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Review Article

Overview on Old and New Biochemical Test for Bacterial Identification

Roy B, Das T, Bhattacharyya S*

Associate Professor Microbiology, AIIH&PH, Kolkata, India.

***Corresponding Author:** Bhattacharyya S. Associate Professor Microbiology, AIIH&PH, Kolkata, India.

Received Date: July 26, 2023; **Accepted Date:** August 05, 2023; **Published Date:** August 15, 2023

Citation: Roy B, Das T, Bhattacharyya S. (2023), Overview on Old and New Biochemical Test for Bacterial Identification, *J, Surgical Case Reports and Images* 6(3); **DOI:10.31579/2690-1897/163**

Abstract

Despite the advent of many newer tests, biochemical tests are still pivotal for bacterial identification. Many biochemical tests are there for bacterial identification and they have to be inoculated depending on which bacteria is suspected. Flow charts have to be prepared sequentially for deducing bacterial identification by biochemical tests. These aspects have been elaborated in this chapter.

Keywords: bacteria; isolates; biochemical

Introduction

Surgical and other infections can be caused by bacteria which need to be identified by phenotypic tests like staining and biochemical tests. Proper identification is the prerequisite for empirical therapy. Newer methods like MALDI-TOF and whole genome sequencing are available now in many places for accurate identification of bacteria from colonies, but biochemical tests are still one of the preferred identification methods for decades, not only just because they are reliable and inexpensive but also because of their quick and accurate results [1]. Identification of unknown microbial culture is a key step in medical, industrial and research institutes, and there are more than thousands of methods of identification of a particular species of microbes. However biochemical tests are still more preferable for many reasons from past till now [2]. Microbial identification at first glance is challenging for experienced research scientists also, and different screening steps are there which make classification schemes smaller and smaller and more specific. Firstly one should go for phenotypic and morphological studies; second, nutrient requirements in different nutrient media; third, Gram staining and acid fact staining ; fourth, biochemical properties of particular strain, fifth is PCR and sequencing [1]. After performing following screening tests, a researcher should have all details about that particular species. Although PCR and sequencing is more specific and expensive among all, mostly microbial origin source is identified in fourth step as well but to confirm the results, PCR and sequencing are done [3].

Biochemical tests are the oldest methods to identify microorganisms, by phenotypic traits. The cornerstone of most biochemical tests is the ability of bacteria to use specific biomolecules, producing valuable organic chemicals for themselves. There are several types of biochemical tests where different bacteria are identified or distinguished on the basis of different criteria. Simple visual confirmation of the organism's growth in the presence of essential nutrients by increasing turbidity in the liquid medium is one of the old approaches that is frequently used. However, in other experiments, the results are dependent on how the medium's colour changes as a result of the medium's pH changes. The way that microorganisms respond to these tests can be used to categorize them into distinct categories. Even down to the species level, several tests allow for the differentiation of microorganisms. . Nevertheless, there are several drawbacks to biochemical tests. Although affordable and providing both quantitative and qualitative data regarding the variety of microorganisms present in a sample, these procedures are time- and labour-intensive, and results take several days to appear. False positive results can occasionally be achieved, especially when similar microbial species are taken into consideration [4].

Different biochemical test and their discovery during time for identification of microbial culture are listed below:

