

Chapter 27

Identification of Isolates

BACKGROUND

The identification of a bacterial isolate involves deciding whether or not its properties are similar enough to those of a described species for it to be considered identical with that species. This depends, of course, on the particular classification adopted.

Most organisms are classified almost solely on morphological criteria, but classifying bacteria into *Bacillus*, *Micrococcus* and *Spirochaeta* doesn't get us very far, so such things as atmosphere required for growth, staining properties and biochemical tests are used.

It was soon realised that characteristics for classification should be as correlated with other characteristics as possible. This means that some characteristics can be used as key characteristics to rapidly identify an organism— eg, rapid indole production for *Escherichia coli*. It also led to the widespread use of keys for identification. However, this approach has its problems: real exceptions occur to most characteristics for most organisms, supposed key characteristics may be shared by quite dissimilar organisms while varying for quite similar ones, and slight variation in technique can cause wrong results and wildly incorrect identifications.

Numerical taxonomy takes an entirely different tack: testing organisms for a large number of characteristics, each of which is given equal weight, and classifying them in clusters of similarity, which form natural taxons. This approach forms the basis of such systems as the API and the various Vitek cards. The 20 or so characteristics chosen for each system were those which had been found to be both highly correlative and most constant for the group of organisms for which the system was designed. These systems now constitute the mainstay of bacterial identifications in the clinical laboratory, but key reactions, many using commercial packages, are also frequently used. For many of those organisms for which no simple packaged system exists, tables and/or keys are available which enable identification.

Genetic methods of classification and identification are making their appearance. These are sometimes useful. Unfortunately, however, genetic classifications are often not very useful clinically. For example, genetically, *Escherichia coli* and *Shigella* should be in the same species.

THE APPROACH TO THE IDENTIFICATION OF BACTERIA IN THE MEDICAL LABORATORY

The importance of knowledge and experience and the consequent 'feel' for a bacterial species cannot be overemphasised. If you know the growth characteristics of an organism, its appearance, smell (if any), perhaps a few key biochemical reactions, likely antibiogram, its usual habitat and the circumstances under which it is likely to be isolated in a clinical laboratory, the identification can be rapid and you are unlikely to be misled into error.

Most clinical specimens are seeded to a number of different types of media and it is important to compare the growth on the different media. For example, an organism growing on blood agar but not enriched chocolate agar with bacitracin is probably Gram positive; one growing on enriched chocolate agar with bacitracin but not on blood agar (except, perhaps, as pinpoint colonies) is probably *Haemophilus*; one growing on blood agar and colistin nalidixic acid agar but not MacConkey is Gram positive; one growing on blood agar, colistin nalidixic acid agar and MacConkey is likely to be either *Enterococcus faecalis* (tiny colonies) or a *Pseudomonas* species; one growing on blood agar but not colistin nalidixic acid agar or MacConkey is probably a non-Enterobacteriaceae Gram negative; etc.

Speed of growth can also be a useful clue. A Gram positive rod appearing overnight, or even in 48 hours, is definitely not a *Mycobacterium*. On the other hand, a *Haemophilus* that takes 48 hours to make a feeble growth on enriched chocolate agar from an eye swab may well be suspected of being *Haemophilus aegyptius* rather than *Haemophilus influenzae*.

Use of colonial characteristics as a criterion has fallen into disfavour in many identification systems. This is largely because such characteristics are difficult to describe in terms that mean the same to all observers, impossible to include in numerical type taxonomies and even difficult to incorporate into keys and tables. However, many bacteria regularly produce colonies that are typical and almost instantly recognisable, reducing identification

procedures to one or two simple confirmatory tests, such as Staphyslide for *Staphylococcus aureus* and indole for *Escherichia coli*. Equally, if an identification system gives you an identification which does not accord with the appearance of the organism as you know it or as it is described in the texts, you should seriously question that identification.

Similar remarks can be made for smell. As proved in a survey with *Streptococcus milleri*, the smell of the growth of some organisms is so characteristic as to approach an absolutely reliable identification procedure.

The Gram stain reaction remains probably the single most correlative characteristic of an organism. This is despite the fact that isolates of some supposedly Gram positive species frequently stain Gram negative. Correlation with the colonial appearance and with the type of media on which the organism is growing may prevent an error in some cases. Also, in many cases, one can learn to recognise microscopically the morphology of species such as *Bacillus* and *Lactobacillus* which frequently overdecolorise, and even to detect the minute difference in the appearance of the cell wall in Gram positive and Gram negative species. The potassium hydroxide string test [Place colony in 3% potassium hydroxide and lightly emulsify. Slowly draw out wire. Gram negatives will form a string from solution to wire.] is a useful supplement in doubtful cases. Unfortunately, it is not infallible, and *Achromobacter*, *Acinetobacter*, *Agrobacterium* and *Moraxella* regularly give false negative reactions, while *Bacillus* species may give a false positive. Where suspicion still exists, vancomycin susceptibility may settle the question; all Gram positives except *Lactobacillus*, *Leuconostoc*, *Pediococcus* and rare strains of *Enterococcus* are sensitive, while *Acinetobacter* and *Moraxella* are the only Gram negatives which may show sensitivity. Nalidixic acid and polymyxin susceptibility also correlate very well (though not perfectly) with 'true' Gram stain reaction— Gram positives are resistant, and Gram negatives susceptible, to both. Again, an oxidase negative and/or large-celled Gram negative bacillus which is penicillin susceptible should be viewed with suspicion unless it has been identified as belonging to a species which includes penicillin susceptible strains.

Slow-growing Gram positive bacilli of fine morphology should be subjected to a modified Ziehl-Neelsen stain.

