Identification of gram negative bacteria using biochemical tests, including api

Science, Biology

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Abstract

Four pure, unidentified cultures of (gram positive cocci) bacteria, labelled A-D were cultured on various agar media. Also an API test was simulated to identify another unidentified bacterium. Identification of bacteria is important when choosing an effective treatment for a microbial-causing illness. This experiment focused on the cultural and biochemical characteristics of bacteria in aid of identification. Under aseptic conditions, each of the four unidentified bacterium were cultured using the bile aesculin, manitol salt and the blood agar plates provided. These were then incubated for over a week and then observed. A catalase and Voges-proskauer were also carried out to verify the identity of the 4 strains of bacteria. Bacteria that produced air bubbles in the catalase test (as oxygen is one of the products formed, in the presence of the enzyme catalase) and a red colour change for the Voges-proskauer (bacteria is able to produce a compound called acetylmethylcarbinol), both indicative of a positive result. For simplicity, the end cultures were compared with a table of results provided in the experiment to confirm the identity of Enterococcus faecalis, Micrococcus luteus, Staphylococcus aureus and Streptococcus pyogenes bacteria. The

firstcultureeasily identified as Streptococcus pyogenes produced a visible ?-haemolysis on blood agar; with an obvious clear zone around the colonies and was also unable to grow on manitol salt agar. The other strains were then determined from the various biochemical tests, as all bacterium possess particular characteristics that distinguish them from other genera. The bacterium used in the API was identified as Staphlyococcus. aureus, by use of an identification table, provided by the manufacturer of the API. However in a normal setting various other tests would have to be conducted to conclude the genus and species of the bacteria.

Introduction

Gram positive and gram negative bacteria have a rigid cell wall called peptidoglycan and this can be used to distinguish between the two groups. Gram positive bacteria have a very thick outer layer of peptidoglycan. They also have the lipopolysaccharide layer absent. (Madigan et al., 2009) Gram positive bacteria usually appear purple and gram negative bacteria can be red to pink in colour with the use of gram staining. (Madigan et al., 2009)

Once established the fact that the bacterium belong to gram positive group, the Dichotomous Key of Gram Positive bacteria can be used to differentiate bacteria by use of various biochemical tests. (Willey et al. 2008)

The isolation and identification of bacteria is an essentialdiagnostictool in microbiology, especially investigating pathogenic bacteria that cause infectious diseases. The clinician and microbiologist work together in this identification process. (Willey et al. 2008) Samples from the suspected

infected area of a patient can be extracted and grown aseptically on agar medium to avoid contamination; these mixed cultures are then separated to produce single colonies of a genus bacterium. The shape of the bacteria can be determined by microscopy (using gram staining or other staining techniques for acid-fast bacteria), and culturing of the bacteria on various media – selective, differential and certain characteristic (metabolic) media. (Willey et al. 2008) Selective media only allow certain bacteria to grow, whilst differential media are used to distinguish bacteria from others, in the presence of some form of dye or indicator. (Madigan et al., 2009) It is also important to note the conditions bacteria are able to grow in, as some may tolerate the presence of oxygen (aerobes) whilst others will not (anaerobes). The presence of specific enzymes enables aerobic bacteria to grow, whilst anaerobic bacteria cannot. (Madigan et al., 2009) Voges-Proskauer tests distinguish bacteria that are able to produce fermentation, especially when they cannot respire aerobically. (Willey et al. 2008)

When microscopy and culturing methods alone are not adequate enough to identify a species, specific biochemical tests are carried out. These tests are used to eliminate the number of possible pathogens causing the illness in question; by comparing the unidentified pathogen with the known metabolic characteristics stored on computer databases. (Madigan et al., 2009) These may include testing for products the bacterium may produce (due to a presence of specific enzyme/s) or even their ability to grow on either selective or differential media or a combination of the two. However some require further investigative tests to identify the bacteria. (Madigan et al.,

2009)An example is the coagulase test, which differentiates S. aureus from S. epidermidis, coagulase has the ability to clot plasma. (Willey et al. 2008) Once the bacteria have been identified, antibiotic sensitivity tests (susceptibility tests) may be performed in order to determine which antibiotic/s would be most effective in treating the illness related to the microorganism. (Willey et al. 2008)

