Bull. Org. mond. Santé | 1960, **23**, 15-35
Bull. Wld Hlth Org. |

Laboratory Diagnosis of Whooping-cough or *Bordetella* Infections *

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CONTENTS

INTRODUCTION

THE *BORDETELLA* GROUP OF ORGANISMS

Whooping-cough is a clinical syndrome and may be caused by three different micro-organisms belonging to the genus *Bordetella*. By far the most common of these organisms is *Bordetella pertussis*, which was described by Bordet & Gengou in 1906 and was for a long time known as *Haemophilus pertussis*. The second of these organisms, *Bordetella parapertussis*, was described in 1937 by Eldering &

* This is one of a series of studies on the laboratory diagnosis of various diseases which, it is hoped, will eventually be revised and published in monograph form. An effort is made to ensure that the diagnostic methods recommended in these studies are as internationally representative and acceptable as possible by securing the co-operation of a number of experts from different countries. A list of the reviewers of the study presented here is given in Annex 2 (page 35). To all of these, and to the authors, the World Health Organization is greatly indebted.—ED.

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Kendrick (1937, 1938) and also by Bradford & Slavin. This organism, which usually causes a very mild type of whooping-cough, has since been reported from many parts of the world,[1] but in most places it does not account for more than about 5% or less of the whooping-cough cases. A much higher rate (20-25%) has been observed in Denmark during epidemics of parapertussis (Lautrop, 1954, and unpublished data). The third organism, formerly known as *Brucella bronchiseptica* but now called *Bordetella bronchiseptica*, was isolated by Brown in 1926 from a child with whooping-cough. He suggested that this organism, which occurs frequently in the respiratory tract of smaller animals (e.g., rabbits, guinea-pigs, dogs and cats), might sometimes cause whooping-cough in humans. A few later reports (Chang, 1950; Medical Research Council, 1951) have substantiated Brown's observation, but their scarcity indicates that human infections are extremely rare. Lacey estimates that they constitute approximately 0.1% of the whooping-cough cases in London.

It would appear that infection with bacteria belonging to other genera such as *Haemophilus influenzae* and *Diplococcus pneumoniae* sometimes produce symptoms indistinguishable from whooping-cough caused by organisms of the *Bordetella* group.

Laboratory studies[2] of the three organisms *Haemophilus pertussis*, *Brucella bronchiseptica* and *Bacillus parapertussis* have shown that they have a number of features in common and it is now generally believed that they represent closely related members of the same bacterial group. Lopez in 1953 proposed introducing the generic term *Bordetella* for this group of organisms and renaming the organisms *Bordetella pertussis*, *Bordetella bronchiseptica* and *Bordetella parapertussis*. This proposal has already received support from some bacteriologists (Pittmann, 1955; Proom, 1955) and is adopted in the latest (seventh) edition of Bergey's *Manual of Determinative Bacteriology* and will be used in this study. The abbreviation *Bord.* is used throughout to indicate *Bordetella*. The table below summarizes the most important differential characters of the three *Bordetella* species.

MOST IMPORTANT DIFFERENTIAL CHARACTERS WITHIN THE GENUS *BORDETELLA*

	Bord. pertussis	*Bord. parapertussis*	*Bord. bronchi-septica*
Cell shape on B-G medium	coccoid	rod	coccoid
Motility	—	—	+
Growth on plain agar medium	—	+	+
Pigment production	—	+	—
Reduction of KNO_3	—	—	+
Splitting of urea	—	+	+
Oxidase	+	—	+
Catalase	+ or —[a]	+	+

[a] Only about 70% of freshly isolated strains contain catalase (Lautrop, 1954; Portwood, 1946).

To avoid nomenclatural confusion the term pertussis should no longer be used synonymously with tussis convulsiva but should be limited to infections caused by *Bord. pertussis* in the same way as the term parapertussis is limited to infections caused by *Bord. parapertussis*. The terms whooping-cough and tussis convulsiva should then be used as clinical terms to describe the classical clinical syndrome, which we now know may be caused by at least three different micro-organisms (Lautrop, 1954).

INDICATIONS FOR BACTERIOLOGICAL EXAMINATION

1. Clinically it is impossible to decide whether a case of whooping-cough is pertussis, parapertussis or a bronchiseptica infection, therefore an etiological diagnosis is dependent on a bacteriological (or serological) examination (Bogdan, 1955; Kendrick & Eldering, 1935; Lautrop, 1954). And only by this means is it possible to demonstrate a simultaneous infection with *Bord. pertussis* and *Bord. parapertussis*, an occurrence which is by no means rare (Lautrop, 1958).

[1] Cruickshank & Knox (1946); Eldering & Kendrick (1952); Forsell et al. (1955); Grigorova & Stoyanova (personal communication from Sofia, Bulgaria, 1956); Lautrop (1954, 1958); Lopez (1952); Marie et al. (1954); Medical Research Council (1951); Miller et al. (1941); Nobechi (1955); Sohier & Fauchet (1949); Syrucek et al. (1954); Vysoká & Syruček (1954); Yoshino (1951).

[2] Andersen (1952, 1953); Bradford & Slavin (1937); Bruckner & Evans (1939); Eldering (1942); Eldering & Kendrick (1938); Eldering et al. (1956); Evans (1940); Evans & Maitland (1939); Ferry & Klix (1918); Ferry & Noble (1918); Fukumi et al. (1953); Kendrick et al. (1953); Kobyashi & Fukumi (1954); Lacey (1951, 1955); Lautrop (1954); Nobechi 1955); Pacagnella (1952); Proom (1955).

2. A case of atypical cough may represent: (*a*) a pertussis infection in the pre-paroxysmal stage; (*b*) a pertussis infection modified by previous vaccination; (*c*) a pertussis infection in a person (usually an adult) with some degree of immunity from a previous infection of the same type; and (*d*) a parapertussis infection. The categories listed as (*b*), (*c*) and (*d*) are much more frequent than was formerly supposed. The only way to reveal the true nature of such clinically uncharacteristic infections is, of course, to make a search for organisms of the genus *Bordetella.*

3. Therapeutic trials and other types of research work must be based on bacteriologically confirmed cases.

4. It is an old dictum that the negative outcome of a single bacteriological examination does not exclude an infection with organisms of the genus *Bordetella.* However, improved methods for collecting specimens and the use of penicillin and other inhibitory substances in the medium to suppress growth of contaminating organisms have enhanced the reliability of the examination to the degree that a consecutive series of three negative nasopharyngeal swabs—obtained and plated correctly and examined in an experienced laboratory—may, for practical purposes, be considered sufficient evidence that a person is not infected or not infective any longer.

TWO DIFFERENT DIAGNOSTIC PROCEDURES

Until recently the success of the bacteriological examination of whooping-cough cases was in most places dependent to a high degree on the personal skill and experience of the investigator. As a consequence—and with truth—it has therefore been claimed that the task requires specialized laboratories. This is true because the training which is necessary for picking the right colonies from a mixed culture and for identifying the organisms by conventional bacteriological methods cannot be obtained except in laboratories which examine a large number of samples. Furthermore, only in such places is it in practice possible to have fresh media available at any time, an essential point in obtaining good results.

In 1954 Lacey described a strongly selective medium for the cultivation of *Bordetella* and advocated the use of serological methods as the principal means for their identification. In principle, Lacey's procedure may be compared with the one used in most places for the isolation and identification of *Salmonella* and *Shigella* from samples of faeces. Using this new procedure Lacey improved his results considerably, and almost equally good results (unpublished) have been obtained in other English laboratories.

As far as is known, Lacey's procedure has not yet been tried outside England. This limited experience at the present moment is deplorable since an evaluation of the efficiency of the method under different conditions is not possible. However, in the author's opinion it is likely that the method will prove to be very useful. The only definite drawback seems to be that the examination often requires an extra 24 hours or more because the selective medium is somewhat growth-inhibiting, especially as regards *Bord. parapertussis.*

Because the author has very little personal experience with Lacey's procedure the following detailed description of the diagnostic procedure is limited to the conventional methods. Details concerning Lacey's procedure are given in Annex 1, worked out by Lacey himself.

It is likely that Lacey's procedure will be of special advantage for laboratories receiving only a small number of samples for examination. One reason for this is that the medium keeps for a longer period than Bordet & Gengou's medium; another is that specially trained personnel are unnecessary. The availability of the right kind of diagnostic sera is, of course, essential; it would appear that the best solution to this problem is to have the sera prepared and checked at a central laboratory.

If circumstances are favourable—i.e., if the personnel are well trained and facilities are present for the preparation of fresh Bordet & Gengou medium twice or thrice weekly—the procedure which is described in the following pages gives very satisfactory results and—a point of no little importance—a report announcing the presence of *Bord. pertussis* or *Bord. parapertussis* can in all cases (with very few exceptions) be made after 72 hours.

