Clinical anaerobic bacteriology

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Simple and direct tests in the laboratory may aid in the investigation of a specimen to provide a presumptive diagnosis of anaerobic infection.

Bordetella pertussis—the laboratory diagnosis of whooping cough

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The laboratory diagnosis of a disease with a World Health Organisation estimate of 60 million cases per annum.
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Introduction
It is well established that anaerobic bacteria can be the cause of serious microbial infection. In some cases that occur as a specific clinical entity, a single anaerobic organism is likely to be the cause. Anaerobic bacteriology can be a time consuming and expensive process and many laboratories question how they can perform this function efficiently and effectively with particular attention to ease of investigation at minimal cost. Also some anaerobic cultures may be characteristically slow and in the presence of complex microbial mixtures, as is often the case, this aids to the complexity of the investigation. Proper collection and transport of specimens for anaerobic culture is extremely important for accurate and reliable results in the laboratory. This subject has been fully discussed elsewhere. In the laboratory itself the investigation may be aided by the use of simple direct tests on the specimen to provide a presumptive diagnosis of anaerobic infection. The use of appropriate enriched and selective media for various groups of anaerobes, with the use of simple preliminary tests to place isolates into easily recognizable groups, help make the investigation easier. Definitive species identification relies on the use of well established conventional tests that may involve long incubation periods together with other tests that require more complex methodology. Commercial kits for anaerobo identification, which rely on patterns given by preformed enzyme activity on substrates contained within plastic cups or wells, have gained popularity with many diagnostic laboratories. The choice of aspans and identification kits relies on local circumstances and preferences but the aim of every diagnostic laboratory should be to operate an efficient system of isolation together with, at least, the capability to reach identification levels that place isolates into easily recognizable ‘primary groups’.

Recent taxonomic changes
The development of chemotaxonomic methods, most importantly those of DNA base composition, DNA homology and RNA sequencing, have resulted in the reclassification or re-assignment of many anaerobes. Changes effecting the clinically important anaerobes are summarised in Table 1. The genus Bacteroides is now restricted to the B. fragilis group and related organisms. 1 The saccharolytic species that were previously included in the B. melaninogenicus-oralis-ruminicola group have been re-assigned to a new genus Prevotella. 2 Pigmented and non-pigmented species occur within this new genus. Asaccharolytic pigmented Gram-negative rods are now given the generic name Porphyromonas. 3 The re-assignment of the B. ureolyticus group and B. gracilis together with some other species awaits further study.

Other taxonomic methods such as the analysis of peptidoglycan composition may also prove useful, as has been shown in studies of the genus Fusobacterium where most species contain meso-lanthionine in the peptidoglycan structure. 4 Other important changes have occurred in the anaerobic Gram-positive cocci and among the clostridia and non-sporing Gram-positive bacilli. The only member of the Peptococcus genus to remain is the black pigmented Peptococcus niger.

Specimens
The best specimens for anaerobic culture consist of aspirated pus samples or excised tissue, both of which should be sent to the laboratory without delay. Swabs are far less satisfactory 5 but may be the only specimens available, in which case an efficient transport medium is of great importance. Rapid delivery is still essential.

Chromically infected sinus tracts are difficult to examine. When looking for Actinomyces species aspirated pus or alternatively the inner portion of the patients dressing will give reasonable results. Swabs from the surface of such sites rarely provide acceptable material for this purpose.

Table 1: Current taxonomic position of clinically important anaerobes.

<table>
<thead>
<tr>
<th>Old nomenclature</th>
<th>New taxonomy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacteroides fragilis and related organisms</td>
<td>B. fragilis</td>
</tr>
<tr>
<td>B. melaninogenicus-oralis-ruminicola group</td>
<td>Prevotella melaninogenicus</td>
</tr>
<tr>
<td>B. asaccharolyticus</td>
<td>Pr. oralis</td>
</tr>
<tr>
<td>B. gingivalis</td>
<td>Pr. buccalis</td>
</tr>
<tr>
<td>B. endodontalis</td>
<td>Pr. oris</td>
</tr>
<tr>
<td>Fusobacterium symbiosum</td>
<td>Porphyromonas asaccharolyticis</td>
</tr>
<tr>
<td>Clostridium botulinum G</td>
<td>P. gingivalis</td>
</tr>
<tr>
<td>some Cl. histolytic</td>
<td>P. endodontalis</td>
</tr>
<tr>
<td>some Cl. subterminale</td>
<td>Clostridium symbiosum</td>
</tr>
<tr>
<td>Ct. barati</td>
<td>Cl. argentinense</td>
</tr>
<tr>
<td>Ct. perrenne</td>
<td></td>
</tr>
<tr>
<td>Ct. paraperlingensis</td>
<td></td>
</tr>
<tr>
<td>Arachnia propionica</td>
<td>Propionibacterium propionicus</td>
</tr>
<tr>
<td>Bifidobacterium ericksonii</td>
<td>Bifido. dentium</td>
</tr>
<tr>
<td>Peptococcus asaccharolyticus</td>
<td>Peptostreptococcus asaccharolyticus</td>
</tr>
<tr>
<td>Pep. Magnus</td>
<td>Pepto. magnus</td>
</tr>
<tr>
<td>Pep. micros</td>
<td>Pepto. micros</td>
</tr>
<tr>
<td>Pep. prevotii</td>
<td>Pepto. prevotii</td>
</tr>
<tr>
<td>Pep. indolicus</td>
<td>Pepto. indolicus</td>
</tr>
<tr>
<td>Gaffkya anaerobis</td>
<td>Peptostreptococcus tetratus</td>
</tr>
</tbody>
</table>