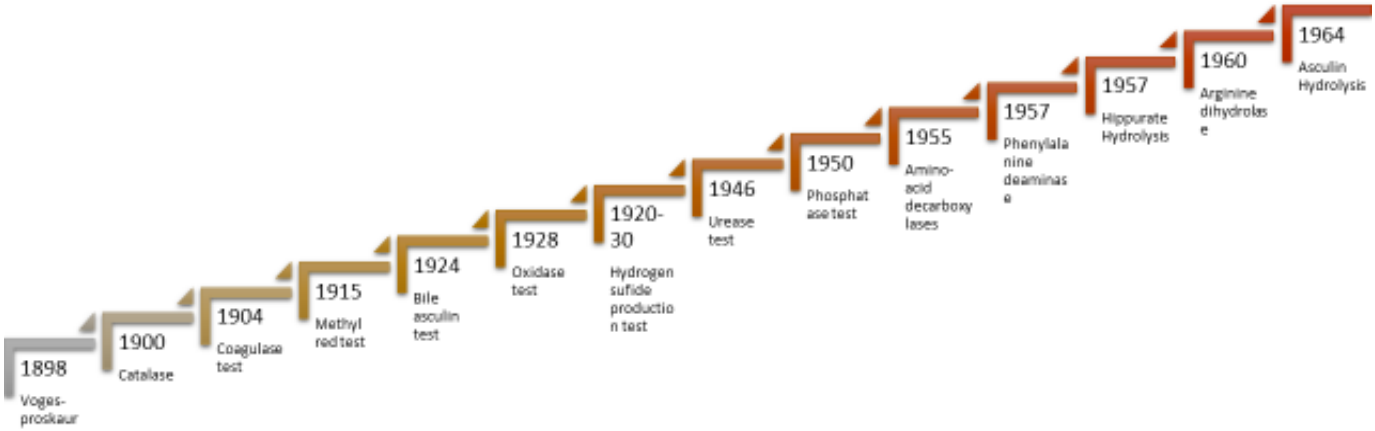

Figure : Evolution of biochemical test for microbial identification (source:- author)

Oxidase test – This test was first postulated by Gordon and McLeod in 1928. The cytochrome oxidase test is useful for identifying bacteria that can manufacture the enzyme. The test aids in identifying the families of Pseudomonaceae that produce oxidase and order Enterobacterales that do not. The mechanism of cytochrome oxidase is the transmission of electrons from the donor (the electron transport chain) to the final acceptor (oxygen), and reduction results in the formation of water. The electron donor will be oxidized by cytochrome oxidase, changing the color to dark purple due to formation of indophenol. This test is carried out by impregnating filter paper with 1 % tetra-methyl-p-phenylenediamine dihydrochloride(TMPPD) which serves as an artificial electron donor, and drying it. The bacterial colonies are applied to a paper strip, and changes in color to blue-purple are detected within ten seconds [5]. Alternatively, the liquid reagent can be poured over the colonies directly and seen for colour change of the liquid. In modified oxidase, 6% TMPPD in DMSO is used for better cell wall penetration, in case of *Micrococcus* spp. Sometimes *Bacillus* spp. and many yeasts also give Oxidase positivity. Oxidase should never be done from colonies on blood agar because hemoglobin of blood has positive oxidase activity. Also, Oxidase should be done by picking the colony by edge of alme sterilized cover slip or glass slide and not nichrome loop, because Nichrome has Iron which has Positive oxidase activity itself. However,. Platinum loop may be used.

Figure. 1. Positive Oxidase disk test in P. aeruginosa

Oxidase positive bacteria	**Oxidase negative bacteria**	**Modified Oxidase positive bacteria (with 6% TMPPD)**
Pseudomonas aeruginosa	*Escherichia coli*	*Micrococcus* spp.
Burkholderia spp.	*Shigella* spp.	-------
Moraxella catarrhalis	*Salmonella* spp.	-------
Vibrio spp.	*Serratia* spp.	-------
Plesiomonas shigelloides	*Enterobacter* spp.	-----
Aeromonas spp.	-----	-------

Table 1. Oxidase positive and negative bacteria of medical importance

Catalase test - Catalase test is used to identify microorganisms that possess the catalase enzymes. These bacteria create the catalase enzymes, which will neutralize the hydrogen peroxide and cause bubbles by producing nascent Oxygen, indicating a positive test result. O. Loew in 1900 discovered Catalase test. Most commonly, facultative anaerobic bacteria and obligate aerobes produce the catalase enzyme. Bacterial colony is mixed with a few drops of 3% H_2O_2 on a slide or in a test tube, and observed for bubble formation within 10 seconds (6). Most bacteria are catalase positive because this enzyme helps in neutralization of oxidizing free radicals. Catalase should never be done from colonies on blood agar due to positive Oxidase activity of hemoglobin . Catalase should be done by picking the colony by edge of sterilized cover slip or glass slide and not nichrome loop, because Nichrome has Iron which has Positive catalase activity itself. However, Platinum loop may be used Also, for Mycobacterium tuberculosis, 30% H2O2 is used for semiquantitative catalase test (tube catalase).

Catalase positive bacteria	**Catalase negative bacteria**
Staphylococcus spp.	*Streptococcus* spp.
Pseudomonas aeruginosa	*Enterococcus* spp.
Mycobacterium tuberculosis	---------

Table 2: Catalase positive and negative bacteria

Coagulase test - The subsequent test is performed first by Loeb in 1904 to identify microorganisms that can produce the coagulase enzyme. It generally aids in detecting *Staphylococcus aureus*, which is a coagulase- and catalase-positive bacterium. Coagulase is one of *S. aureus*'s virulence-inducing components. During the reaction process, the coagulase enzyme will cause the blood plasma to coagulate by converting fibrinogen into fibrin. In order to perform this test, rabbit plasma and bacterial colonies in saline suspension are mixed. Bacteria will produce the coagulase enzyme, which will cause the plasma to coagulate as an indication of a positive reaction [7]. There are 2 types of coagulase tests :- free (tube) and bound(slide). Tube coagulase positivity is indicated by gellification of the plasma within 4-6 hours when incubated with liquid bacterial culture. It is to be note here that the gel again liquifies after 18-24 hours if kept at 37 degree C. Rabbit plasma is best for coagulase test but pooled human plasma can also be used. *Staphylococcus aureus* is positive for both slide and tube coagulase tests.

