THE LABORATORY DIAGNOSIS OF BORDETELLA PERTUSSIS: A CRITICAL EVALUATION

by

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INTRODUCTION

Etiology of whooping cough

Whooping cough has been an important disease of young children since it was first recorded in the medical literature of the middle ages (Bradford, 1965). Thousands died and many more were left incapacitated from complications of the disease. The causative agent was discovered in the sputum of children affected with whooping cough in the early part of the twentieth century (Bordet and Gengou, 1906). The organism was isolated on glycerinated potato blood agar (Bordet-Gengou), a culture medium still in use today. Bordet (1907, 1909 and 1910) showed that suspensions of the organism were agglutinated and fixed complement in the presence of convalescent serum, providing further evidence of the etiological role of the newly discovered bacillus.

For many years the bacterium was known as the Bordet-Gengou bacillus, and later as Haemophilus pertussis because a culture medium rich in blood was needed for its primary isolation. However, Moreno-Lopez (1952) created a special genus to encompass three serologically related Gram negative bacilli causing localized respiratory infections of man and animals. He called this genus Bordetella to honour Bordet. The three species of this genus are B. pertussis, B. parapertussis and B. bronchiseptica, of which B. pertussis is the most important member.

Following the discovery and cultivation of B. pertussis attempts were made to develop an effective vaccine for whooping cough. Many were unsuccessful until Sauer (1933) and Kendrick and Eldering (1939) succeeded in producing an effective vaccine. The success was largely due to the observation made by Leslie and Gardner (1931) that B. pertussis cells did not always grow in a smooth phase (phase 1) but

occurred also in a rough (phase 4) or in intermediate forms (phase 2 and 3). Only smooth forms were found suitable for vaccine production. After the difficult problems of growing, killing, detoxifying, preserving and standardizing the cell suspensions were solved, a practical and effective vaccine was finally produced.

Pathogenesis

Whooping cough is predominantly an infection of the respiratory mucosa, running a protracted course of four to eight weeks after an incubation period of seven to fourteen days. The bacilli can be demonstrated adhering to the cilia and luminal surface of the epithelium of the bronchioles, bronchi and trachea. They grow only on the surface of ciliated epithelium, without invading the mucosa more deeply; they cause damage to the cilia and consequently initiate the irritation that induces increased secretion of mucus and the stimulus for the paroxysmal cough and bronchospasm. Some workers, however, believe that the syndrome may be related to the production of a neurotoxin by the bacillus. The organisms are expelled in droplets during coughing and in scanty viscid sputum, and are most easily demonstrable in the first two or three weeks of infection. Later, toxic damage may affect the submucosa and extend into lung tissue accompanied by peribronchiolar infiltration with lymphocytes and polymorphonuclear cells. Blocked bronchioles lead to areas of lung collapse and patches of emphysema. Secondary bronchopneumonia due to infection with pyogenic cocci may ensue and sometimes causes death, or bronchietasis may develop as a long-term sequel. Convulsions and, more rarely, encephalopathy may occur in severe infections of young children and may be related to anoxaemia. An early leucopenia is soon followed by leucocytosis (total white blood cells: 15,000 to 30,000 per cmm. with 70 to 80 percent lymphocytes) related to a lymphocytic - stimulating factor (Cruickshank, 1973).

Whooping cough is predominantly a disease of infants and children. It affects some 70% of children in unvaccinated urban communities, and

is an infection of early childhood since there seems to be little or no passive immunity by antibodies from the mother. The early infections are the most severe, so that most of the deaths from whooping cough occur in the first year of life.

Characterization of Bordetella Pertussis

It is generally agreed that Bordetella pertussis is the causative agent of whooping cough and Bordetella parapertussis causes a milder form of the disease. Bordetella bronchiseptica, a third member of the genus, is basically found in the respiratory tract of animals. It is isolated from humans only after close contacts with rabbits, guinea pigs, cats and other domestic animals (Brooksaler 1967). The three species of the genus Bordetella belong to the family Brucellaceae.

B. pertussis is a small, aerobic, non motile, ovoid, Gram negative bacillus (0.3 x 0.5 x 0.5 to 1 μm) (Plate 1) which does not form spores and has a tendency to show bipolar staining. Lawson (1933) described a capsule or sheath, but this is not a prominent feature of most strains. Growth of B. pertussis on Bordet-Gengou agar usually occurs by the fourth day. Typical colonies have a pearly appearance. They are small, grayish-white, glistening, and butyrous with smooth edges. Usually they produce a partial, diffuse hemolysis on Bordet-Gengou agar, but this is difficult to see around young colonies. On prolonged incubation the colonies continue to grow and the hemolysis becomes more obvious.

B. pertussis does not ferment carbohydrates, and utilizes amino acids as a source of energy. During its growth in media containing amino acids, the pH rises markedly from the release of alkaline metabolites. The growth requirements are relatively simple, but nicotinic acid (Hornibrook, 1940) and amino acids (Proom 1955) are essential. Inhibitors, particularly colloidal sulphur and colloidal copper sulphide, may prevent growth of B. pertussis (Proom 1955). Blood, charcoal and ion-exchange resins have been used to neutralize these inhibitors (Pittman, 1970).

Like many bacteria, B. pertussis has the ability to mutate from a smooth virulent form (phase 1) to a rough form (phase 4) which is non-virulent and usually grows on plain nutrient agar. Intermediate forms (phase 2 and 3) have also been described (Leslie and Gardner 1931). Metabolic, morphologic and antigenic changes accompany the transformation or modulation. The smooth forms are typically coccobacilli which agglutinate with antisera to smooth cultures. They also have the ability to induce immunity and to sensitize mice to various agents such as histamine, serotonin, and various types of shock. The rough cultures, on the other hand, usually consist of longer pleomorphic rods with filamentous forms, which do not specifically agglutinate with antisera to smooth forms and lack the ability to protect mice from infection or to sensitize them to histamine and various other shock-inducing substances. Some antigens found in smooth cells are absent from rough strains, but many common antigens, including the heat labile ($56^{o}C$ for ½ hour) toxin, are retained. Gel diffusion tests, using antiserum raised with a smooth culture, and extracts from smooth and rough cultures, showed that some of the unique and common antigens are present (Munoz, 1963).

Many biologically active substances have been demonstrated in B. pertussis. At least eight agglutinogens, a hemagglutinating substance, an endotoxin (lipopolysaccharide), and a heat labile ($56^{o}C$ for ½ hour) toxin have been found. Furthermore, a histamine sensitizing factor, a mouse protective antigen, a lymphocytosis - promoting factor, and a heat labile ($80^{o}C$ for ½ hour) adjuvant activity can be demonstrated in cells from smooth cultures. It appears that these last four activities are due to one substance for which Munoz (1976) proposed the name "pertussigen".

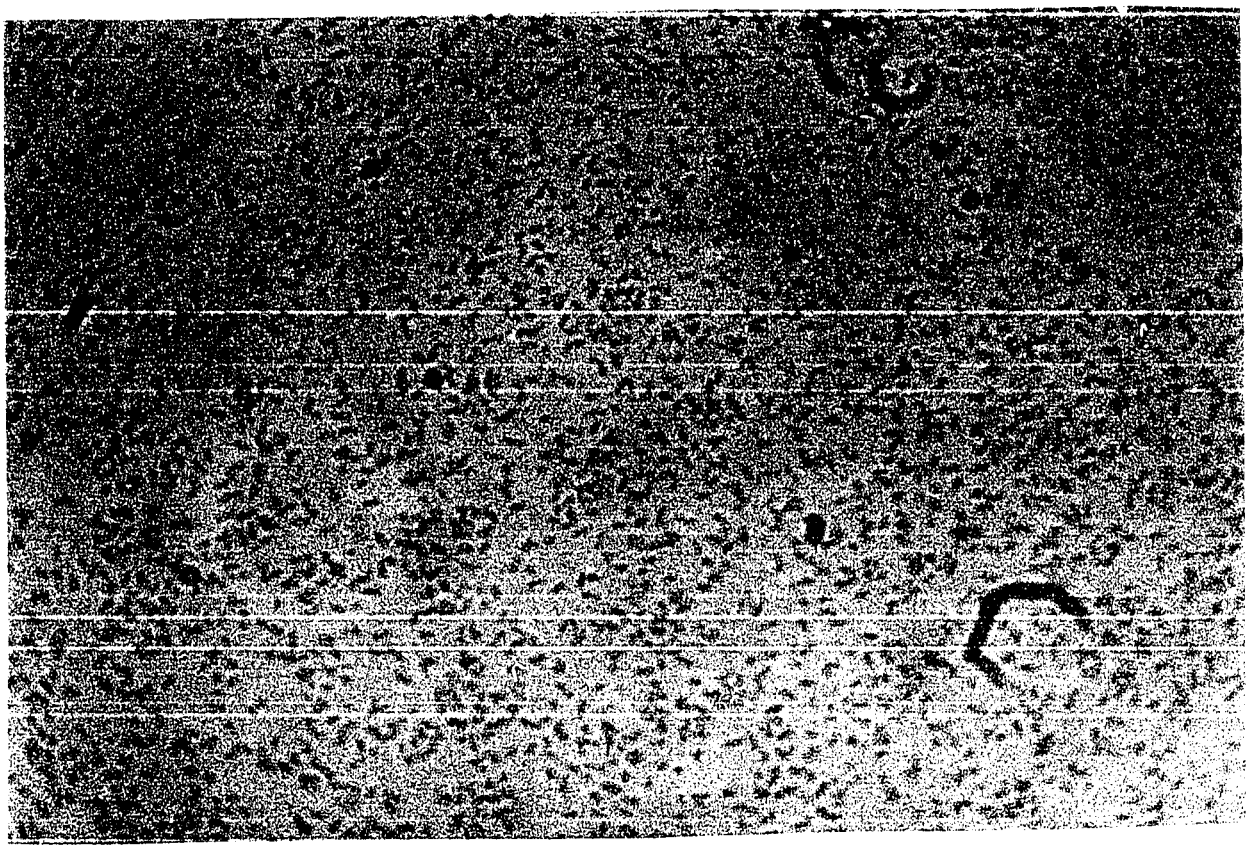
Apart from these biologically active substances, B. pertussis can be differentiated from the other Bordetella species by the following characteristics. (Table 1).

Table 1

Differential characteristics of Bordetella species

Characteristics	B. pertussis	B. parapertussis	B. bronchiseptica
Growth on blood-free peptone agar	-	+	+
Browning of peptone agar	-	+	-
Production of urease	-	+	+ 4 hrs.
Reduction of nitrates	-	-	+
Motility	-	-	+

Plate 1



Gram stained film of Bordetella pertussis

Agglutinogens of Bordetella and serotypes of B. pertussis

The antigenic complexity of the agglutinogens of Bordetella was not fully realized until Andersen (1953) studied the heat stable 'O' antigens and heat labile 'K' antigens. She found the somatic 'O' antigen to be common to the three organisms B. pertussis, B. parapertussis and B. bronchiseptica, but found a variation in the heat labile 'K' antigen. For B. pertussis she suggested a scheme for these 'K' antigens using the numbers 1 to 5, factor 1 being common to all the strains tested. She also found 'K' antigens which were common to the other Bordetella species. Eldering, Hornbeck and Baker (1957), working with Andersen's strains as a basis for comparison, added a sixth agglutinogen to the B. pertussis scheme, postulated a further eight (7-14) to explain the serological relationships of B. parapertussis and B. bronchiseptica. Of these, factor 7 is found in all Bordetella cultures, factor 14 is specific for B. parapertussis, 12 is specific for B. bronchiseptica and 8,9,10 may be found in both B. parapertussis and B. bronchiseptica.

Eldering, Eveland and Kendrick (1962) reported an analysis of antigenic factors of Bordetella cultures, as shown in Table 2.

Table 2

Analysis of antigenic factors of Bordetella cultures

Culture	Factors
B. pertussis	
5373	7,1,3,6,13
5374	7,1,2,5,6,13
5375	7,1,2,4,13
B. parapertussis	
17-903	7,8,9,10,14
B. bronchiseptica	
5376	7,8,9,12,13
214	7,9,12,13
899	7,8,10,11,12

They also found that antisera to agglutinogen 1 could be conjugated with fluorescein isothiocyanate and that this conjugate was capable of staining B. pertussis cells, while antisera specific to agglutinogen factors 2-5, similarly conjugated, were not capable of staining cells containing those factors. It is not clear whether the specific sera conjugated with fluorescein isothiocyanate were still capable of agglutinating the cells. These observations have been interpreted by Pittman (1970) as indicating that factor 1 agglutinogen is a molecule with radicals of different specificities corresponding to the other serologic factors, but this would not explain the failure to stain with fluorescent antibody. As Pittman (1970) suggests, radicals on the agglutinogen 1 could give it different specificities which would be detected as the other factors. By plating strains of B. pertussis on slightly modified Cohen and Wheeler and on Bordet-Gengou media, Cameron (1967) observed marked colonial variation in the growth which in turn was correlated with differences in the agglutinogenic composition of the variants. He concluded that the results suggest that type 1,2,3 might be regarded as the parent from which the others namely, types 1,2; 1,3; or 1 are derived. Stanbridge and Preston (1974) show that these organisms can mutate in both directions, since factor 1 strain can give rise to strains having 1,2 or 1,3 agglutinogens or strains 1,2 or 1,3 can change to strains with agglutinogens 1,2,3.

With the recognition of these agglutinogens a serotyping scheme has been established, based on the identification of the species group antigen (antigen "1") and type specific antigens "2" to "6" (Bradford, 1965).

Antigens "1", "2" and "3" are generally regarded as the major ones (Preston, 1965). The most important serotypes are therefore serotype 1,2; serotype 1,2,3 and serotype 1,3.

Laboratory diagnosis of B. pertussis

The following methods are available for the laboratory diagnosis of B. pertussis.

(I) Cultures

(II) Immunofluorescence

(III) Serological tests

However, the types of specimens collected and the use of different culture media may greatly affect the results.

Specimens

1. Cough plate

Chievitz and Meyer (1916) described this method of collection of specimens, still in use in many laboratories. A plate of Bordet-Gengou medium is held about four inches from the patient's mouth during several bouts of expulsive coughs, so that the spray from the cough is collected on the medium. The plate is then covered and sent to the laboratory for incubation and examination. Disadvantages of this method include the difficulty in getting patients, especially very young infants, to produce a satisfactory cough, and the frequent over growth of the culture by bacterial and fungal contaminants. Results are generally poor.

2. Pernasal swab

The specimen is collected from the nasopharynx on a small sterile cotton swab mounted on a thin flexible wire. (Bradford and Slavin 1940). To obtain the specimen, the child's head is immobilized, and the swab is bent into a downward arc and passed through the nostril into the nasopharynx. The inoculated swab is withdrawn, and then streaked on a Bordet-Gengou plate. The procedure provides satisfactory specimens, but can be dangerous.

3. Auger nasopharyngeal suction

Auger (1939) described this method for obtaining nasopharyngeal secretion by suction with a syringe using a soft nasal catheter. The child does not need to cough, and usually sufficient and satisfactory specimen can be obtained. This is reputed to be the method of choice.

Culture Media

1. Bordet-Gengou

Bordet-Gengou (1906) obtained growth of B. pertussis on a solid medium containing glycerinated potato extract and 50% (V/V) blood. Since then a number of modifications of the medium have been proposed. It appears that the modification by Kendrick and Eldering (1934) is most widely used.

2. Modified Bordet-Gengou

Kendrick and Eldering (1934) reduced the blood content from 50% to about 20%. The salt content of the medium was adjusted to prevent lysis of blood. With this concentration of sheep blood, colonies of B. pertussis develope a hemoltyic zone, which is useful in identifying the organisms. Penicillin in a concentration of 0.25 to 0.50 unit per ml. was subsequently used to reduce the growth of commensal flora. The modified dehydrated Bordet-Gengou medium is available commercially.

3. Charcoal agar

Sutcliffe and Abbot (1972) described a medium containing Oxoid charcoal agar (CM119) with 10% horse blood, 1% Difco proteose peptone no. 3, and 40 mcg/ml of cephalexin for isolation of B. pertussis and

B. parapertussis. Plates are easier to read due to the reduction in the number of commensals. The charcoal absorbs toxic metabolic products, yielding larger characteristic shiny colonies against the dark background of the medium.

Immunofluorescence

A disadvantage of the cultural method is the time needed for a positive culture report: a minimum of three, and occasionally seven days are required. Donaldson and Whitaker (1960) and Kendrick et al (1961) studied the use of fluorescent antibody staining of nasopharyngeal smears for the identification of B. pertussis. Holwerda and Eldering (1963) examined 517 nasopharyngeal swabs from suspected whooping cough patients by culture and by fluorescent antibody (FA) staining procedures applied to direct slide preparations. A total of 138 were positive by both methods, 25 by culture only, and 25 by FA only. The FA technique was also used in the identification of young colonies. It was shown that a positive preliminary report could be issued at least a day earlier.

Serological Tests

Serological diagnosis of whooping cough has been attempted by the use of complement fixation and agglutination tests on paired blood sera. The second specimen of blood has to be taken as late as possible in convalescence because of the slow development of demonstrable antibodies. A combined Scottish study (1970) revealed that for patients under six months of age isolation of B. pertussis was much commoner than positive serological findings, and for patients over one year of age serological tests were more often positive than were cultures. However, serological

tests are of limited diagnostic value since circulating antibodies occur late in the infection. Also, the results can be misleading due to previous vaccinations. Because of these disadvantages serological tests have not found general acceptance in the laboratory diagnosis of B. pertussis infections. However, they have been used to evaluate the responses of children to vaccination (Abbott et al 1971).

Incidence and Control Measures

Whooping cough was once a serious childhood disease. The disease in its typical form is prolonged and debilitating and may affect 70 to 80 percent of unprotected children with a high incidence and severity of attack in infants under two years of age. It was more prevalent than tuberculosis, diphtheria, measles and scarlet fever over the ten year period of 1925 - 1934 in the United States (Kendrick, 1935). This infection killed some 40,000 children in England and Wales in the decade 1921 to 1930 (Cruickshank, 1973).