Direct examination
There are certain direct examinations of clinical material that will yield presumptive evidence of anaerobic infection. The presence of a foul odour is considered by some authorities to be definitive evidence of the presence of anaerobic bacteria. The absence of foul odour, however, does not necessarily mean that anaerobes are absent. The use of a direct Gram stain using Kopellof’s modification 1 may be helpful particularly when certain typical morphotypes are present (e.g. fusobacteria). Gas Liquid Chromatography (GLC) has been used extensively in the past as presumptive evidence of anaerobic infection. Multiple acid peaks are taken as evidence of the presence of anaerobes and the rare occurrence of false negatives (i.e. no acids detected but anaerobes grown on culture) makes this a useful procedure. However, it can be argued that in many instances GLC affords no more information above that which can be obtained from the combined results of the smell of the specimen and a well performed Gram stain.

Similarly the use of ultra violet light to detect the brick red fluorescence of pus samples containing some species of Prevotella or Porphyromonas does not provide absolute information since many such samples fail to fluoresce.
Selective media

The use of certain selective media is essential to begin to separate the various anaerobes that are likely to occur in mixed infections. Unfortunately there is still no all-purpose medium for the culture of all types of anaerobes and the use of more than one medium is helpful in the preliminary investigation. Selective media traditionally contain aminoglycosides (usually neomycin or kanamycin). Neomycin has been used to recover clstridia and anaerobic Gram-negative rods. However some anaerobes, especially cocci and asaccharolytic Gram-negative bacilli, will fail to grow. Kanamycin is slightly more inhibitory, and, whilst retaining selectivity for clostridia, other anaerobes fail to grow (notably fusobacteria and some Gram-positive cocci). Addition of vancomycin to these media render them selective for Gram-negative anaerobes. The inhibitory nature of aminoglycosides in agar media may be overcome by substituting nalidixic acid alone or nalidixic acid with vancomycin. The growth of nonosping Gram-negative anaerobes is better on media containing nalidixic acid and vancomycin. Gram-positive anaerobic cocci grow better on agar media containing nalidixic acid alone. Clostridia however are still best recovered using aminoglycoside media. The use of nalidixic acid media is more fully discussed elsewhere.8

Table 2: Selective media used in anaerobic bacteriology.

<table>
<thead>
<tr>
<th>Medium</th>
<th>Antibiotics</th>
<th>Organisms selected</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neomycin agar</td>
<td>Neomycin</td>
<td>Clostridia, Bacteroides, Anaerobic cocci</td>
</tr>
<tr>
<td>Kanamycin agar</td>
<td>Kanamycin</td>
<td>Clostridia</td>
</tr>
<tr>
<td>Neomycin – Vancomycin agar</td>
<td>Neomycin</td>
<td>Bacteroides, Prevotella</td>
</tr>
<tr>
<td>Kanamycin – Vancomycin agar</td>
<td>Kanamycin</td>
<td>Bacteroides, Prevotella</td>
</tr>
<tr>
<td>Nalidixic acid agar + Tween 80</td>
<td>Nalidixic acid</td>
<td>Bacteroides, Prevotella</td>
</tr>
<tr>
<td>Nalidixic acid – Vancomycin agar</td>
<td>Nalidixic acid</td>
<td>Fusobacteria</td>
</tr>
<tr>
<td>Brain Heart Infusion agar (+Nal &amp; Met)</td>
<td>Nalidixic acid</td>
<td>Veillonella</td>
</tr>
<tr>
<td>CCFA</td>
<td>Cefoxitin</td>
<td>Cl. difficile</td>
</tr>
</tbody>
</table>