The ability of bacteria to produce catalase is an important biochemical characteristic, aerobic bacteria are able to secrete specific enzymes this characteristic can be manipulated in identification. (Madigan et al., 2009) Aerobic bacteria are able to neutralise hydrogen peroxide (that would otherwise be toxic to it) by converting it to water and oxygen. Bubble formation would indicate a positive result of this reaction taking place. (Greenwood et al., 2007) This test helps to identify streptococcus from staphylococcus. (Willey et al. 2008) Further more some bacteria may have the ability of secreting other enzymes like superoxide dismutase and peroxidise. This depends on the growth conditions the bacteria require, to neutralise free (unpaired) oxygen radicals that would otherwise destroy the normal functioning of bacterial cells. These radicals are the result of oxygen being reduced in the electron transport chain. (Willey et al., 2008)

Indicator medium of blood agar (usually containing horse blood) is used for the haemolysis test to indicate if the bacterium produces a specific toxin (haemolysin) this is a common virulence factor that pathogenic bacteria possess. A positive result indicates the bacterium possesses this toxin. (Willey et al. 2008) The toxin is able to lyse erythrocytes by forming pores in

the cell surface, releasing its contents – haemoglobin and other ions. (Willey et al., 2008) This can be observed on blood-agar as a clear halo with no distinct colour around the colonies, called ?-haemolysis. Partial (?) haemolysis leaves a slight green discolouration, as hydrogen peroxide oxidises haemoglobin to methaemoglobin. (Greenwood et al., 2007)

Bile aesculin agar is selective and differential, black formation on the culture plate would indicate the ability of the bacterium to hydrolyse aesculin and mix with ferric citrate. (Mahon and Manuselis, 2000) The manitol salt agar is an example of selective media that only allows growth of specific bacteria to grow, thus it can be used in biochemical tests. This is due to the high concentration of salt within this medium, which inhibits most bacteria from growing. (Mahon and Manuselis, 2000)

Rapid identification of a microorganism can be determined by the use of an API (Analytical Profile Index) or manual ' kit' (Willey et al. 2008)that contains 20 microtubules with dehydrated substrates, once inoculated with bacteria and left to incubate; the various wells produce colour changes when reagents are added. These colour changes are related to the metabolic characteristics of specific bacteria that can be matched to an identification table.

The use of currenttechnologyenables one to study the genomic and antigenic structure of microorganisms and is thus useful in identification. The use of PCR and electrophoresis can be used in Multilocus Sequence Typing (MLST) and genomic fingerprinting. (Willey et al. 2008)Also the various surface

proteins especially antigens can be identified for its interaction with particular antibodies by immunofluorescence or agglutination technique. This technique may yield rapid results and streptococci associated with sore throats can be identified this way; however these tests are not as accurate as the culturing techniques. (Champoux et al., 2004) New and more accurate technologies are being studied such as the use of Biosensors. (Willey et al., 2008)

Staphylococci have a round shape (from the Greek word ' kokkos' meaning a berry.) these bacteria form clusters like grapes (derived from the Greek word ' staphule') Staphylococci also have a slime layer, and are mainly found on the surface of skin.(Heritage et al., 1999) These aerotolerant anaerobe are able to grow in either aerobic or anaerobic conditions. Although Staphylococcus aureus is harmless living on the surface of the skin, it is able to cause serious illness like septicaemia when it enters open wounds. (Mandal et al., 1996) This bacterium can also become an opportunistic pathogen, responsible for epidemics like MRSA due to resistance of the antibiotic methicillin and emerging resistance to vancomycin. (Willey et al., 2008) A quick biochemical test called Staphaurex can also be used. (Willey et al., 2008)

Streptococci are facultative anaerobes and do not form any gas products, as they produce lactic acid fermentation and will therefore catalase negative. (Willey et al., 2008) The streptococcus genera cover an extensive group of bacteria – the cocci that are spherical in shape and thus placed into 3 groups: pyogenic, oral and other (colon) streptococci. (Greenwood et al.,

2007) Virulence factors produced by the pathogenic bacteria (pyogenic) like the presence of streptolysin, have the ability to lyse erythrocytes and can inhibit the host's immune response as it kills leukocytes. Haemolysis is a key step to identify pyogenic (harmful) streptococci from other streptococci. (Willey et al., 2008)