Whooping cough due to Bordetella pertussis can be controlled, if not wholly prevented, by prophylactic vaccination. A series of controlled trials of different vaccines was carried out in Britain during the 1950's under the sponsorship of the Medical Research Council. The main findings as summarized in the final Medical Research Council Report (1959) were as follows:

> "The results of the trials clearly showed that it was possible by vaccination to produce a high degree of protection against the disease, as shown by the substantial reduction in the attack rate amongst home contacts, and, in those cases where vaccination failed to give complete protection, to reduce the severity and duration of the disease. The results also showed that the different vaccines employed varied a great deal in their protective action; the poorest gave an attack rate in home contacts of 87 percent, and the most effective an attack rate of 4 percent."

As a result of childhood immunization the incidence of whooping cough in vaccinated countries has been declining yearly. However, it is still a serious problem in underdeveloped countries where vaccination

programs have not yet been well established. In Kenya in the 1960's it was the fifth common cause of death among children, and it was estimated that about 9 percent of all deaths in children in Western Nigerian hospitals were due to whooping cough and its complications. Similar findings were reported in Uganda (Bwibo, 1971).

In vaccinated countries, although the incidence of whooping cough has greatly declined, yet the disease is far from being extinct. In England and Wales, there were about 40 cases notified to the Registrar General each week (Br. Med. J. 1975). In the United States and Canada, there are still many cases of this disease, documented in epidemiological, morbidity and mortality reports. The Ontario Ministry of Health reported 652 cases in 1971, 345 cases in 1972, 407 cases in 1973 and 793 cases in 1974. At the Hospital for Sick Children in Toronto, more than 100 strains of Bordetella pertussis have been isolated regularly every year (Fleming, 1975). They are isolated from pertussis syndromes early in the disease, from Auger suction material from the nasopharynx primarily of infants, a high proportion of whom have either not had, or have not completed, a full course of immunization.

Although virtually no isolation of Bordetella pertussis was made prior to 1975, the National Capital Region is by no means exempted from the disease. The Ottawa-Carleton Health Unit reported 114 cases in 1971, 35 cases in 1972, 46 cases in 1973 and 163 cases in 1974.

It is however notorious that whooping cough is under reported, either because of negligence or lack of bacteriological confirmation. The actual incidence of whooping cough is certainly higher than the figures presented.

As a control measure it could be important to know the serotype of the Bordetella pertussis strains circulating in the community. It has been suggested that immunizing agents containing the types found in a community would provide improved immunity.(Preston 1970, 1976; Blaskett et al 1971).

The possible role of viruses in the etiology of the whooping cough syndrome

A number of studies have suggested that viruses may cause whooping cough. McCordock and Smith (1934) described inclusion bodies in the epithelial cells of the upper respiratory tract of children who had died of pneumonia following whooping cough. The inclusions which they described resembled those produced by some viruses. Following the introduction of tissue culture techniques, Olson, Miller and Hanshaw (1964) isolated adenovirus type 12 from the throats of four children with whooping cough. Infection was confirmed by subsequent rises in neutralizing antibodies, and in each patient cultures for Bordetella pertussis were negative. In a prospective study conducted at the Edinburgh City Hospital, Urquhart, Moffat, Calder and Cruickshank (1965) recovered viruses, a third of which were adenoviruses, from 15 of 49 children with clinical whooping cough. B. pertussis was isolated from only 2 of the 49 patients. Collier, Connor and Irving (1966) isolated adenovirus type 5 from the liver, lungs and kidney of a four year old who died following a whooping-cough-like illness complicated by pneumonia. Connor (1970) isolated adenoviruses (type 1,2,3 and 5) from 11 of 13 sporadic cases of whooping cough in which there was no evidence of infection with B. pertussis by culture or serology.

Pereira and Candeias (1971) isolated 37 strains of virus, almost a third of which were adenoviruses, from 136 Brazilian children with whooping cough. Although B. pertussis was recovered from 29 children, the virus isolation rate was twice as high in the group from whom B. pertussis was not isolated.

About the same time, Sturdy, Court and Gardner (1971) reported their studies on 34 children with whooping cough admitted to hospitals in the Newcastle area. B. pertussis was not isolated from any of these children whereas viruses, mainly respiratory syncytial virus and

adenovirus, were obtained from 20. These authors suggested that, in view of the controversy existing about the etiology of whooping cough, a large scale prospective study should be undertaken.

A combined Scottish study on clinical, bacteriological and virological findings in children with suspected whooping cough from 1966 to 1968 was published ("What causes whooping cough?", 1970). It indicated that Bordetella pertussis was much more clearly associated with whooping cough than viral agents. Of 210 patients in this study from whom pernasal swabs and paired sera were received, 102 (49%) yielded B. pertussis and/or showed rising antibody titres to B. pertussis. Positive virological findings were obtained in 42 (20%) of the 210 patients; 25 of these 42 also had current B. pertussis infection and 17 were negative for B. pertussis. The commonest virological infections identified were adenovirus in 16 patients (9 positive and 7 negative for B. pertussis) and respiratory syncytial virus in 16 patients (8 positive and 8 negative for B. pertussis). The authors stressed that in Scotland B. pertussis was much more closely associated with clinical whooping cough than any single viral agent or group of recognized viral agents.

Lewis, Gust and Bennett (1973) reported a five year study of routine bacterial and viral investigations on 483 patients with whooping cough in a hospital in Australia. A total of 175 (36.2%) B. pertussis strains were isolated, compared to 116 (24%) viruses. If, as postulated, respiratory viruses had an important role in the etiology of whooping cough, it would be expected that they would be isolated more frequently from patients from whom B. pertussis was not isolated. But there was no

significant difference between the viral isolation rate in the two groups. The authors concluded that although viruses may be implicated in occasional cases of whooping cough syndrome, there was no evidence to suggest that they are responsible for a large proportion of cases.

In recent years there has been a tendency to emphasize the importance of viruses in the etiology of whooping cough and to dismiss the role of B. pertussis. It appears that the main reason for this is the general disappointingly low rate of isolation of B. pertussis in the past.

In most of these studies single pernasal swab specimens were taken and cultured on Bordet-Gengou plates containing penicillin. The techniques of specimen taking and culture for Bordetella pertussis were not given in great details. Moreover, it is not clear at what stage of the disease the specimens were taken. These factors are very important, as success in isolating Bordetella pertussis depends on availability of a good posterior nasopharyngeal specimen taken at the early onset of the disease and upon culturing of the specimen without delay on a proper medium. Bass et al (1975) disagreed with the concept that adenoviruses play an independent role in the pathogenesis of the pertussis syndrome and emphasized the importance of technique for recovery of Bordetella pertussis. It was noted by Bass et al (1975) that this species could be recovered from 52% to 81% of patients with clinical pertussis without previous antibiotic therapy.

SPECIFIC AIMS OF THE STUDY

Although precise data are not available, it appears that a proper search for Bordetella pertussis is not made in most clinical microbiological laboratories (Holwerda, 1971). This may partly explain the low rate of isolation of Bordetella, despite clinical and/or epidemiological evidence to the contrary. There were 358 cases of whooping cough reported by the Ottawa-Carleton Regional Health Unit from 1971 to 1974, but the isolation of B. pertussis was practically nil in all the hospitals in Ottawa.

In view of the information presented before it could be suspected that:

(1) Bordetella pertussis was still present in the Ottawa area, but was undetected.

(2) The true number of B. pertussis cases was unknown due to the lack of laboratory confirmation.

(3) The failure to isolate this organism by clinical microbiology laboratories was due to improper specimens and poor selective culture media in addition to factors such as previous antibiotic therapy and collection of specimens at a late stage in the disease.

The opening of the Children's Hospital of Eastern Ontario in July 1974 made it possible to investigate these problems from 1975 to 1976. The specific aims of the study were:

(1) Evaluation of commercial reagents and media.

(2) Correlation of isolation of Bordetella pertussis and Bordetella parapertussis with isolation of other pathogens (bacterial and viral).

(3) Investigation of the epidemiology of pertussis infections in the Ottawa-Carleton area.

(4) Correlation of microbiological and clinical data in pertussis.

EVALUATION OF METHODS

Evaluation of immunofluorescence tests for the identification of B. pertussis and B. parapertussis

Direct immunofluorescence

1. Specificity

Difco FA - B. pertussis and FA - B. parapertussis antisera were the only commercially available reagents at the time of our study. These were globulins (exact details not available from Difco) from immunized chicken, and conjugated with fluorescein isothiocyanate.

Six strains of B. pertussis (R019, R333, R411, R455, R603 and R660) and two strains of B. parapertussis (R1378 and R30036) were obtained from Dr. P.C. Fleming, Hospital for Sick Children, Toronto. These cultures were smooth cultures maintained on charcoal agar and stock cultures at their third to fifth subculture were kept by suspending fresh cultures in litmus milk and then freezing at -70°C.

The technique followed was the one recommended by the manufacturers. Smears of cultures of B. pertussis, B. parapertussis and some common organisms were made by emulsifying a colony in a drop of 0.85% saline on a slide. The smears were allowed to dry and were then fixed by gentle heating. FA - B. pertussis and FA - B. parapertussis conjugates were added to corresponding smears and incubated at room temperature in a moist chamber for 30 minutes. After incubation the conjugate was rinsed off with phosphate buffered saline, pH 7.2 (Difco FA-buffer). The slides were placed in FA-buffer for 10 minutes each with two changes of buffer. They were then removed, dried, and mounted with Difco FA mounting fluid. The slides were examined using two Leitz SM-LUX microscopes with a comparison bridge, allowing for simultaneous comparison between test and control slides. U.V. source lights were HBO 50 watts with KP 500 filters and using incident illumination.

Table 3

FA-staining on B. pertussis, B. parapertussis and some common organisms

Organism	Fluorescence with FA-B. pertussis antiserum	Fluorescence with FA-B. parapertussis antiserum
B. pertussis (R019)	++++	++
B. pertussis (R333)	++++	++
B. pertussis (R411)	++++	++
B. pertussis (R455)	++++	++
B. pertussis (R603)	++++	++
B. pertussis (R660)	++++	++
B. parapertussis (R1378)	++	++++
B. parapertussis (R30036)	++	++++
Haemophilus influenzae	+	+
Neisseria species	+	+
Alpha-hemolytic Streptococcus	+	+
E. Coli	+	+
Staph aureus	+	+

+ → ++++ = increasing degree of fluorescence.

Table 3 shows the results of the initial trial of FA-staining on B. pertussis, B. parapertussis, and common organisms found in the nasopharynx, using the procedures recommended by the manufacturer.

Too thick smears were found to be the main reason for a light to a moderate degree of non-specific fluorescence with common organisms. However, apart from making thinner smears, the conjugated sera were diluted in FA-buffer to find out if results could still be improved. Table 4 shows the effect of dilution of conjugated sera on fluorescence.

Diluting the FA antiserum did reduce the degree of specific fluorescence, while non-specific fluorescence was still present with control organisms.

A similar experiment was carried out using undiluted FA antiserum, thin smears and an additional 10 minutes washing in FA-buffer. This combination gave the best results. Specific organisms were stained with a high degree of fluorescence (4+), and non-specific fluorescence with control organisms was kept to a minimum.

2. Sensitivity

Sensitivity is an important factor of the immunofluorescence method for a reliable and rapid laboratory test in the diagnosis of pertussis. To test the sensitivity of the immunofluorescence method, a suspension of a fresh culture of B. pertussis (R019) grown on charcoal agar was prepared in saline to match the turbidity of the 0.5 McFarland turbidity standard. The suspension was mixed with a vortex mixer and examined in an improved Neubauer counting

Table 4

FA-staining using diluted antiserum as an attempt to reduce non-specific fluorescence.

rganisms	Fluorescence with FA - B. pertussis antiserum			Fluorescence with FA - B. parapertussis antiserum		
	Undiluted	1:4 dilution	1:8 dilution	Undiluted	1:4 dilution	1:8 dilution
. pertussis (R019)	++++	+++	++	+	trace	trace
. pertussis (R333)	++++	+++	++	+	trace	trace
. pertussis (R411)	++++	+++	++	+	trace	trace
. pertussis (R455)	++++	+++	++	+	trace	trace
. pertussis (R603)	++++	+++	++	+	trace	trace
. pertussis (R660)	++++	+++	++	+	trace	trace
. parapertussis (R1378)	+	trace	trace	++++	+++	++
. parapertussis (R30036)	+	trace	trace	++++	+++	++
laemophilus influenzae	+	trace	trace	+	trace	trace
leisseria species	+	trace	trace	trace	trace	trace
lpha-hemolytic Streptococcus	trace	trace	trace	trace	trace	trace
. Coli	trace	trace	trace	trace	trace	trace
taph. aureus	trace	trace	trace	trace	trace	trace

+ → ++++ = increasing degree of fluorescence

chamber. The organisms were found to be monodispersed and total counts were made from the suspension. Ten-fold dilutions of this suspension were also made in saline and a loopful (using a calibrated loop of 0.01 ml.) of each dilution was spread on a slide. The slides thus prepared were heat fixed and stained by FA - B. pertussis antiserum. At the same time a loopful of each dilution was spread on a charcoal agar plate without antibiotic for viable counts. The plates were incubated at $36^{o}C$ in a moist atmosphere for seven days. The colonies were counted for those dilutions showing 10 to 100 colonies on the plate. The number of total and viable organisms in the original suspension was thus determined to asses the sensitivity of the FA test. Results of the test are listed in Table 5.

The results indicated that 10^{-2} dilution of the original suspension was the highest dilution which would still be recorded as a positive FA test. This was equivalent to a concentration of $(1.8 \pm 0.07) \times 10^{6}$ total organisms/ml or $(7.5 \pm 0.34) \times 10^{4}$ viable organisms/ml. In other words the FA test would be negative if there were fewer than $(1.8 \pm 0.07) \times 10^{6}$ total organisms/ml or $(7.5 \pm 0.34) \times 10^{4}$ viable organisms/ml in the specimen if one used the test system described.

The low sensitivity suggested that not all B. pertussis cells, dead or viable, stained readily with the commercial fluorescent antiserum. The viable count experiment yielded growth of B. pertussis colonies on every plate up to a 10^{-4} dilution of the original suspension and indicated culture is a much more sensitive method than direct immunofluorescence.

Table 5

Sensitivity of FA test for *B. pertussis*

Dilution	Total organisms/ml.	Viable organism/ml.	No. of fluorescent organism per 40 X objective field	Interpretat of FA resul
Undiluted	$(1.8 \pm 0.07) \times 10^8$	$(7.5 \pm 0.34) \times 10^6$	numerous	++++
10^{-1}	$(1.8 \pm 0.07) \times 10^7$	$(7.5 \pm 0.34) \times 10^5$	approx. 200	++
10^{-2}	$(1.8 \pm 0.07) \times 10^6$	$(7.5 \pm 0.34) \times 10^4$	20 - 30	+
10^{-3}	$(1.8 \pm 0.07) \times 10^5$	$(7.5 \pm 0.34) \times 10^3$	doubtful	doubtfu
10^{-4}	$(1.8 \pm 0.07) \times 10^4$	$(7.5 \pm 0.34) \times 10^2$	not seen	negativ
10^{-5}	$(1.8 \pm 0.07) \times 10^3$	$(7.5 \pm 0.34) \times 10$	not seen	negativ

Concentration of total organisms in the undiluted suspension was obtained by using improved Neubauer counting chambers. These results gave a mean count from six counts, 1/250 cmm per chamber, of 719 ± 27.2 at the 95% confidence level of this volume, or $(1.8 \pm 0.07) \times 10^8$ total organisms/ml.

Concentration of viable organisms in the undiluted suspension was calculated from the data obtained at the 10^{-3} dilution level. These results gave a mean count on three plates, 0.01 ml. per plate, of 75 ± 3.4 at the 95% confidence level per 0.01 ml., or $(7.5 \pm 0.34) \times 10^6$ viable organisms/ml. in the undiluted suspension (see Table 10).

Indirect immunofluorescence

We suspected the low sensitivity of direct immunofluorescence could be due to the inefficiency of the Difco reagents. Indirect immunofluorescence is generally considered to be more sensitive than the direct method due to the additional combining sites which are made available by the antibody molecules of the middle layer acting as antigen for the fluorescent antiglobulin.

An attempt to adapt indirect immunofluorescence was made. Known positive smears made from B. pertussis cultures and known negative smears made from E. coli cultures were prepared. These slides were covered with diferent dilutions of B. pertussis antiserum (Wellcome, rabbit absorbed specific antiserum) and incubated at room temperature for 30 minutes. The slides were rinsed and placed in FA-buffer for 10 minutes with three changes of buffer for a total of 30 minutes. The air-dried slides were then further stained with different dilutions of fluorescein anti-rabbit antiserum (Wellcome, purified gammaglobulin, anti IgG) for 30 minutes at room temperature in a moist chamber. After three washings of 10 minutes each in FA-buffer the slides were air dried, mounted in FA-mounting fluid and examined.

A chessboard titration experiment was performed to find the optimal dilutions of B. pertussis antiserum (rabbit) and fluorescein anti-rabbit antiserum for subsequent use. Results of the titration are shown in Table 6(A) and Table 6(B).

From this experiment, a dilution of 1:8 of B. pertussis antiserum (rabbit) and a dilution of 1:16 of fluorescein anti-rabbit antiserum were the optimal combinations to carry out subsequent indirect immunofluorescence examinations.

Table 6(A)

Chessboard titration of B. pertussis antiserum (rabbit) and fluorescein anti-rabbit antiserum, using known B. pertussis positive control slides.

Dilution of fluorescein anti-rabbit antiserum \ Dilution of B. pertussis antiserum (rabbit)	1:4	1:8	1:16	1:32	1:64
1:4	++++	++++	+++	+	trace
1:8	++++	++++	+++	+	trace
1:16	++++	(++++)*	+++	+	trace
1:32	++++	++	+	trace	trace

* Optimal combination of B. pertussis antiserum (rabbit) and fluorescein anti-rabbit antiserum.

Table 6(B)

Chessboard titration of B. pertussis antiserum (rabbit) and fluorescein anti-rabbit antiserum, using known E. coli negative control slides.

Dilution of Fluorescein anti-rabbit antiserum \ Dilution of B. pertussis antiserum (rabbit)	1:4	1:8	1:16	1:32	1:64
1:4	-	-	-	-	-
1:8	-	-	-	-	-
1:16	-	-	-	-	-
1:32	-	-	-	-	-

FA buffer was used as diluent.

- = no fluorescence

+ to ++++ denote increasing degree of fluorescence

A study on the sensitivity of indirect immunofluorescence using this combination of antisera on known B. pertussis slides was then carried out. This was similar to the study on sensitivity of direct immunofluorescence (see Table 5). Results were not improved by adopting indirect immunofluorescence, and positive tests could not be recorded for less than $(1.8 \pm 0.07) \times 10^8$ total organisms/ml, or $(7.5 \pm 0.34) \times 10^6$ viable organisms/ml.