The actual morphology of an organism is frequently characteristic and can sometimes be virtually diagnostic. It is important not to minimise the usefulness of this information. In some cases, it may be difficult to decide if an organism is a rod or a coccus. The appearance of cells grown in the presence of a β -lactam to which they are susceptible (eg, from the zone edge around a penicillin disc) can often be useful in deciding this; cocci tend to enlarge and disrupt spherically, while rods are prone to elongate.

Other important properties that can be almost instantly determined are the catalase and oxidase (Kovacs method using a platinum (never nichrome) loop to inoculate an 18-24 hours old colony from a non-selective and non-differential medium to freshly prepared 1% tetramethyl-p-phenyldiamine dihydrochloride (reacts with cytochrome c to form a blue coloured compound; positive reaction must occur in 10 seconds) is the most satisfactory method) reactions. Motility may also be apparent in a simple wet preparation.

The single most important biochemical characteristic is undoubtedly the O-F reaction. Whether an organism utilises glucose fermentatively, oxidatively or not at all is a highly correlative criterion. For most organisms, Hugh-Leifson's medium should be used for the purpose. [Hugh-Leifson medium differs from carbohydrate fermentation media by decrease of peptone concentration from 1% to 0.2%, increase of carbohydrate concentration from 0.5 to 1% and decrease of agar concentration from 0.3% to 0.2%.] However, for more fastidious organisms, such as coryneforms, *Neisseria*, *Moraxella*, etc, it may be necessary to use cysteine tryptone agar sugars to establish this criterion. It is important to realise that nonfermentative organisms are strict aerobes and vice versa.

Given just the above criteria, Cowan and Steele's initial tables purport to group all the bacteria one is likely to encounter in a clinical microbiology into a number of groups which lead on to further tables eventually allowing a firm identification. In many cases, this is true. However, blindly following the scheme can readily lead one into error. This is because of the broad groupings, with lack of due notice given to important exceptions; the fact that absolute positive and negative values of characteristics are given at the 85% level, which gives a fairly high probability of encountering an exception; because descriptions of genera are sketchy and sometimes wrong in failing to note important exceptions, while descriptions of species are virtually nonexistent; such basic properties as colonial and cellular morphology are rarely mentioned. So, anyone using Cowan and Steel should check the identification carefully against a description in Balows or Bergey.

The tables in Balows are more complete, frequently quote percentages, and are usually accompanied by clear descriptions of species. The problem with Balows is that it largely presupposes enough knowledge to be able to get to the right table. The three keys— 'Nonenterobacteriaceae Fermentative Gram Negative Bacilli', 'Non-fermenting Gram Negative Bacilli' and 'Fastidious Gram Negative Bacilli'— require only urea, indole, nitrate and lactose as additional tests and are very useful but there are problems getting there: How do you know a fermentative Gram negative bacillus is non-Enterobacteriaceae? Why isn't *Pseudomonas* in 'Non-fermenting Gram Negative Bacilli'? Why does 'Fastidious Gram Negative Bacilli' not include *Haemophilus*, *Brucella*, etc?

Probably the best scheme for identification of nonfermenting and fastidious Gram negative bacilli is the Weaver-Hollis scheme. However, even here there are problems: misread any one of the three prime separating criteria (O-F, MacConkey, oxidase) and you'll quickly be right off the track; many of the tests are not ones normally used in the laboratory; some organisms are far more quickly and definitively identified by alternative procedures; referral to fuller descriptions of organisms is still required.

The packaged identification systems, such as Vitek and API, can be extremely useful if used within their limitations, but it is essential to know what the limitations of each of these systems are. These limitations can arise because the necessary data are not in the data base, because the tests employed have insufficient discrimination for particular organisms, or because a test gives incorrect results. All these systems should be used strictly as directed. Eg., reading an API before 14 hours can definitely give false results, as can employing the wrong inoculum density in the Vitek. It is possible to use reactions obtained in these systems to 'manually' identify organisms. However, a great deal of caution must be applied here since different results may well be obtained using different methods— something that must be borne in mind whatever method you are using. Reactions obtained after prolonged incubation (> 12 h) in the GNI cannot be trusted.

[API20E: With the API20E, the basic inoculation time is 14-24 hours. Attempts to read results earlier will frequently result in misidentification. It is always wise to set up the standard extra tests (motility, nitrate, O-F glucose, MacConkey) on any oxidase positive organism; also, any organism which shows only a few reactions after overnight incubation should be reincubated for a further 24 hours and the extra tests set up. The API20E correctly identifies about 93% of common Enterobacteriaceae. The identification of any organism by the API20E must be critically regarded, particularly when it is based on only a few characteristics. In inoculating the API20E, a single well-isolated colony suspended in 5 mL saline should be used. For organisms which do not grow on MacConkey or on the usual susceptibility test agars, the addition of a few drops of sterile serum to the saline will improve the test. A purity plate should be set up from the suspension inoculated. Though API literature suggests performing oxidase and nitrate tests on the strip, these are much better performed externally: the oxidase test using the Kovacs oxidase test on filter paper, the nitrate test in a tube of nitrate broth. [In reading the nitrate test, a red colouration (diazo dye complex) on the addition of the nitrate A (sulphanilic acid) and B (?-naphthylamine) reagents indicates reduction of nitrate to nitrite. If no red colouration appears, add a small amount of zinc dust; a red colouration indicates no reduction of nitrate, while no red colouration indicates reduction of nitrate to nitrogen gas.] The API32E is virtually 100% accurate for common Enterobacteriaceae, but the API10S correctly identifies only about 70%. It may be worth noting that, except for the gelatinase reaction, all the tests utilised in the API20E are readily adapted to a self-prepared microtitre formula, which should give excellent agreement (~ 97%) with results obtained using the commercial system.