COLLECTION AND INCUBATION OF SPECIMENS

The causative organism of whooping-cough is found in the respiratory tract of the patient and only there.

While the organism is easily demonstrated during the catarrhal stage in almost all patients, the percentage of patients harbouring bacilli drops gradually during the first five weeks of the convulsive stage (Christensen, 1955). After the fifth week a positive culture is rare, although it may be obtained as late as during the tenth or twelfth week. These figures apply to *Bord. pertussis*. In parapertussis the infective period is usually shorter, but even *Bord. parapertussis* may in rare cases be found many weeks after the onset (Lautrop, 1954).

During antibiotic therapy the number of *Bordetella* present in the respiratory tract is considerably reduced, and their demonstration is correspondingly more difficult. Individuals who are partly immune, whether as a result of natural infection or immunization, seem to harbour the organisms for a much shorter period, but exact data are not available.

SWAB METHOD

Mucus from the posterior wall of the nasal part of the retropharyngeal cavity is a suitable material for examination and may be obtained either by the aid of a cotton-wool swab on a bent rod introduced through the mouth and pushed up behind the soft palate (peroral or post-nasal) (Bradford & Slavin, 1940) or by a swab on a straight but flexible wire introduced through the nose (pernasal) (Cruickshank, 1944). The peroral swab usually picks up more "contaminants" than the pernasal one. The "contaminants" may obscure or suppress the growth of *Bordetella* on the Bordet & Gengou medium and therefore the pernasal swab is considered the method of choice (Cockburn & Holt, 1948), especially in very young children (Bradford & Brooks, 1941). With media which include inhibiting substances for all the more important "contaminants" the two methods seem to give equally good results (Lacey, 1954).

When the peroral route is used much care should be taken to avoid contamination from the oral cavity, and to facilitate this the bent part of the rod should be short, about 1 cm. The swab must rest firmly against the posterior wall of the pharynx and then be moved a little up and down a few times. By the pernasal route the swab is pushed gently backwards through one of the nostrils until it touches the posterior wall and is then rotated axially. Care must be taken not to direct the swab upwards but to let it creep along the floor of the nasal cavity. Obstructions, which may be due to a deviation of the septum, must not be forced, but the other side should be tried. It is absolutely necessary for the child's head to be held firmly by an assistant during swabbing, irrespective of the route.

Before sterilization it should be ascertained that the cotton wool of the swab is safely fixed to the wire. Some types of cotton contain bactericidal substances (Pollock, 1949) and must be avoided. The ideal time for swabbing seems to be in the morning, before the first meal.

Plating of the swab is a very important part of the technique (Freundt, 1951; Lautrop, 1954). Since the swab holds an unknown—often very large—number of bacteria of many kinds, a streaking method which ensures a continuous variation from heavily to lightly inoculated areas on the plate is required. The largest possible surface of the swab—not only the tip—is placed against the medium at the top of the plate and moved back and forth a few times; visible lumps of mucus should be deposited here. The remaining part of the plate is then covered by a number of closely set streaks, performed from side to side, care being taken to utilize the surface completely. This method of plating applies to conditions where no laboratory facilities are at hand. In the laboratory it is usual to streak only half of the plate directly with the swab and spread this inoculum further with a bent glass rod. It is recommended that at least two plates of Bordet & Gengou medium be used for each swab. With more highly selective media one plate or part of one plate may be sufficient.

The survival time of pertussis organisms on a swab is usually short, whereas some of the "contaminants" probably keep alive for long periods and may even multiply at room temperature (Freundt, 1951). Therefore, immediate plating of the swab is strongly recommended. If the swab has to be mailed to the laboratory it should be placed either in an empty sterile tube or, preferably, in a

tube with a slanted solid medium to prevent drying (Cruickshank, 1944). Carrier tubes with Lacey's medium are probably even better.

COUGH METHOD

The cough method is not as effective as the swab method. This is most evident when few organisms are present as, for instance, in the later stages of the disease (Bradford & Slavin, 1949; Brooks et al., 1942; Cruickshank, 1944; Freundt, 1951; Miller et al., 1943; Saito et al., 1942). In the catarrhal stage, however, the cough method yields excellent results.

The use of the cough method has the special advantage that the plates function as carrier medium. Once organisms of the genus *Bordetella* are deposited on the medium they remain viable for long periods even if multiplication does not take place; therefore it is possible to mail the inoculated plates over long distances before actual incubation starts. To make handling easy and safe in these circumstances the Petri dishes—both top and bottom—should be made of aluminium, and to prevent slipping of the medium during transportation—usually by mail—two ridges in the form of concentric circles are embossed on the bottom part of the dish. Another advantage of the cough method is that the sampling may be left to the mother. The doctor orders the plates to be sent directly to the patient's home and instructs the mother, who, after exposing the plates during typical paroxysms, returns them by mail to the laboratory.

Such a procedure is simple and time-saving for everybody involved and explains why in Denmark, for instance, the cough method is still extensively used by practising physicians during the early stages of the disease.

During a cough-paroxysm the opened Petri dish is held in front of the patient's mouth at a distance of 15-20 cm. In this way bacteria-containing droplets impinge on the surface and thereby inoculate the medium (Chievitz & Meyer, 1916). Visible lumps of mucus and vomiting have to be avoided.

Repeated exposure of a plate is recommended if the paroxysms are only weak ones, and in cases where no spontaneous paroxysms occur they may be provoked by artificial means—for instance, by a tongue depressor or by exerting a slight pressure on the larynx from without.

It is recommended that two plates be exposed for each patient.

OTHER METHODS

Other methods for collecting specimens have been recommended. One is to suck up mucus from the pharyngeal or supralaryngeal area by means of a thin rubber hose mounted on a syringe (Herzog, 1954; Silverthorne et al., 1945). This method may be useful under special circumstances—for instance, in the newborn—or as a supplement to one of the other methods, but as a routine method it is unnecessarily cumbersome. Swabbing from the supralaryngeal area has also been recommended and may sometimes yield positive results when other methods have failed.

INCUBATION OF SPECIMENS

In the laboratory, incubation should take place in a thermostat, preferably at a temperature of 35°C (±1°C) (Bradford & Brooks, 1941; Lacey, 1954). The conventional temperature of 37°C for incubation of human pathogenic bacteria may be used, but is not optimal for *Bord. pertussis*. A high degree of humidity favours growth; one way to secure this is to use an air-tight incubator with a tray of water at the bottom (Lacey, 1954); another way is to stack the dishes in tightly closed metal containers (Lautrop, unpublished data).

The necessary incubation period for the development of recognizable colonies depends upon various factors, of which the quality of the medium is the most important. Working with an ideal medium a trained observer may detect the colonies after 48 hours, but under average conditions about 72 hours are required. On a less satisfactory medium colonies may not become visible until after four, five or six days. As a routine measure it is recommended that the first examination be made on the third day, and all negative plates re-incubated and examined again on the fourth day. Local experience must decide if examination later than the fourth day is necessary to detect all positives. Daily inspection of the plates with the object of cutting out colonies of contaminating moulds is used in some laboratories.

MEDIA FOR CULTIVATION

BORDET & GENGOU'S MEDIUM

Most people still consider Bordet & Gengou's original medium or modifications of it to be the best medium for the cultivation of *Bord. pertussis*, which is by far the most fastidious of the *Bordetella* organisms (Kendrick et al., 19--; Lautrop, 1954; Marie et al., 1954; Tunevall, 1946). (In the following Bordet & Gengou's medium will be abbreviated as B-G medium).

The following method of preparing B-G medium has been used at the Statens Seruminstitut in Copenhagen for more than 40 years, and in spite of many attempts to improve the growth-promoting capacity, this has never been achieved. Potatoes are cleaned, peeled and sliced and 1000 g are boiled in 2 litres of tap water containing 80 ml of glycerol until the slices fall apart. The loss of water in boiling is made up. The juice is separated by mashing through a sieve, followed by squeezing through a towel. To this concentrated potato juice is added 3 volumes of a 0.6% solution of sodium chloride in tap water and the diluted juice is distributed in 200-ml amounts in flasks; agar—usually 4.25 g per 100 ml of diluted juice, but varied according to the quality of the agar—is added and the flasks are sterilized by autoclaving. Growth is best on a rather soft medium, but if plates are to be posted they must be harder. The potato base should have a pH of about 7.4.

From this potato base, which will keep for months in the refrigerator, the final medium is prepared by the addition of an equal volume of defibrinated blood. The mixing of the potato-agar base and the blood is a very important step in the preparation. The difficulty is to keep the temperature at a level high enough to ensure satisfactory pouring but low enough to avoid destruction of heat-labile growth factors in the blood (Bailey, 1933; Lautrop, 1954). The following procedure may be recommended: The base is melted, cooled down and kept at 45°C in a water-bath. Blood drawn the same day is left at room temperature and blood kept in the refrigerator is transferred to room temperature two or three hours before use and left there. The contents of each bottle of blood should not exceed 200 ml. About 15-20 minutes before mixing, the bottle is placed in the water-bath at 45°C. Mixing of the two components and pouring of the plates must then take place as quickly as possible.