Attempts to produce antisera

Since immunofluorescence with commercial reagents did not seem to give a high degree of sensitivity an attempt was made to produce an antiserum of our own. Since rabbits may have natural antibodies against Bordetella bronchiseptica, and these cross react with B. pertussis and B. parapertussis, the plan was to select rabbits which did not have antibodies towards Bordetella and then immunized these rabbits with killed B. pertussis culture.

Six rabbits were acquired. They were bled from the marginal vein of the ear. The blood was allowed to clot and the serum separated by centrifugation.

B. pertussis, B. parapertussis and B. bronchiseptica antigens (B. bronchiseptica strain was obtained from a quality control program and maintained as a stock culture) were prepared by growing the organisms on charcoal agar and the growth suspended in saline to give an opacity of No. 7 McFarland standard (approximately 2×10^9 organisms/ml). These suspensions were killed at 60^0C for one hour and used as antigens for agglutination tests to detect antibodies in the rabbit blood towards the three species of Bordetella.

Serial doubling dilutions of rabbit serum were made in 0.85% saline, in 0.2 ml amounts, over a range of 1:2 to 1:128. Equal volumes of killed bacterial suspensions were added so that the final serum dilutions ranged from 1:4 to 1:256. Positive controls using known B. pertussis and B. parapertussis antisera at a final dilution of 1:40 were used. B. bronchiseptica antiserum was not available and a positive control could not be included. Negative controls consisted of mixture of saline with the three killed bacterial suspensions.

The tubes were incubated in a 37^{o}C waterbath overnight and agglutinations read the next morning.

The end point of agglutination was taken as 50% (or ++) agglutination. As shown in Table 7, all six rabbits showed antibodies towards all three species of Bordetella, up to a dilution of 1:8.

Personal communication with Central Public Health Laboratories revealed that pre-existing antibodies were problems in producing B. pertussis antiserum in Toronto. The staff in the Central Public Health Laboratories found that only eight out of about one hundred rabbits were free from Bordetella antibodies. Our experiment might have become too involved and too expensive and the plan of producing our own antiserum was therefore discontinued.

Table 7

Agglutination tests to detect Bordetella antibodies in rabbit blood.

	Tube number	1	2	3	4	Positive Control	Negative Control
	Final dilution of serum	1:4	1:8	1:16	1:32	1:40	
	Antigen						
Rabbit 1	B. pertussis	++++	+++	-	-	+++	-
	B. parapertussis	++++	++++	+++	+	+++	-
	B. bronchiseptica	+++	++	+	+	not applicable	-
Rabbit 2	B. pertussis	+++	++	-	-		
	B. parapertussis	+++	+++	++	-		
	B. bronchiseptica	+++	++	+	+		
Rabbit 3	B. pertussis	++++	+++	++	-		
	B. parapertussis	+++	++	+	-		
	B. bronchiseptica	++++	++++	+++	++		
Rabbit 4	B. pertussis	+++	+++	+	-		
	B. parapertussis	++++	++++	++++	+++		
	B. bronchiseptica	++++	++++	+++	++		
Rabbit 5	B. pertussis	+++	++	+	-		
	B. parapertussis	++++	++++	++++	++		
	B. bronchiseptica	++++	++++	+++	++		
Rabbit 6	B. pertussis	++++	++++	++	-		
	B. parapertussis	++++	+++	++	-		
	B. bronchiseptica	++++	++++	+++	++		

- = no agglutination
+ = 25% agglutination
++ = 50% agglutination
+++ = 75% agglutination
++++ = 100% agglutination

Evaluation of Culture Media for Bordetella

Ability to support growth

The following three culture media were compared for their ability to support growth:

1. Bordet-Gengou glycerol potato agar (Cruickshank 1975)
2. Bordet-Gengou (Kendrick and Eldering 1934)
3. Charcoal agar (Sutcliffe and Abbott 1972)

1. Bordet-Gengou glycerol potato agar (Cruickshank)

Potato Slices	250 g.
Sodium Chloride, NaCl	9 g.
Water	2 L.
Agar	45 g.
Glycerol	20 ml.
Proteose peptone (Difco)	20 g.

Potatoes were cleaned and peeled, and cut into thin slices. The slices were boiled with salt in 500 ml. of water until they fell into pieces. The water lost in boiling was made up to volume, and filtered through linen and adjusted to pH7. Agar was dissolved in 1500 ml. of water by heat and were added with the potato extract, glycerol and peptone. The mixture was distributed in bottles and heated in the autoclave with free steam at about 100°C for one hour, then at 115°C for 10 minutes. The bottles were stored until use.

Preparation of Complete Medium

Glycerol potato agar	2 volumes
Sterile defibrinated horse blood	1 volume

The agar in bottle was steamed for one hour, and then cooled to about 55°C. The blood was warmed by placing it in the 55°C bath for two to three minutes. The blood was added, mixed with the agar and the medium poured into plates.

2. Bordet-Gengou (Kendrick and Eldering)

Bordet-Gengou Agar Base (BBL) was available in dehydrated form. Formula for per litre medium was as follows:

Potato, Infusion from (solids)	4.5 g.
Polypeptone peptone	10.0 g.
Sodium chloride	5.5 g.
Agar	20.0 g.

For use, 40 g. of the powder were suspended in 1,000 ml of a 1% solution of glycerol in purified water. The suspension was thoroughly mixed and heated with occasional agitation. The medium was boiled for one minute to obtain solution, then autoclaved at 121°C for 15 minutes. After the medium had been cooled to about 45 - 50°C sheep blood was added to give a concentration of 20%. The medium was thoroughly mixed after the addition of blood, and poured into plates.

3. Charcoal Agar (Sutcliffe and Abbott)

Oxoid Charcoal agar (CM119) was used

Formula per litre:

Lab - Lemco powder (Oxoid L29)	10.0 g.
Peptone (Oxoid L37)	10.0 g.
Starch	10.0 g.
Charcoal Bacteriological (Oxoid L9)	4.0 g.
Sodium chloride	5.0 g.
Nicotinic acid (Niacin)	0.001 g.

Agar No. 3 (Oxoid L13) 12.0 g.

pH 7.4 approx.

For use, 51 g. of powder were suspended in 1 litre of distilled water. The mixture was brought to boil to dissolve completely, and then autoclaved at 121°C for 15 minutes. After autoclaving, the medium was cooled to about 50°C and 10% of defibrinated horse blood was added. The medium was well mixed and then poured into plates.

Known fresh B. pertussis and B. parapertussis cultures were diluted to about 10^8 organisms/ml in saline and a loopful of 0.001 ml. was plated onto the three different media. The plates were incubated at 36°C in a moist atmosphere with 5% CO_2.

Luxurious growth was obtained on all three media after three days. On prolonged incubation colonies on Bordet-Gengou glycerol potato agar were considerably larger (at least 1 mm. or more in diameter), but not as glistening as the colonies on the other two media. Colonies were haemolytic on Bordet-Gengou (Kendrick and Eldering) medium with a clear zone of haemolysis around the colony. B. parapertussis produced a brown pigment on all three media, even though it was least obvious on the charcoal agar because of the black background.

The large Bordetella colonies referred to above (about 2 mm diameter) were only obtained on raw potato medium when the potatoes were prepared fresh. A repeat experiment using raw potatoes prepared a few weeks previously and kept in bottles as mentioned did not produce the same size of colonies. The colonies became dramatically smaller (smaller than those on the other two media) when using raw potatoes prepared and kept for a

few weeks. The added fact that Bordetella colonies were not as glistening on raw potato medium and therefore made them more difficult to recognize made this medium inferior to the other two, and not practical in a busy diagnostic bacteriology laboratory. Keeping these factors in mind, the most logical thing to do was then to compare Bordet-Gengou medium (Kendrick and Eldering) and the charcoal agar medium and to select the better medium.

Experiment on 64 Bordetella isolates

Over a two year period (1975-1976), 61 strains of B. pertussis and three strains of B. parapertussis were isolated. These isolates were kept in litmus milk frozen at -70°C.

The ability to support growth of B. pertussis and B. parapertussis by Bordet-Gengou medium and charcoal agar was compared on these 64 strains grown under poor biological conditions. The 64 strains of Bordetella were subcultured and incubated at 36°C in a moist atmosphere for two weeks so that the cultures were deteriorating by aging, gradual loss of moisture, depletion of nutrients from the medium, and accumulation of toxic metabolic products. Suspensions of organisms in saline to match 0.5 McFarland standard were prepared. These were further diluted 1:100 in saline and a loopful of 0.001 ml of this dilution was inoculated onto Bordet-Gengou and charcoal plates. The plates were incubated at 36°C for one week. Results are shown in Table 8.

Table 8

Ability of Bordet-Gengou medium and charcoal agar to grow colonies from aging cultures of Bordetella.

	Bordet-Gengou	Charcoal agar
B. pertussis grown	0	41
Total B. pertussis grown/ Total B. pertussis inoculated	0/61	41/61
B. parapertussis grown	3	3
Total B. parapertussis grown/ Total B. parapertussis inoculated	3/3	3/3

It was surprising that commercial Bordet-Gengou medium did not produce one single colony out of the 61 aging B. pertussis cultures, while on charcoal agar 67.2% (41/61) of the strains were recovered. All three B. parapertussis strains were isolated on both media, but the absolute number of colonies for all three strains were fewer on Bordet-Gengou medium than on charcoal agar.

This experiment showed that in practical situations, such as patients in the late stage of the disease, or receiving antibiotics for treatment, or delay in shipment and/or culture of specimens, it would be most likely that the organisms could be isolated on charcoal agar but not on the Bordet-Gengou medium.

Isolation of B. pertussis from mixed cultures

Overgrowth by other bacterial flora has been a problem of isolating Bordetella. Benzyl penicillin was and still is widely used as a selective agent in media for the isolation of Bordetella pertussis and B. parapertussis. However, Sutcliffe and Abbott (1972) reported the incorporation of cephalexin at a concentration of 40 mcg/ml in Oxoid charcoal agar. They concluded that the isolation rate was as good on this medium as on the routine medium containing penicillin, and that plates were easier to read due to the reduction in the numbers of commensals.

A comparative study was made with penicillin and cephalexin in charcoal agar. The conventional Bordet-Gengou medium with penicillin was also included for comparison.

A stock strain of B. pertussis was deliberately mixed with organisms commonly found in the upper respiratory tract (Table 9) and then the mixture was inoculated onto the three types of media. All organisms were suspended individually in saline to approximate 0.5 McFarland turbidity standard, and equal volumes of bacterial suspensions were mixed as listed in Table 9. A loopful of 0.001 ml. of mixed suspension was spread on the three types of media. The plates inoculated with the mixtures of organisms were incubated at 36°C for seven days and examined for the presence of B. pertussis colonies.

Charcoal agar with 40 mcg/ml of cephalexin was the only medium which isolated B. pertussis colonies from all mixtures. While most of the plates of the other two media with penicillin produced B. pertussis colonies, the majority of these colonies were mixed with unwanted organisms. With the exception of mixture no. 6, where Haemophilus influenzae managed to grow with B. pertussis colonies, all the other eight mixtures on charcoal agar with 40 mcg/ml of cephalexin yielded only heavy pure growth of B. pertussis. This experiment showed that cephalexin was superior to penicillin for the selection of B. pertussis from mixed cultures.

Colony Counts

Colony count experiments were carried out on charcoal agar with different concentrations of cephalexin, on commercial plain Bordet-Gengou medium and on commercial Bordet-Gengou medium with 35 mcg/ml of cephalexin (plates of the last two media being available from a commercial firm). B. pertussis (R019) was the strain used for this experiment.

Table 9

Recovery of B. pertussis from mixed cultures in the presence of penicillin or cephalexin.

Mixture No.	Composition of culture	Bordet-Gengou with penicillin 0.25 unit /ml.	Charcoal agar with penicillin 0.25 unit /ml.	Charcoal with ceph 40 mcg/ml
1	B. pertussis & Staph. aureus	-	+	+
2	B. pertussis & Hemolytic Streptococcus (not group A)	+	+	+
3	B. pertussis & Neisseria Sp.	+	+	+
4	B. pertussis & D. pneumoniae	+	+	+
5	B. pertussis & Alpha hemolytic Streptococcus	+	+	+
6	B. pertussis & H. influenzae	+	+	+
7	B. pertussis & E. coli	-	-	+
8	B. pertussis & Neisseria Sp. & D. pneumoniae	+	+	+
9	B. pertussis & Neisseria Sp. & Alpha hemolytic Streptococcus	+	+	+

+ = B. pertussis isolated

- = B. pertussis not isolated

The strain of B. pertussis was grown on plain charcoal agar at 36°C for three days. A suspension of the organism was made in saline to approximate 0.5 McFarland standard. The suspension was well mixed with a Vortex mixer and 10-fold dilutions of the suspension was made in saline. A loopful (0.01 ml using a standardized loop) was spread in triplicate on each individual medium. The inoculated plates were incubated at 36°C for seven days and the colonies were counted.

Table 10 shows the results of the counts.

The colony count experiment showed that plain charcoal agar supported the growth of B. pertussis much better than plain Bordet-Gengou medium. A mean count of 75 ± 3.4 at 10^{-3} dilution on plain charcoal agar generated about ten times more colonies when compared with 86.66 ± 56.88 at 10^{-2} dilution on plain Bordet-Gengou medium, at 95% confidence limits.

A plot of the colony count results against different concentrations of cephalexin (Figure 1) indicated the strain of B. pertussis was not inhibited by 35 mcg/ml of cephalexin. There was slight inhibition at 40 mcg/ml, and greater inhibition above this concentration.

Commercial Bordet-Gengou medium with 35 mcg/ml of cephalexin, at least this batch tested, did not grow B. pertussis.

A similar colony count experiment was also carried out for B. parapertussis at the same time (see Table 11). B. parapertussis (R1378) was the strain used. Plain charcoal agar yielded a mean of 117.33 ± 4.47 at 10^{-3} dilution. There was no significant difference when compared with a mean count of 126.66 ± 5.71 on commercial plain Bordet-Gengou agar, at 95% confidence limits.

Table 10

Colony count of *B. pertussis* on charcoal agar and Bordet-Gengou medium

Medium	Cephalexin mcg/ml.	Dilution of suspension and gorwth (or count) per 0.01 ml. with 95% confidence limits							
		Undiluted	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
Charcoal	0	confluent	heavy	moderate	78	6	-	-	-
		confluent	heavy	moderate	75	8	1	-	-
		confluent	heavy	moderate	72	11	1	-	-
				Mean:	75 ± 3.4				
Charcoal	10	confluent	heavy	moderate	77	10	-	-	-
		confluent	heavy	moderate	65	9	4	-	-
		confluent	heavy	moderate	68	8	-	-	-
				Mean:	70 ± 7.08				
Charcoal	20	confluent	heavy	moderate	79	2	-	-	-
		confluent	heavy	moderate	62	4	-	-	-
		confluent	heavy	moderate	74	8	-	-	-
				Mean:	71.67 ± 9.9				
Charcoal	30	confluent	heavy	moderate	70	7	-	-	-
		confluent	heavy	moderate	67	9	1	-	-
		confluent	heavy	moderate	74	9	-	-	-
				Mean:	70.33 ± 3.98				
Charcoal	40	confluent	heavy	moderate	60	3	-	-	-
		confluent	heavy	moderate	74	4	1	-	-
		confluent	heavy	moderate	66	5	-	-	-
				Mean:	66.67 ± 7.96				
Charcoal	50	confluent	heavy	moderate	55	6	-	-	-
		confluent	heavy	moderate	57	77	-	-	-
		confluent	heavy	moderate	36	3	1	-	-
				Mean:	49.33 ± 13.09				
Charcoal	60	confluent	heavy	moderate	41	5	-	-	-
		confluent	heavy	moderate	51	3	-	-	-
		confluent	heavy	moderate	46	4	-	-	-
				Mean:	46 ± 5.67				
Charcoal	70	confluent	heavy	moderate	29	3	-	-	-
		confluent	heavy	moderate	27	-	-	-	-
		confluent	heavy	moderate	29	-	-	-	-
				Mean:	28.33 ± 1.31				
Charcoal	80	confluent	heavy	moderate	14	-	-	-	-
		confluent	heavy	moderate	23	3	-	-	-
		confluent	heavy	moderate	21	-	-	-	-
				Mean:	19.33 ± 5.35				
Charcoal	90	heavy	moderate	30	7	-	-	-	-
		heavy	moderate	38	1	1	-	-	-
		heavy	moderate	19	1	-	-	-	-
				Mean:	29 ± 13.49				
Charcoal	100	heavy	moderate	10	-	-	-	-	-
		heavy	moderate	8	1	-	-	-	-
		heavy	moderate	5	1	-	-	-	-
				Mean:	7.66 ± 2.31				
Bordet-Gengou (commercial plates)	0	confluent	moderate	117	12	-	-	-	-
		confluent	moderate	95	7	-	-	-	-
		confluent	moderate	39	-	-	-	-	-
				Mean:	83.66 ± 56.88				
Bordet-Gengou (commercial plates)	35	-	-	-	-	-	-	-	-
		-	-	-	-	-	-	-	-
		-	-	-	-	-	-	-	-

Figure 1

Colony count of B. pertussis on charcoal agar (Plot from Table 10). The bars represent the standard errors of the means at the 95% confidence level.