REPLICATOR TECHNIQUES may be used in identification, giving a 95% correlation with API20E.

IDENTIFICATION OF NONFERMENTATIVE AND OXIDASE POSITIVE FERMENTATIVE BACTERIA: Commercial systems are not particularly reliable, the Oxi/Ferm system being 80% accurate, the Minitek 72% and the API20E 61%. However, failure is usually due to a failure of generated codes to appear in the compendium, rather than of misidentification. These systems can still be usefully employed if used very critically.

VITEK: The Vitek can identify a variety of Gram negative and Gram positive organisms in 3-13 hours and perform susceptibility testing in 3-10 hours (4% very major errors). Identification of common Enterobacteriaceae is virtually 100% accurate. Vitek 1 accurately identifies *Burkholderia pseudomallei* but Vitek 2 does not. Direct identification and susceptibility testing of a suspension of centrifuged organisms from positive blood cultures is possible in many cases (93% accuracy overall); however, it will not work with such organisms as pneumococci, *Neisseria* and *Haemophilus* and

may give erroneous results for oxacillin sensitivity of *Staphylococcus aureus*, several antimicrobial agents with enterococci, and ampicillin and cephalosporins with *Citrobacter*, *Enterobacter* and *Serratia*.

OTHER SYSTEMS: Of the other common systems, Microscan correctly identifies about 98% of common Enterobacteriaceae, Microbact 24 about 90%, Microbact 12 about 57%, and BBL Crystal about 79%.

THE IDENTIFICATION OF FAECAL ISOLATES is usually a matter of following a simple protocol, which will allow the identification of virtually all likely significant isolates. The rapid SST strip is a rapid, accurate and cost-effective method of enteric pathogen screening.

On xylose lysine deoxycholate medium, *Salmonella* appears as distinct black colonies due to H₂S production, and on Salmonella-Shigella agar as clear colonies with some H₂S production. Test first for urease production [converts urea to ammonium carbonate, giving an alkaline reaction; spot test positive in 2 minutes, tube test in 2 hours or less]. If positive, this is not *Salmonella* (probably *Proteus*). If urea negative, identify the isolate using the Vitek or API system. Also set up a nutrient agar plate for agglutinations. A heavy suspension is made of the suspected *Salmonella* in formal saline from the nutrient agar plate. To drops of this suspension are added 1 drop of polyvalent A-G and/or polyvalent A-S (somatic O antigens), polyvalent H (flagellar antigen) and Vi (capsular antigen) respectively. If polyvalent A-G and/or A-S and polyvalent H are positive and Vi negative, the organism is a *Salmonella* other than *Salmonella typhi* and can be further identified by specific agglutinations. If the somatic O antigens are negative, the suspension should be boiled and the agglutinations repeated. If the Vi reaction is positive, boil the suspension for 15 minutes and repeat the agglutinations. *Salmonella typhi* will agglutinate in poly H and Vi but usually not polyvalent O or group D specific sera before heating (VW strains will agglutinate in both Vi and group D without heating; the heating destroys the capsular (Vi) antigen which masks the somatic O antigen).

Shigella does not ferment xylose and appears as red, sometimes crenated, colonies on xylose lysine deoxycholate agar, clear on Salmonella-Shigella agar. *Shigella* is usually associated with leucocytes and erythrocytes in the faeces. It may be identified using the Vitek or API.

Colonies of *Aeromonas hydrophila* are large, rhizoid, non-xylose fermenting and oxidase positive. When subcultured onto blood agar, they are ?-haemolytic. Identification is by Vitek or API.

Plesiomonas shigelloides is non-xylose fermenting, oxidase positive, non-haemolytic on blood agar. Identification is by API or Vitek.

Campylobacter is a microaerophilic Gram negative bacillus which grows at 42°C. On Skirrow's medium (blood agar with vancomycin, polymyxin B and trimethoprim), the colonial morphology ranges from small discrete colonies through to swarming colonies which may cover the entire surface of the plate in a uniform film and can be easily missed. *Campylobacter* is oxidase and catalase positive and appears in a Gram stain as Gram negative delicate 'seagull-like' rods. Rapid hippurate discs are used to differentiate between *Campylobacter jejuni* (positive) and other thermophilic *Campylobacter* species (negative).

Typical colonies of *Yersinia enterocolitica* on CIN medium are small, dark red colonies with a clear border, usually ? 1 mm. On xylose lysine deoxycholate medium they appear as tiny clear colonies. A rapid screening test using urea/phenylalanine deaminase discs can be performed (urea positive, phenylalanine deaminase negative organisms are presumptive *Yersinia enterocolitica*; confirm by Vitek or API).

Vibrio grows on thiosulphate citrate bile sucrose agar after 24 hours as ? 2mm colonies (*Vibrio cholerae* (sucrose fermenter): 2-3 mm yellow; *Vibrio parahaemolyticus* (lactose fermenter): 3-5 mm green). Faecal isolates are oxidase positive but this cannot be tested from thiosulphate citrate bile sucrose agar as false negatives occur. Full identification is made using the Vitek or API system and agglutination tests.

Colonies of *Clostridium difficile* on blood agar + cycloserine + cefoxitin agar after 48 hours of anaerobic incubation at 37°C are large, grey, irregular and have a distinctive putrid smell.