Originally Bordet used human or rabbit blood. Today sheep's blood is used in many places, and in Denmark and some other places horse blood is preferred. As to growth-promoting capacity, these four types of blood seem to be almost equal—at least, no single type of blood has been unanimously recommended. Human blood has the disadvantage that haemolysis is sometimes absent or atypical until the incubated plate has been refrigerated for a short time (Lautrop, 1954). Horse blood has the advantage that it gives a clear-cut morphological differentiation of *Bord. pertussis* and *Bord. parapertussis* (Lautrop, 1954). Rabbit and sheep blood have the advantage that haemolysis develops on them better than on horse blood (Lautrop, 1954).

According to Bordet's original formula 50% blood should be used, but the amount has in many places been reduced to about 16-17% or even less. In Denmark 50% is still adhered to and considered essential. If for various reasons 50% cannot be used, the reduction must be the smallest possible one and less than 30% should probably not be used (Dawson et al., 1951). The use of a high concentration of blood will more or less obscure haemolysis, but if opaque dishes of aluminium are used the value of haemolysis in diagnosis is already limited. The blood should be as fresh as possible, preferably drawn the same day and probably not more than one or two days old. If 30% to 50% fresh blood is used the pH of the finished medium should be about 7.6 (Kendrick & Eldering, 1934; Lautrop, 1954).

Before mixing, a penicillin solution is added to the melted base (Bradford et al., 1946; Fleming & Maclean, 1930; Maclean, 1937; Tunevall, 1946). A final concentration of 0.5 units per ml of finished medium is recommended. The addition of nicotinamide (Hornibrook, 1940) to a final concentration of 1 mg per litre of total medium is also sometimes recommended (Tunewall, 1946) because all species of *Bordetella* require this vitamin; if, however, 50% blood is used, it seems to be unnecessary.

Peptone in the medium is recommended in some widely used formulae, but since at least some brands of peptone have a decidedly inhibitory effect on the growth (Dawson et al., 1951; Lautrop, unpublished data) it should be left out.

Plates should be thick; it is recommended that they be made not less than 5 mm (Saito et al., 1942; Sauer & Hambrecht, 1930).

The poured plates are stored in the refrigerator at +4°C and a sterility test is performed on one plate from each flask by incubation at 37°C overnight. After this period the plates are ready for use and should be used as soon as possible. Plates more than five days old at the time when incubation in the laboratory is started give inferior results (Lautrop, 1954).

A perfect plate has a bright-red colour and should keep this colour almost unchanged throughout incubation (Bailey, 1933; Lautrop, unpublished data). Dark plates usually indicate that something is wrong—for example, the blood used may have been too old or have been heated too much, or the plates may have been stored for too long a period.

OTHER MEDIA

A number of more or less modified B-G media (Bertoye, 1950; Madsen, 1937; Silverthorne & Cameron, 1942) and other types of media (Dawson et al., 1951; Nicholson & Turner, 1954; Pollock, 1947) have in the course of time been devised to replace Bordet & Gengou's original medium. In spite of favourable reports from the originators none of these media has obtained universal recognition, probably because the postulated improvement was either insignificant or did not regularly show up in the hands of others.

With a view to vaccine production much effort has been spent in the elaboration of a synthetic or semi-synthetic fluid medium that would support growth of *Bord. pertussis* (Cohen & Wheeler, 1946; Hornibrook, 1939; Mishulow et al., 1953; Nobechi, 1955; Powel, 1951; Verwey & Sage, 1945; Verwey et al., 1949; Wilson, 1945). During this work much has been learned about the growth requirements of *Bord. pertussis* (Dawson et al., 1951; Fisher, 1948; Jebb & Tomlinson, 1951, 1955; Mazloum & Rowley, 1955; Pollock, 1947, 1949; Rowat, 1955; Ungar et al., 1950; Watanabe et al., 1952) but up to now the acquired knowledge has not resulted in better solid media for primary isolation. For a review of this problem see Rowat (1957a, 1957b).

EXAMINATION OF SPECIMENS

Examination of colonies on the primary plate and microscopic examination of a Gram-stained slide preparation of suspected colonies represent the first stage in the examination of a specimen. This stage is here called the preliminary examination. The next stage—if necessary—includes a number of cultural and biochemical tests and in some cases a serological test. These tests will be described later.

For macroscopic examination it is recommended that the plate be held obliquely below a shaded electric-light bulb and the naked eye used for a primary inspection and a hand lens for closer observation. The use of a plate microscope is sometimes recommended for the detection of colonies at the earliest possible moment.

CULTURAL AND OTHER CHARACTERISTICS OF THE *BORDETELLA* SPECIES

The following detailed description of the cultural characteristics of the *Bordetella* species on B-G medium provides the criteria on which the picking of colonies on the primary plate must be based.

Colony appearance

Traditionally, colonies of *Bord. pertussis* are described as looking like small droplets of mercury or a bisected pearl, i.e., they are small, raised, shiny, smooth, grey-white colonies with an entire circular edge. Their appearance, however, may be rather variable, depending on the medium; a flatter, whiter and not so shiny type is frequent. Also the size—after a fixed incubation period—varies with the quality of the medium and neighbouring colonies on the plate. Furthermore, some strains of *Bord. pertussis* have inherently a smaller colony type than others (Lautrop, unpublished data). It is important to know that colonies invisible to the naked eye at a certain time may 24 hours later have a diameter of about 1 mm (Kline, 1933; Lautrop, unpublished data).

Colonies of *Bord. parapertussis* are larger than those of *Bord. pertussis*; after 3-4 days' incubation their diameter is usually about one-and-a-half times as big (Lautrop, 1954), but on continued incubation this difference is reduced. In addition the surface of *Bord. parapertussis* colonies is as a rule duller and a slight tint of yellow or green may be present.

Colonies of *Bord. bronchiseptica* on primary plates are described as being indistinguishable from *Bord. pertussis* colonies (Chang, 1950). Subcultures of *Bord. bronchiseptica* strains on B-G medium sometimes reveal two colony types: one like a rather large *Bord. pertussis* colony, the other more flattened but with a raised centre. The latter colony-type contains predominantly motile cells, the former predominantly non-motile ones (Lautrop, unpublished data). In subculture rough variants of *Bord. bronchiseptica* are quite common (Lautrop, unpublished data).

Changes in the medium : haemolysis and pigmentation

All three species of *Bordetella* produce zones of haemolysis on B-G medium. The size of the zone around single colonies varies with the type of blood used and its concentration; furthermore, the pH of the medium (Lautrop, 1954) is of importance.

Some investigators stress the diagnostic value of a big haemolytic zone around the colony and for that reason prefer a medium with a rather low blood concentration; others find that haemolysis is not essential in identification and use a high concentration of blood in order not to impair the growth-promoting quality. The use of a B-G medium with 50% blood is a definite advantage in the preliminary differentiation of *Bord. pertussis* and *Bord. parapertussis* (Lautrop, 1954). On this medium after three days' incubation *Bord. parapertussis* usually shows a narrow zone of haemolysis around the colonies, whereas usually no zones are visible around *Bord. pertussis* colonies. The difference may be very striking and is then one of the best criteria in the preliminary differentiation, but since it is a difference of degree it may happen that at the time of inspecting the plates the zones have not yet developed around *Bord. parapertussis* colonies or are already present around *Bord. pertussis* colonies (Lautrop, 1954). Plates with 50% blood are relatively soft and colonies of *Bord. parapertussis* are often observed lying at the bottom of a small pit produced by the collapse of the haemolysed red corpuscles (Lautrop, 1954). Even if this is not in any way a specific characteristic of *Bord. parapertussis*—it may be produced by any haemolytic organism on a soft blood-medium—it sometimes facilitates their differentiation from *Bord. pertussis*, which do not produce this phenomenon until after further incubation.

Bord. parapertussis differs from the two other *Bordetella* species by producing—under certain conditions—a brown or black pigmentation of the medium (Bradford & Slavin, 1937; Eldering & Kendrick, 1938). The pigment is of the melanine type and it is supposed to be formed from tyrosine, which is present in the medium through the activity of the enzyme tyrosinase, produced by the bacteria (Ensminger, 1953).