The species E. faecalis can be found in the intestinal tract, it has the ability to cause opportunistic infections like urinary tract infections (UTI) and also is able to grow in 6. 5% sodium chloride, and can resist certain antibiotics. (Willey et al., 2008) The enterococcus group are closely related to the streptococcus group, but are associated more within the intestinal area. (Champoux et al., 2004) The species M. luteus are obligate aerobes in that they rely completely on oxygen to survive and so can be found on one's own microbiota, the surface of skin. (Madigan et al., 2009)

Method

A week before identification, 4 unidentified pure strains labelled (A-D) were each cultured on blood, bile aesculin and manitol salt agar that corresponded to each letter. The streak-plate technique was applied, a loop used to transfer the bacteria to the agar plates was sterilised under an open flame and left to cool, before each set of streaks. After a week, the agar plates were all examined and the type of results they produced was recorded. A single colony (seen by naked eye) was removed from the original (ordinary) agar plates. Each of these was inoculated over a few days and used for the Voges-Proskauer test. The reagents alpha napthol and 40% KOH were added, the tubes were then observed for colour changes. Also a catalase test was

carried out, an inoculated loop was used to transfer a small amount from each strain (from the ordinary agar plates) to a microscope slide and hydrogen peroxide was added. Those that bubbled were noted as positive. All results from the various biochemical tests were compiled in table format – the catalase; Voges-Proskauer; haemolysis (blood agar); ability to produce aesculetin (bile agar) and ability to grow (on manitol salt agar). The 4 strains of bacteria were thus identified.

Separately, an API test was simulated of an ' unidentified' staphylococci bacterium. Each well of the incubation box for the API was filled with distilled water followed by an ampoule of the bacteria which was inoculated and prepared to the correct McFarland standard tube of 0. 5. Mineral oil filled the outlined wells. The box was incubated for a few days; reagents were added to the corresponding wells and after 10 minutes observed for colour changes. Reagents VP1 and VP2 were added to the VP well; NIT1 and NIT2 to NIT well and lastly Zym A and Zym B to PAL well. The test colour result for each well was then noted (either positive or negative) on an API Staph strip and matched with the identification table of the various Staphylococcus species. The staphylococcus species was thus identified.

Results

The 4 unidentified strains (labelled A-D) were exposed to various biochemical tests, the results from these are given below.

Table 1: Results from the gram positive strains

Results from the API test:

A bacterium was then identified by the use of API test, a colour indication table was also provided to determine if the results were positive or negative. These results were jotted down on a test strip and compared with a test table to identify the species of Staphyloccocus.

Figure 1: Test strip

Figure 2: Identification Table of Staphylococcus species (Provided by API Manufacturer)

Discussion

Observationof the colour and characteristics of the pathogen, with the use of various biochemical tests can identify the bacterium causing the infection. (Madigan et al., 2009) This can be applied in this experiment.

Referring to Table 1: The ability to produce haemolysis is dependent on bacteria to secrete a toxic substance called haemolysin, which is able to lyse red bloods cells. Thus blood agar is used which is a differential medium. (Willey et al., 2008) The type of haemolysis bacteria produce can be observed by the naked eye, as clearing zones around the colonies. A ?-haemolysis results in distinct, colourless clear zones of colonies, as the erythrocytes (of the blood agar) have completely lysed. The species S. pyogenes has the ability to secrete exotoxins, depending incubation conditions it will either secrete Streptolysin-O (anaerobic) or Streptolysin-S (aerobic). The pyogenic bacteria are distinguished from other streptococci by

producing ?- haemolysis. (Willey et al., 2008) Whilst ?-haemolysis is the partial destruction of erythrocytes with some clearing and slight green discolouration, it is not as distinct as ?-haemolysis. The green tinge is a result of haemoglobulin being oxidised. Conversely M luteus and E. Faecalis produce ?-haemolysis i. e. no colour change or clearing zone on the agar as the bacteria are unable to produce haemolysin. (Greenwood et al., 2007)

Furthermore the Lancefield method together with haemolysis testing can be used to identify pathogenic streptococci from other less evasive streptococci. (Greenwood et al., 2007) The Lancefield method involves the agglutination of antibodies with the cell wall antigens (C polysaccharide) each serotype is classified A-T, depending on the sort of antigen-polysaccharide nature of this reaction. (Willey et al., 2008).