PRESUMPTIVE IDENTIFICATION OF URINE ISOLATES: One can also easily recognise the great majority of urinary isolates and identify them with well known, straightforward methods. 85% of urines with 10⁵ organisms/mL produce recognisable growth after 4-6 hours incubation. The organisms can be presumptively identified with > 90% accuracy by a combination of colonial morphology, Gram stain and simple tests (eg, *Staphylococcus aureus* and coagulase negative staphylococci: slide coagulase or agglutination; *Proteus mirabilis*: non-lactose fermenting, oxidase and indole negative, urease positive; *Escherichia coli*: primary isolation plate colonial morphology + spot indole identifies 69% of

isolates but spot indole can only be done from blood agar, fluorogenic β -glucuronidase assay identifies 87-94% in < 1 hour, usual method indole broth containing tryptophane (produces indole which reacts with p-dimethylaminobenzaldehyde, added after 2-4 hours incubation, to give a red colour.)

GROWTH CHARACTERISTICS ON CYSTINE LACTOSE ELECTROLYTE DEFICIENT MEDIUM AFTER 18 HOURS INCUBATION:

Escherichia coli: yellow, opaque colonies with a slightly deeper coloured centre, about 1.25 mm diameter (non-lactose fermenting strains: blue colonies)

Klebsiella: extremely mucoid colonies varying in colour from yellow to whitish blue

Proteus: translucent blue colonies usually smaller than *Escherichia coli*

Salmonella: flat blue colonies

Pseudomonas: green colonies with typical matt surface and rough periphery

Enterococcus faecalis: yellow opaque colonies about 0.5 mm diameter

Staphylococcus aureus: deep yellow colonies about 0.75 mm diameter, uniform in colour

Coagulase negative staphylococci: pale yellow or white, more opaque than *Enterococcus faecalis*, often with paler periphery

Corynebacterium: very small grey colonies

Lactobacillus: similar to *Corynebacterium* but with a rougher surface

Candida: small clear white to blue colonies

Streptococcus agalactiae: tiny clear blue colonies, often quite hard to see and easily missed after overnight incubation

IDENTIFICATION OF URINARY ISOLATES FROM CYSTINE LACTOSE ELECTROLYTE DEFICIENT AGAR:

A. Gram negative bacilli:

1. Smell, colonial appearance and oxidase reaction:

a. ? *Escherichia coli* (flat, non-mucoid lactose fermenting colonies): inoculate indole medium and

read at 4-6 hours

indole positive = *Escherichia coli*

indole negative: inoculate MICROID, ATB32E, API20E or Vitek GNI

b. ? *Proteus*: inoculate urea broth, ornithine decarboxylase (inoculate one tube of ornithine

decarboxylase medium and one of basal medium for each test; overlay each tube with paraffin so that it is anaerobic; if, after incubation, the basal medium is blue, the test is invalid), blood agar and indole and read at 4-6 hours

urea positive:

ODC positive (ODC tube blue, basal medium yellow), spreading on blood agar, indole negative = *Proteus mirabilis*

ODC positive, not spreading on blood agar, indole positive = *Morganella morganii*

ODC negative (ODC and basal medium tubes both yellow), spreading on blood agar, indole positive = *Proteus vulgaris*

ODC negative, not spreading on blood agar: inoculate API20E or Vitek GNI

c. ? *Pseudomonas*: perform oxidase test

oxidase positive: inoculate API20E + O-F glucose, nitrate, MacConkey and motility, or Vitek GNI

2. Other Gram negative bacilli: perform oxidase test and inoculate MICROID (oxidase negative only), ATB32E, API or Vitek GNI

B. Gram positive cocci: perform catalase test

1. Catalase positive = staphylococci and micrococci; perform Staphyslide test

a. Staphyslide positive = *Staphylococcus aureus*

b. Staphyslide negative: test novobiocin susceptibility and set up glucose O-F

i. Novobiocin susceptible = coagulase negative staphylococcus other than *S.saprophyticus*

ii. Novobiocin resistant, fermentative = *Staphylococcus saprophyticus*

iii. Novobiocin resistant, oxidative = *Micrococcus*

2. Catalase negative: inoculate bile esculin, 6.5% NaCl, blood agar

a. bile esculin positive, 6.5% NaCl positive = *Enterococcus*

b. bile esculin positive, 6.5% NaCl negative = non-enterococcal group D *Streptococcus*

c. bile esculin negative, 6.5% NaCl negative = other streptococci; use blood agar plate for grouping

d. bile esculin negative, 6.5% NaCl positive = probably misidentified *Staphylococcus*; possibly *Aerococcus* (? -haemolytic on blood agar)

C. Yeasts: germ tube test verifies *Candida albicans* in 2-4 hours.

[MICROID: This system is intended to be used within the stipulated time span of 4-6 hours. This necessitates the use of a fairly heavy inoculum (usually several colonies), which in turn means that it should be used only where one is reasonably certain of being able to obtain an inoculum consisting entirely of one organism (especially since no purity check will be available when the strip is read). Be alert for insufficient inoculum density or incubation time. The majority of reactions should be clear-cut and sharp. Note particularly that *Escherichia coli* will give reactions of *Shigella* if insufficient reaction occurs. Always be suspicious of an identification (in any system) based only on a few characteristics. In the case of the MICROID, it is particularly important to check the oxidase reaction, especially where only nitrate and one or two other reactions are positive. If an unlikely identification or no identification occurs, check purity and set up an API20E or other system.]

IDENTIFICATION OF ISOLATES FROM GENITAL AND RESPIRATORY SPECIMENS is simplified by the facts that, as with faeces, one is looking for a restricted range of pathogens and it is entirely possible to quickly become familiar with the appearance of both normal flora and pathogens from these sites on the various media employed (always look at the combination of media rather than making snap judgments based on the appearance on one). As long as one is alert to such rarities as *Haemophilus* in a urethral swab or *Pasteurella* in a sputum, the vast majority of isolates can be easily identified by the standard methods.