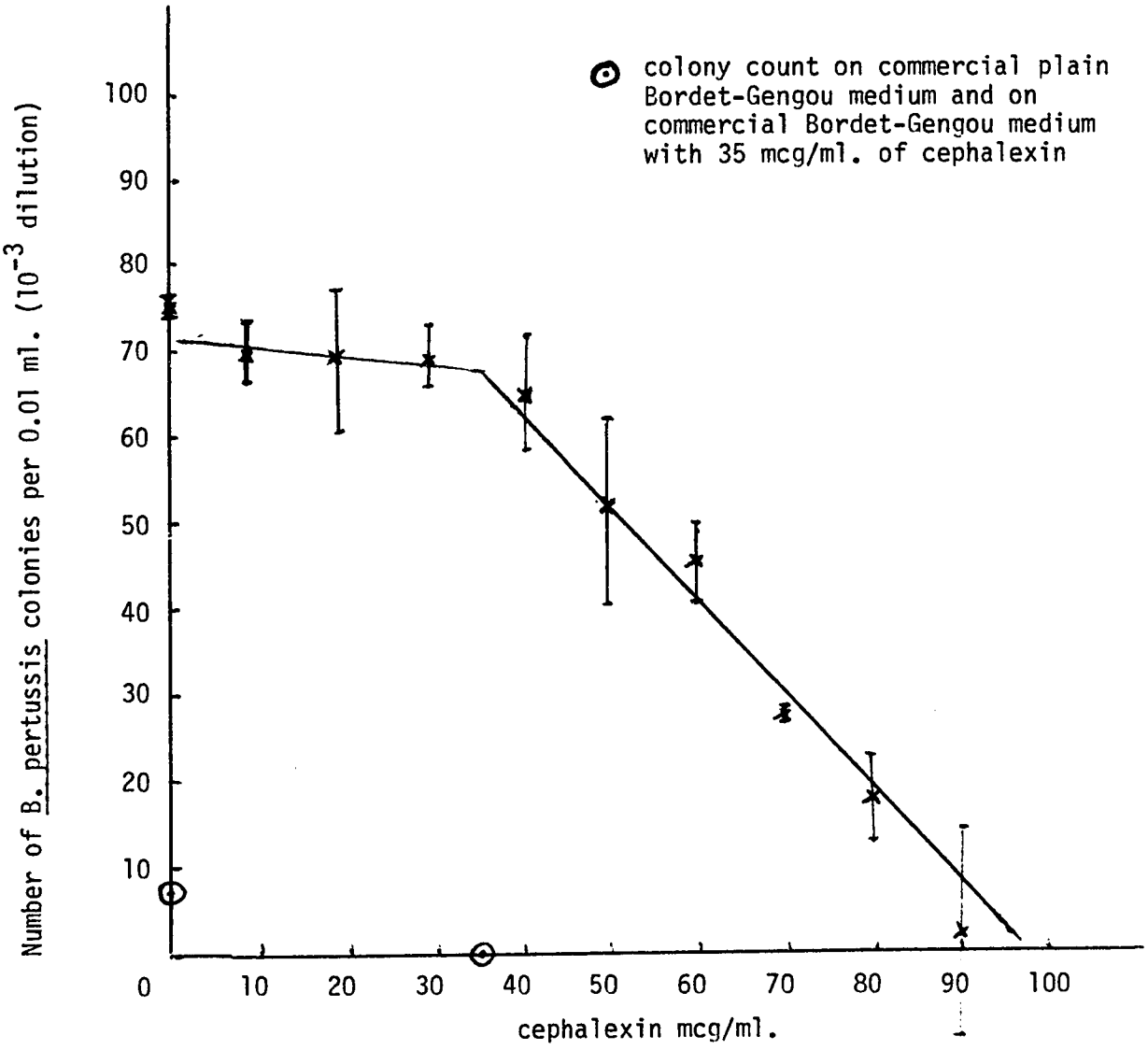


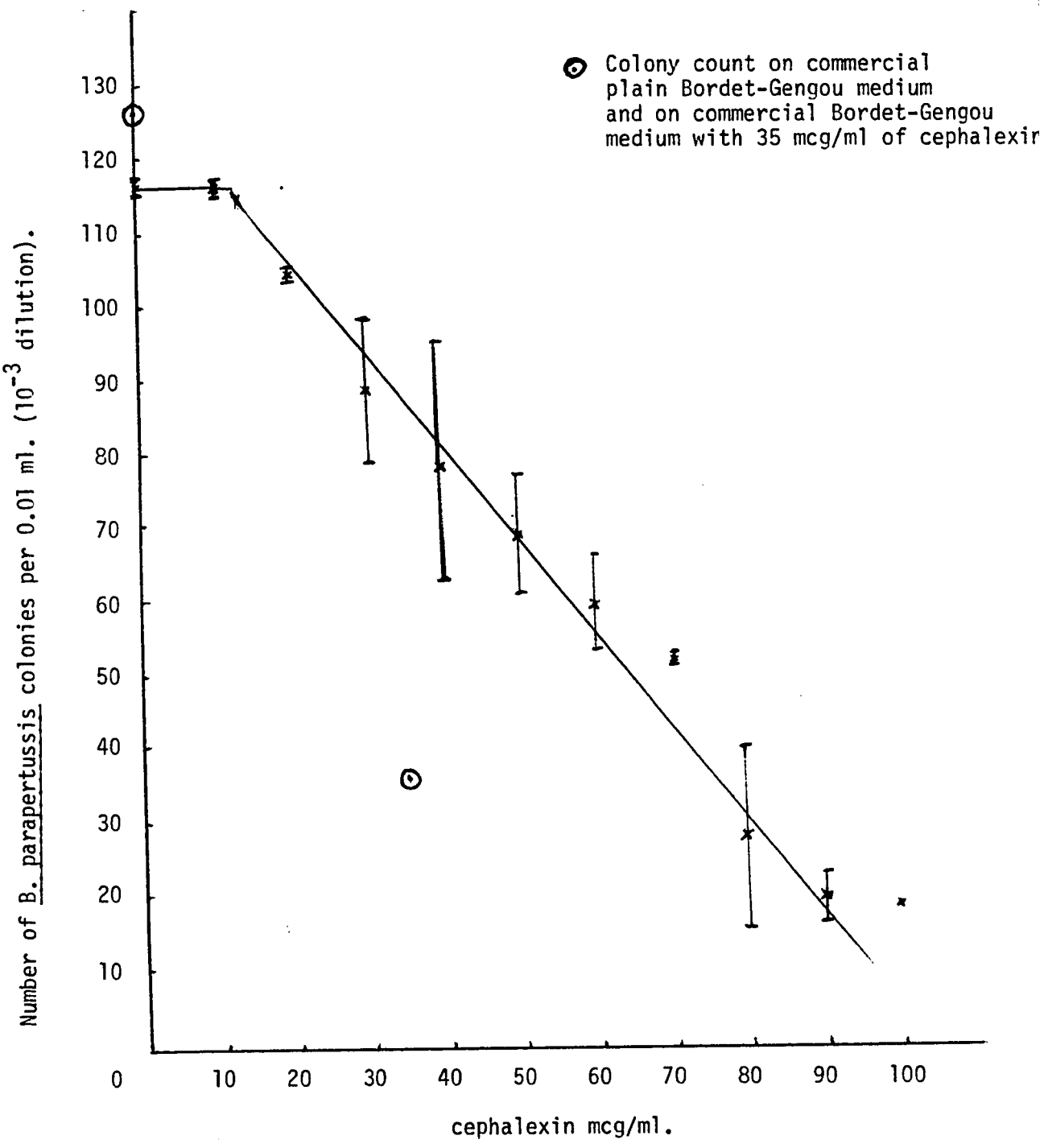
Table 11

Colony count of B. parapertussis on charcoal agar and Bordet-Gengou medium

Medium	Cephalexin mcg/ml.	Dilution of suspension and growth (or count) per 0.01 ml. with 95% confidence limits							
		Undiluted	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
Charcoal	0	confluent	heavy	moderate	121	17	-	-	-
		confluent	heavy	moderate	115	15	-	-	-
		confluent	heavy	moderate	116	16	1	-	-
					Mean: 117.33 ± 4.47				
Charcoal	10	confluent	heavy	moderate	118	21	-	-	-
		confluent	heavy	moderate	120	19	2	-	-
		confluent	heavy	moderate	117	17	2	-	-
					Mean: 118.33 ± 2.16				
Charcoal	20	confluent	heavy	moderate	103	17	-	-	-
		confluent	heavy	moderate	107	16	-	-	-
		confluent	heavy	moderate	103	12	2	-	-
					Mean: 104.33 ± 3.52				
Charcoal	30	confluent	heavy	moderate	91	16	2	-	-
		confluent	heavy	moderate	97	10	1	-	-
		confluent	heavy	moderate	81	14	2	-	-
					Mean: 89.66 ± 11.43				
Charcoal	40	confluent	heavy	moderate	85	12	1	-	-
		confluent	heavy	moderate	88	18	-	-	-
		confluent	heavy	moderate	64	6	-	-	-
					Mean: 79 ± 18.5				
Charcoal	50	confluent	heavy	moderate	76	2	-	1	-
		confluent	heavy	moderate	68	5	3	-	-
		confluent	heavy	moderate	65	3	-	-	-
					Mean: 69.66 ± 8.03				
Charcoal	60	confluent	heavy	moderate	58	5	-	-	-
		confluent	heavy	moderate	68	6	-	-	-
		confluent	heavy	moderate	60	6	-	-	-
					Mean: 62 ± 7.48				
Charcoal	70	confluent	heavy	moderate	53	7	2	-	-
		confluent	heavy	moderate	54	4	1	-	-
		confluent	heavy	moderate	52	10	1	-	-
					Mean: 53 ± 1.41				
Charcoal	80	confluent	heavy	moderate	17	1	-	-	-
		confluent	heavy	moderate	33	-	-	-	-
		confluent	heavy	moderate	36	1	-	-	-
					Mean: 28.66 ± 14.42				
Charcoal	90	confluent	heavy	139	18	-	-	-	-
				148	16	3	-	-	-
				181	30	-	-	-	-
					Mean: 21.33 ± 4.17				
Charcoal	100	confluent	heavy	144	22	2	-	-	-
		confluent	heavy	124	21	1	-	-	-
		confluent	heavy	135	21	-	-	-	-
					Mean: 21.33 ± 0.45				
Bordet-Gengou (commercial plates)	0	confluent	heavy	moderate	131	18	1	1	-
		confluent	heavy	moderate	123	17	-	1	-
		confluent	heavy	moderate	126	24	-	-	-
					Mean: 126.66 ± 5.71				
Bordet-Gengou	35	confluent	heavy	moderate	42	1	-	-	-
		confluent	heavy	moderate	35	-	-	-	-
		confluent	heavy	moderate	28	-	-	-	-
					Mean: 35 ± 9.90				

Figure 2

Colony count of B. parapertussis on charcoal agar (Plot from Table 11). The bars represent standard errors of the means at the 95% confidence level.



There was a slight inhibition on this strain of B. parapertussis above 15 mcg/ml of cephalexin (Figure 2). Strains of B. parapertussis were reported to be more sensitive to cephalexin than B. pertussis (Sutcliffe and Abbott, 1972), but the inhibition was usually slight by 40 mcg/ml. At 35 mcg/ml of cephalexin, charcoal agar yield more than twice the number of colonies of this strain of B. parapertussis when compared with Bordet-Gengou medium.

Amino Acids Contents

Bordetella pertussis does not use any of the common sugars either aerobically or anaerobically and amino acids must be supplied for growth. Glutamic acid is one of the amino acids used (Ungar et al, 1950) and when it has disappeared from the medium, growth stops. (Jebb and Tomlinson, 1951). Proom (1955) found that in simple mixtures of amino acids glutamate could be replaced by succinate or alpha-ketoglutarate.

Ungar et al (1950) found that aspartic acid, serine, glycine, threonine, alanine and proline as well as glutamic acid disappeared from media during growth of Bordetella pertussis. Proom (1955) reported the utilization of cystine, serine, glutamic acid, alanine, proline and leucine; aspartic acid, glycine and threonin were not mentioned. Hornibrook (1940) grew Bordetella pertussis in a defined medium containing glutamate, tyrosine, glycine, proline, histidine, arginine and cystine, in addition to starch, salts and nicotinic acid.

Lane (1970) examined the amino acid consumption by B. pertussis growing in broth containing casein hydrolysate. It was found that serine, proline, alanine, glycine, aspartate and glutamate were rapidly consumed, in a manner which suggested that they supplied the energy requirements

of the organisms. Exhaustion of the energy source appeared to be the main factor limiting the yield of cells. There was no correlation between the utilization of individual amino acids and the phase of growth, and uptake appeared to depend only upon relative concentrations. The addition of glutamic acid to supplement the fluid medium resulted in an increase of cell yield by an average of 43.5%.

In view of the above information, an attempt was made to analyse the amino acid contents of charcoal agar and Bordet-Gengou medium.

Dehydrated charcoal agar (Oxoid) and Bordet-Gengou medium (BBL) were weighed out precisely on a chemical balance according to formula to make up 100 ml medium. The contents were suspended in 100 ml distilled water, and allowed to dissolve by mixing. The agar in the medium was allowed to settle after mixing, and the supernatant filtered through a filter unit (millipore filter 0.2 micron) to ensure the separation of agar.

The amino acids contents of the preparations were analysed in the Biochemistry Laboratory, Children's Hospital of Eastern Ontario, Ottawa. The preparations were deproteinized by filtering through membrane cones by centrifugation at 1,000 r.p.m.

The filtrates were then passed through resin columns, and amino acids eluted at the appropriate pH. The amino acid contents were analysed with a Technicon Amino Acid Analyser, using the ninhydrin reaction. Four separate experiments with different batches of culture media were carried out and the means calculated. Results of amino acid analyses are given in Table 12.

Table T2

Amino acid contents of Charcoal agar (Oxoid) and Bordet-Gengou medium (BBL). (Micromole per 0.3 ml. with the 95% confidence limits).

Amino Acid	Charcoal agar				Mean	Bordet-Gengou				Mean
Taurine	0.08	0.09	0.07	0.08	0.08 ± 0.01	0.07	0.08	0.07	0.07	0.07 ± 0.004
Aspartic Acid	0.14	0.29	0.29	0.25	0.24 ± 0.06	0.31	0.47	0.47	0.60	0.46 ± 0.10
Threonine	0.19	0.32	0.31	0.33	0.29 ± 0.06	0.32	0.54	0.50	0.58	0.49 ± 0.10
Serine	0.30	0.48	0.46	0.48	0.43 ± 0.07	0.35	0.52	0.47	0.53	0.47 ± 0.07
Asparagine	0.18	0.20	0.22	0.22	0.21 ± 0.02	0.60	0.79	0.74	0.82	0.74 ± 0.08
Glutamic Acid	0.43	0.72	0.75	0.76	0.67 ± 0.14	0.44	0.64	0.62	0.68	0.60 ± 0.09
Glycine	0.56	>0.72	>0.78	>0.72	>0.70 ± 0.08	0.74	>0.84	>0.93	>0.80	>0.83 ± 0.07
Alanine	0.21	>0.57	>0.62	>0.57	>0.49 ± 0.16	>0.35	>0.48	>0.53	>0.66	>0.50 ± 0.11
Valine	N.D.	N.T.	N.T.	N.T.		0.12	N.T.	N.T.	N.T.	0.12
Cystine	0.09	0.24	0.30	0.25	0.22 ± 0.08	>0.17	0.66	0.49	0.65	>0.49 ± 0.19
Cystothionine	0.08	N.T.	N.T.	N.T.	0.08	N.T.	N.T.	N.T.	N.T.	
Methionine	0.11	0.29	0.34	0.29	0.26 ± 0.09	0.33	0.58	0.49	0.50	0.48 ± 0.09
Isoleucine	0.18	0.35	0.39	0.42	0.34 ± 0.09	0.55	>0.75	>0.83	>0.86	>0.75 ± 0.12
Tyrosine	0.09	0.12	0.10	0.12	0.11 ± 0.01	0.19	0.33	0.30	0.33	0.29 ± 0.06
Lysine	0.41	0.49	0.49	0.47	0.47 ± 0.03	1.85	>2.36	>2.31	>2.22	>2.19 ± 0.20
Histidine	0.09	N.T.	N.T.	N.T.	0.09	N.D.	N.T.	N.T.	N.T.	
Carnosine	N.D.	N.T.	N.T.	N.T.		N.D.	N.T.	N.T.	N.T.	
Arginine	0.21	0.35	0.40	0.35	0.33 ± 0.07	0.54	1.34	1.21	1.37	1.11 ± 0.34
Phenylalanine	0.12	0.38	0.33	0.32	0.29 ± 0.09	0.43	1.32	1.09	1.32	1.04 ± 0.36
Leucine	0.45	>0.71	>0.76	>0.82	>0.69 ± 0.14	>1.3	>1.37	>1.37	>1.41	>1.36 ± 0.04
Ornithine	N.D.	N.T.	N.T.	N.T.		0.15	N.T.	N.T.	N.T.	0.15

N.D. = < lower detectable limit

Table 12 shows glutamic acid, the most important amino acid, is present in charcoal agar and Bordet-Gengou medium in comparable amounts (0.67 ± 0.14 and 0.60 ± 0.09 μ mole per 0.3 ml respectively). The other amino acids were found in higher concentration in Bordet-Gengou than in charcoal medium.

Amino acids may be required by bacteria for non-specific functions; for example, buffering the medium or chelating metals such as iron and preventing their precipitation when the medium is heated (Rowatt, 1957). Their functions become more important as the medium is simplified. Proom (1955) thought that apart from glutamic acid, proline, leucine and alanine were either essential for growth or could only be replaced by complex mixtures of amino acids and almost any combination of three or four amino acids would do to supplement these three.

The fact that charcoal agar, the medium less rich in amino acid contents, yielded better growth of B. pertussis indicates amino acid contents in the medium were not a major factor for its superiority over Bordet-Gengou medium. The presence of some twenty amino acids, including glutamic acid, would enable both media to grow B. pertussis in as far as amino acids are concerned.

However, Sutcliffe and Abbott (1972) described some strains of B. parapertussis showed marked variation of colony size on certain batches of charcoal-blood-agar containing no antibiotic. They found this inhibitory effect of some batches of charcoal-blood-agar was removed by the addition of 1% Difco proteose peptone no. 3, and incorporated this amount of peptone into their charcoal agar medium.

LABORATORY PROCEDURES ON SPECIMENS FROM PATIENTS AND COLLECTION OF CLINICAL DATA

Difco FA - B. pertussis and Difco FA - B. Parapertussis were the reagents chosen for direct immunofluorescence examination on specimen slides. Charcoal agar containing 10% horse blood, 1% Difco proteose peptone no. 3, and 35 mcg/ml of cephalexin was the chosen medium for culture of Bordetella. The time of study covered a complete two-year period, from January 1, 1975 to December 31, 1976.

Commercial Bordet-Gengou medium containing 35 mcg/ml of cephalexin did not support growth of B. pertussis at the time of the colony counts experiment, but subsequent batches tested did manage to grow B. pertussis colonies. Commercial Bordet-Gengou medium, in addition to the charcoal agar, was used for comparison in field conditions for a period of 11 months.