Identification of anaerobes

Identification of clinically important anaerobic bacteria can be achieved at two levels. Every laboratory should have the capability of performing simple tests to recognize certain common clinically significant anaerobes. Sixty-five to seventy-five percent of anaerobes recovered from clinical material consists of the B. fragilis group, Peptostreptococcus, F. nucleatum, Peptostreptococci sp, and C. perfringens. Such simple tests include colonial type (including pitting of the agar), Gram stain morphology (including the presence of spores), the use of certain antibiotic discs, the Nagler test and other egg yolk agar reactions and the reverse CAMP test. Further information may be gained from stimulation of growth and formate-fumarate mixtures, the spot indole test, the nitrate reduction disc test and growth on, and hydrolysis of, bile aesculin agar. Results are summarised in Table 3.

The B. fragilis group is often involved in serious infection, and is the most common group of anaerobes to be recovered from clinical material. Some workers consider the prognosis to be poorer when these organisms are present. Identification, therefore, may be important. The B. fragilis group grows on and hydrolyse bile aesculin agar.

Not all pigmented Gram-negative rods tolerate bile and may fail to grow on bile aesculin agar. B. ureolyticus and B. gracilis both give rise to colonies that pit the surface of blood agar plates. B. gracilis is more resistant to antibiotics than B. ureolyticus but both are sensitive to kanamycin. Fusobacterium nucleatum, the most common fusobacterium in clinical material, is sensitive to kanamycin and gives the appearance of needles on Gram stain from colonies that most often appear 'breadcrumb-like' on blood agar. The spot indole test is positive.

The use of discs containing brilliant green has been advocated by some workers for differentiating bacteriae (which are sensitive) from the fusobacteria (resistant).11 Care must be taken when reading these tests since the zones of inhibition are small and, in some cases, not easily seen.

C. perfringens produces colonies showing double zones of haemolysis and the conventional Nagler test is positive. A more recently described test, the reverse CAMP test is specific to C. perfringens and reveals enhanced haemolysis of group B streptococci (Figure 1). C. sordellii and C. bifurcumen are both negative in this test.

Definitive identification of anaerobic

Table 3: ‘Preliminary group’ identification of anaerobes.

<table>
<thead>
<tr>
<th>Gram negative anaerobes</th>
<th>Kana</th>
<th>Vanc</th>
<th>Collistin</th>
<th>Bile Aesc</th>
<th>F/F</th>
<th>NO3</th>
<th>Motility</th>
<th>Pitting of agar</th>
<th>Pigment</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. fragilis gp</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>+</td>
<td>F/F</td>
<td>-</td>
<td>Motile</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Peptostreptococcus sp</td>
<td>R</td>
<td>V</td>
<td>V</td>
<td>-</td>
<td>F/F</td>
<td>-</td>
<td>Motile</td>
<td>+</td>
<td>V</td>
</tr>
<tr>
<td>Porphromonas spp</td>
<td>R</td>
<td>V</td>
<td>V</td>
<td>-</td>
<td>F/F</td>
<td>-</td>
<td>Motile</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>B. ureolyticus spp</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Motile</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Fusobacterium spp</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>Motile</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Veillonella spp</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>Motile</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Gram positive anaerobes</th>
<th>Spores</th>
<th>SPS</th>
<th>Indole</th>
<th>Cat</th>
<th>Nagler</th>
<th>Rev CAMP</th>
<th>Grows in CO2</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. perfringens</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>Cat</td>
<td>Nagler</td>
<td>Rev CAMP</td>
<td>Grows in CO2</td>
</tr>
<tr>
<td>C. bifermentans</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>Cat</td>
<td>Nagler</td>
<td>Rev CAMP</td>
<td>Grows in CO2</td>
</tr>
<tr>
<td>C. sordellii</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>Cat</td>
<td>Nagler</td>
<td>Rev CAMP</td>
<td>Grows in CO2</td>
</tr>
<tr>
<td>C. species</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>Cat</td>
<td>Nagler</td>
<td>Rev CAMP</td>
<td>Grows in CO2</td>
</tr>
<tr>
<td>Peptostreptococcus spp</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>Cat</td>
<td>Nagler</td>
<td>Rev CAMP</td>
<td>Grows in CO2</td>
</tr>
<tr>
<td>Peptostreptococcus spp</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>Cat</td>
<td>Nagler</td>
<td>Rev CAMP</td>
<td>Grows in CO2</td>
</tr>
<tr>
<td>Propion. acnes</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>Cat</td>
<td>Nagler</td>
<td>Rev CAMP</td>
<td>Grows in CO2</td>
</tr>
<tr>
<td>Propion. spp</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>Cat</td>
<td>Nagler</td>
<td>Rev CAMP</td>
<td>Grows in CO2</td>
</tr>
<tr>
<td>Other NSGPB</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>Cat</td>
<td>Nagler</td>
<td>Rev CAMP</td>
<td>Grows in CO2</td>
</tr>
</tbody>
</table>