The simple scheme below will allow the identification of the great majority of isolates of **anaerobes**, at least to the degree required. *Bacteroides fragilis* may be identified rapidly by gas-liquid chromatography. The AnIdent and RapId ANA systems provide rapid identification of a variety of anaerobes, though with only ? 84% agreement with conventional systems.

A SIMPLE SCHEME FOR IDENTIFICATION OF ANAEROBES

Growth on BA + vitamin K, BA + vitamin K + vancomycin + kanamycin; no growth on CNA; metronidazole susceptible; Gram stain ? Gram negative bacilli

Vancomycin R kanamycin R colistin R(S)

Growth on bile esculin agar = *Bacteroides fragilis* group

No growth on bile esculin agar = *Bacteroides spp*

Vancomycin R kanamycin R colistin V, black pigment present = *Prevotella melaninogenica*

Vancomycin R kanamycin S colistin S

Pitting colonies

Urease positive = *Bacteroides ureolyticus*

Urease negative = *Bacteroides gracilis*

Colonies not pitting

Indole pos, growth on bile esculin pos, esculin neg, lipase neg = *Fusobacterium varium*

Indole positive, no growth on bile esculin, lipase pos = *Fusobacterium necrophorum*

Indole pos, no growth on bile esculin, lipase neg = *Fusobacterium nucleatum*

Indole neg, growth on bile esculin, esculin pos, lipase neg = *Fusobacterium mortiferum*

Growth on BA + vitamin K; no growth on BA + vitamin K + vancomycin + kanamycin, CNA; metronidazole susceptible; Gram stain ? Gram negative cocci

Nitrate pos = *Veillonella*

Nitrate neg = *Acidaminococcus, Megasphaera*

Growth on BA + vitamin K, CNA; no growth on BA + vitamin K + vancomycin + kanamycin; metronidazole susceptibility variable; Gram stain ? Gram positive cocci

SPS S = *Peptostreptococcus anaerobius*

SPS R

Indole pos = *Peptostreptococcus asaccharolyticus*

Indole neg = other *Peptostreptococcus spp*

Growth on BA + vitamin K, CNA; no growth on BA + vitamin K + vancomycin + kanamycin; metronidazole susceptibility variable; Gram stain ? Gram positive bacilli

Diphtheroid-like

Catalase pos

Indole pos = *Propionibacterium*

Indole neg = *Rothia, Bifidobacterium*

Catalase neg = *Lactobacillus, Eubacterium*

Actinomyces-like ? further testing

Clostridium-like

Double zone of haemolysis present, Nagler pos = *Clostridium perfringens*

Double zone of haemolysis absent

Heavy swarming

Spores terminal = *Clostridium tetani*

Spores subterminal = *Clostridium septicum*

Little or no swarming

Urease pos = *Clostridium sordellii*

Urease neg = other *Clostridium spp*

Most problems are likely to occur with aerobes isolated from specimens from sites other than those listed above. Even here, of course, the vast majority of isolates can be readily recognised by colonial appearance on the different media employed and identified in the usual simplistic manner.

GRAM NEGATIVE BACILLI

Growth on MacConkey

Coliforms can usually be recognised by appearance and smell, and identified by GNI or API. Note that both systems have problems with *Enterobacter/Klebsiella*; in doubtful cases, a motility test may settle the question. Capsular swelling (the swelling of capsule on the surface of the bacterium in the presence of specific antiserum) may also be useful in identifying *Klebsiella*.

Pseudomonas aeruginosa is usually recognisable by appearance (it commonly produces a green pigment) and distinctive odour. It is, of course, oxidase positive. It grows on blood agar, enriched chocolate agar with bacitracin and on MacConkey agar, but not on colistin nalidixic agar. It can, if necessary, be identified by GNI or API. [FN medium includes magnesium sulphate as activator of fluorescein production and nitrate to detect reduction of nitrate to nitrogen gas and helps differentiate *Pseudomonas* from other nonfermentative gram negative bacilli.]

Aeromonas can be recognised by its coliform appearance (it grows on MacConkey agar as a non-lactose fermenter, sometimes with a pink halo), α -haemolysis on blood agar, oxidase positivity and characteristic odour. It can be identified with GNI or API, though the GNI probably performs better overall. *Aeromonas* is always resistant to ampicillin.

One can also learn to recognise such organisms as *Flavobacterium*, *Burkholderia cepacia* and *Stenotrophomonas maltophilia* by appearance and smell.

Flavobacterium, *Alcaligenes* and *Achromobacter* are all oxidase positive organisms that are often resistant to aminoglycosides.

A Gram stain should be the first step for organisms whose probable identity is unknown. Curved rods are probably *Vibrio*. The GNI is now probably more reliable than the API for these organisms (the reverse of the previous case), but the identification may still need to be checked with such tests as growth on TCBS, O/129 susceptibility, salt tests, antibiotic susceptibilities (consult Balows).

Oxidase positive coccoid rods are probably *Moraxella*, which can possibly be identified with an NHL. In some cases, manual tests may be necessary.

Oxidase negative rods of similar morphology are probably *Acinetobacter*. The GNI will identify both biovars, but with very few positive reactions; such a result should be checked by Gram stain if this has not already been done. The API identifies *anitratus* but not *polymorpha*; however, a coccoid Gram negative bacillus which grows on MacConkey, is oxidase negative and gives no reactions in the API can be accepted as being *Acinetobacter calcoaceticus var polymorpha*. Note that both biovars are always resistant to penicillin.