Pigment formation requires the presence of oxygen (air) and an alkaline reaction (Lautrop, 1954). On a B-G plate a sufficiently alkaline reaction is obtained only when growth is confluent. In that case the medium acquires a greenish-black or greenish-brown colour in an ill-defined zone stretching somewhat beyond the growth area. Single colonies do not produce a sufficient change in pH to make pigmentation visible and any dark zone around a single colony of *Bord. parapertussis* on a B-G plate is due to haemolysis, which is favoured by a neutral and still more by an acid reaction (Lautrop, 1954). Therefore, the otherwise characteristic pigment of *Bord. parapertussis* is rarely of any diagnostic importance during the preliminary examination, but in subculture where confluent growth can be produced at will and other conditions made favourable for the appearance of pigmentation, it represents the most characteristic and most reliable single sign indicative of *Bord. parapertussis*.

Consistency of the colonies

The consistency is observed when colonies are picked for microscopic examination and transferred to a drop of water on the slide.

Usually, colonies of all three *Bordetella* species are butyrous or a little sticky, but the growth suspends easily and evenly in water. Sometimes, however, colonies are more brittle and when touched with the needle may be pushed in their entirety on the surface or break in larger pieces. Such colonies are of the white, flat type and a suspension in water is not quite homogeneous.

The ease with which suspension in water takes place is reflected in the general pattern of the microscopic picture (see below).

Gram-staining technique

The staining may be performed by any one of the usually recommended methods. For counter-staining the use of carbolfuchsin is recommended. Because of the rather slow uptake of the dyes used in counter-staining, this part of the procedure should be extended somewhat. Two minutes are recommended. All *Bordetella* species are Gram-negative.

Cell shape and size

Typically, *Bord. pertussis* has a coccoid cell-shape, the length and breadth being of almost equal size, about 0.5μ-1μ. Cells in the stage of division may look like diplococci or short rods. A high degree of uniformity as to cell shape and size is the predominating impression. Cultures may, however, show some degree of variation. The most frequent variation is the occurrence of a small number of long rods, with a uniform diameter which does not exceed the breadth of the normal cell-type or does so only to a very slight extent, and the occurrence of faintly stained, structureless spheres much larger than the typical elements. On aged or haemolysed media or under other sub-optimal growth conditions the cells tend to elongate and consequently show a more definite rod shape. In Copenhagen we have found that a certain variety of *Bord. pertussis*—representing 20-25% of the isolates—is on the primary plate almost constantly more rod-shaped than coccoid (Lautrop, unpublished data). This variety is further characterized by smaller colonies than usual on B-G medium, a higher degree of fastidiousness as to growth requirements than other *Bord. pertussis* organisms, lack of catalase, and a more pronounced tendency to auto-agglutination in water (Lautrop, unpublished data).

The definitely rod-shaped cells of *Bord. parapertussis*, about 1-2μ long, are morphologically clearly distinct from the coccoid cells of the majority of *Bord. pertussis* organisms. The rod-shaped variety of *Bord. pertussis* is usually a little shorter than a *Bord. parapertussis* cell, but the difference is too small to be relied on in differentiation. In this situation it is fortunate that the difference in colony size is even more pronounced than usual, because of the small colonies of the rod-shaped variety of *Bord. pertussis*.

In a Gram-stained preparation, *Bord. bronchiseptica* looks like a typical *Bord. pertussis*, with perhaps a tendency to be a little more elongated.

It should be pointed out that the above description applies to cultures on B-G media with horse blood. On media prepared with other types of blood the morphological differences are blurred, all types becoming so short that the differences although still present are much more difficult to recognize (Lautrop, 1954).

General microscopic pattern

In a microscopic field *Bord. pertussis* does not possess any single characteristic feature by which it can be recognized immediately; on the other hand, the field presents a constant pattern of distribution with great regularity which thereby becomes a valuable aid in diagnosis. The cells are arranged in rather loose clusters of variable size, and between the clusters a number of organisms are scattered in such a way that their mutual distance usually increases with increased distance from the cluster (" thumb-print " distribution).

Butyrous colonies show a more soft and homogeneous pattern than the brittle ones, which are more " rugged " in their microscopic appearance and have larger and denser clusters.

Bord. parapertussis organisms are distributed in a similar way but their rod shape allows for the occurrence—to a limited degree—of palisade arrangements, V-formed pairs and intersecting cells, all of which contribute to create a somewhat different pattern which facilitates the immediate microscopic differentiation between *pertussis* and *parapertussis* organisms.

The microscopic appearance of *Bord. bronchiseptica* is like that of *Bord. pertussis*.

DIAGNOSIS OF *BORD. PERTUSSIS* AND *BORD. PARAPERTUSSIS*

The first step in the diagnostic procedure is to is to select colonies for microscopic examination. The general attitude should be to pick any colony which, according to the experience of the investigator, cannot immediately and definitely be rejected as being not *Bordetella*. Growing experience will reduce the number of " false " colonies picked, but even an experienced investigator will quite often select colonies which on microscopic examination turn out to be something else.

The organisms whose colonies most frequently give rise to mistakes are *Haemophilus* spp., *Corynebacterium* spp., *Neisseria* spp., and the group of organisms known at present under a variety of names such as *Bacterium anitratum*, *Mima polymorpha*, *Herellea vaginicola*, or *Diplococcus mucosus*. A mistake is especially apt to occur if only a single small colony is present. Usually the mistake is easily cleared up by the microscopic examination, but in rare cases the differentiation between a *Bordetella* and a *Haemophilus* or a *Bacterium anitratum* may present difficulties even after microscopy has been performed. *Bacterium anitratum*, which usually has a quite characteristic morphology—rather large, oblong or almost spherical cells often interspersed with grotesquely shaped involution forms—may

occur as a short slender rod looking very much like *Bord. parapertussis*. Typically *H. influenzae* and *H. parainfluenzae* present a pleomorphic picture, showing besides the small coccoids, which look exactly like *Bord. pertussis*, a varying number of long rods, filaments and swollen cells of irregular shape which are never seen in *Bord. pertussis*. Therefore gross mistakes are not very likely to occur. But if a colony looking like a *Bordetella* is picked from a crowded area on a plate and microscopic examination reveals besides typical coccoids a limited number of elements characteristic of *H. influenzae*, then one has to be careful and make sure if this could not be a mixture of cells from a *Bordetella* colony and an *H. influenzae* colony growing very close together on the plate.

No attempt will be made to mention all the other different organisms which may be mistaken macroscopically on a B-G plate for a *Bordetella*. Microscopic examination usually suffices to show that they are not so, but some non-fermenting Gram-negative rods—in most cases probably belonging to *Pseudomonas* or to the ill-defined genera *Alcaligenes* and *Achromobacter*—should be mentioned because their cells, although usually too big to be considered as *Bordetella*, may occur in a shape and size which make it difficult to distinguish them from the more rod-shaped varieties of *Bordetella* and consequently call for further examination.

By the end of the preliminary examination the task is reduced to the question whether a demonstrated Gram-negative small coccoid or rod-shaped bacterium is a *Bordetella* or not, and to a decision as to what type of *Bordetella* it may be. (The term "*Bordetella*-looking organism" is used from now on to describe those bacteria which, on the basis of the preliminary examination, are selected for further consideration as *Bordetella* whether this diagnosis be eventually confirmed or not.)

Theoretically at least, even this limited task requires the regular use of a number of additional tests. However, experience has shown that the very nature of the source material limits the diagnostic problem somewhat and if—in routine diagnosis—the recognition of the very rarely occurring *Bord. bronchiseptica* is disregarded, the situation is very much simplified; so much indeed that an experienced investigator will be in a position to make a diagnosis of *Bord. pertussis* and *Bord. parapertussis* in the majority of all cases without further examination.

The decision of the experienced investigator is based partly on the previously described characteristics of the organisms and partly on very subtle traits in colony appearance and morphology, learned through trial and error, and almost impossible to describe.

Usually the results of the preliminary examination may be grouped in four categories:

(1) almost certainly *Bord. pertussis* ;

(2) almost certainly *Bord. parapertussis* ;

(3) almost certainly *Bordetella*, but differentiation as to type uncertain;

(4) probably *Bordetella*, but not quite typical.

For each category the following further procedure is recommended:

(1) no further examination necessary;

(2) a subculture on tyrosine-agar recommended;

(3) } subcultures on tyrosine-agar and B-G

(4) } medium obligatory.

Some laboratories prefer to confirm the diagnosis even of culturally typical *Bord. pertussis* by a serological test. This seems unnecessary as a routine procedure in laboratories with much experience, but must be strongly recommended in laboratories without previous experience. It should be remembered, however, that a slide agglutination test with a culture from the primary B-G plate is not regularly feasible because too many strains turn out to be auto-agglutinable, and to produce a subculture from which an antigen for tube agglutination can be prepared means a delay of the report for at least two days.