Specimen Collection

Auger suction (Auger, 1939) is preferable to either a cough plate or a pernasal swab, and was the method chosen to collect nasopharyngeal secretions from suspected cases of whooping cough. The same specimen was used for bacteriological and virological investigations.

The equipment for the procedure included the following items:

Sterile - gloves
- 20 cc syringe
- feeding catheter, 8 Fr, 15" long
- 4 oz. plastic specimen container
- gauze square

The nursing staff of the Children's Hospital of Eastern Ontario, Ottawa, collected the speciemsn according to the following protocol.

"Put on sterile gloves and attach the catheter to syringe. Holding the syringe with one hand, use the other hand to insert the catheter through the nose towards the nasopharynx. For insertion of catheter to correct length, measure from nose to ear. If the length is selected, the tip of the catheter should not appear beyond the soft palate. When the catheter tip is well into the nasopharynx, apply suction to the syringe. Repeat the process to collect as much secretion as possible releasing the syringe briefly, then re-applying suction with syringe.

When secretions have been collected, withdraw catheter through a sterile gauze square. Separate catheter from the syringe and place the tubing in the sterile 4 oz. plastic specimen container. Seal lid and send specimen to laboratory."

Leaving the nasopharyngeal secretions in the catheter ensured an uncontaminated specimen and prevented loss when only small amounts had been obtained. No transport medium was used since all specimens were collected in the hospital and were normally processed within two to four hours of collection. Only one specimen was investigated for each suspected case of whooping cough.

Fresh specimens were

(1) stained by haemotoxylin-eosin and examined for the presence of ciliated cells, to check the quality of the specimens,

(2) examined by direct immunofluorescence for B. pertussis and B. parapertussis,

(3) cultured for B. pertussis, B. parapertussis and other microorganisms,

(4) inoculated into tissue cultures for virological studies.

Direct Immunofluorescence

Direct immunofluorescence for B. pertussis and B. parapertussis was carried out, using Difco FA - B. pertussis and FA - B. parapertussis antisera as previously described. A purulent part of the Auger nasopharyngeal suction specimen was selected and two thin smears made. One was treated with FA - B. pertussis conjugate and the other with FA - B. parapertussis conjugate.

A positive and a negative control were included. The positive control consisted of a smear of B. pertussis culture and the negative control a smear of E. coli culture.

Culture and Identification for Bordetella

Specimens were inoculated onto freshly prepared charcoal agar plates, made from Oxoid medium CM 119, supplemented with 10% horse blood, 1% Difco proteose peptone No. 3 and 35 mcg/ml of cephalexin. For a period of 11 months, an additional Bordet-Gengou agar plate containing 35 mcg/ml of cephalexin was also included for a comparative study between the two media in field conditions.

The plates were incubated for seven days at 36^{o}C in a 5% CO_2 moist atmosphere and examined on day 3, 5 and 7 for the presence of the typical dew-like colonies of B. pertussis and B. parapertussis. Final identification was made by Gram stain, ability to grow on ordinary blood agar, presence or absence of brown pigment, production of urease, direct immunofluorescence, and slide agglutination tests using Difco B. pertussis and B. parapertussis antisera on the colonies.

Routine Bacteriological Culture

Auger suction specimens were also inoculated on two blood agar plates (Trypticase soy agar with 5% sheep blood) for common pathogens. One plate was incubated in 5% CO_2 moist atmosphere and the other plate incubated anaerobically. The plates were incubated at 36°C and read at 24 and 48 hours. Organisms were identified by standardized bacteriological procedures.

Viral Studies

The suction tubes were washed-out with culture medium and the specimens inoculated into African green monkey kidney and human fetal lung tissue cultures. Hemadsorption tests were carried out on the 2nd day and repeated on the 10th day, while the cultures were examined for the presence of a cytopathic effect (CPE) for 14 days. Positive cultures were further identified by complement fixation, neutralization tests and electron microscopy. Viral serology was not performed because no early blood specimen was available in most cases.

Age and immunization status of the patients

Data on immunization status were gathered from the case histories and confirmed later through a questionnaire sent to the parents several weeks or months after the child's illness. It proved impossible to verify from the immunization records the type, origin or batch number of the vaccine given. However, since practically all vaccinations had been carried out by physicians in private practice in Ottawa, it can safely be assumed that the vaccine most often used was the Connaught combined diphtheria, pertussis, tetanus and poliomyelitis vaccine without adjuvant.

Identification of B. pertussis in relation to onset of symptoms and antibiotic treatment

Information on onset of symptoms and antibiotic treatment was obtained from case histories available from the Medical Records Department of the hospital.

White blood cell counts

Haematological examination was performed on most of the suspected whooping cough patients when they were seen at the hospital. Blood and Auger suction specimens were usually taken on the same day. Results of the haematological examination were available on the patient's charts.

RESULTS

Over a two year period (1975 - 1976) 231 single specimens of nasopharyngeal secretions were collected from suspected cases of whooping cough in the National Capital Region. Of these 231 cases, 144 were in-patients and 87 were out-patients. 194 patients were residents in Ontario, mainly from Ottawa, while 37 patients were from Quebec.

Presence of ciliated cells and quality of the specimens

Only 21% of the specimens contained ciliated cells, which suggests a poor sampling technique, considering that 65% of the specimens were collected in the catarrhal stage of the disease. At this stage, there is desquamation and necrotizing inflammation of the superficial epithelial layers of the entire respiratory tract (Pittman, 1970).

Direct immunofluorescence

When insufficient material was submitted, the specimen was cultured and direct immunofluorescence was omitted. Enough material was available in 192 Auger suction specimens to allow for culture as well as direct immunofluorescence (FA) to be processed. (See Plates 2, 3 and 4) . A total of 34 positive FA were obtained in these 192 specimens (17.7%). A comparison between FA and cultures is presented in Table 13.

Table 13

Comparison between direct FA and cultures in 192 Auger suction specimens from cases of whooping cough or pertussis syndrome.

A.

	B. pertussis Positive culture	B. pertussis Negative culture	Total
FA positive	28	6	34
FA negative	24	134	158
Total	52	140	192

A specimen positive by FA had a higher probability to yield a positive culture than a specimen negative by FA.
$X^2 = 60.5$, $p < 0.001$.

B.

Total positives=58

Positive FA and culture = 28 (48.3%)

Positive culture only = 24 (41.4%)

Positive FA only = 6 (10.3%)

Total positive culture = 52 (89.7%)

Total positive FA = 34 (58.6%)

Table 13 shows that out of 58 positives, only 34 (58.6%) were identified by the FA method, whereas 52 (89.7%) were positive by culture. Only six specimens were positive by FA alone. All these six specimens were from patients who had received antibiotic treatment. This represented a gain of 10.3% in the identification rate. However, a drop of 31.1% in the identification rate would have resulted if only the FA method had been used.

Plate 2

Direct immunofluorescence on Auger nasopharyngeal suction specimen (negative reading).

Plate 3

Direct immunofluorescence on Auger nasopharyngeal suction specimen (positive reading).

Note fluorescent coccobacillary forms of Bordetella pertussis.

Plate 2

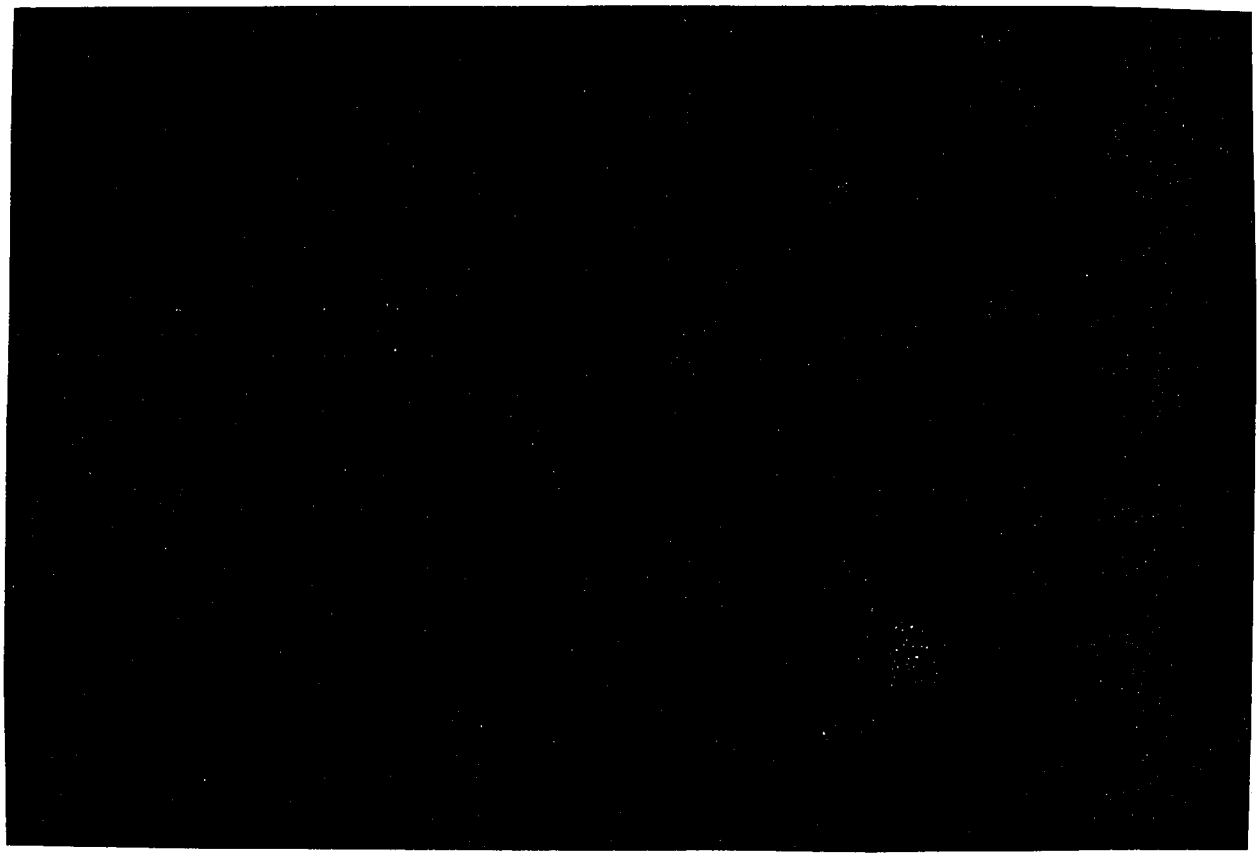


Plate 3

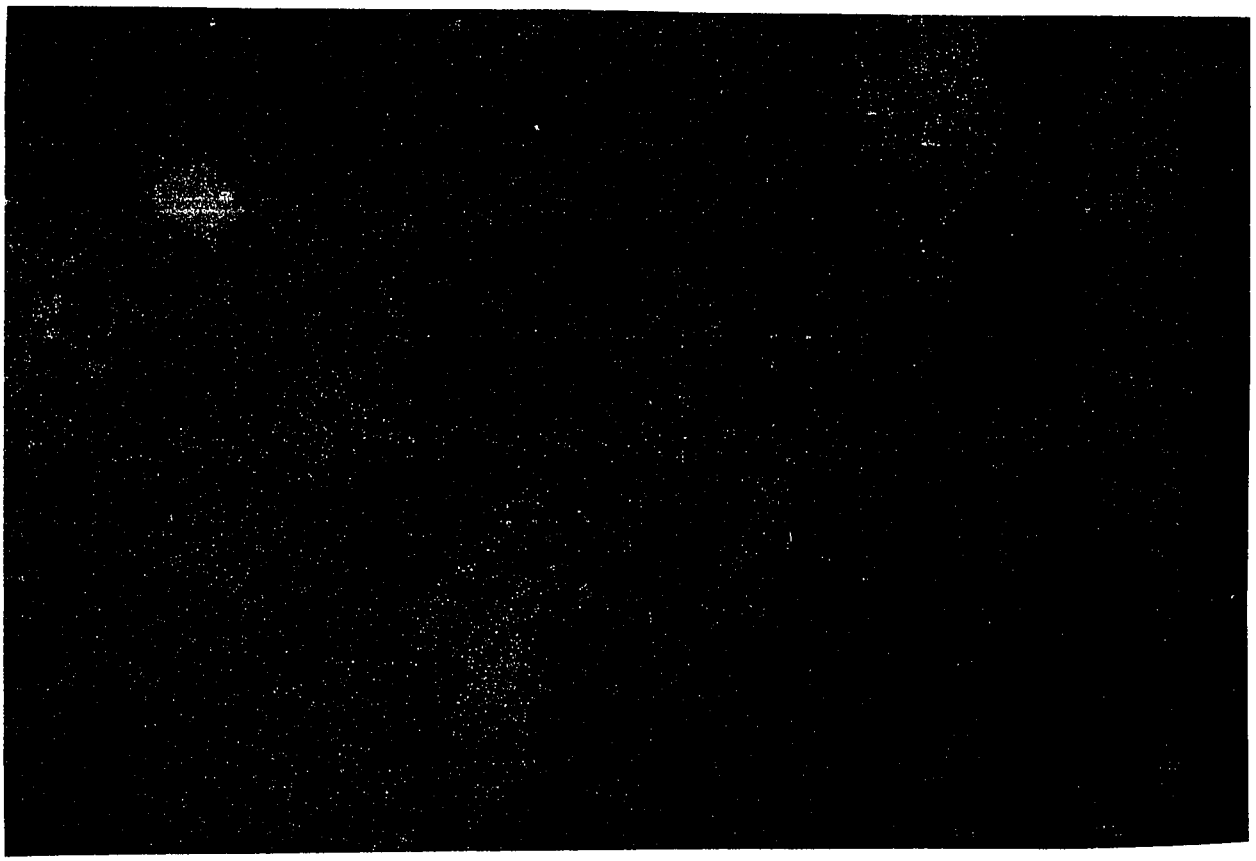
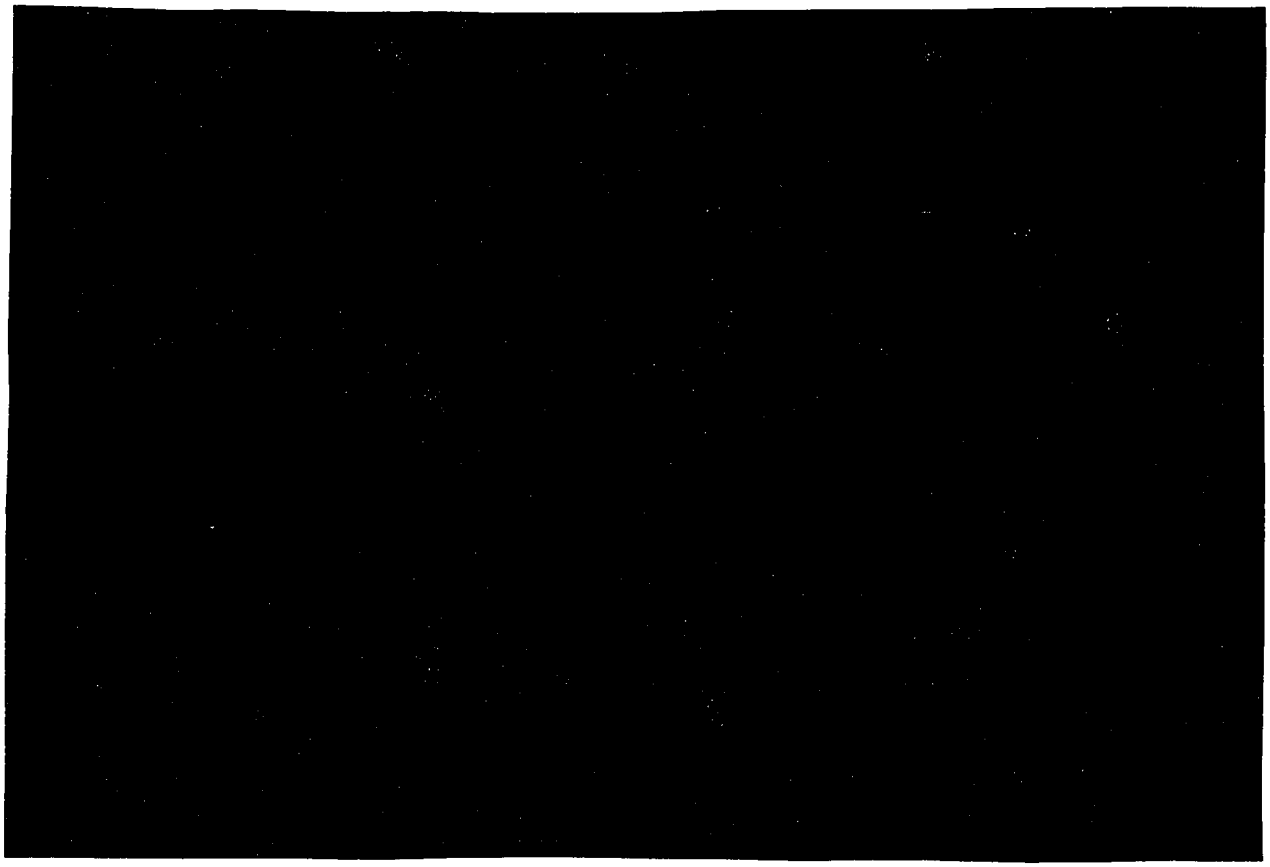


Plate 4



Direct immunofluorescence on Bordetella pertussis culture.

Indirect Immunofluroescence

Indirect immunofluorescence was performed on a random portion of specimens in 1976, in the hope that it might improve the diagnostic rate. A dilution of 1:8 of B. pertussis antiserum (rabbit) and a dilution of 1:16 of fluorescein anti-rabbit antiserum were the chosen combinations to carry out indirect immunofluorescence examination on direct smears from Auger suction specimens (see Table 6 (A)). A comparison between direct immunofluorescence, indirect immunofluorescence and cultural results is presented in Table 14.

Table 14

Comparison between direct immunofluorescence, indirect immunofluorescence and cultural results on 23 specimens. Figures shown are those of positive findings.

Direct FA only	Indirect FA only	Culture only	Culture and Direct FA	Culture and Indirect FA	Total by all methods
1	0	4	2	0	7

As shown in Table 14, a diagnosis could not be obtained from indirect immunofluorescence alone. Also, indirect immunofluorescence could not provide an earlier answer on specimens that yielded positive cultures, and gave poorer results than direct immunofluorescence.