V = Variable, SPS = Liquid, S = Sensitive, R = Resistant, ↔ = Most strains negative, F/F = Stimulation of growth (formate and fumarate), NSGPB = Non-sporing Gram-positive bacilli.
bacteria may be achieved in two ways. The use of conventional biochemical tests, coupled with end product analysis and comparing the results with a recognised text, such as the VP Anaerobe manual, is regarded as the gold standard. More recently, the use of commercially produced identification kits which compare the preformed enzyme profiles to a computerised database has become popular. Many laboratories are turning away from conventional tests in preference for these kits which offer convenience and speed, the results being available after four hours of aerobic incubation. The two kits most widely used are the Rapid ID 32A, the Rapid ID ANA II system. Both of these kits successfully identify the common anaerobes found in clinical material and to some extent have been successful also in identifying certain strains that have failed to give satisfactory answers in conventional tests. The identification achieved by the kit should agree with that given by the preliminary grouping tests. These kits require practice in both setting up and correctly interpreting the results (Figure 2).

Clostridium difficile colitis

Laboratory resources are best reserved for the examination of faecal supernatant for C. difficile cytotoxin using cell culture or by the use of a more rapid but more expensive EIA for enterotoxin. Isolation of the organism itself must be followed up by testing the isolate for toxigenicity. Isolation may be necessary however, even from asymptomatic patients, if there is a suspicion of cross infection and where the typing of the isolates is necessary. C. perfringens enterotoxin may also cause antibiotic associated diarrhoea and can be detected in faecal supernatant using a reverse passive latex agglutination assay.

Clinical aspects

Many of these anaerobic organisms are well known causes of disease in patients. However, newer associations are being found from time to time. It is now clear, for example, that C. perfringens may also be associated with antibiotic associated diarrhoea and should be borne in mind in addition to C. difficile. The association of fusobacteria (mostly F. nucleatum) with amniotic fluid is another interesting finding, with the organism being recovered from both amniotic fluid and the neonatal gastric aspirate. Pseudotrepitococcus magnus is commonly found in infective processes, particularly gangrenous ulcers in diabetic patients and in infected prosthetic joints. Both of these last two examples reflect the advances made in cultural practices and particularly the improvements in selective agar media.

With more immunocompromised patients entering hospital, organisms usually found in normal flora are beginning to be associated with sepsis in patients suffering from the effects of alcoholism, diabetes, leukaemia, carcinoma, lymphoma and AIDS. Such organisms have included Wolinella, Selenomonas, Desulfovibrio, Anaerobiospirillum and Leptotrichia. Various sources have included pleural fluid, transtracheal aspirate, wound drainage and blood. More common isolates such as C. septicum have been shown to be closely associated with carcinoma.

We have recently isolated strains of Leptotrichia from the blood of pyrexial leukaemia patients (Figure 3). The clinical significance of these isolations is somewhat uncertain but they may reflect a growing pattern of 'rarer' organisms beginning to be found in such patients. Such infections are probably due in part to the changes occurring in modern medical and surgical practice.

Future developments

Rapid detection of anaerobes may be achieved with the use of nucleic acid probes or immunofluorescent labelled monoclonal antibody. Use of such tests for the rapid diagnosis of infection involving toxins such as those of C. botulinum or C. difficile as well as the presence of overwhelming pathogens, for example F. necrophorum, would have beneficial effects on patient care. With the progression of newer technology the development of such techniques will undoubtedly alter the practice of clinical anaerobic bacteriology and will raise the question of whether identification and susceptibility testing would be better served by specialist centres, with the diagnostic laboratory concentrating on rapid diagnosis and isolation.

References


Figure 2: Commercial kits for anaerobe identification Rapid ID 32A, (top), and RapID ANA II, before and after addition of reagents, (bottom).

Figure 3: Leptotrichia buccalis on blood agar anaerobically (72 hours).
Bordetella pertussis—the laboratory diagnosis of whooping cough

JE Hoppe, Director, Bacteriology Laboratory, University Children's Hospital, Tübingen, Germany.

The World Health Organisation estimates that the annual number of pertussis cases is 60 million, and that the disease is responsible for half a million to one million deaths per annum. Mortality is high in infants and particularly so during the first few months of life. At least half of the deaths from pertussis occur in this age group.