Figure 3. Picture of positive slide coagulase test (image: author)

Indole production test - The test described below is useful in identifying microorganisms with the capacity to produce the tryptophanase enzyme. Tryptophan is an amino acid that gets transformed into indole and Indole acetic acid by this enzyme. As a result, it can be tested by introducing various reagents like Ehrlich's reagent or Kovac's reagent (which gives red colour). Kovac's reagent has paradimethyl amino benzaldehyde(pDMAB) in isoamyl alcohol and concentrated HCl, whereas Ehrlich's have ethanol instead of isoamyl alcohol. As a result of the reagent and indole reaction, red rosindole dye is produced, a sign of positive test.

Indole positive	**Indole negative**
Escherichia coli (in 90-95%) cases	*Salmonella* spp.
Citrobacter koseri	*Klebsiella pneumoniae*

Klebsiella oxytoca	*Citrobacter freundii*
Shigella dysenteriae	Most strains of *Shigella flexneri*

Table 2: Table showing Indole test results

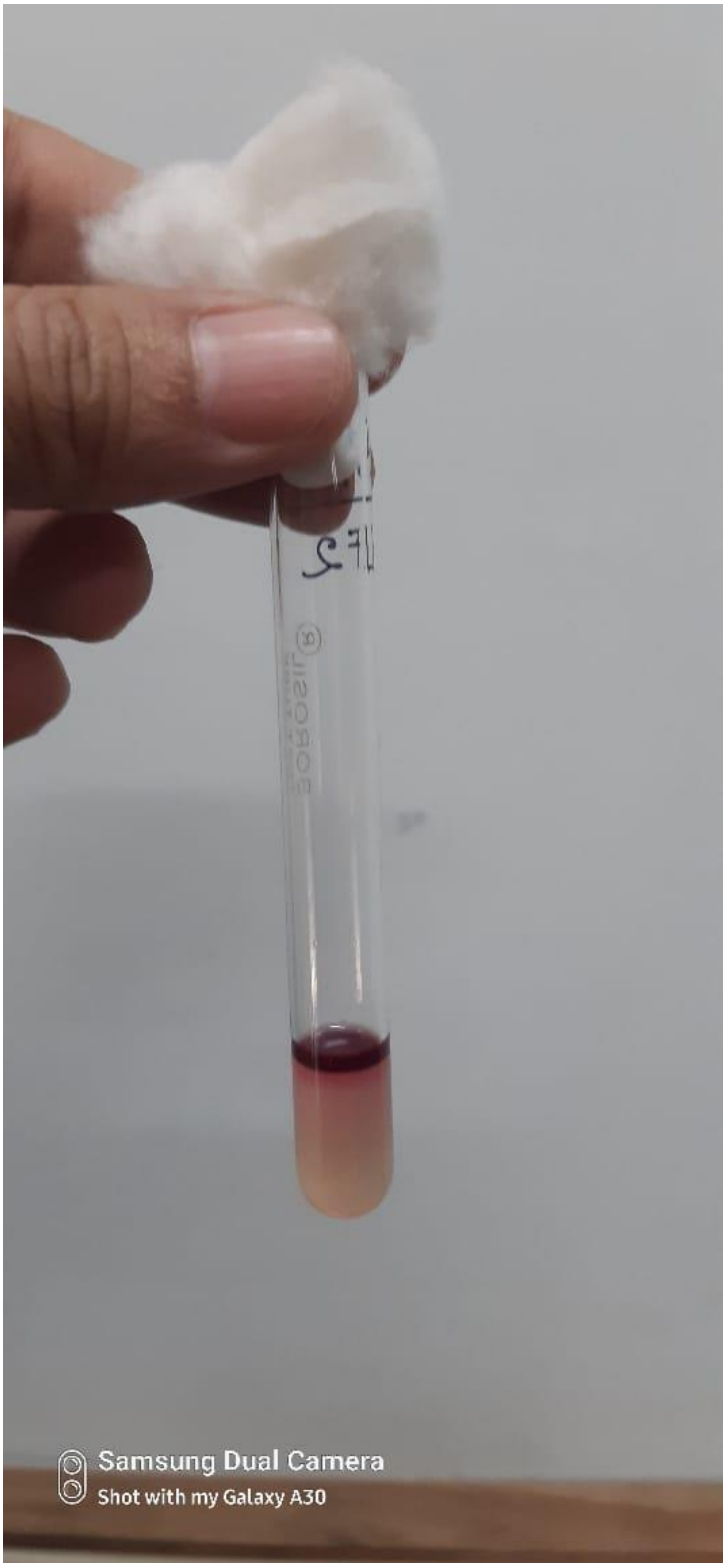

Figure 4: Positive Indole test (image: authors)

Figure 5: Negative Indole test (image: authors)

Spot indole test:- Here cinnamaldehyde is used instead of pDMAB. It is used mostly for anaerobic bacteria, and the isolate is rubbed on a filter paper containing cinnamaldehyde. Development of a dark brown to reddish colour within seconds indicates positive result.

Urease test - A urease test aids in identifying microorganisms that can produce the urease enzyme. Christensen in 1946 threw light on urease rest. Urease is a member of the superfamilies of amidohydrolases and phosphodoesterases. Urea is hydrolysed by urease enzyme into NH_3 and Carbon dioxide. The ammonia production will cause the medium's pH to shift to an alkaline level and its colour to pink at pH 8.1 provided phenol red is used as pH indicator, which indicates a positive result. *Helicobacter pylori* , which is urease positive can be detected rapidly with this test. The bacterium uses the urease enzyme to produce an alkaline environment to tackle gastric acid. Urease test is carried out by inoculating bacterial colonies in urea broth or urea agar. This test needs 18 to 22 hours of incubation for results but for *H. pylori* and *Proteus* spp., positive results may appear as rapidly as 4-6 hours after inoculating [9].