Culture for Bordetella

Over a two year period (1975 - 1976) 231 single specimens of nasopharyngeal secretions were examined for Bordetella. 61 strains of B. pertussis and three strains of B. parapertussis were isolated. All isolates were obtained by growth of organisms on enriched charcoal agar

with 35 mcg/ml. of cephalexin.

129 Auger suction specimens were cultured on freshly prepared charcoal agar and Bordet-Gengou agar, both with 35 mcg/ml. of cephalexin, as a comparative field study on the two media. The difference in results for the two media is shown in Table 15.

Table 15

Comparison between charcoal agar and Bordet-Gengou agar on 129 specimens

	B. pertussis positive culture	B. pertussis negative culture
Charcoal agar	25	104
Bordet-Gengou agar	4	125

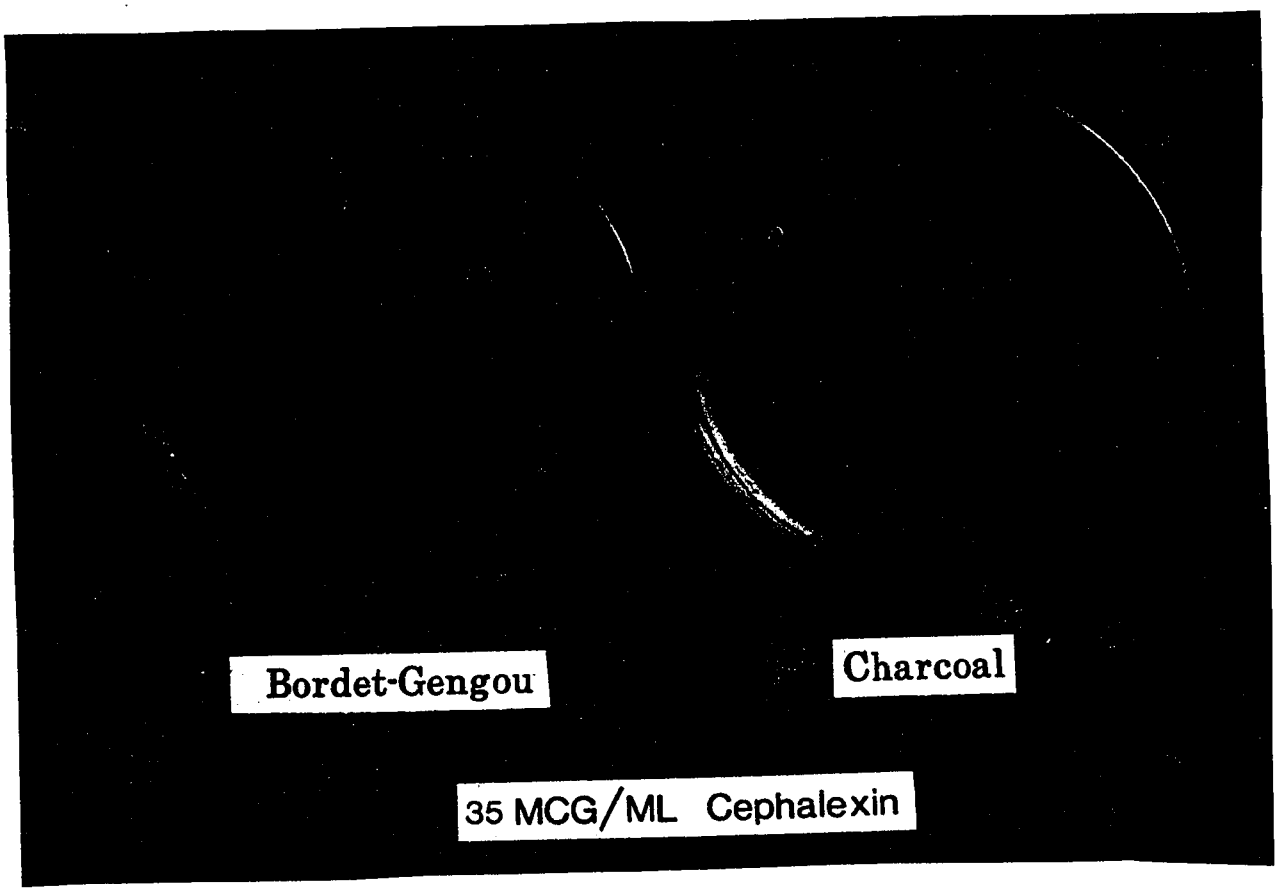
$X^2 = 15.53$, $P < 0.001$

A total of 25 B. pertussis strains were isolated out of these 129 Auger suction specimens. Four were isolated on both media, and 21 were isolated on charcoal agar only. (See Plate 5). The Bordet-Gengou plates failed to grow any strain which did not grow on charcoal agar. This difference between the two culture media was statistically significant, $P < 0.001$.

Routine Bacteriological Culture

Routine bacteriological culture was carried out on all 231 Auger suction specimens. A total of 20 organisms, mainly flora of the upper respiratory tract, were isolated. Out of these 20 organisms, only alpha-hemolytic Streptococcus, Streptococcus pneumoniae and Neisseria species were present in more than 10% of the specimens. There was no difference in the isolation rate of these organisms between the B. pertussis positive and the B. pertussis negative groups, $P > 0.1$. See Table 16 (A).

Plate 5



Auger suction specimen inoculated on Bordet-Gengou medium and charcoal agar plate, both with 35 mcg/ml. of cephalexin.

Only a few colonies of Bordetella pertussis grew on the Bordet-Gengou medium, but luxurious growth was obtained on charcoal agar.

Table 16 (A)

Routine bacteriological culture on 231 Auger suction specimens.

Organisms other than B. pertussis	B. pertussis positive culture (total = 61)	B. pertussis negative culture (total = 170)	
Alpha-hemolytic Streptococcus	30/61 (49.2%)	73/170 (42.9%)	$X^2 = 0.596$, $p > 0.1$
Diplococcus pneumoniae	17/61 (27.9%)	41/170 (24.1%)	$X^2 = 0.205$, $p > 0.1$
Neisseria sp.	36/61 (59.0%)	105/170 (61.8%)	$X^2 = 0.008$, $p > 0.1$

There was a great reduction in growth of commensals on the selective charcoal agar, which facilitated the examination of plates. Table 16 (B) shows the effectiveness of the selective medium.

Table 16 (B)

Inhibition of commensals of 231 Auger suction specimens on selective charcoal agar.

Organisms other than B. pertussis	B. pertussis positive culture (total = 61)	B. pertussis negative culture (total = 170)
None	34	111
1 - 15 colonies	7	20
16 - 50 colonies	8	20
51 - 200 colonies	8	12
over 200 colonies	4	7

Viral studies

There have been suggestions that viruses, particularly adenoviruses, may cause whooping cough (Olson, Miller and Hanshaw, 1964; Urquhart, Moffat, Calder and Cruickshank, 1965; Collier, Connor and Irving, 1966; Connor, 1970; Pereira and Candeias, 1971). In our study, viral investigations were carried out in nasopharyngeal secretions from 204 patients with a diagnosis of whooping cough or whooping cough syndrome, and in 1,621 nasopharyngeal secretions received from other non whooping cough patients. The virological findings in these two groups of patients are summarized in Table 17.

The proportion of virus isolates from cases of whooping cough in which B. pertussis was not isolated was not significantly different

Table 17

Virological findings in nasopharyngeal secretions from 204 whooping cough cases and from 1,621 non whooping cough patients.

A.

Viral isolates	Whooping cough cases with		Other Patients (1,621)
	Positive cultures for B. pertussis (53)	Negative cultures for B. pertussis (151)	
Adenoviruses	0	2	10
Coxsackie A or B	1	0	27
E. C.H.O.	0	0	25
Enteroviruses (untyped)	0	0	13
Cytomegalovirus	0	0	1
Herpes simplex	0	1	19
Influenza A/Victoria/75	0	1	19
Parainfluenza	0	3	14
Respiratory syncytial virus	1	3	16
Rubella	0	0	3
Number of isolates and isolation rate	2 (3.7%)	10 (6.6%)	147 (9.06%)

B.

	with whooping cough	without whooping cough
Virus positive	12	147
Virus negative	192	1474

$X^2 = 2.73$, not significant at the 0.05 level, i.e. there is no relationship between virus and whooping cough, $P = 0.05$.

from the proportion of virus isolates in those of proven B. pertussis etiology, $p > 0.05$.

Likewise, the proportion of virus isolates in the whooping cough cases was not significantly different from the proportion of isolates in the non-pertussis controls. Our results show there was no relationship between virus and whooping cough, $X^2 = 2.73$, $p = 0.05$

Identification of B. pertussis in relation to age and immunization status of the patient (Table 18)

Data on immunization status were gathered from the case histories and confirmed later through a questionnaire sent to the parents several weeks or months after the child's illness. Of the 231 Auger suction specimens from patients with a clinical diagnosis of whooping cough or whooping cough syndrome, B. pertussis was identified in 67 (29%); 91% of the identifications were made by culture and the others by direct immunofluorescence. While 12 identifications (17.9% of the 67) were made in completely immunized children, at least half were made in patients who were either less than one year old (53%) or incompletely immunized (61%). Among those who were incompletely immunized, 15 (22%) were less than three months old and therefore could not have been expected to have received any vaccine.

Identification of B. pertussis in relation to onset of symptoms and antibiotic treatment (Table 19)

In the six instances in which only the immunofluorescence test results were positive antibiotics (penicillin, ampicillin or erythromycin) had been administered. Few isolations were made beyond the fifth week of the disease, irrespective of the administration of an

Table 18

Identification of 67 strains of B. pertussis in relation to age and immunization status of the patient.

Immunization Status	Patient's age: No.* of identifications						
	3 mo.	3-6 mo.	6-12 mo.	1-2 yr.	2-4 yr.	> 4 yr.	Total no. (and %)
Complete immunization +	0	0	1	2	3	6	12 (17.9)
Primary course ++	0	0	4	3	1	0	8 (12.0)
Incomplete immunization	15 (2)	8 (1)	7	7	2	2 (2)	41 (61.1)
No information	0	1 (1)	0	1	0	4	6 (9.0)
Total no. (and %)	15 (22.4)	9 (13.4)	12 (17.9)	13 (19.4)	6 (9.0)	12 (17.9)	67 (100)

* Numbers of strains that could not be identified by culture but only by direct immunofluorescence are in parenthesis.

\+ Primary course of three injections of B. pertussis vaccine and one or more booster injections.

++ Three injections only.

Table 19

Identification of 67 strains of *B. pertussis* in relation to onset of symptoms and antibiotic treatment.

Status of antibiotic treatment	Interval between onset of symptoms and identification (wk.): no.* of identifications				
	< 2	2 - 5	> 5	No information	Total
Antibiotic given	14 (3)	7 (3)	0	2	23
Antibiotic not given	25	6	2	0	33
No information	5	3	0	3	11
Total no.	44	16	2	5	67

* Numbers of strains that could not be identified by culture but only by direct immunofluorescence are in parenthesis.

antibiotic. Most of the isolates were obtained in the catarrhal stage. However, 16 identifications (23.9%) were made during the paroxysmal stage of the disease.

Of the patients with negative results of culture for B. pertussis 57.1% had received antibiotics, as compared with 24.6% of those with positive results - a significant difference ($P < 0.001$) that may account for the negative cultures in 151 instances.

White Blood Cell Counts

White blood cell counts were performed on 188 patients about the time when the Auger suction specimens were taken. B. pertussis was isolated from 52 patients, while 136 patients were negative for B. pertussis culture. Only 17 of the 52 positive patients (32.7%) had a W.B.C. count of more than 12,000 per cmm., and 70% or more of lymphocytes. There was no significant relationship between B. pertussis infection and W.B.C. count, $X^2 = 3.35$, $p = 0.05$; but significant relationship could be demonstrated between B. pertussis infection and lymphocytosis, $X^2 = 9.16$, $p = 0.01$ (Table 20).

Antibiotic sensitivity testing of Bordetella isolates

Bass et al (1969) described a method for testing the sensitivity of B. pertussis to antibiotics on charcoal medium. Charcoal does not inactivate the test drugs, and it has the advantage of circumventing the need for the large quantities of blood required when Bordet-Gengou medium is used.

Charcoal agar as for culturing B. pertussis, but without the incorporation of cephalexin, was the medium used. Fresh cultures of

Table 20

W.B.C. count of 188 patients with whooping cough or whooping cough syndrome.

A.

	W.B.C. under 12,000/cmm.		W.B.C. 12,000/cmm. and over		Total Number (and %)
	Lymphocytes under 70%	Lymphocytes 70% and over	Lymphocytes under 70%	Lymphocytes 70% and over	
B. pertussis positive culture					
Number of patient (and %)	13 (25.0)	9 (17.3)	13 (25.0)	17 (32.7)	52 (100.0)
B. pertussis negative culture					
Number of patient (and %)	61 (44.9)	15 (11.0)	37 (27.2)	23 (16.9)	136 (100.0)

B.

	W.B.C. $< 12,000$/cmm.	W.B.C. $\geq 12,000$/cmm.
B. pertussis positive culture	22	30
B. pertussis negative culture	76	60

$X^2 = 3.35$ No significant relationship between B. pertussis infection and W.B.C. count, $P = 0.05$

C.

	Lymphocytes $< 70\%$	Lymphocytes $\geq 70\%$
B. pertussis positive culture	26	26
B. pertussis negative culture	98	38

$X^2 = 9.16$ Significant relationship between B. pertussis infection and lymphocytosis, $P = 0.01$

the 64 Bordetella isolates were suspended in saline to match a turbidity standard prepared by adding 0.5 ml. of 1% $BaCl_2$ to 99.5 ml. of 1% H_2SO_4 (0.36 N). The suspensions were mixed with a vortex mixer and then inoculated with sterile swabs on charcoal agar plates. High concentration antibiotic discs were added and the plates were inoculated at 36°C in a moist atmosphere for three days. The zone sizes given by the different antibiotics were then measured and the results interpreted according to Bauer et al (1966). All 61 strains of B. pertussis were sensitive to tetracycline and all but one B. pertussis strains were sensitive to ampicillin, cephalothin, clindamycin, erythromycin, penicillin G, and chloramphenicol. All three strains of B. parapertussis were resistant to clindamycin and penicillin G, but sensitive to ampicillin, cephalothin, erythromycin, tetracycline and chloramphenicol (Table 21).

Penicillin M.I.C. of Bordetella isolates

The fact that almost all strains of B. pertussis (60 out of 61) were found sensitive to penicillin G by the disc method makes one question the validity of incorporating penicillin G in the medium to make it selective for B. pertussis. Penicillin G has been usually used in the range of 0.25 to 0.50 unit/ml. in Bordet-Gengou medium (Holwerda, 1971). Some laboratories may use even a concentration of up to 2 units/ml. (Baker, 1962). Therefore, we decided to check the penicillin M.I.C. of our Bordetella isolates, and to find the maximum concentration of penicillin that could be incorporated into culture media for isolation of the organisms.

Table 21

Antibiotic sensitivity of 64 Bordetella isolates by the disc method.

Number of strains sensitive to antibiotics out of no. of strains tested.							
	Ampicillin	Cephalothin	Clindamycin	Erythromycin	Penicillin G	Tetracycline	Chloramphenicol
B. pertussis	60/61	60/61	60/61	60/61	60/61	61/61	60/61
B. parapertussis	3/3	3/3	0/3	3/3	0/3	3/3	3/3

Note: The same strain of "B. pertussis" (Isolate no. 56) was resistant to ampicillin, cephalothin, clindamycin, erythromycin, penicillin G and chloramphenicol. The characteristics of this organism are discussed in detail under "Serotyping of B. pertussis".

An agar dilution method was used for the study of minimal inhibitory concentration (M.I.C.) of penicillin incorporated into both charcoal agar and Bordet-Gengou medium. Fresh cultures of the Bordetella isolates were suspended in saline to give a turbidity matching to 0.5 McFarland Standard (approximately 10^8 organisms/ml.). These suspensions were further diluted 1:100 in saline and inoculated with a 0.001 ml. loop onto the charcoal and Bordet-Gengou agar plates with different concentrations of penicillin. Plain charcoal and plain Bordet-Gengou plates were also inoculated. These plates without penicillin served as growth controls. All the inoculated plates were inoculated at $36^{o}C$ in a moist atmosphere for seven days and examined for growth.

The control plates without penicillin grew in excess of 200 Bordetella colonies for all 64 strains. At a concentration of 0.31 unit/ml. of penicillin in the Bordet-Gengou medium, there was inhibition on B. pertussis as the size of the colonies were smaller compared with those on the control plates. At a concentration of 0.62 unit/ml. of penicillin, fewer strains of B. pertussis (48 out of 61) grew on the Bordet-Gengou medium, as compared to charcoal agar (61 out of 61), and the B. pertussis colonies, if grown, were markedly inhibited and could hardly be recognized as B. pertussis colonies. Only two strains of B. pertussis managed to grow at a concentration of 1.25 unit/ml. of penicillin on Bordet-Gengou, as compared to 19 strains on charcoal agar at this concentration. (See Plates 6, 7, 8 and 9).

While colonial inhibition of B. pertussis was prominent on Bordet-Gengou medium with increasing concentrations of penicillin, the phenomenon was not demonstrated on charcoal agar. If grown, B. pertussis

colonies on charcoal agar were characteristic and could be readily recognized, in the presence of a high concentration of penicillin.

The experiment demonstrates that firstly, penicillin can only be used at a low concentration as a selective agent, and secondly charcoal agar is superior to Bordet-Gengou medium as a selective medium to isolate Bordetella pertussis. With the incorporation of penicillin, B. pertussis colonies were characteristic on the charcoal agar, but those on Bordet-Gengou medium might easily be dismissed as they could hardly be recognized as B. pertussis colonies.

Only one strain of B. pertussis (Isolate No. 56, see Table 21) managed to grow at a concentration of, and higher than 2.5 units/ml. of penicillin. All three strains of B. parapertussis were resistant to penicillin, surviving 10 units/ml., the highest concentration tested.

The reduction in number of colonies, as concentration of penicillin increased, was observed on both charcoal and Bordet-Gengou agar for B. pertussis. This process continued from a penicillin concentration of 0.31 unit/ml. to 1.25 units/ml., when absolutely no growth could be obtained at a concentration of 2.5 units/ml. (except Isolate no. 56, to be further discussed with serotyping results). For convenient comparison of results, growth of less than 10 colonies was regarded as "no growth". Results of this experiment is summarized in Table 22.