Epidemiology
Pertussis is an endemic disease with periodic epidemics. In Europe, the annual incidence rate varies widely. In countries with strictly enforced vaccination schemes, the incidence rate may be as low as 1 per 100,000 population (e.g. in the former German Democratic Republic) whilst in countries without vaccination programmes the incidence rate may be >100 per 100,000, eg. the Swedish rate in 1985 was 3200 per 100,000. In West Germany where pertussis is no longer a notifiable disease and where very few children are vaccinated it is estimated that about 100,000 cases occur annually.

Infection with B. pertussis is highly contagious. Secondary attack rates in unvaccinated populations are high, ranging from 25% to 50% in schools to 70% to 100% in susceptible household contacts. In unvaccinated populations, pertussis is primarily a disease of childhood. However, pertussis in adults is far more common than previously thought. In many instances the disease in adults is atypical or even asymptomatic. Adult pertussis can occur despite a prior history of full immunisation and even in persons with a prior history of the natural disease.

Toxins and other virulence factors
Pertussis toxin (PT)
PT is also known as lymphocytosis-promoting factor and islet-activating protein. PT is an envelope protein antigen which is a heat-agglutinin.

PT is a unique exotoxin of B. pertussis; it is not produced by B. parapertussis or B. bronchiseptica. Chemically, it is an oligomeric protein containing six subunits, five of which are different. PT is a typical A-B toxin. The enzymatically active (A) portion (the S-1 subunit) catalyses the adenosine 5'-diphosphate ribosylation of a 41,000-molecular-weight membrane protein in a variety of cell types. This membrane protein is involved in inhibitory control of mammalian cellular adenylyl cyclase. The action of the A portion results in enhanced activity of the mammalian cell adenylyl cyclase.

The B (binding) portion of PT is required for binding PT to specific cell receptors thus enabling the A portion to reach its site of action in the epithelial cells along the respiratory tract of the host.

PT has many biologic activities in animals, e.g. sensitisation to histamine, lymphocytosis promotion (both B and T cells increased in the circulation) and enhancement of insulin secretion.

Filamentous haemagglutinin (FHA) FHA is a filament-like surface protein of B. pertussis cells. FHA does not have toxic activity but is an important factor in the attachment of B. pertussis to ciliated epithelial cells.

Adenylyl cyclase (AC)
In contrast to PT, AC is produced not only by B. pertussis but also by B. parapertussis and B. bronchiseptica. AC enzymatically induces the accumulation of cyclic adenosine 3',5'-phosphate (cAMP) in various mammalian cell types, including neutrophils, lymphocytes, and monocytes. High levels of intracellular cAMP impair the bactericidal functions of PMN and macrophages thus inhibiting host defenses of the tracheobronchial tree. AC may play a role in the establishment of the initial pertussis infection.

Tracheal Cytotoxin (TCT)
TCT, a glycopeptide, shows specificity for ciliated respiratory epithelial cells, the site of initial attachment of B. pertussis. The toxin is released during log-phase growth of the organism and acts by inhibition of DNA synthesis, thus causing ciliary stasis and impairing cellular regeneration.

Lipoplysaccharide (endotoxin)
The lipoplysaccharide of B. pertussis has the same biological activities as enterobacterial endotoxin (except a lower pyrogenic activity) but it has a different chemical structure. The role of B. pertussis lipoplysaccharide in the pathogenesis of the disease remains largely unknown.

Heat-labile Toxin (dermonecrotic toxin)
This toxin is composed of four polypeptides. It is destroyed by heat (56°C) and causes inflammation and necrotic skin lesions in various animals. Its mode of action and its role in pathogenesis have not yet been elucidated.

Agglutinogens
Agglutinogens are protein surface antigens of Bordetella species. On the basis of agglutination of B. pertussis organisms by specific antibodies, six agglutinogens can be differentiated. Agglutinogens 1 to 3 have been termed "major". All virulent strains of B. pertussis belong to one of three serotypes according to their contents of major agglutinogens: 1, 2, 3, 1.2, 1.3. This is evidence suggesting that agglutinogens 2 and 6 are located on the lipoflagella (pili) of the bacteria and that agglutinogens 6 and 3 may play a role in virulence, possibly as adhesins.

69KDA Outer membrane protein
This protein has been identified as an agglutinogen and exhibits eukaryotic cell-binding activity. The name 'pertactin' has been proposed for this and related proteins. According to recent evidence, the 69KDA protein is an important antigen in the pathogenesis of pertussis.

