new rapid diagnostic tests for the diagnosis of pertussis.

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