Serotyping of B. pertussis

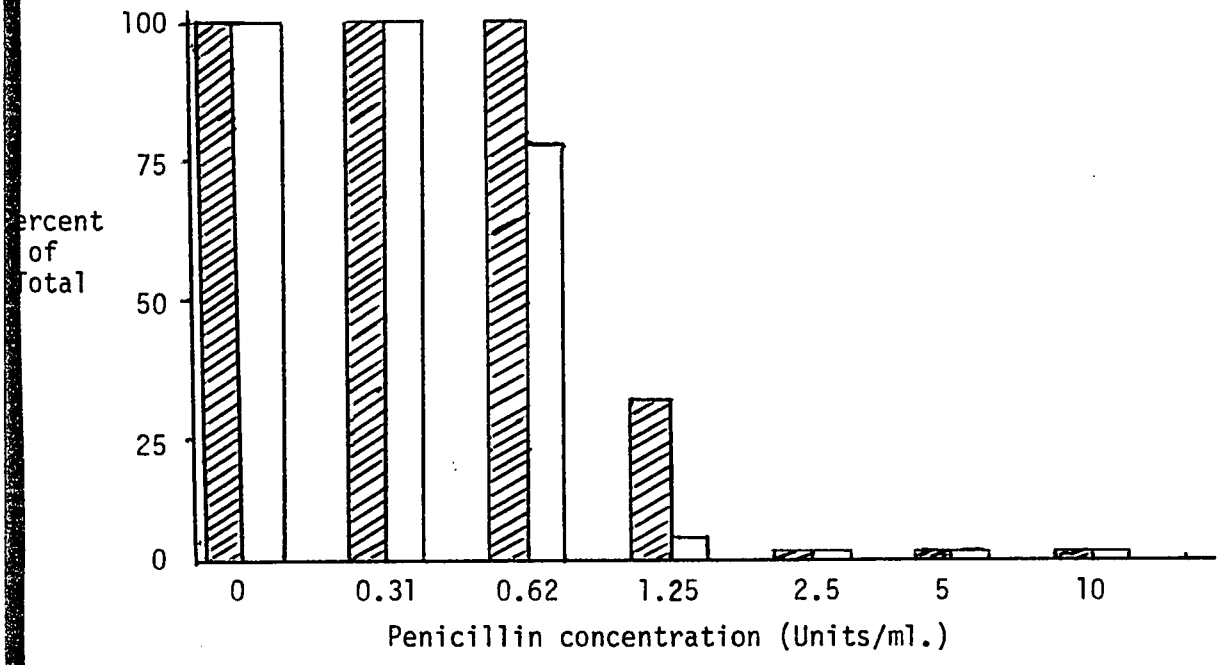
B. pertussis isolates were sent to three independent laboratories for serotyping. 55 isolates in their second to fourth subculture were found to be serotype 1,3 by all three laboratories. Two of the three laboratories serotyped all 61 B. pertussis isolated. 60 out of the 61 strains were

Table 22

Penicillin M.I.C. of 64 Bordetella isolates on charcoal agar and Bordet-Gengou medium.

No. of strains growing out of no. of strains tested							
	Penicillin Concentration (Units/ml.)						
	0	0.31	0.62	1.25	2.5	5	10
B. pertussis							
Charcoal agar	61/61	61/61	61/61	19/61	1/61	1/61	1/61
Bordet-Gengou	61/61	61/61	48/61	2/61	1/61	1/61	1/61
B. parapertussis							
Charcoal agar	3/3	3/3	3/3	3/3	3/3	3/3	3/3
Bordet-Gengou	3/3	3/3	3/3	3/3	3/3	3/3	3/3

Figure 3



Penicillin M.I.C. of 61 B. pertussis isolates on charcoal agar and Bordet-Gengou medium.

Plate 6

Penicillin M.I.C. of four strains of Bordetella pertussis on Bordet-Gengou medium and charcoal agar.

Penicillin concentration = 0 unit/ml.

Note characteristic and luxurious growth on charcoal agar.

Plate 7

Penicillin M.I.C. of four strains of Bordetella pertussis on Bordet-Gengou medium and charcoal agar.

Penicillin concentration = 0.31 unit/ml.

Note inhibition of growth on Bordet-Gengou medium at this concentration of penicillin.

Plate 6

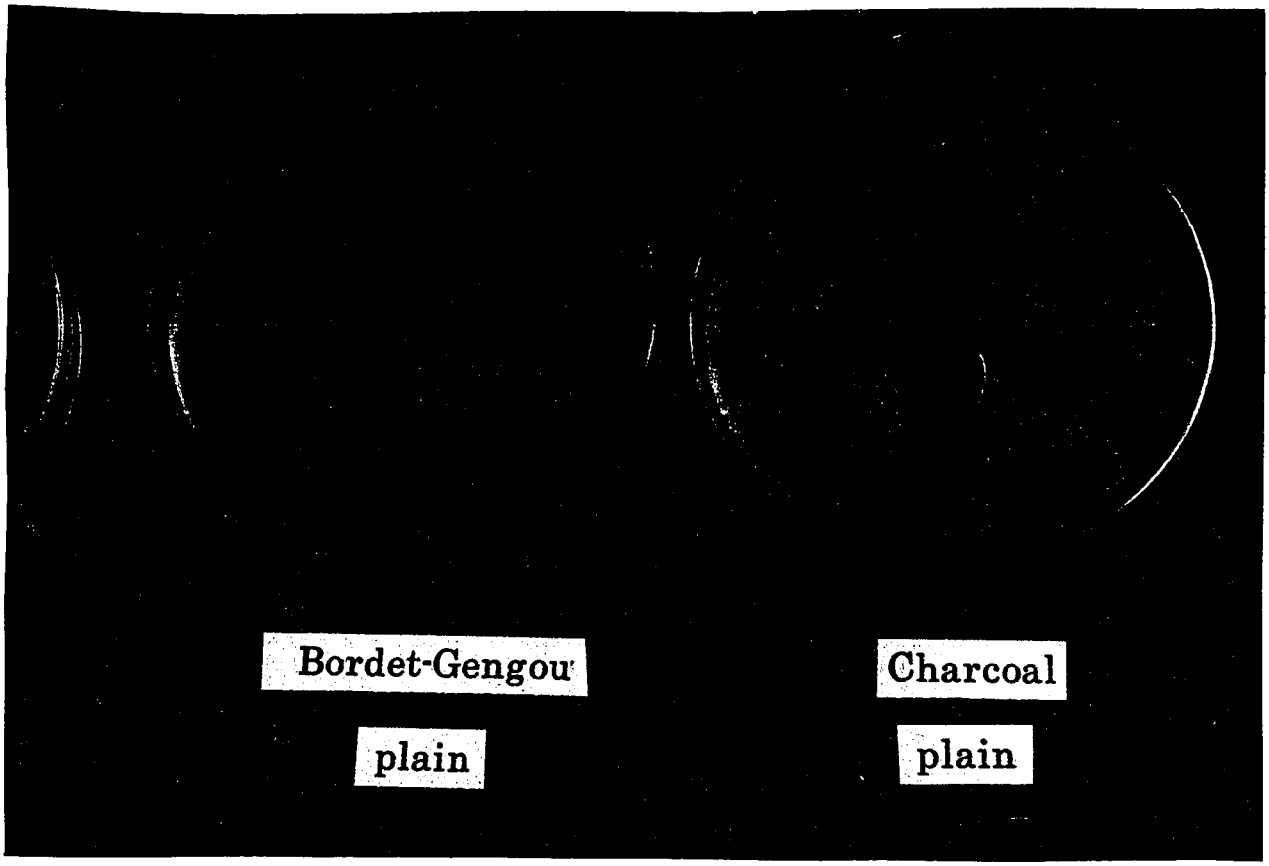


Plate 7

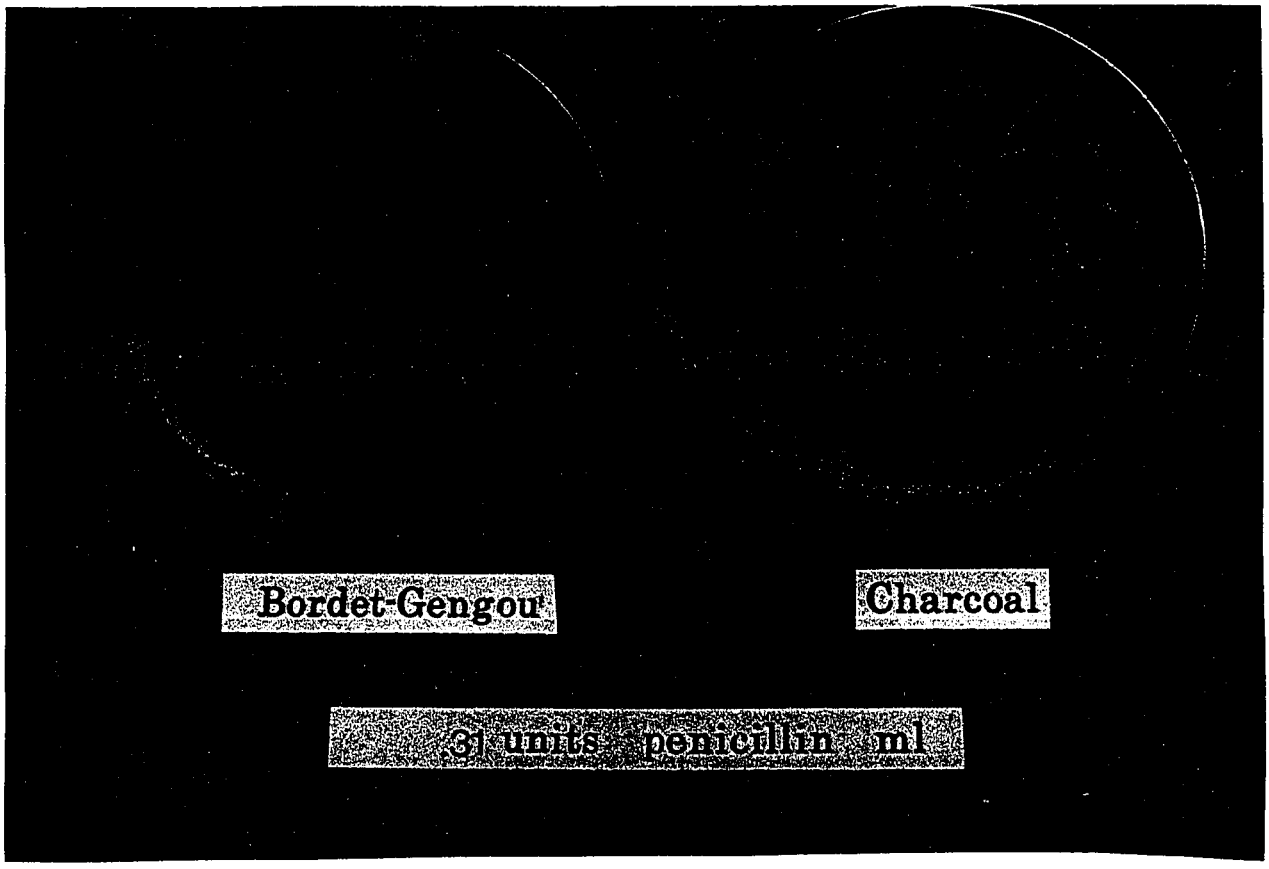


Plate 8

Penicillin M.I.C. of four strains of Bordetella pertussis on Bordet-Gengou medium and charcoal agar.

Penicillin concentration = 0.62 unit/ml.

Note only one strain of B. pertussis can be recognized on Bordet-Gengou medium at this concentration of penicillin.

Plate 9

Penicillin M.I.C. of four strains of Bordetella pertussis on Bordet-Gengou medium and charcoal agar.

Penicillin concentration = 1.25 unit/ml.

Note absence of growth on Bordet-Gengou medium.

All four strains of B. pertussis managed to grow on charcoal agar at this concentration of penicillin, with one strain still exhibiting its characteristic colonial morphology.

Plate 8

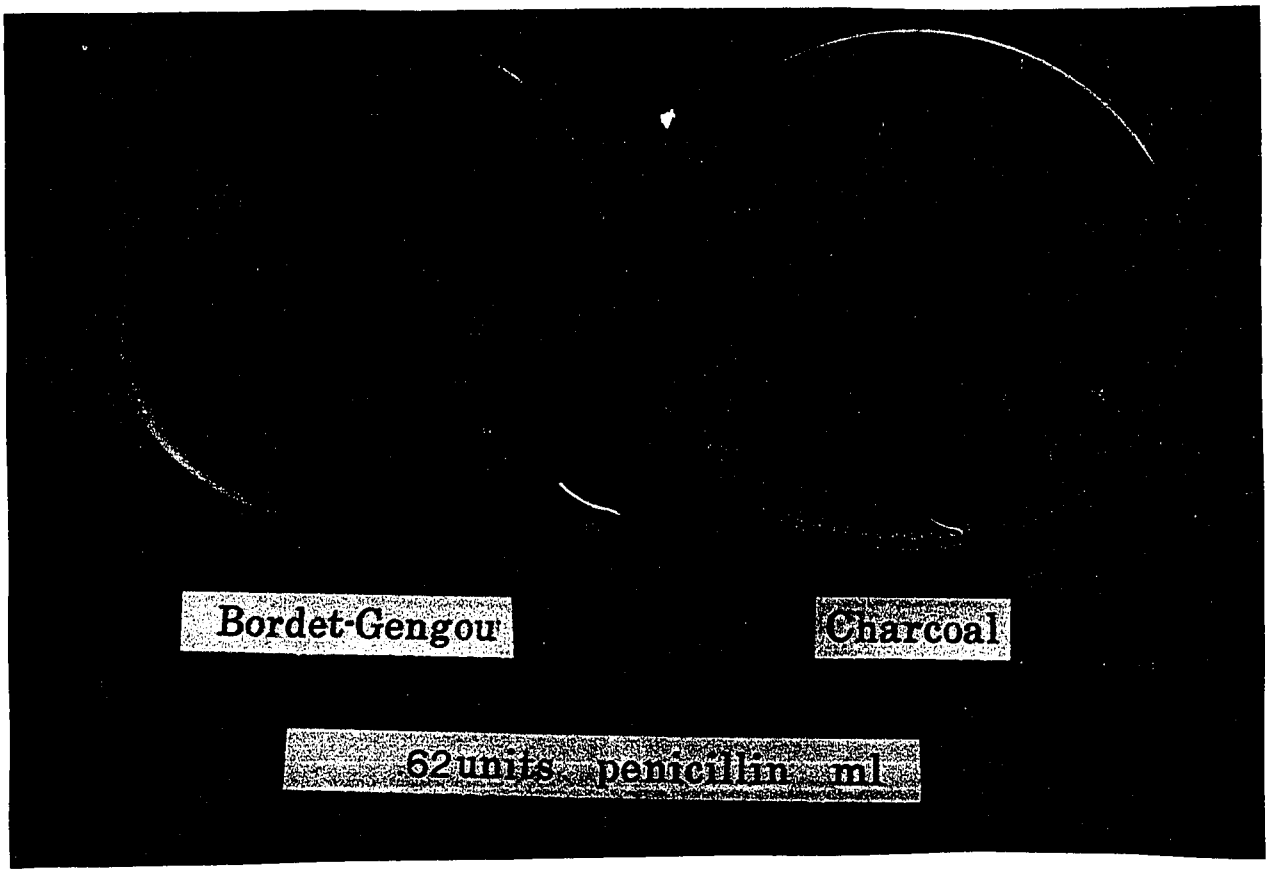
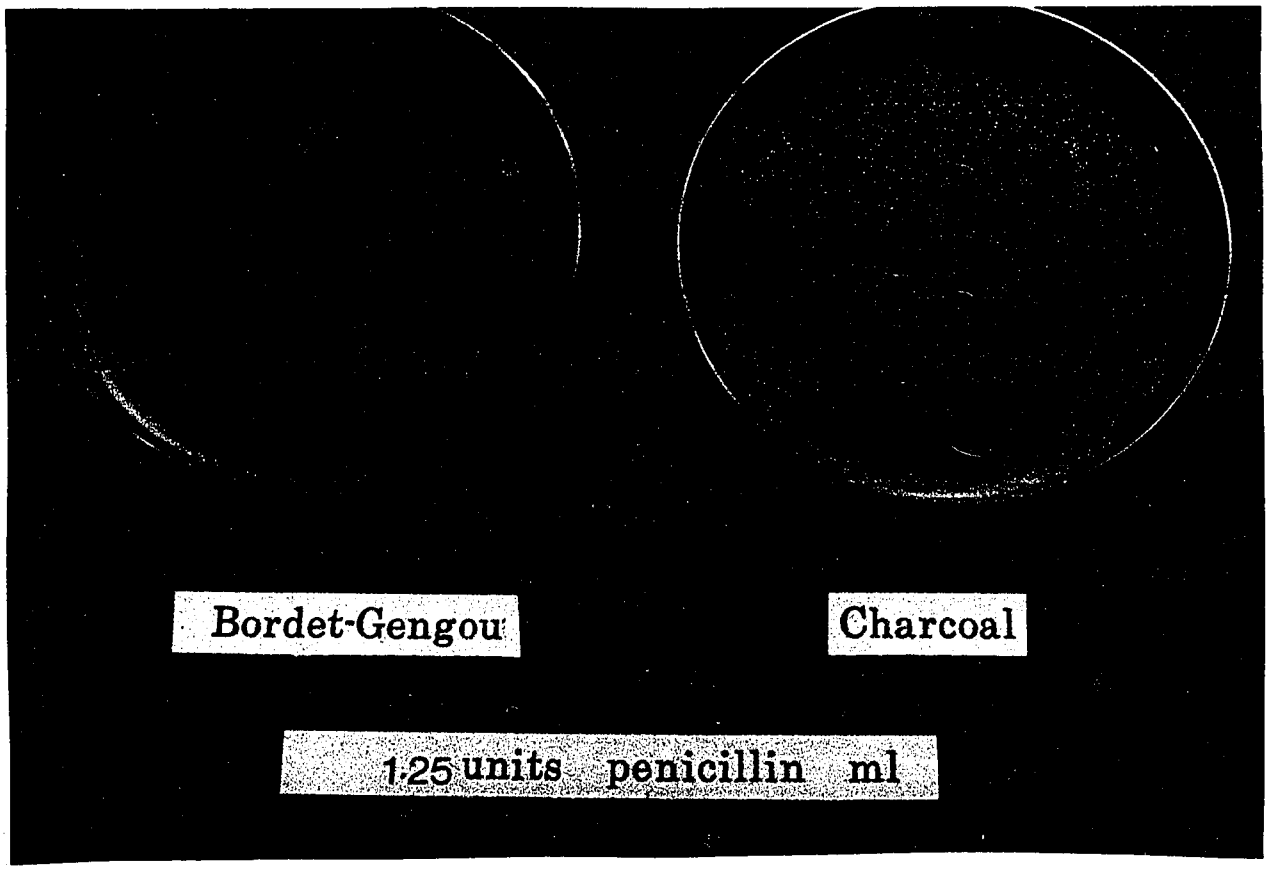


Plate 9



found to be serotype 1,3 by both laboratories, and one strain (Isolate no. 56) could not be serotyped.