Diagnosis of pertussis
Culture
Reported data on the sensitivity of the culture methods used vary from very low rates to 93%. All the quoted figures are inaccurate since no available method can detect 100% of pertussis cases. This inaccuracy also refers to the clinical picture which is often equivocal in atypical or mild cases and is, therefore, only of limited value as a reference standard for laboratory methods.

Many factors affect the sensitivity of positive B. pertussis cultures, such as the time between the disease onset and the initial specimen collection, the age and immunisation status of the patient, any preceding antimicrobial therapy and technical problems with the collection, transport, and culturing of specimens. For achieving the best possible results, every detail of specimen collection and culture technique must be optimal. In this way it is possible to increase the survival of the fastidious Bordetella organisms.

Collection of specimens
Nasopharyngeal (NP) swabs yield higher isolation rates of B. pertussis than cough plates and they are the most commonly used method of specimen collection. Duplicate swabs from both nostrils are recommended. Throat or anterior nasal swabs are not useful since B. pertussis has a strict tropism for ciliated respiratory epithelium which is not found in the anterior nose and the pharynx.
Calcium alginate is superior as swab material to dacron, rayon and cotton wool. Methods of obtaining NP secretions by aspiration such as Auger's suction are likely to increase the yield, as is shown indirectly by studies on direct antigen detection (see below). However, large systematic comparative studies between NP aspiration and NP swabs are lacking. Methods for obtaining NP aspirates can be adapted to the conditions present in physicians' offices, where most pertussis patients are seen.10,11

Transport of specimens

Direct specimen plating onto selective media at the bedside is the method of choice. Direct plating is also feasible in an office setting if the physician has some experience with basic bacteriological techniques (e.g., many physicians culture for group A streptococci). Direct plating in the office has been shown to significantly increase the yield of B. pertussis when compared with the use of Regan-Lowe transport medium (see below). In addition, the diagnosis is established more rapidly.12 Plates are incubated in the office and are only sent to a bacteriological laboratory if bacterial growth is observed on them. Thus, identification of the colonies remains in the hands of the bacteriologist.

If direct plating is not possible, a transport medium (TM) has to be used. Non-nutritive antibiotic-free transport media such as Stuart's or Amies' are suitable for short duration transport only, if they are used, they should contain charcoal. If transport exceeds 24 hours, Regan-Lowe transport medium (RLTM) should be used which acts as an enrichment medium for B. pertussis whilst growth of the normal NP flora is suppressed.13 RLTM consists of half-strength Charcoal Agar with 10% whole defibrinated horse blood and 40 mg/ml cephalaxin. Poured tubes of RLTM have a shelf-life of 4-8 weeks if stored at +4°C; they are not commercially available in most countries.

Multiplication of cephalexin-resistant NP flora during transport in RLTM poses a larger problem than if specimens are plated directly.12 The temperature at which the transport systems (i.e., swab in TM) are kept prior to and during transport is of importance. Inhibition of B. pertussis by growth of NP flora has been reported when TM is held at ambient temperatures.14 Preincubation of swabs at 35°C before shipment can increase B. pertussis numbers but will also allow overgrowth of NP flora. When transport systems are stored at 4°C, overgrowth is suppressed but B. pertussis colony numbers decrease by >75%.14 The higher concentration rate after preincubation has to be weighed against the substantial loss of B. pertussis organisms during refrigeration, which may render isolation of small inocula impossible. Thus, the shipping temperature remains controversial but in our experience preincubation is preferable.

For long transport runs, transport by mail at ambient temperatures after preincubation is of course much easier to perform than refrigeration during shipment.

Culture media

Agar media

Numerous studies have demonstrated the superiority of the cephalexin-supplement-

ed charcoal horse blood agar (CHB-C) to Bordet-Gengou agar (BG) (potato infusion agar with 10% glycerol and 20% sheep blood) for the isolation of B. pertussis from clinical specimens. Optimal results, however, are achieved by the combined use of both media.15,16 Ready-poured plates of CHB-C with a shelf-life of 4-8 weeks are commercially available.

Buffered charcoal yeast extract agar, a medium developed for Legionella, and cycloheximide solid medium, an entirely synthetic agar developed in Japan, were found to be inferior to CHB-C.14,17

Omission of blood from CHB-C significantly decreases the Bordetella isolation rate. One study compared horse blood with sheep blood18: growth of B. pertussis on the horse blood agar was more abundant and more rapid and the colony morphology was more typical. Donated human blood containing an anticoagulant is a much less suitable agar supplement than either horse or sheep blood.19 However, donated human blood is often the only available source of blood in bacteriology laboratories in developing countries. A field study in Nigeria showed that B. pertussis isolation on charcoal human blood agar from children with suspected whooping cough is possible; the isolation rate was 16% (Hoppe et al, manuscript submitted to 'Tropical and Geographical Medicine').