For other isolates, perform oxidase and catalase tests and set up O-F, motility, urea and indole tests. Setting up a GNI or API may provide an identification, but this may well be incorrect. On the other hand, using the above few tests in Cowan and Steele's tables, Balow's keys and/or the Weaver-Hollis scheme will lead directly to an identification in many cases and provide a sure path to identification in most others.

No Growth on MacConkey, Growth on Blood Agar

The appearance and smell may well give a clue to the organism's identity. The Gram stain will also often be very helpful, being quite characteristic for many organisms in this group.

If you know the organism is an obligate aerobe, a combination of oxidase test, Gram stain and motility will soon tell you what genus you have. An oxidase negative organism will either be a *Pseudomonas* species or *Bordetella parapertussis*. These can be readily distinguished on Gram stain. An oxidase positive organism will either be *Pseudomonas*, *Flavobacterium*, *Bordetella parapertussis*, *Bordetella bronchiseptica* or *Moraxella*. The latter three organisms have quite characteristic Gram staining reactions. *Bordetella parapertussis* can be identified by serological reaction. *Bordetella bronchiseptica* gives a positive spot urease test in two minutes or a positive tube test in < 4 hours. *Moraxella* can be quite coccoid and may give a false negative string test. It can be loosely identified by NHL, more strictly by use of tables. *Pseudomonas* (motile) and *Flavobacterium* (nonmotile) can be separated on motility. They can be identified by GNI, though by no means all species are covered. Failing this, recourse must, if necessary, be made to one or other of the tables; the API is weak in this area.

If you know the organism is oxidative, the only choices are *Pseudomonas* and *Flavobacterium*.

Most people quickly learn to recognise *Pasteurella multocida* by sight and smell; it identifies well in the GNI and usually in the API, though addition of serum may be required.

Eikenella corrodens again is usually easily recognised by colonial appearance and smell, though it can be mistaken for a streptococcus. It can be identified in the API or NHI, but the easiest and most definitive identification is given by its unique requirement for X factor aerobically but not anaerobically.

Other organisms in this group for which Gram stain recognition is important are *Gardnerella*, *Brucella*, *Campylobacter* and salt-requiring *Vibrio* species. *Gardnerella* produces tiny non-haemolytic colonies resembling lactobacilli on blood agar but tiny α -haemolytic colonies on special *Gardnerella* medium. It is usually susceptible to metronidazole and always resistant to sulphonamides. *Vibrio* species can be identified by GNI or API, but the identification should be verified by other tests as given in Balows. Methods of identifying other organisms are well documented.

For other organisms which do not meet the above criteria, oxidase and catalase tests should be performed, O-F, indole, urea and nitrate tests set up and the appropriate keys and tables followed.

No Growth on Blood Agar, Growth on Enriched Chocolate Agar

Gram negative bacilli which may not grow on either MacConkey agar or blood agar but which grow on enriched chocolate agar are *Brucella*, *Campylobacter*, *Haemophilus* and *Streptobacillus moniliformis*. These can all be readily separated on cellular morphology and identified appropriately. *Haemophilus* is usually identified by its requirement for X and/or V factors. Water-soluble factors (X, V and X+V) are impregnated into discs or filter paper strips or rings and placed on a medium deficient in these factors (brain heart infusion or trypticase soy agar) which has been inoculated with the organism. Growth around a disc indicates a requirement. The porphyrin test is regarded as a more reliable test for X factor requirement than the X factor disc method. Organisms not requiring X factor convert α -aminolevulinic acid to porphobilinogen, which is detected by fluorescence under UV or reaction with modified Ehrlich's reagent. A tube test for porphyrin production confirms *Haemophilus influenzae* in 4 hours. The Vitek NHI card correctly identifies 94% of *Haemophilus influenzae* and 96% of *Haemophilus parainfluenzae* isolates but only about half of the *Haemophilus* strains are correctly identified.

GRAM NEGATIVE COCCI

Aerobic Gram negative cocci are *Neisseria* or *Moraxella* (*Branhamella*). All species are strict aerobes and oxidase positive and have characteristic colonial and cellular morphology. *Neisseria gonorrhoeae*, *Neisseria meningitidis* and *Neisseria lactamica* are the only *Neisseria* species that regularly grow on New York City medium, while *Neisseria gonorrhoeae* and *Neisseria meningitidis* do not grow on nutrient agar. However, note that a high inoculum density can produce a false result in these tests and that nutrient agar means a nutrient agar such as brain heart infusion agar, not an enriched nutrient agar such as Columbia agar (on which *Neisseria meningitidis* will grow). *Neisseria* species can be identified by a rapid carbohydrate utilisation test in which balanced phosphate buffered saline containing phenol red indicator and drops (or discs) of carbohydrates is heavily inoculated and the reaction read at 4 hours; these tests are not, however, always completely reliable. The ONPG (o-nitrophenyl- β -galactopyranosidase) test detects delayed lactose fermenters by the production of yellow o-nitrophenol by β -galactosidase and is frequently preferred to lactose fermentation. Gonocheck 11 has 100% sensitivity and 99.6% specificity. The Phadebact Monoclonal GC test may be used to verify an isolate as *Neisseria gonorrhoeae* but the reaction should be definitely positive and only in one of the two reagents. Used in this manner, it has a sensitivity of 97% and specificity of 100%. Otherwise, an NHI should be used, as it should be for other *Neisseria* species. The Vitek NHI card correctly identifies 100% of *N.cinerea*, 100% of *N.lactamica*, 99% of *N.gonorrhoeae* and 92% of *N.meningitidis*, but 0-9% of other *Neisseria* species and will not identify *Moraxella catarrhalis*. *Moraxella catarrhalis* is easily identified with the tributyrin test. Some strains of *Neisseria meningitidis* will not ferment carbohydrates on primary isolation but will often do so after a series of rapid subcultures; these isolates can also be definitively be identified by PCR at a reference laboratory.