Use of the oxidase test as described by Kovacs (1956) is sometimes helpful in the differentiation between *Bord. pertussis* and *Bord. parapertussis* on the primary plate (Lautrop, unpublished data). The reagent (tetramethylparaphenylenediamine dihydrochloride) is sucked on to filter-paper and the colonies streaked on to the impregnated paper. *Bord. pertussis* gives a positive reaction, i.e., a blue colour within 15 seconds; *Bord. parapertussis* gives a negative reaction. Difficulties encountered in this procedure are due to the small size of the colonies and the false positive reaction which may result from the blood in the medium. *Bord. bronchiseptica* (and *Alcaligenes faecalis*) give a positive reaction.

A subculture on tyrosine-agar is one of the means by which a final differentiation between *Bord. pertussis* and *Bord. parapertussis* may be obtained. It is a very simple test to perform and has the

advantage that the combined occurrence of growth and typical pigment formation on this medium is practically proof that *Bord. parapertussis* is present. No growth on the tyrosine-agar subculture suggests that the organism in question is *Bord. pertussis*, but the result is of course not conclusive. Therefore a subculture on B-G medium should be included whenever *Bord. pertussis* is a reasonable possibility. Often *Bord. pertussis* looks atypical on the primary plate because the colonies are influenced by surrounding " contaminants ". In pure culture they regain their typical appearance and make possible a definite diagnosis which was not possible previously. If the diagnosis is still doubtful a serological test is resorted to, and fortunately the tendency to auto-agglutination is much less in a dense subculture.

If for some reason (for example, lack of experience or inadequate media) the immediate differentiation of *Bord. pertussis* and *Bord. parapertussis* gives rise to uncertainty, almost all results will be in category (3). In this situation a routinely performed subculture on tyrosine-agar of all *Bordetella*-looking organisms is recommended until differentiation on the basis of the preliminary examination has been learned. A subculture on B-G medium would serve the same purpose, but the results would be further delayed and the cost higher. To use both media in these circumstances seems unnecessary.

DIAGNOSIS OF *BORD. BRONCHISEPTICA*

To disregard the diagnosis of *Bord. bronchiseptica* completely—as was done in the previous section—is of course a questionable procedure, particularly as the postulated infrequency of this organism as a cause of whooping-cough may alone be a reflection of the fact that further tests, which are not performed routinely in most places, are required after the preliminary examination to distinguish *Bord. bronchiseptica* from *Bord. pertussis*. This section on the diagnosis of *Bord. bronchiseptica* is therefore included for the convenience of laboratories which decide to make a search for this organism part of their regular routine.

From the information in the table on page 16 it emerges that the preliminary examination does not allow of a differentiation between *Bord. pertussis* and *Bord. bronchiseptica*. It also emerges that a simple screening test for the latter organism is to make a subculture on a plain agar medium, which will support the growth of *Bord. bronchiseptica*, but not of *Bord. pertussis*. Any meat extract or nutritive agar will do, but since the tyrosine-agar is already included among the diagnostic requisites it is recommended that this medium be used throughout and the confirmative test for *Bord. parapertussis* thus combined with the screening test for *Bord. bronchiseptica*. If all *Bordetella*-looking organisms are subcultured on tyrosine-agar, the search for *Bord. bronchiseptica* may be limited to such cultures as grow on the tyrosine-agar but do not produce the brown pigmentation.

The following tests must be performed on all such cultures to prove or disprove a diagnosis of *Bord. bronchiseptica* :

(1) a test for motility;

(2) a test for acid production from glucose;

(3) a test for nitrate reduction; and

(4) a test for urea splitting.

Bord. bronchiseptica is motile by peritrichous flagella, but it seems that completely immotile variants occur (Lacey, 1953; Lautrop, unpublished data), and that in some strains or under some conditions the number of motile cells may be so small that motility is difficult to demonstrate (Lacey, 1955; Lautrop, unpublished data). Growth in semi-solid agar at room temperature or at 30°C is recommended to make certain whether a strain is motile or not. Acid is not produced from glucose by *Bord. bronchiseptica*. The majority of strains examined reduce nitrate to nitrite, but exceptions occur. Splitting of urea has been found a very constant property in all strains. A few other characteristics of *Bord. bronchiseptica* cultures should be mentioned. All strains contain a catalase and an oxidase, and they do not produce indole or H_2S. No growth occurs in a vitamin-free synthetic medium, but follows on the addition of nicotinic acid.

The tests for acid production from glucose and motility will exclude a variety of organisms and limit considerations to Gram-negative, motile rods which grow well on a simple agar medium at 37°C and at a lower temperature, and do not form acids from glucose, but even so the task is by no means a simple one.

More or less well-known organisms which must be differentiated from *Bord. bronchiseptica* are the peritrichously flagellated *Alcaligenes faecalis*, other *Achromobacter* species, and a number of polarly flagellated, non-pigmented organisms such as *Lophomonas alcaligenes* (Galarneault & Leifson, 1956), *Pseudomonas diminuta* (Leifson & Hugh, 1954), and

others. Members of the last-mentioned group, which according to the author's experience are quite often found on whooping-cough plates, do not present difficulties if a flagella stain is performed. Peritrichously flagellated organisms which deserve the specific name "*Alcaligenes faecalis* (Petruschky) Bergey et al." seem to be rare. The most constant difference between this organism and *Bord. bronchiseptica* is probably that the last-mentioned contains urease and cannot grow without nicotinic acid in a synthetic medium. However, it is difficult to tell how constant and reliable this difference is because *Alcaligenes faecalis* is at present not a clearly delineated group of organisms (Nyberg, 1935; Thibault et al., 1955; Türck, 1952; Ulrich & Needham, 1953).

The occurrence in the agar subculture of other organisms more or less similar to *Bord. bronchiseptica* but not easily identifiable with known species may be anticipated. A direct comparison of the unknown strain and a typical *Bord. bronchiseptica* strain on various media and by various tests is the recommended procedure in such cases.

From the preceding it will be realized that often no more than a tentative dignosis of *Bord. bronchiseptica* is possible by cultural and biochemical means, and therefore it is recommended that no definite diagnosis be made without serological confirmation.

DETAILS OF CULTURAL AND BIOCHEMICAL TESTS

Subculture on tyrosine-agar (Lautrop, unpublished data)

The medium is a meat extract or meat infusion agar with 0.1% tyrosine. To avoid precipitation of tyrosine in the final medium a solution of tyrosine is made in a small volume of water acidified with a little HCl. After sterilization for 10 minutes by steaming, the tyrosine solution is mixed with the melted agar base and the pH is adjusted to about 7.5. The final alkaline reaction is important to obtain vigorous pigmentation. A light-coloured agar base is preferable to facilitate early recognition of the pigmentation, and for the same reason plates should be poured in a thin layer. If only a few subcultures are expected slants may be more economical.

Inoculation is made with one whole colony on a small area of the medium, to obtain heavy confluent growth. If performed in this way, it can be relied on that the subculture will show at least a trace of pigmentation after overnight incubation; on continued incubation the slight brownish tint of the medium develops into a very conspiciuous dark-brown colour, which spreads beyond the inoculated area. As many as 20-30 culture spots may be accommodated on one plate. As the pigment is not produced under anaerobic conditions, care must be taken to ascertain that the lid and the bottom part of the Petri dish do not adhere to each other. To give early evidence of pigmentation, slants should be inoculated in the upper end, where the layer of medium is thin.

Test for acid production from glucose

For the demonstration of a fermentative breakdown of glucose, any of the conventional methods might do, but to make sure that an oxidative breakdown also is recognized, it is recommended that the medium of Hugh & Leifson (1953), which is especially made for this purpose and contains a relatively high carbohydrate concentration and a low peptone concentration, be used. The composition is as follows: peptone, 0.2%; NaCl, 0.5%; K_2HPO_4, 0.03%; agar, 0.3%; bromothymol blue, 0.003%; glucose, 1.0%; pH 7.1. The acid reaction produced by an oxidative organism is apparent first at the surface and extends gradually downwards into the medium. Where the oxidation is weak or slow, it is usual to observe an initial alkaline reaction. This may persist for a variable length of time, up to several days of incubation, before turning acid and must not be mistaken for a negative reaction. The acid reaction of fermentative organisms in this medium starts from the bottom. Inoculation is made by stabbing.

Test for motility

Hugh & Leifson's semi-solid glucose-agar referred to above also serves as a medium for the demonstration of motility. Motile organisms growing below the surface can be seen to swarm out in the medium away from the stab, while non-motile organisms will remain along the stab. Strictly aerobic organisms growing only on the surface should be examined by direct microscopic observation of the culture.