Urease positive bacteria	Urease negative bacteria
Staphylococcus aureus	*Escherichia coli*
Klebsiella pneumoniae	Salmonella spp.
Mycobacterium tuberculosis (in broth)	*Mycobacterium* avium (in broth)
Proteus spp, Providencia spp, Morganella spp.	Pseudomonas aeruginosa

Table 3 : List of urease positive and negative bacteria

Figure 6: Urease positive (left) and negative tubes(right). (image: authors)

Citrate utilization test (CUT) - The identification of microorganisms with the capacity to use citrate as a single source of Carbon, is made easier with the use of this test. For this test, Simmons' citrate agar is employed, which contains inorganic ammonium and citrate as sources of Nitrogen and Carbon, respectiveily. The CUT test is useful in identifying microorganisms that make the enzyme citrate permease, which transforms citrate into pyruvate and subsequently enters the metabolic cycle of organisms to generate energy and growth on culture media. The ammonium ions are converted into NH_3 when microorganisms use citrate, raising the pH of the medium. When the pH rises above 7.6, bromothymol blue's colour will change from green to blue due to the pH change. Bromothymol blue is used as an indicator. As a result it should form colonies on the top of the slant and change colour into royal blue. If colour has been changer but colonies does not appear it also contemplated as negative. Colonies must be there to deduce a positive result [10]. Also inoculum on Citrate agar should be light and colonies should first be suspended in normal saline before inoculation. Colonies should never be directly inoculated form solid media on Citrate agar, because colonies may also contain dead bacterial cells and living bacterial cells may utilise Carbon from dead cells to give false positive Citrate utilization result.

Citrate utilization positive bacteria	**Citrate utilization negative bacteria**
Pseudomonas aeruginosa	*Escherichia coli*
Klebsiella spp.	*Salmonella* Typhi

Table 4:- examples of Citrate utilization positive and negative important bacteria.