Broth media

The semi-solid RLTM is often used for enrichment of small B. pertussis inocula. One study evaluated charcoal horse blood broth with cephalexin and noted problems with heavy overgrowth of cephalexin-resistant NP flora on the subculture plates.19 Much better results were obtained with antibiotic-free heptakis-supplemented Stainer-Scholle broth.20 Inclusion of this broth allowed the isolation of a considerable number of additional isolates which did not primarily grow on charcoal media. Overgrowth of NP flora was not a problem with this broth.

Broth media which allow rapid multiplication of even small inocula of B. pertussis might play a role in future by enhancing the sensitivity of rapid antigen detection tests.

Figure 1: Plastic box to prevent desiccation of Bordetella colonies during incubation.

Figure 2: Colonies of B. parapertussis on charcoal horse blood agar.

Inhibitors of NP flora in agar media

At present, cephalexin is the selective agent of choice for inhibition of the normal NP flora present in clinical specimens. Cephalexin was found to be superior to penicillin, methicillin, lincomycin, and cefalexin.21 Cefbiufen and cefetamet are possible alternatives to cephalexin that have yet to be studied.22 Since overgrowth by cephalexin-resistant flora is not uncommon in daily practice, progress in this field would be desirable.

Some strains of B. pertussis are said to be inhibited by cephalexin, therefore both selective and non-selective media should be used for isolation.2 A recent study, however, found no cephalexin-sensitive strains.21

Culture and identification of Bordetella

Incubation in normal air is preferable to an atmosphere with CO₂ enrichment.18 The optimal temperature is 35-36°C, not 37°C. Prevention of desiccation during incubation is of utmost importance. Simple plastic boxes with tightly closing lids suffice for this purpose (Figure 1). Mature colonies of B. parapertussis (Figure 2) usually become visible after two to three days of incubation and colonies of B. pertussis after three to four days. Some strains of B. pertussis may grow more slowly, therefore, plates without visible
growth should be incubated for seven days before being discarded.

Fresh, clinical isolates of B. pertussis have a typical morphology on CHI5-C. They are smooth, round and shiny and have a characteristic anthracite colour (mercury droplet-like appearance) (Figure 3) which turns into grey as the colonies grow older. Subcultures of B. pertussis have grey colonies from the beginning, like the colonies of B. parapertussis which become brownish-green on ageing. Gram stains of Bordetella colonies reveal small Gram-negative coccoid rods.

Colonies of typical appearance are further differentiated with the oxidase reaction (B. pertussis: positive, B. parapertussis: negative). Final identification is achieved with specific antisera. Both agglutinating and fluorescent antisera are commercially available. For slide agglutination, only fresh colonies can be used since older (>5 d) colonies tend to be sticky and do not emulsify in saline. If this problem is encountered, a subculture must be prepared. B. pertussis grows more rapidly on subculture plates (48 hours) than the original isolates on the primary plates.

Biochemical tests for identification of B. pertussis are needed in exceptional cases only.

Direct detection of B. pertussis or its components in clinical specimens

Direct fluorescent antibody test

The direct fluorescent antibody (DFA) technique uses fluorescein-labelled antibodies against B. pertussis and B. parapertussis for detection of the organisms in NP secretions. Polyclonal antibodies raised in rabbits and directed against whole bacteria are commercially available.

When compared with culture techniques, the DFA test has a striking advantage of rapidity (several hours vs 3-4 days). Also the DFA test can detect bordetellae that are no longer viable due to antimicrobial therapy.

The DFA test has, however, several important drawbacks which limit its usefulness considerably. If the number of organisms in the specimen is low, the test may be false-negative. More important is the high number of false-positive results. These may be due to the inexperience of the technologist reading the test (reader variation may be substantial) to poor-quality slides or to non-specific reagents. Cross-reactivity of DFA reagents with other organisms which share common antigenic determinants has been reported.

Some of the current problems with DFA testing may be overcome in the future by use of monoclonal antibodies directed against specific B. pertussis surface components. At present, the DFA test should be used as an adjunct to culture techniques only, it cannot replace them.

DNA probes

A DNA probe for B. pertussis has been developed using a cloned fragment from a mutant strain. A preliminary evaluation with clinical specimens showed promising results. The sensitivity depended on the method of specimen collection: it was 43% for NP-swabs and 85% for NP-aspirates. The polymerase chain reaction (PCR) may be used to increase the sensitivity of DNA probes. This technique has also been applied to detection of B. pertussis in clinical specimens. In one study, PCR detected 63 out of 66 culture-positive specimens. In addition, 33 culture- and DFA-negative specimens were positive by the PCR assay, many of which appeared to be true- rather than false-positives. The PCR assay has the additional advantage of rapidity (5 hours).