Test for nitrate reduction

The following medium is distributed in a layer 5 cm high in test-tubes: peptone, 0.5%; KNO_3, 0.1%; distilled water; pH 7.2-7.4. From the tyrosine-agar enough culture is suspended in the medium to produce visible turbidity. After incubation at 37°C overnight about 1 ml is transferred to an

empty tube and tested for the presence of nitrite. The test reagent is a fresh mixture of equal parts of a 0.8% solution of sulfanilic acid in 5N acetic acid and a 0.5% solution of α-naphthylamine in 5N acetic acid. If nitrite has been formed the addition of a few drops of the reagent will almost immediately produce a red colour (a positive test for nitrate reduction). If a red colour does not appear it is not evidence that nitrate reduction has not taken place; three possibilities can occur: (1) poor growth or slow formation of the adaptive enzyme may be the reason why nitrite is not yet present in demonstrable amounts; (2) nitrite has been formed, but a further breakdown has already taken place; (3) the organism in question does not possess the ability to reduce nitrate under the conditions of the test (a true negative test). To distinguish between these possibilities a small amount of zinc dust free from nitrate and nitrite is added; if a red colour then develops the presence of non-reduced nitrate is indicated, and such tubes should be incubated further and tested again. No colour indicates that no nitrate is left in the tube and consequently a reduction must have taken place in this case also.

Test for urea splitting

A modification of the method of Ferguson & Hook (1943) is recommended. The following solution is used: KH_2PO_4, 0.1%; K_2HPO_4, 0.1%; NaCl, 0.5%; urea, 1.0%; phenol red, 1 : 100 000; pH 7.0. The base is sterilized by autoclaving. A 20% urea solution is sterilized by Seitz filtering and the necessary amount is added to the base to give a final concentration of 1.0%. The same base without the addition of urea is used as a control. Both solutions are kept in the ice-box and, according to need, 0.5-ml amounts of each are pipetted off in small narrow test-tubes. From the tyrosine-agar or any other subculture heavy suspensions are made in tubes with the urea solution and the control solution. The tubes are left at 37°C and read after two hours and again on the following day. Splitting of urea with the formation of NH_3 is followed by a shift of the reaction to the alkaline side, and consequently in a change of the indicator from yellow or orange to definitely rose. The control tube should retain its original colour so that a clear-cut difference between urea tube and control tube is present. In most cases a positive reaction is manifest after two hours. (Late and weak reactions may indicate that the culture is a mixture of a non-urea-splitting organism and a urea-splitting one, and appropriate steps must then be taken to separate the two organisms.) The outlined technique, in which a resting cell suspension is used, has the advantage that it can also be applied to *Bord. pertussis*, which does not grow on the media recommended for other types of urease tests.

DETAILS OF SEROLOGICAL TESTS

General remarks about the serology of the genus Bordetella

Several studies on the serology of the genus *Bordetella* have been made (Andersen, 1952, 1953; Bradford & Slavin, 1937; Eldering, 1942; Eldering & Kendrick, 1938; Eldering et al., 1956; Kendrick et al., 1953; Nobechi, 1955; Pacagnella, 1952), but a generally accepted, detailed antigenic scheme does not exist. However, enough knowledge has accumulated to make a serological test a very valuable means of confirming a diagnosis of *Bordetella* and of differentiating between the three *Bordetella* species.

There are at least three different kinds of antigens in the genus *Bordetella*: H antigens or flagellar antigens, which are, however, limited to *Bord. bronchiseptica* strains; K antigens, which are relatively heat-labile, surface antigens; and O antigens, which are heat-stable. All *Bordetella* strains seem to contain an identical O antigen. For practical diagnostic purposes interest may at present be limited to the K antigens.

The K antigen of a particular strain seems to be composed of a number of K-partial antigens of which one dominates and may be considered the major K antigen of the strain. The serological identification and species-differentiation of strains is based on the fact that the major K antigens are identical in all strains within each species and that each of the three species has a different major K antigen. The minor K antigens in *Bordetella* strains are not finally mapped out as to number and relationships, but it is definitely known that one is common to all strains and causes cross-agglutination between the species in unabsorbed sera; others are species specific or common to only two species. Among *Bord. pertussis* and *Bord. bronchiseptica* strains the minor K antigens occur in different combinations, and this has provided the basis for a serological subdivision of such strains. In contrast, all *Bord. parapertussis* strains seem to be identical with respect to all their K antigens.

To make a serological diagnosis of an unknown strain suspected of being a *Bordetella*, three different K sera are required—i.e., one serum for each species prepared in such a way that it contains antibodies against the homologous major K antigen to a high titre. The preparation of such sera usually does not present much difficulty. It should, however, be realized that antigenic variations induced by growth conditions occur (Lacey, 1951, 1955) and therefore strict adherence to the standardized conditions outlined below for the preparation of sera and antigens is essential. Also, rough variants (Leslie & Gardner, 1931) must be avoided in serological work. Cross-agglutination owing to common minor K factors is eliminated either by absorption or—more easily—by appropriate dilution of the sera. If Lacey's medium is used a special serum (polymodal serum) is necessary (see Annex 1).

Production of diagnostic sera

(*a*) *Vaccine.* A typical and smooth strain of each of the *Bordetella* species is grown on B-G medium at 37°C for two or three days. The growth is scraped off and suspended homogeneously in saline. Filtration through cotton may be useful to remove agar particles. The suspension is diluted to contain about 10×10^9 organisms per ml by comparison with some kind of standard—for example, the International Opacity Reference Preparation. Formol is added to a final concentration of 0.15% or ethylmercurithiosalicylate (thiomersal) in a dilution of 1 : 10 000, and the vaccine is left at room temperature for a few days before it is used.

(*b*) *Animals.* Natural infection with *Bord. bronchiseptica* is common among some stocks of rabbits. Thus a preliminary serological examination is necessary to exclude animals with pre-formed antibodies.

(*c*) *Injections.* Four injections (0.25 ml, 0.50 ml, 1.0 ml, 1.0 ml) are given intravenously at weekly intervals and one week after the last injection a trial bleeding is made. A K titre of about 10 000 is satisfactory and if not attained after the first four injections further injections of 1 ml at weekly intervals should be given until this titre has been reached. If after two months the titre is still unsatisfactory, it is not likely that continued immunization will raise the titre further and the injections are discontinued. It is recommended that two or three rabbits be used for each vaccine and the sera mixed. Sera are stored in the ice-box at + 4°C after addition of thiomersal (1 : 10 000) or in the frozen state.

Agglutination tests

(*a*) *Tube agglutination.* From a subculture on B-G medium of the strain to be examined an antigen suspension is prepared in exactly the same way as the vaccine. Serum dilutions of the three diagnostic sera in 0.2-ml amounts are mixed with 0.3 ml of antigens in small test-tubes. Equal amounts of serum dilution and antigen suspension—e.g., 0.5 ml of each—are used in some laboratories. The first dilution should be well above the titre for cross-agglutination between the species, and the last dilution at least one step higher than the titre with the vaccine strain. A rack with the tubes is placed in a shaking-machine, preferably a rotating disc tilted at an angle of 45° to the horizontal (modified from Miller & Silverberg, 1939). After one hour's shaking at room temperature the tubes are read. The agglutination is of a finely granular type and the end-point is usually very clear-cut. As antigen control a tube without serum is always included and at intervals the sera must be controlled by the inclusion of antigens prepared from known strains. Incubation of the tubes in a water-bath is less satisfactory, because after short incubation the titre is too low and after long incubation agglutination is not as specific. However, incubation in a water-bath is the most widely used method. Agglutination to about the titre given by the vaccine strain in one of the sera, without any reaction in the two others, is the usual type of result and establishes the diagnosis definitely. Not infrequently *Bord. pertussis* strains are encountered which react to a considerably lower titre than expected, but also a low specific titre confirms the diagnosis.

(*b*) *Slide agglutination.* Performed with colonies from the primary B-G plate this technique (Kendrick, 1933) has only limited value because many strains turn out to be auto-agglutinable under the conditions of the test. But in cases where the suspension in a drop of saline on a slide is stable the procedure represents a time-saving short-cut.

The sera should be used in a dilution in which the two other species do not cross-agglutinate on a slide. A serum from a non-immunized rabbit without pre-formed *Bordetella* antibodies should be included as a control.

Specific agglutination occurs almost immediately, and only such immediate reactions should be considered positive.

Demonstration of antibodies in patients' sera

During infection with *Bord. pertussis* specific humoral antibodies are usually formed. In most cases they begin to appear between the third and the fourth week and reach a maximum about the seventh to the eighth week. The decline is often slow and protracted. It would appear that during this stage of decline non-specific stimuli—e.g., other infections—can produce a new rise in antibody titre.

The demonstration of specific antibodies may be of diagnostic value. However, their late occurrence limits the usefulness of the serological tests to cases which are already in an advanced stage; and their long standing and the possibility of a secondary, non-specific rise in titre makes it difficult to evaluate a positive finding in an individual case.