Figure 7: Citrate utilization positive (right) and negative (left) tubes

Triple sugar iron test

This test is useful in identifying microorganisms that are members of the Enterbacteriacea family. The test medium contains three sugars: lactose, sucrose, and glucose, each at a concentration of 1% (no, glucose is as 0.1%). As indicators, phenol red and ferric ammonium citrate are used. Sodium thiosulphate is also there as source of Sulphur. The medium is butted and slanted and both are inoculated. Since almost all inoculated bacteria use glucose, the concentration of glucose is kept low in comparison to other sugars. Within 16-18 hours of inoculation, the colour of the slant and butt will change to yellow as a result of acid production if the bacteria can use glucose in both aerobic and anaerobic circumstances. The media will continue to be yellow and acid production will continue if the bacteria can use the sucrose and lactose. If the bacteria are unable to use lactose or sucrose, they begin to use amino acids, which turns the medium alkaline and causes it to turn red from phenol red. If the bacteria is a strict aerobe, the colour of the butt remains the same, and the reaction will only happen in slant. Both will react if the bacteria is a facultative anaerobe. Agar media may rise or crack as a result of the production of hydrogen peroxide gas by reduction of thiosulfate by certain bacterial species [11]. TSI is interpreted as a/a and k/k , depending on acid formation in slant and butt. Slant reaction is used as numerator and butt reaction in denominator in a/a and k/k. H_2S is detected by formation of black colour due to formation of Iron-sulfur complex.

Mannitol motility test

Mannitol motility test is required for confirmation of weather the bacteria are motile or not. Mannitol is a sugar which are fermented by some bacteria and it turns into pink in colour, if Andrade's indicator is used in this medium. For identification of *Bacillus cereus*, mannitol should not be fermented and motility should be positive. It takes 18 to 22 hours of incubation for showing result. It is also useful for detecting *Enterococcus casseliflavus*.

Voges-Proskaur (VP) Test

This is an expansion of the methyl red test that locates organisms with the capacity to create the product butylene. The intermediate of this reaction, discovered by the use of alpha-naphthol and 40% KOH, is acetoin. Medium used is Glucose phosphate broth and the isolate needs to be grown for 48 hours at least. If KOH is present, acetoin will oxidize to diacetyl. Diacetyl will react with the guanidine component of peptone in the presence of alpha-naphthol, producing a deep red colour that indicates a positive result. The Methyl Red (MR) test is used for this. First identified during 1898. [12]. Write in table examples of VP positive and negative important bacteria. Examples of VP positive bacteria: *Enterobacter* spp., *Klebsiella* spp. and *Staphylococcus aureus*. *Staphylococcus aureus* is positive for both MR and VP. However, a bacterium that is positive for MR test is not usually positive for VP test.

Bile asculin agar test

This test was first described by Rochaix in 1924 and Swan first introduce the use of bile Esculin agar in 1954, is used to determine which bacteria hydrolyze esculin when bile is present. This test is a selective and differentiating medium for enterococcus identification. Bile and sodium azide are the selective media, while esculin is the differential medium. In contrast to sodium azide, bile will prevent the growth of Gram-ve bacteria, with the exception of enterococci and a few species of streptococci. When bile is present, some bacteria can hydrolyze esculin to produce esculetin, which reacts with ferric citrate in the medium to form phenolic iron complex, changing the color of agar from dark brown to black to indicate a positive test, such as for *Enterococcus* and *Streptococcus bovis*. The color will not alter in negative case [13]. Write in table examples of Bile aesculin hydrolysis positive and negative important bacteria.

Methyl Red-

Discovered by Clark and Lubs in 1915. This test used to identify coliform bacteria and their potential to produce acid from the glucose. And ultimately lowered the pH at about 4. Medium used is Glucose phosphate broth. A few drops of methyl red as an indicator when added to the 48-hour old microbial culture, a bright red colour is contemplated as positive. Shades fall intermediate between yellow and red result as doubtful positive. Methyl red indicator is prepared by dissolving 0.1 g methyl red in 300 ml 95% ethyl alcohol, which is then diluted to 500 ml with distilled water [14].

β-Galactosidase (ONPG)- This test is to identify late-lactose-fermenting paracolon organisms (like *Enterobacter* spp., *Citrobacter* spp., *Shigella sonnei*) known by their unique β-galactosidase activity which make them different from non lactose- fermenting bacteria like *Salmonella* and *Proteus* spp. From **o-**nitrophenyl-/3-D-galactopyranoside (ONPG), β – Galactosidase releases o-nitrophenol. Test organism should be grown in ONPG broth made by adding 250ml of ONPG solution to 750 ml of peptone water and stored at 4°C before use. The ONPG solution contains **0.6%** ONPG in 0.01M Na^2HPO^4 buffer at pH 7.5. Alternatively, it can be done with ONPG disk. Normal

saline is taken in 2 ml amounts and inoculated with loopful of the test bacterial culture previously grown on agar and ONPG disk. If there is yellow colour within 4-5 hours, it means that o-nitrophenol is formed, which indicates β -galactosidase activity. The colour may be change to bright yellow within 3 hours of incubation at 37°C. Tubes showing no colour change between 24 h considered as negative [14]. It is useful for detecting Late lactose fermenters like *Shigella sonnei, Citrobacter* spp. and some *Enterobacter* species([4].