GRAM POSITIVE COCCI

The catalase test (5 seconds slide test) is, of course, the basic test for differentiating micrococci and staphylococci from streptococci. It is possible to get a false positive catalase test with *Enterococcus faecalis* taken from blood agar or enriched chocolate agar or with other streptococci by picking up blood cells with the colony from a blood agar plate. The fact that the organism does not grow on mannitol salt agar will usually make this mistake apparent.

Micrococcus is fairly easily differentiated from *Staphylococcus* because it is strictly aerobic and oxidative, while *Staphylococcus* will grow anaerobically and is fermentative (glucose fermentation performed in yeast extract + 1% peptone). Cowan and Steele's table states that *Micrococcus* is oxidase positive. This is true, but only using a special procedure; using the normal method, it is negative.

Staphylococcus aureus grows on blood agar and colistin nalidixic acid agar and is catalase positive and coagulase positive. It is easily identified with Staphyslide. The negative control latex must always be used. If the negative control latex agglutinates, the test is invalid and a tube coagulase must be performed. False positives are very rare (make sure the organism is a *Staphylococcus*; organisms giving false positives include *Candida* and *Enterococcus faecalis*). False negatives are also uncommon but negative results should be checked with a tube coagulase if the colonial morphology or the clinical picture suggests a *Staphylococcus aureus*. There are reported instances of *Staphylococcus aureus* (usually associated with gentamicin treatment) which will not produce positive reactions in either of these tests or will do so only under such special circumstances as room temperature incubation or incubation in CO₂. These can be confirmed biochemically as *Staphylococcus aureus* using, eg, the API Staph. Unfortunately, the Vitek GPI depends on being told whether the isolate is coagulase positive or negative and is no help in these cases.

Coagulase negative staphylococci which are reported without further identification should be reported as such, not as *Staphylococcus epidermidis*. All reported isolates from blood cultures and hospital specimens should be identified using the GPI. There are some doubts about the absolute accuracy of some of these identifications, but at least it is more or less consistent and allows correlation between specimens and consequent information about possible sources of isolates from blood cultures.

Staphylococcus saprophyticus is readily differentiated from other staphylococci by novobiocin resistance. Some *Micrococcus* species are also novobiocin resistant; this rarely causes a problem but the two can be differentiated by anaerobic growth and O-F reaction if necessary. *Staphylococcus saprophyticus* regularly gives a positive reaction to both test and control reagents in the Staphyslide,

Stomatococcus produces large, adherent colonies on blood agar, looks like a *Staphylococcus* in a Gram stain but is either catalase negative or weakly positive. Unlike micrococci and staphylococci, it will not grow in 6.5% NaCl.

The other catalase negative Gram positive cocci are *Enterococcus*, *Streptococcus*, *Lactococcus*, *Aerococcus*, *Gemella*, *Pediococcus* and *Leuconostoc*. *Enterococcus*, *Streptococcus*, *Lactococcus* and *Leuconostoc* all have similar morphology, though *Leuconostoc* tends to be coccobacillary. *Aerococcus*, *Gemella* and *Pediococcus* all have staphylococcal-like morphology. *Aerococcus* grows poorly anaerobically. *Enterococcus*, *Streptococcus*, *Lactococcus*, *Aerococcus* and *Gemella* can all be identified using the API Strep. A heavy suspension (MacFarlane #3) is made in 2-5 mL saline and the strip inoculated per instructions. A purity plate on blood agar is essential as a mixed inoculum makes any results invalid. An esculinase tube test verifies *Streptococcus pneumoniae* and group D streptococci in 30 minutes but bile solubility (tube method confirms *Streptococcus pneumoniae* in 5-15 minutes) and optochin tests are the most reliable for identification of *Streptococcus pneumoniae*. Capsular swelling (the swelling of capsule on the surface of the bacterium in the presence of specific antiserum) may also be useful. The bile-esculin test is used to differentiate group D streptococci from other streptococci, while the salt tolerance (6.5% sodium chloride) test is used in conjunction with the bile-esculin test to differentiate enterococci (*Enterococcus faecalis*, *E.faecium*, *E.durans*, *E.avium*) from non-enterococci (*Streptococcus bovis* and *S.equinus*). *Streptococcus pyogenes* is α -haemolytic on blood agar and colistin nalidixic acid agar and does not grow on MacConkey. A grouping is required for further identification. When used with isolation plates or broth cultures, Streptex is both sensitive and specific for grouping of α -haemolytic streptococci of groups A, B, C, F and G. However, use of grouping without other tests in speciation may give false results. Note especially that 'minute colony' strains of *Streptococcus milleri* (*Streptococcus anginosus*) may group as A, C, F, G (or not at all); these should be clearly differentiated from 'classical' representatives of groups A, C and G. Note also that not all α -haemolytic colonies from throat swabs are streptococci; they may be *Haemophilus*, staphylococci, *Neisseria* and others. Further, Streptex and similar systems may give misleading results with β - or γ -haemolytic streptococci; in particular *Streptococcus pneumoniae* may group as group C. *Pediococcus* and *Leuconostoc* are both invariably resistant to vancomycin. It should, perhaps, be pointed out that the bile-esculin and 6.5% NaCl tests are by no means definitive for enterococci; most lactococci, aerococci and *Leuconostoc*, and many pediococci, also give positive results for both tests. In addition, 95% of *Pediococcus* strains and 35% of *Leuconostoc* react with group D antiserum.