There are only a few reports dealing with the diagnostic importance of the demonstration of antibodies and no general agreement concerning the usefulness of the procedure. If the bacteriological service is adequate it will rarely be necessary to resort to the demonstration of antibodies, but now and then situations occur which make it necessary for the laboratory to be able to perform a serological examination of the patient's serum.

Antibodies may be demonstrated by agglutination (Donald, 1938; Evans & Maitland, 1939b), by complement fixation (Donald, 1938; Gundel & Schlüter, 1933-34), by precipitation (Aufdermauer & Grumbach, 1944), and by the opsonocytophage test (Kendrick et al., 1937). It seems that agglutination (especially slide agglutination) is a more sensitive test than complement fixation, but the latter has, according to the literature, been used more widely. Until a few years ago it was believed that all *Bord. pertussis* strains were serologically alike, but in 1953 Andersen demonstrated that serological subtypes exist and this was confirmed by Eldering, Hornbeck & Baker in 1957. This fact has not been considered in relation to the demonstration of antibodies in the sera from patients, and yet it seems likely from a comparison with animal experiments that a demonstration may now and then fail if antigen and infecting strain are not of the same serological subtype.

The tube agglutination test described in a previous section can be used. The lowest serum dilution should be 1 : 10 or 1 : 20. According to Donald (1938) most positive sera do not have a titre higher than 1/180. A slightly different technique is advocated by Evans & Maitland (1939b). A rise in antibody titre during the course of infection is considered to be of higher diagnostic value than just a raised antibody level.

Experience with the complement-fixation test is described by Gundel & Schlüter (1933-34) and by Donald (1938). A detailed description of this technique is out of place here since the general principle is the same as in all other complement-fixation tests and the same rigid control system is necessary to obtain reliable results. The antigen Donald used was a suspension of live organisms harvested from a B-G medium, and he employed a concentration corresponding to 2×10^9 organisms per ml. Preliminary testing with selected positive sera is required to find the optimal antigen concentration for a sensitive test (see, for instance, Boyd (1956). It seems worth while considering whether the use of a mixed antigen containing representatives of all the serological subtypes would not represent an improvement. The same applies to the antigen used in the agglutination test.

REPORTING OF RESULTS

A report saying that *Bord. pertussis* or *Bord. parapertussis* has been found or not found can in the majority of cases be made immediately after the preliminary examination if the diagnosis of *Bord. bronchiseptica* is not considered. If further incubation of the primary plate is considered necessary, no report is made until the end of the observation period, which may vary according to local circumstances. If further examination is required to distinguish *Bord. pertussis* and *Bord. parapertussis* a report is made to the effect that *Bordetella* or whooping-cough bacteria have been found and that the result of species determination will be forwarded later. If no definite diagnosis can be made after the preliminary examination, no report is made until a decision has been reached on the basis of further examination. Laboratories which include examination for *Bord. bronchiseptica* in their routine may have to substitute a report of *Bord. bronchiseptica* for their previous report of *Bord. pertussis* if this diagnosis is established by the further examination of the strain.

Sterile plates, plates overgrown with moulds or bacteria and streaked plates only exhibiting areas of very crowded growth should not be reported as " no *Bordetella* found ", but should be reported as " unsatisfactory " and the reason given. In some places it might be convenient to post immediately a new set of plates to obtain another specimen.

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Annex 1

CULTURAL DIAGNOSIS OF WHOOPING COUGH *

Principles

Soon after its introduction in 1916, Chievitz & Meyer's cough plate method superseded Bordet's original method of sputum culture. But the use of post-nasal swabs (Maclean, 1937; Cruickshank, 1944), per-nasal swabs (Bradford & Slavin, 1940) and supralaryngeal swabs (Bogdan, 1951) has now superseded the cough plate method. With swabs and an adequately selective medium the organisms of whooping cough may be isolated as easily from symptomless contacts (usually those incubating the disease) or from persons with mild second attacks or atypical attacks after vaccination, as from persons with the typical disease. Organisms can often be isolated from the pharynx or larynx when not detectable in the nose or nasopharynx and vice versa. A combination of pharyngeal and per-nasal swabs is therefore desirable. Although both Bordet & Gengou's medium with penicillin and Nicholson & Turner's (1954) medium give fair results with per-nasal swabs, a more selective medium yields more positives with per-nasal swabs

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and is necessary for adequate culture of swabs of the pharynx or larynx, sputum or vomit.

All three species of *Bordetella* which cause pertussis, or pertussis-like disease (*pertussis* approx. 95%: *parapertussis* approx. 5%: *bronchiseptica* approx. 0.1%) die within a few hours on dry plain or serum swabs. A suitable carrier medium however will maintain full viability for up to 72 hours at room temperature. With a combination of pharyngeal and per-nasal swabs, and the use of carrier medium, a negative result on the diamidine-penicillin-fluoride (DPF) medium (Lacey, 1954), described below, presumptively excludes the presence of *Bordetella*.

The antigenic structure of all three species varies with conditions of growth (Lacey, 1953). *B. pertussis* has three potential antigenic forms (X, I and C modes) each with a characteristic surface antigen. The three modes are easily distinguished by their agglutinability on a slide. On Bordet & Gengou's medium the mode of growth of *B. pertussis* between 35 and 37°C is almost always the same (X mode) whereas on DPF medium any mode may appear according to the exact temperature of incubation and composition of the particular plate. For this reason a serum with antibodies to all three modes (polymodal serum) is essential for recognition of *B. pertussis* on primary DPF cultures. About one in five rabbits produces useful amounts of antibody to all three modes when inoculated with growth from DPF medium.

Several serotypes of *B. pertussis* can be recognized in X mode (see Andersen, 1953). Andersen types "2, 3, 4" and "2, 4" are the most common in England. Diagnostic sera specific for *B. pertussis* in X mode should have antibodies to factors 2, 3 and 4. All serotypes are indistinguishable in I and C modes.

On DPF medium at 35°C *B. parapertussis* grows in X mode, *B. bronchiseptica* in C mode. On Bordet & Gengou's medium at 35°C both grow in X mode. *B. bronchiseptica* is motile in C mode, but non-motile and non-flagellated in X mode. No mode of *B. parapertussis* or *B. bronchiseptica* overlaps antigenically with the I and C modes of *B. pertussis* but all three species overlap in X mode.

Preparation of DPF medium

(NOTE: The complete medium, with the exception of the penicillin solution and horse blood, is obtainable from Southern Group Laboratory, Hither Green Hospital, London S.E.13.)

(*a*) *Containers.* Use stainless steel, hard glass or glass *not previously used for holding tellurite solutions.* Clean stainless steel with soap and water only and glass with the help of only soda and hydrochloric acid.

(*b*) *Base*

New Zealand Agar (Davis Gelatine Co.) (or 1.3 times amount of any agar used in 5% blood agar)	about	14.5 g
Potato starch (British Drug Houses) (B.D.H.)		15.0 g
Glycerol AR (B.D.H.)		5.0 ml
DL-alpha alanine (Hopkin and Williams) (H. & W.)		1.0 g
L-glutamic acid (H. & W.)		3.7 g
Sodium fluoride puriss (B.D.H.)		0.5 g
Sodium chloride AR (H. & W.)		0.5 g
Potassium chloride AR (H. & W.)		3.3 g
Tap water	to	1070.0 ml

Add the agar and starch to about 600 ml tap water and dissolve in the steamer shaking occasionally. Add all the other ingredients to about 100 ml tap water, dissolve with heat and neutralize (to orange with phenol red) with 10% potassium hydroxide AR (about 17 ml). Add this to the agar and starch solution. Make up 1070 ml and adjust to pH 7.2. Distribute in 100 ml amounts in 200 ml screw-capped bottles and autoclave at 110°C for 10 min. Store at room temperature. Discard after one year.

(*c*) *Cysteine-magnesium salt mixture*

Magnesium lactate (H. & W.)		7.6 g
Fumaric acid (H. & W.)		2.0 g
Malonic acid (H. & W.)		2.4 g
L- or DL-cysteine hydrochloride (H. & W.)		2.0 g
Magnesium hydroxide 8.5% suspension (Phillips' Magnesia Co.)	qs:	about 44.0 ml
Distilled water	to	170.0 ml

Add the solids to 90 ml water in a Pyrex flask and bring to the boil to dissolve. Cool to about 80°C and add the magnesium hydroxide suspension with shaking until a slight excess persists for at least 3 min. Make up to 170 ml and filter through Whatman's No. 1 paper in a glass funnel. Bring to the boil, simmer for 3 min. and transfer to a sterile screw-capped bottle which will admit a 10 ml pipette. Store at 5°C without further sterilization. Discard after six months.