Aesculin Hydrolysis

Discovered by Gemmell and Hodgkiss in 1964 to detect lactobacilli by incorporating 1% aesculin into NA media. Some microorganism has ability to hydrolyse glycoside to aesculetin and glucose, this product is detected by using 0.1% aesculin into a suitable liquid or agar nutrient medium that supports good growth of the organism and 0.05% ferric citrate is added to the medium**.** A positive reaction is displayed by the occurrence of a brownish-black colour, produced by aesculetin in combination with Iron. Coral like crystals appear after hydrolysis, which are assumed to be aesculetin. If acid or gas produce from glucose moiety, it was indication of aesculin decomposition [14]. Sometimes small amounts of aesculetin produced is insufficient to turn medium blackish, and then the medium should be kept in UV light. A lack of fluorescence implies that Aesculin has been broken down.

Hippurate Hydrolysis

This test was first put forward by Thirst in late 1957. Some Hippurate hydrolyzing bacteria are *Gardnerella vaginalis*, *Campylobacter jejuni*, *Listeria monocytogenes* and group of B Streptococci. Test is based on fact that hippurate is hydrolysed to form benzoate and glycine by the action of hippuricase. Result can be identified by adding acidic $FeCl_3$ to a broth culture grown in the presence of hippurate. . The test is performed by adding 1% sodium hippurate to a liquid nutrient media,(0.1 ml) aliquots of the acidic $FeCl_3$ reagent (12%) $FeC1_3.6H_2O$ and 2.5% conc. HCl in distilled water) are added to 1 ml of the uninoculated medium until the hippurate ppt just re-dissolves. The equal volume of the reagent is then added in to 1 ml of the clear centrifuged supernatant of incubated culture. It works on the principle that benzoate is less soluble than hippurate. The development of a precipitate in the test indicates that the less soluble benzoate has been produced. This identification method makes it very long process about 48 hr. For rapid detection ninhydrin as a indicator used to detect of glycerine formation, it takes only 2 hours [14].

Aromatic Ring cleavage mechanism

Some organisms obtain energy by metabolizing aromatic compounds like benzene ring; the cleavage usually occurs at either the meta or ortho positions, but sometimes both mechanisms appear to be involved. Colorimetric tests is used to detect different intermediate metabolic compounds that result from these cleavages. The test organisms have to grow in agar media to which 1g of sodium *p*-hydroxybenzoate is added previously. After good bacterial growth has been noticed, the organisms are suspended in 2 ml of 0.02 M-tris buffer (pH 8.0**)** before adding 0-5 ml toluene and 20pmoles of a solution of sodium protocatechuate than the tubes are shaken and the emergence of a bright yellow colouration within a few minutes indicates that meta cleavage of the substrate has taken place. If the result appear negative, the tube is shaken periodically for 1 h at 30°C until coloration develop. In next step for identification of ortho cleavage, about 1 gm of$(NH_4)_2S0_4$ crystals are added followed by one drop of 1% sodium nitroprusside solution and then by about 0.5 ml 0.880 sp. gr. ammonia. After mixing, the development of a deep violet colouration due to the presence of, 8-ketoadipate indicates the ortho cleavage of the substrate. The tests, has been used to differentiate pseudomonads by Stanier from other groups of organisms [14].

Hydrogen Sulphide production

The test was first sighted around 1920s to 1930s. Most of the microorganisms produce hidrogen sulphide gas when grown on media containing organic sulphur compounds. There are numerous tests known to be used to detect this H_2S production and these vary widely in sensitivity. The relatively insensitive tests differentiate the strong H_2S producers from the others. The test organism may be grown in a medium having sulphur (e.g. peptone) and an indicator of sulphide production (0.05% lead acetate, or 0.025% ferric ammonium citrate, or 0.015% ferrous acetate); the medium then turns black if H_2S is produced. The culture should be incubated for 7 days. Growth at 22°C or 30°C instead of 37°C may positively enhance H_2S production by some bacteria [14]. H_2S can also be detected in TSI and also by putting a lead acetate paper above the liquid culture of the bacterium without touching it. The paper turns black overnight due to formation of lead acetate.