Figure 3: Mercury droplet-like morphology of young (72 hour) colonies of B. pertussis on charcoal horse blood agar.

New Selective Supplement – For the isolation of Legionella from Water Samples

Unipath Ltd have introduced a new supplement to the Oxoid range of freeze-dried supplements for the isolation of Legionella spp. from environmental water samples.

Legionella (GVPC) Selective Supplement (Code SR1S2) is formulated for use with Legionella CYE Agar Base and Legionella BCYEn Growth Supplement to make GVPC medium. Legionella (GVPC) supplement contains glycine, vancomycin and polymyxin to inhibit most Gram-positive and Gram-negative bacteria and cycloheximide to suppress fungal overgrowth.

This selective supplement is reported to be the most efficient in vitro method for the isolation of L. pneumophila when used in conjunction with acid or heat pre-treatments. It is available in packs of 10 vials, each vial is sufficient to supplement 500mls of medium.

For further information contact: Mrs V Kene, Unipath Limited, Wade Road, Basingstoke, Hampshire RG24 6PW England Tel: (0256) 841144. Telex: 656790.
Various showed a sensitivity of 92% as compared with Wirsing vonExcellent. Adenylate cy clase
Confer and Eaton have developed an assay for detection of B. pertussis via its enzyme. With in-vitro studies, this test detected as few as 100 B.pertussis organisms. A clinical evaluation using cal modulin-supplemented Stainer-Scholte broth was conducted by Wirsing von König et al. The assayed showed a sensitivity of 92% as compared with culture. The measurement of AC activity still need a radioactive tracer but deserves further study.

Serology
Various serologic tests have been used to demonstrate infection with B. pertussis. The older methods such as agglutination, complement fixation, bacteridal assays, indirect haemagglutination, and indirect immunofluorescence are quite insensitive. Today, enzyme-linked immunosorbent assays (ELISAs) are the technique of choice because of their improved sensitivity. Pertussis-specific antibodies of the immunoglobulin (Ig) classes A, M and G can be measured by ELISA. Antigens used in ELISAs include whole cells of B.pertussis, whole-cell sonic extracts and purified antigens such as PT and FHA. Use of whole cells or their sonic extracts may increase the risk of cross-reactivity with other related organisms, whereas the use of purified antigens produced by B.pertussis only (such as PT) would make a highly specific assay.

ELISAs which require antibody production by the patient are not suitable for diagnosis of early pertussis (catarrhal stage) because they become positive during the convulsive stage only. They are most useful for a differential evaluation of long-lasting cough and for epidemiological studies.

Since pertussis patients usually are ambulatory, it is often difficult to obtain paired specimens of acute- and convalescence-phase sera. In most cases, only a single sample can be obtained when the patient presents to his physician at the end of the catarrhal or the beginning of the convulsive stage. Accordingly, the titre may be 'borderline' and difficult to interpret.

The fact that the immune response to pertussis differs in vaccinated and unvaccinated persons further complicates the interpretation of serological tests. Infected previously vaccinated individuals usually do not react with a rise of IgM antibodies, although recent vaccination and recent infection of an unvaccinated person may lead to elevated IgM and IgG titres. Production of pertussis-specific IgA is observed only after natural infection, not after vaccination. Young infants, however, are frequently unable to produce detectable amounts of IgA antibodies.

A Finnish ELISA for detection of pertussis-specific IgA, IgM and IgG is commercially available in some countries. The detection of secretory mucosal antibodies—which probably reflect the immunostatus in pertussis more accurately than serum antibodies—is still in an experimental stage.23

References

Bibliographic references

New Listeria Selective Supplement (MOX)
The recent addition of Listeria Selective Supplement (MOX) (Code SH157) further enhances the comprehensive range of Oxoid media for the isolation and identification of Listeria from foods. Used with Listeria Agar Base (Oxford) (Code CM856), Listeria Selective Supplement (MOX) is recommended for the isolation and identification of Listeria monocytogenes from processed meat and poultry products.

Moxalactam and colistin which are incorporated in Listeria Selective Supplement (MOX) suppress the growth of other micro-organisms that may form part of the normal flora of the specimen e.g. Staphylococcus, Proteus and Pseudomonas spp. It is available in packs of 10 vials each vial is sufficient to supplement 500mls of medium.

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