This culture, (Isolate no. 56) presented the features of B. pertussis on primary isolation. The growth rate was similar to B. pertussis on charcoal agar, and the colonies, though less glistening, resembled those of B. pertussis. It was a Gram negative bacillus, more slender when compared with a typical B. pertussis culture. It gave a positive FA test with Difco FA-B. pertussis antiserum, and agglutinated in Difco B. pertussis antiserum, though the agglutination was not as strong as that of a known B. pertussis strain. The culture was sent to Central Public Health Laboratory, Toronto, where it could not be identified. It was referred to the National Reference Laboratory at L.C.D.C., Ottawa, and again it was not identified. This culture gave a resistance pattern on antibiotic disc sensitivity testing (Table 20), and grew in the presence of 10 units/ml. of penicillin in an agar dilution test (Table 21). With respect to these two features Isolate no. 56 differed from the 60 typical isolates of B. pertussis.

DISCUSSION

This study has demonstrated the presence of B. pertussis in the National Capital Region. The rate of identification of the organism increased from practically 0 in 358 patients with whooping cough from 1971 to 1974, to 67 in 231 patients from 1975-1976.

The success of the isolation of B. pertussis from these patients appears to be chiefly due to two major factors, namely, the use of Auger nasopharyngeal suction specimens and enriched charcoal agar with 35 mcg/ml of cephalexin as the culture medium. Auger suction specimens are far superior to cough plates, as more material can be collected in the nasopharyngeal catheter using the suction technique. Though the Auger suction specimens actually received in the laboratory were generally poor, they still provided us with many positive results.

With respect to culture, charcoal agar proved to be the best medium for the isolation of B. pertussis and B. parapertussis. It yielded more colonies as indicated in the experiments on viable counts (Table 10 and 11). It supported the recovery of the majority of B. pertussis cultures aged under suboptimal conditions (Table 8) and, with the incorporation of 35 mcg/ml. of cephalexin compared with Bordet-Gengou medium it best selected B. pertussis from mixed cultures (Table 9). Furthermore, field studies in parallel with Bordet-Gengou medium demonstrated the superiority of charcoal agar to Bordet-Gengou medium in the rate of isolation of B. pertussis colonies from actual specimens (Table 15).

While medium prepared from raw potatoes supported the growth of B. pertussis and yielded large colonies, storage of the potato suspensions according to recommended procedures resulted in poor growth and small colonies. It is probable that the starch present in the potatoes underwent hydrolysis on storage. With dehydrated Bordet-Gengou medium, containing potato

extracted by infusion, poor growth and reduction in size of B. pertussis colonies was also observed, especially when the plates were not freshly prepared, and the medium contained an antibiotic. The poor growth and small colonies on these Bordet-Gengou plates are not characteristic of normal B. pertussis colonies, and the plates can easily be dismissed as culture negative.

The phenomenon of poor growth and colony size reduction, however, was not shown on the charcoal agar, even in the presence of antibiotics or when the plates were not freshly prepared.

During growth B. pertussis forms a substance resembling oleic acid, and thus inhibits its own growth (Pollock 1947; Rowatt 1957). Charcoal has the ability to remove the inhibitory substance, thus enabling B. pertussis to continue to grow. It is the presence of charcoal that makes the charcoal agar superior to Bordet-Gengou medium.

In addition to charcoal, the charcoal medium also contains nicotinic acid, in specified amounts in the formula of the dehydrated medium. Hornibrook (1940) reported the finding of nicotinic acid as a growth factor for B. pertussis, and managed to grow B. pertussis in a defined medium with the addition of nicotinic acid. The formula of dehydrated Bordet-Gengou medium does not specify the amount of nicotinic acid included in the medium, and it is not known whether it is present.

Lacey (1954) incorporated a mixture of amino acids (including glutamic acid) in his medium to isolate B. pertussis. The medium, although excellent for isolation, is difficult to prepare. It contains eleven extra compounds, and four solutions including blood, which must be added aseptically to the autoclaved base. Although its qualities

are thoroughly appreciated, it is not widely used because of the difficulty of preparation.

The analysis of amino acid content in different batches of charcoal agar and Bordet-Gengou medium (Table 12) showed that both media contained comparable amounts of glutamic acid (0.67 ± 0.14 vs. 0.60 ± 0.09 μ mole per 0.3 ml.). Furthermore, a total of 17 other amino acids were present in both media, more than sufficient to grow B. pertussis in this regard. The incorporation of 1% Difco proteose peptone no. 3 to the charcoal agar provided additional supply of amino acids, but it was not necessary to grow B. pertussis. Sutcliffe and Abbott (1972) found addition of the peptone removed the inhibitory effect of some batches of charcoal agar on some B. parapertussis strains.

Direct immunofluorescence was found to be a far less sensitive technique than culture in the identification for B. pertussis. It needs a high concentration of organisms (1.8 ± 0.07) x 10^6 organisms/ml) for a definite positive reading. It appears that not all B. pertussis cells stained readily with commercial fluorescent antiserum (Table 5). During field studies, direct immunofluorescence detected only about half of the positive cultures (Table 13). This is contrary to some reports (Donaldson et al 1960: Chalvardjian, 1966).

When testing the specificity of the commercial FA - B. pertussis and FA - B. parapertussis conjugates, it was found that a few organisms stained non-specifically. Neisseria species, Haemophilus influenzae, Pseudomonas species and Klebsiella species produced non-specific fluorescence. The high proportion of false positive results with immunofluorescence was also mentioned by Linnemann (1974) as a problem

in diagnosing pertussis. In the staining of smears made from Auger nasopharyngeal suction specimens, it was also found that tissue cells might fluoresce. This increased the difficulty in the reading of slides for direct immunofluorescence.

The fact that direct immunofluorescence could only identify about half of the positive cases made us suspect the possible poor quality and inefficiency of the commercial reagents. An attempt was made to produce antisera instead of using these commercial reagents. Due to the complications of existing antibodies against all three Bordetella species in all six unimmunized rabbits, the plan was discontinued because of the cost involved in the search for rabbits free from Bordetella antibodies.

As an alternative to the above, indirect immunofluorescence was attempted on known B. pertussis smears. The working dilutions of rabbit antiserum and fluorescent sheep anti-rabbit conjugate were chosen by chessboard titration. The chosen combination was applied on a random proportion of smears made from Auger nasopharyngeal suction specimens. Indirect immunofluorescence performed on these specimens did not improve the diagnostic rate.

Routine bacteriological culture revealed that Diplococcus pneumoniae was the most common pathogen or potential pathogen isolated from these whooping cough patients. It was grown from 27.9% of the Auger suction specimens which yielded B. pertussis, and 24.1% from specimens which did not grow B. pertussis. There is no statistical significance for the presence of D. pneumoniae in the B. pertussis positive and negative culture groups ($X^2 = 0.205$, $p>0.1$, Table 16A). Culture performed on 216 Auger

suction specimens from non-whooping cough patients in our routine bacteriology laboratory also revealed that 55 isolates of pneumococci (25.5%) were present in these specimens. Auger (1939) found the pneumococci particularly numerous in the nasopharynx in the early stages of childhood pneumonia, and felt that the nasopharynx is a breeding ground for pneumococci and is probably the source by which pneumococci reach the lung. Other pathogens or potential pathogens found in the routine bacteriological culture included Staphylococcus aureus, Haemophilus influenzae, Beta hemolytic streptococcus (group A and NOT group A), and Klebsiella pneumoniae. Compared with pneumococci, these organisms were present in a much less prevalent amount (less than 10%). Neisseria species and alpha-hemolytic Streptococcus constituted most of the commensal flora, each being isolated in about 50% of the specimens, and often isolated together.

Charcoal agar plates were set up routinely on all Auger suction specimens, irrespective of the clinical diagnosis. Over a period of two years (1975-1976) 2,148 Auger suction specimens without a diagnosis of whooping cough were received. Out of these 2,148 specimens, mostly from patients with a history of upper respiratory infection, two B. pertussis and three B. parapertussis were isolated. Considering that a large number of specimens consisting essentially of single specimens from patients, it appears that whooping cough was not underdiagnosed, since only two B. pertussis infections escaped clinical attention. All three B. parapertussis infections were not diagnosed as whooping cough, suggesting this milder form of infection did not present the characteristic symptoms of whooping cough, and would have escaped diagnosis if a charcoal

agar plate had not been set up as a routine procedure for culture of Auger suction specimens.

Virologic findings in nasopharyngeal secretions from 204 patients with whooping cough and from 1,621 other patients - mostly with a respiratory tract infection other than whooping cough - revealed that adenovirus and respiratory syncytial virus did not play an important role in these syndromes (Table 17). The parallel studies of bacteriological and virologic examinations on these nasopharyngeal secretion specimens clearly indicated that B. pertussis was the organism chiefly responsible for whooping cough. Many laboratories failed to obtain positive B. pertussis cultures from whooping cough patients and this had led to the search for other possible agents, in particular, viruses. It is very important to start with a properly taken specimen before one can obtain a positive culture of B. pertussis. The Auger nasopharyngeal suction technique, when properly carried out, will provide the laboratory with a satisfactory specimen. It is unfortunate that most laboratories are not employing the Auger suction technique. While the cough plate method is totally unsatisfactory, the pernasal swab is probably the only other acceptable alternative. Pernasal swab specimens should be taken from the posterior nasopharyngeal area, that is, "through the nose until touching the posterior nasopharyngeal wall". This is very important inasmuch as pertussis organisms are not invasive, but primarily infect the ciliated margins of the respiratory tract epithelium and proliferate off into the overlying mucous bed. If they are recovered in the mouth, the anterior oropharynx, or in the anterior nasopharynx, it is because they have contaminated these areas from their primary focus of infection. The

posterior nasopharyngeal area is as much a primary focus as the ciliated epithelium and overlying mucous bed of the lower tracheobronchial tree. Specimen collection at the posterior nasopharyngeal area is undoubtedly the single best source for recovery of B. pertussis organisms in patients with pertussis. Bass et al (1975) reported they repeatedly obtained positive cultures using this technique following negative results by inexperienced house staff who have attempted to culture the organisms from an anterior nasopharyngeal swab.

In this study the identification rate of B. pertussis is 29% (67 out of 231 single specimens from patients). The result is far from perfect, as quality of specimen material was generally poor, and most patients were previously treated with antibiotics. Only 192 immunofluorescence tests were carried out on these 231 specimens (Table 13). Immunofluorescence examination was not performed on 39 specimens, mainly because there was not sufficient material. There was no growth obtained from 33 specimens on routine bacteriological culture. This suggests poor sampling and/or previous antibiotic treatment.

Only 21 strains of B. pertussis (15 by culture, and six by FA only; see Table 19) could be identified after patients had received antibiotic treatment. In the group of patients from whom B. pertussis could not be cultured, 57% (97 out of 170) was previously treated with antibiotics (mainly penicillin, ampicillin or erythromycin). Antibiotic testing of our isolates showed that B. pertussis was sensitive to these antibiotics (Table 21) and could explain our negative findings for these patients.

Considering the usual poor quality of the specimens and the fact that only single specimens were studied, there should be little doubt

that B. pertussis could be identified in more than 50% of the cases if better specimens were obtained, especially before patients received antibiotic treatments.

Islur, Anglin and Middleton (1975) reviewed the findings in 251 children admitted to the Hospital for Sick Children in Toronto for suspected whooping cough. B. pertussis was cultured from nasopharyngeal secretions collected at the time of admission and on days 2, 4, 6 and 10 from 175 of the children (69.7%). However, as impressive as that figure might be, clearly quintuplicate sampling is prohibitively expensive and impossible to do in daily practice. The good results we obtained with single specimens could be attributed to the fact that they were cultivated on charcoal agar plates containing 35 mcg/ml of cephalexin rather than on Bordet-Gengou plates containing 0.25 - 0.50 units/ml of penicillin G. We have shown that on the latter medium a few colonies of B. pertussis can easily be overgrown by throat commensals or inhibited by this concentration of penicillin G (Chan and Rossier, 1977).

Leucocytosis with a major increase in lymphocytes was observed only in 32.7% of patients (17 out of 52) with positive B. pertussis cultures, and in 16.9% of patients (23 out of 136) with negative B. pertussis cultures (Table 20). There was no significant relationship between positive B. pertussis infection and W.B.C. count ($X^2 = 3.35$, $p = 0.05$), but significant relationship between B. pertussis infection and lymphocytosis was demonstrated ($X^2 = 9.16$, $p = 0.01$).

It appears that the season of whooping cough is summer and fall. Out of 61 strains of B. pertussis 24 (39%) were isolated from specimens submitted in the months of August and September. The Hospital for Sick

Children in Toronto reviewed the monthly incidence of whooping cough from 1969 to 1972 and also found whooping cough primarily a summer and late fall disease (Effectiveness of Pertussis Vaccine, 1973).

More than half of the 67 strains of B. pertussis were identified in patients who were less than one year old. Among these patients 15 were less than three months of age (Table 18).

The examination of 2,148 specimens from non-whooping cough patients at the children's Hospital of Eastern Ontario resulted in the isolation of two B. pertussis and three B. parapertussis, and indicated that the organisms were not significantly carried in children. A similar study by Linnemann et al (1968) also did not reveal asymptomatic carriers. Although the possibility of adult carriage (Anonymous, 1973) has been brought to the attention of the medical authorities, there were no adult specimens received at the Children's Hospital of Eastern Ontario. However, there were reports of B. pertussis and B. parapertussis isolated from adults in Toronto (Regan and Lowe, 1977) and recently, the Ontario Ministry of Health reported that B. pertussis was isolated from many adults in an outbreak (Epidemiology Report November 18, 1977).

Table 18 shows that while 12 identifications (17.9% of the 67) were made in completely immunized children, at least half were made in patients who were either less than one year old (53%) or incompletely immunized (61%). All isolates of B. pertussis belonged to serotype 1,3.

In many countries over the past few years B. pertussis serotypes have changed from a mixture of types 1,2,3 and 1,2 to a predominance of type 1,3. This change is related to the introduction of mass vaccination with vaccines rich in antigens 1 and 2 but weak in or devoid of antigen 3.

Furthermore there have been outbreaks of type 1,3 infections in fully vaccinated children (Preston, 1976). The National Capital Region is no exception: although this is the first time serotyping on B. pertussis is performed in this area, type 1,3 is also the prevalent serotype here, in keeping with other findings in Ontario (Chalvardjian, 1965), (Magus, 1976) and Nova Scotia (Preston, 1976).

Immunization against whooping cough was introduced in Canada in the 1940's and thereafter the reported incidence of the disease decreased (White and Varughese, 1977). The few available serotyping results on B. pertussis indicated that type 1,3 is predominant in Canada, a similar pattern as experienced in other countries following mass-vaccination with material rich in antigens 1 and 2 but weak in, or devoid of, antigen 3.

As in other countries (Stewart, 1977) there is controversy in Canada about the need for and the safety and efficacy of pertussis vaccination. Apart from the well known under reporting of the disease there are remarkably few laboratory data available about the true incidence of B. pertussis and its serotypes across the country, although our investigation has demonstrated that they can be obtained in field conditions. Such data are necessary before a national policy for or against pertussis vaccination can be established in Canada. (Rossier and Chan, 1977).

SUMMARY AND CONCLUSIONS

Over a two-year period 67 strains of Bordetella pertussis were identified in 231 single specimens of nasopharyngeal secretions submitted from patients suspected to have whooping cough in the National Capital Region.

192 specimens were examined for B. pertussis by direct immunofluorescence and culture. 28 (14.6%) were positive by both methods, 24 (12.5%) were positive by culture alone, and 6 (3.1%) were positive by direct immunofluorescence only. 134 (69.8%) were negative by both methods. Indirect immunofluorescence performed on a random portion of these specimens did not improve the diagnostic rate.

129 specimens were cultured on enriched charcoal agar and Bordet-Gengou plates, both containing 35 mcg/ml of cephalexin. Out of 25 strains of B. pertussis four were isolated on both media, while 21 were isolated on charcoal agar only. The Bordet-Gencou plates failed to grow any strain which did not grow on the charcoal medium. Colony count experiments on charcoal agar and Bordet-Gengou plates with and without cephalexin supported these findings. The conventional Bordet-Gengou medium, with the incorporation of penicillin G in the concentration of 0.25 - 0.50 unit/ml., was found to be inhibitory to some strains of B. pertussis, so that colonies of the inhibited B. pertussis cultures, if grown, could hardly be recognized.

More than half of the 67 strains of B. pertussis were identified in patients who were less than one year old, and most of the patients with bacteriologically confirmed whooping cough had not been fully immunized. The highest isolation rate was obtained during the first two weeks of the disease, in the absence of antibiotic treatment. The

prevalent serotype was 1,3, and all of them were sensitive to ampicillin, cephalothin, clindamycin, erythromycin, penicillin G, tetracycline and chloramphenicol.

Viral studies were carried out in parallel with bacteriological investigations in 204 of these 231 cases of whooping cough. There was no evidence that adenoviruses or other viruses played any important etiologic role in whooping cough or whooping cough syndrome.

This study demonstrates that there is no evidence of a carrier state of B. pertussis in children, and that B. pertussis can be isolated from a single specimen of nasopharyngeal secretions in about one of three suspected cases of the disease, even in suboptimal conditions. These findings are in sharp contrast with those from previous years, when virutally no B. pertussis isolate was made in the National Capital Region. This appears to be due mainly to the collection of proper specimens by Auger suction, and the use of a high quality selective medium, namely, enriched charcoal agar with 35 mcg/ml. of cephalexin.

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