Phenylalanine deaminase

Many bacteria deaminate phenylalanine to produce phenylpyruvic acid. In later 1957, Ewing and his team formulated a medium to test organisms that has the capacity to deaminate the phenylalanine oxidatively [4]. Organisms typically associated with this test are of *Proteus, Morganella* and *Providencia* genera (Tribe Proteae). The test organism should be grown on phenylalanine containing agar plates and than incubated overnight. If bacterial culture produces phenylpyruvic acid it will turns a $FeC1_3$ indicator solution green. Therefore when, the slant of growing culture on Phenylalanine agar is flooded with 0.2 ml of a 10% aqueous solution of $FeCl_3$ which turns parrot- green if deamination has occurred [14].

Amino-acid decarboxylases

The test was first introduce by Moeller in 1955. Respective test is used to identify members of the *Enterobacteriaceae.*Tests is based on bacterial decarboxylation of lysine, arginine, ornithine and glutamic acid**.** L-amino acid (L(+)-lysine dihydrochloride**,(** +)- arginine monohydrochloride, L(+)-ornithine dihydrochloride, or L(+)- glutamic acid each 1%, is incorporated in a suitable medium containing peptone, meat extract ,pyridoxal,glucose, 0.2% solution of bromothymol blue, 0.2% solution of cresol red and pH adjusted 6.0**.** An inoculum from the test organism culture grown on this media is introduced with a straight wire through the paraffin layer and examined daily for up to 4 days. As soon as bacteria ferment the glucose in the medium, the indicator emits yellow color. The control tube, without amino acid, will then remain yellow. The tube express change in color from yellow to violet or purple later in the tests defines that alkaline degradation products have been produced in the course of decarboxylation of the particular amino-acid [14].

Arginine dihydrolase

Certain organisms have the ability to produce an alkaline reaction in arginine-containing media under relatively anaerobic conditions. First demonstrated by Thornley (1960) to differentiate between Gram-negative aerobic bacteria, especially *Pseudomonas* spp and other. The alkaline reaction is thought as a result of the production of ornithine, CO_2 and NH_3 from the arginine. Medium should be placed in screw capped vials with test organisms are stab inoculated and sealed. After 7 days of incubation period, if color changes occur from yellowish-orange to red, means the given culture is positive for arginine dihydrolase [14].

Proteinases

Some microorganisms are proteolytic in nature and can be encounter by incubated in Loeffler's serum slope will eventually liquefy the medium, they form clear zones while incubated in milk agar or even in egg yolk agar also due to decomposition of protein. A saccharolytic and proteolytic combined effect will appear in litmus milk as media [14]. Protease can also be detected by development of clearing around colonies on egg yolk agar.

Lipase

It is denoted by appearance of shiny colonies on Egg yolk agar. It is present in *C. botulinum* and many other bacteria also. It is also produced by *Candida albicans* and helps differentiate it from *Candida dubliniensis*.

Lecithinase

It is produced by *Bacillus cereus* and also many other pathogenic bacteria, and is expressed as development of a zone of opacity or haziness around colonies on Egg yolk agar. It occurs due to breakdown of lecithin of egg by the enzyme lecithinase in the bacteria, followed by liberation of free fatty acid, combination of free fatty acid with Calcium of egg and formation of opaque Calcium-fatty acid complex.

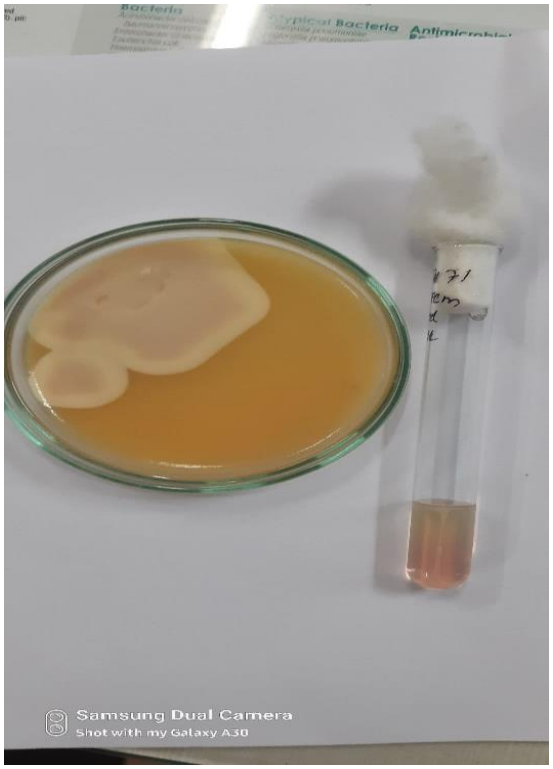

Figure 8. Lecithinase (on egg yolk agar) and no mannitol breakdown in *Bacillus cereus* (image: author)

Amylase

Amylase producing bacteria are spotted easily on starch containing media. After the growth of bacterial colony occurs, plates should eventually flooded with dilute iodine solution, a clear zones around the viable colony forming unit displayed the the test organism produse amylase as its biomolecule and starch present in the media is hydrolyzed by it. In others, that don't contain amylase will not ultimately hydrolyze the starch, develop deep blue coloration with iodine [14].