Voges-proskauer is used to indicate if the bacteria in question produce fermentation, this would depend on their culture needs – especially anaerobic bacteria which are unable to respire without the electron transport chain. (Willey et al., 2008) The red colour produced is a test positive for the production of acetoin or acetylmethylcarbinol in glucose fermentation. (Champoux et al., 2004) Referring to Table 1, S. aureus tests positive as it's a facultative anaerobe. (Willey et al., 2008) Whilst M. luteus and Str. pyogenes can grow in aerobic conditions and so do not require the principles of fermentation, they test negative.

Conversely, unlike anaerobes ability to produce fermentation, most aerobes possess the enzyme catalase. A positive catalase test results in bubble

formation when hydrogen peroxide is added to a bacterium. The enzyme catalase is able to form water and oxygen from hydrogen peroxide. (Madigan et al., 2009) The Streptoccocus pyogenes produce no gas, and instead utilise lactic acid to break down sugars. (Willey et al., 2008) They catalase negative as the enzyme catalase is not present; so cannot break down hydrogen peroxide to form water and oxygen. (Greenwood et al., 2007) Staphylococcus tests positive and can utilise glucose to form acidic products. (Madigan et al., 2009) It's also an aerotolerant anaerobe, it may lack the enzyme superoxide dismutase which can break down superoxide radicals, but can make use of manganese ions instead. This may have been an adaptative mechanism when the very first forms of bacteria were exposed to oxygen. (Madigan et al., 2009) This enzyme is common in most pathogenic bacteria, and increases their virulence by neutralising the otherwise toxic hydrogen peroxide and minimizing death by phagocytosis by host cells. (Champoux et al., 2004) M. luteus grow in aerobic conditions and can only utilise glucose in these conditions, this would explain why it would catalase positive, to neutralise toxic hydrogen peroxide. (Madigan et al., 2009)

Bile aesculin agar is selective and differential, black formation on the culture plate would indicate the ability of the bacterium to hydrolyse aesculin and mix with ferric citrate. (Mahon and Manuselis, 2000) The presence of bile salts will inhibit some types of bacteria like S. pyogenes and M. luteus (as seen on Table 1)

Manitol is a selective media, only allowing some bacteria to tolerate it, like S. aureus and E. faecalis. They are both able to utilise manitol by fermenting it

to produce acid, thus lowering the pH the agar changes from red to a yellow colour as a result. Incubation of S. aureus is slightly longer, and so a coagulase test can also be implemented. (Mahon and Manuselis, 2000) Whilst haemolysis identifies pathogenic streptococci like Str. pyogenes, the manitol agar identifies pathogenic staphylococci. Also Str. pyogenes cannot grow on this agar, and so no visible colonies are formed. (Willey et al., 2008) While M. luteus has the ability to grow on manitol salt agar (visible colonies), so one would assume that it cannot utilise manitol, as there is no colour change present as it cannot produce acid.

As mentioned, there are various API tests available; this experiment used an API Staph Test which identified Staphylococcus, micrococcus and kocuria genera. (CITATION) The test kit was compared with the colour change table of the various substrates (when reagents are added) and the API test strip was marked accordingly for a positive or negative result. The test strip (Figure 1) was then compared to the identification table (Figure 2) and the unknown bacterium was identified as S. aureus. The limitations of this test is that a pure culture of bacteria must be used and that API's are specific for a particular genera of bacteria, various API tests are available (http://www. biotech. ug. edu. pl/odl/biochem/api. html) these include an API 20E to identify Enterobacteriaceae (Willey et al., 2008) Also any experimental error like not adding reagents correctly to specific well can also give false positives, thus not correctly identifying the species.

Conclusion

Identification of gram positive bacteria can be achieved by carrying out various biochemical tests. Differential media like blood agar is useful in identifying the type of haemolysis and thus the pathogenicity of various bacteria (streptococci). Selective media like manitol salt agar inhibits growth of certain bacteria like streptococci, whilst also determining the presence of particular enzymes by the end products produced, this can be observed by colour changes. Various other biochemical tests are available and can produce rapid results – like the API. The simulation of identifying bacteria in this experiment, accentuated how vital these tests are in order to treat patients effectively. However it should be noted in realistic settings further biochemical tests and the use of modern technologies may be required to correctly identify microorganisms.

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