(*d*) *1% May & Baker compound No. 938 (M. & B. 938).* Weigh out 100 mg of 4:4′-diamidino diphenylamine dihydrochloride $2H_2O$ on a sterile watch glass or filter paper and add to 10 ml of sterile distilled water in a 30 ml screw-capped bottle. Store at 5°C. The solution can be used for up to six weeks provided no deposit has formed. Dilute one in ten before adding to the rest of the medium.

(*e*) *Penicillin solution.* Prepare a solution containing 50 units/ml in sterile 0.25% trisodium hydrogen citrate

AR ($Na_3C_6H_5O_7.2H_2O$) (B.D.H.) from crystalline sodium or potassium benzyl penicillinate (e.g. Buffered Penicillin G. of B.D.H.). Keep at 5°C and use for up to 14 days.

Preparation of plates and carrier tubes

Melt 100 ml of DPF base in the steamer and cool to 45°C, preferably in a water bath for 45 min. Add in the following order, mixing after each addition:

8.0 ml cysteine-magnesium salt mixture
1.25 ml 0.1% M. & B. 938
0.75 ml 50 unit/ml penicillin
60.0 ml defibrinated horse blood: less than 7 days old, well mixed, and at room temperature

Use the whole amount to pour four or five 9 or 9.5 cm plates, preferably in stainless steel dishes. (Stainless steel dishes of improved design are available from Taw Manufacturing Co., Campsbourne Works, The Campsbourne, High Street, Hornsey, London N.8.) Leave the plates undisturbed overnight: store undried at 5°C for up to 10 days and dry for a minimum time before use.

For carrier tubes: distribute the 170 ml of complete medium in 3 ml amounts (approx. 1¼ ") in 4 × ½ " tubes plugged with cotton wool. Allow to set while standing vertically, store at 5°C and use up to six weeks old (or at room temperature for up to three weeks).

Preparation of swabs

Remove the dressing from 3 denier nylon staple fibre (British Nylon Spinners, 25 Upper Brook Street, London W.1) by rinsing well in five volumes of a 50 : 50 mixture of ether and distilled water. Remove excess ether and water by gentle squezing and allow to dry protected from dust.

For per-nasal swabs: form a smooth head 2.5 mm in diameter on the roughened end of a 15 cm length of 21 SWG (0.81 mm diameter) copper or nichrome wire. Curve the wire slightly along its whole length. For pharyngeal swabs: form a similar head 4 mm in diameter on 18 SWG (1.22 mm diameter) wire and bend the wire to an angle of about 135° immediately behind the head. Insert the swabs with cotton wool plugs into 4 × ½" tubes and sterilize in the autoclave.

Swabs made of ether-washed cotton wool and sterilized with hot air are probably only slightly less efficient.

Collection of swabs and use of carrier medium

Insert a per-nasal swab, with convexity upwards, into one nostril and push it gently backwards as far as possible. With this swab still in position, swab the pharynx or supralaryngeal region through the mouth with a pharyngeal swab, using a tongue depressor if necessary. Remove the per-nasal swab last. Plunge each swab to the bottom of a column of carrier medium if more than three hours must elapse between taking and examination. A cardboard box holding a pair of swabs and two carrier tubes, and ready to post, is a help to general practitioners. Suitable boxes 7½" × 2½" × 1½" overall are obtainable from Hospital and Laboratory Supplies, 12 Charterhouse Square, London E.C.1.

Culture

Inoculate a quadrant of DPF medium directly with any specimen, whether received in carrier medium or not. Streak a second quadrant from this inoculum if the swabs are from patients receiving chemotherapy, or if the specimen is sputum or vomit. Keep the plates at 35°C, in a humid atmosphere, in a water-jacketed incubator, for five days. Raise the humidity with a tray of dilute copper sulphate solution if less than half the incubator space is filled with plates. (Many anhydric incubators are not adequately thermostatic and incubator rooms are almost always too drying. A temperature of 35°C is suitable for all routine diagnostic bacteriology.)

Replace swabs received in carrier medium in their carrier tubes and keep on the bench. Inoculate, with secondary and tertiary streaking, a half or whole plate of fresh DPF medium with any such swab yielding many contaminants during the first forty hours incubation.

Examination of plates

Inspect the DPF plates daily and examine any visible growth with a plate microscope. Confluent or separate colonies of *Bordetella* species have a convex mirror-like surface which reflects a brilliant image of an electric-light bulb filament. Colonies of enterococci, fungi or species of *Aerobacter*, *Proteus* or *Pseudomonas* appear in about 12% of post-nasal swab cultures, but they are seldom confluent, tend to appear earlier and have a coarser surface. Growth of *B. pertussis* is often visible at 48 hours as a confluent mass or profusion of tiny translucent colonies. An immediate conclusive diagnosis is then usually possible by agglutination on the slide. Separate colonies of *B. pertussis* are usually agglutinable by 72 hours and almost always by 96 hours. On DPF medium *B. parapertussis* grows more slowly than *B. pertussis* and very rarely

as a confluent mass; but separate colonies are generally visible by 96 hours and agglutinable the following day. This organism can be isolated within 48 hours, and with slightly more certainty, by using 15% blood digest agar containing a final concentration of 5 μg of streptomycin and 1 unit of penicillin/ml (Lacey, 1954).

Identification

A presumptive diagnosis of the species is usually possible from the appearances of the primary DPF colonies, and as a rule a final diagnosis can be made in a few minutes, with the help of appropriate sera, according to the following table:

CHARACTERS OF *BORDETELLA* SPECIES GROWN ON DPF MEDIUM AT 35°C

	B. pertussis	*B. parapertussis*	*B. bronchiseptica*
Growth time (hours)	48-96	72-120	36-48
Colonies: diameters (mm)	1.0-2.5	0.5-1.0	2.0-4.0
consistency	Butyrous	Viscid	Butyrous
oxidase reaction (*a*)	Pos. (30 secs.)	Neg. (5 min.)	Pos. (30 secs).
Motility	—	—	+
Agglutination on slide by:			
polymodal pertussis serum (*b*)	+	+ or −	−
specific pertussis (X mode) serum (*b*) (*c*) (*d*)	+ or −	—	−
specific parapertussis (X mode) serum (*b*) (*c*)	−	+	−
polyvalent and polymodal bronchiseptica serum (*b*)	+ or −	+	+

Notes

(*a*) Aqueous 1% NN′-tetramethyl-*p*-phenylenediamine hydrochloride (H. & W.) prepared monthly and kept at 5°C. One loopful applied to growth and observed for 2 minutes.

(*b*) Obtainable from Department of Bacteriology, Westminster Medical School, London S.W.1.

(*c*) Obtainable from Standards Laboratory, Central Public Health Laboratory, Colindale, London N.W.9.

(*d*) Unless otherwise stated, sera may lack antibodies to Andersen's factor " 3 " and hence agglutinate serotype " 3 " poorly or not at all.

On rare occasions the diagnosis cannot be established from the primary culture, either because suspensions of colonies agglutinate spontaneously, or because growth of contaminants restricts the growth of single colonies. In either case the suspect colonies should be sub-cultured, with a straight wire and secondary streaking, on to both 5% blood digest agar and fresh DPF medium. Small Gram-negative coccobacilli which are spontaneously agglutinable from DPF medium sometimes prove to be *B. parapertussis* but are usually unidentifiable.

Methods of slide agglutination

Shake a portion of confluent growth, or the whole of one or more colonies, in three drops of physiological saline in a small tube until the suspension is homogeneous. With a Pasteur pipette, add one drop of suspension to a 2 mm loopful of serum on one platform of a Murray slide (Murray, 1944) and place a second, as a control, on an adjacent platform. Rock the slide continuously and observe with oblique illumination. With polymodal pertussis serum any mode of *B. pertussis* (i.e. any growth from DPF medium) is obviously and completely agglutinated within three minutes. It is very seldom necessary to test *B. pertussis* with any other serum. Most batches of polymodal serum also agglutinate *B. parapertussis*, but usually more slowly.

Difficulties

Although DPF medium is easier to make and use than Bordet & Gengou's medium with added penicillin, the following difficulties have been met with on occasions:

(a) Loss of selectivity, with growth of streptococci and diphtheroids, from the use of inactive penicillin solution.

(b) Poor growth of *B. pertussis*, with rapid darkening of blood over the whole plate, from incomplete neutralization of the cysteine-magnesium salt mixture, insufficient cooling of the base before adding blood, or the use of old, contaminated, or icteric blood.

(c) Absence of growth of *B. pertussis* in a zone (usually at the edge of a plate) from which disinfectant, detergent or tellurite has not been removed.

(d) Overgrowth by *Aerobacter* or *Proteus* species in culture of swabs taken

(i) during or shortly after chemotherapy, or

(ii) from contaminated carrier medium.

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This Broadsheet has been prepared by the Author at the invitation of the Committee on Technical Methods. The methods have been tested on behalf of the Association and found to be satisfactory.

Annex 2

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