Haemolysis

Microorganisms producing hemolytic enzymes by various hemolysins, can be identified by hemolysis test. When the test organism is incubated in media having sheep, horse or goat blood, it may show 3 types of zones based on their ability to break hemoglobin. Clearly defined zone show complete hemolysis called β-hemolysis mainly produced by Streptococci on horse and sheep blood agar, and also *Staphylococcus aureus*, some enteropathogenic strains of *Escherichia coli* and *Bacillus cereus*. Greenish discoloration of blood agar medium around a colony denotes α-hemolysis, due to incomplete lysis of the RBCs, typically produced by *Streptococcus pneumoniae*, Streptococci of the viridans group (or oral Streptococci) and *Enterococcus* spp. and intermediate ill-defined zones without discoloration are termed gamma-hemolysis [14]. Gamma-hemolysis means no hemolysis. Also, a highly well-defined zone of hemolysis may actually be hemodigestion, as seen in *Bacillus* spp.

Phosphatase- The test was discovered by Stuart S.Kind in 1950. Phosphatase is the enzyme which cleave phosphate group from compound by hydrolyzing ester bond. Some bacteria containing this enzyme when grown on phenolphthalein diphosphate supplemented media at 45^0C liberate free phenolphthalein, detected by placing ammonia in lid of inverted plate, colonies liberate phenolphathalein turns pink in color when exposed to ammonia [14]. It is one of the most specific tests to identify *Staphylococcus aureus*.

KCN tolerance test:- Some bacteria like Enterobacter spp. and *Citrobacter* spp. Can tolerate Potassium cyanide and grow in broth containing the chemical. This also helps in diagnosis.

Nitrate reduction test: It contains Nutrient broth with Potassium nitrate (KNO_3). Bacteria like *Escherichia coli* and *Pseudomonas aeruginosa* reduce Nitrate to nitrite, which can be detected by adding Sulfanilic acid and N,N-dimethyl alpha naphthyl amine. A red color indicates positive Nitrate reduction. Modifications have come like Greiss' rapid nitrate reduction test. *Acinetobacter* spp. do not reduce Nitrate to Nitrite.

Discussion

Surgical site infections and many other infections can be caused by medically important bacteria. Despite new identification methods like PCR and sequencing, phenotypic and biochemical tests still hold the key for laboratory identification due to their lower cost and ease in carrying out and interpretation. Newer modifications of biochemical tests have come like Rapid carbohydrate utilization test, API -20E and others, but they have not been able to replace old conventional biochemical tests. Hence they are still very important to know and discuss. Tests like

Catalase, coagulase and Indole production are still the mainstay for proper laboratory diagnosis of many bacterial infections. Also, one needs to form flow charts for proper and accurate identification, like if one gets Gram positive cocci in clusters and golden-yellow pigmented colonies on Nutrient agar or Salt-milk agar, and if the isolate is catalase and coagulase positive, it is most likely to be Staphylococcus aureus. Likewise if one gets Lactose fermenting Gram negative bacilli and the isolate is catalase positive and Indole positive with no Citrate utilization and Urease activities, and in TSI one gets a/a with gas but no H2S, it may be *Escherichia coli*. Such flow charts help in more precise and accurate identification. Also sometimes 2 or 3 biochemical tests can be combined in 1 medium or tube to reduce workload, like SIM medium (Sulfide-Indole-motility), MIU (Motility-Indole-Urease) medium and others. They also yield satisfactory results.

Conclusion

Among the biochemical tests, assays such as catalase test, amylase and nitrate test are used to identify the gram positive bacteria while oxidase test, urease test, indole test, arginine dihydrolase test, hydrogen sulfide test, methyl red test, and voges-proskauer test are used to encounter gram negative bacteria. Some special test like heamolysis test, phosphatase test, amino acid decarboxylase test, phenylalanine deaminase test and aromatic ring cleavage test is used to illuminate some special groups of bacteria carrying unique properties. Through this paper, one can get a clear overview of biochemical test and their advancement with time can be understandable easily. Biochemical test is the key for easily microbial identification for researchers in past, present and also in future, from various sites of infections.

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DOI: 10.31579/2690-1897/163