GRAM POSITIVE BACILLI

Perhaps the first thing that should be said is that the first stage table for Gram positive bacilli in Cowan and Steele contains a large number of errors of fact and should not be used.

Large, sporeforming, catalase positive, Gram positive bacilli are members of the genus *Bacillus*. They all grow aerobically and may or may not grow anaerobically. *Clostridium* is easily distinguished from *Bacillus* by being catalase negative and, except for *C.carnis*, *C.histolyticum* and *C.tertium*, by being strict anaerobes.

The problem in identifying *Bacillus* arises when such isolates are Gram negative, don't spore and are smaller than usual. The string test was actually developed to solve this problem but false positives do occur. However, unlike Gram negatives with which they could be confused, they are vancomycin susceptible. Procedures exist to induce sporing but these are somewhat cumbersome and don't always produce results. Identifying *Bacillus* species, when this is necessary, has been simplified by the advent of the Vitek *Bacillus* card.

Gram positive bacilli appearing in 48 hours or less are definitely not *Mycobacterium*, *Nocardia* or *Actinomyces*.

Slow-growing, fine, weakly Gram staining bacilli should be suspected of being *Mycobacterium* or *Nocardia* and a modified Ziehl-Neelsen stain performed. *Nocardia* will usually show some branching but a squash preparation may be necessary to show this, since it easily fragments. It may be difficult to demonstrate acid-fastness unless the isolate is growing on a high protein medium such as Lowenstein-Jensen or casein medium. *Actinomyces* may show somewhat similar morphology to *Mycobacterium* or *Nocardia* but grows anaerobically, whereas *Mycobacterium* and *Nocardia* are strict aerobes. So is *Streptomyces*, which, however, tends to have thicker filaments which show little fragmentation, and is never acid-fast. *Nocardia* has a very earthy odour, while the earthy odour of *Streptomyces* is almost overpowering. A number of other nocardiform species may be encountered, usually as environmental contaminants.

Dematophilus is another strict aerobe which consists of branching filaments. However, the rather bizarre appearance of the filaments, which branch at right angles, and the production of motile coccoid forms serve to distinguish it from the other organisms mentioned above.

Oerskovia also produces extensively branching filaments which break up into motile rods and coccoid elements. However, its appearance on Gram stain is quite dissimilar to *Dematophilus* and it grows anaerobically. It is interesting in that it grows much better on blood agar than on enriched chocolate agar and is catalase positive when grown aerobically but negative when grown anaerobically.

As before, I am neglecting the strict anaerobes. Aerotolerant clostridia have already been mentioned. Of the other genera with aerotolerant species or strains, it can be said that, if catalase positive they will be *Propionibacterium*, while if catalase negative they will be *Actinomyces* or *Lactobacillus*. Colonial and cellular morphology should enable separation of the latter two genera. Lactobacilli can be mistaken for streptococci.

The non-acid-fast, non-sporing, regular, Gram positive rods include *Listeria* and *Erysipelothrix* as the most important genera. *Listeria* consists of small coccoid rods, is usually β -haemolytic, is catalase, esculin (an esculinase tube can be read after 30 minutes) and Voges-Proskauer [organism produces acetoin, which reacts with oxygen and 40% potassium hydroxide to produce diacetyl which produces a red colour with β -naphthol] positive and shows tumbling motility at 25°C; it can be identified with the GPI. *Erysipelothrix* is β -haemolytic, catalase negative and produces H₂S in triple sugar iron agar. It can be mistaken for a streptococcus. It can be identified in the GPI; unfortunately, while it seems to be always correctly identified with this system, other organisms, including streptococci, are sometimes identified as *Erysipelothrix*. Two other genera usually considered in this group are *Brocothrix* and *Kurthia*; these are of little, if any, clinical significance. *Brocothrix* is very similar to *Listeria* but is nonmotile and does not grow at 37°C. *Kurthia* is a strict aerobe, oxidase positive, esculin negative and glucose negative.

The corynebacteria and related coryneforms frequently cause problems. Any Gram positive rods which are not sporing, branching, filamentous or acid-fast, show some degree of pleomorphism and tend to stain irregularly are described as coryneform. This can include a lot of genera other than *Corynebacterium*, and many corynebacteria are quite regular both in cellular morphology and in Gram staining reaction. All one can do is to be sure that the organism is not an unusual representative of one of the other genera mentioned above and then attempt to identify it using the table in Balows, which includes all the species of *Corynebacterium* and related species of any medical relevance. If identification is not possible by this means, all that remains is usually to label it a 'diphtheroid'. This includes a large number of environmental and plant pathogen species of *Corynebacterium*, as well as such environmental and dairy genera as *Caseobacter*, *Aureobacterium*, *Microbacterium*, *Agromyces*, *Arthrobacter*, *Brevibacterium*, *Cellulomonas* and *Micromonospora*. *Arthrobacter* can be identified, with some difficulty, by its rod \rightarrow cocci \rightarrow rod cycle and other properties. Identification of the other genera usually requires such exotic methods as cell wall analysis, fatty acid analysis, G+C content of DNA, DNA-DNA hybridisation.

CONCLUSION

With the approach outlined above, the great majority of bacteria isolated in the clinical laboratory can be identified. This approach can be characterised as a systematic one guided by knowledge and verified by close attention to the properties of the organism, with stress being placed on such basic properties as colonial and cellular morphology, smell, growth characteristics, possession of an oxidative or fermentative metabolism, oxidase and catalase reactions, and such other biochemical reactions as are known to be close to invariant for the organism. It is important not to be misled by a single anomalous test, whether this is due to poor technique, poor information or the nature of the organism. It is also necessary to realise the limits of one's expertise and when to yell for help.