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RESEARCH ARTICLE

IDENTIFICATION OF MICROBIAL FLORA FROM UTI PATIENTS VIA BIOCHEMICAL REACTIONS

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ABSTRACT

Urinary Tract Infection (UTI) is one of the most common infectious disease ranking next to upper respiratory tract infection is the cause of morbidity and mortality in human. They are mostly caused by bacteria. 50-80% women experience UTI at least once or twice in their lives. Enteric pathogens (e.g. E.coli.) are most commonly responsible, it is well established that for UTI, but Klebsiella sp., Enterobacter sp. and Pseudomonas aeruginosa, Proteus sp. are also responsible grams positive organisms including Enterococcus sp. Staphylococci and Streptococci have also been found to cause severe infections in human being. Therefore, studying and identifying bacterial pathogens causing UTI through biochemical test is the highest priorty.

INTRODUCTION

Urine in the human bladder is normally sterile. The presence of bacteria in the urine is called bacteriuria (Ramzan *et al*., 2004). Bacteria normally present in the colon and hence, on the perineum, may enter the urethral opening from the skin around the anus and genitals. Women are more susceptible to UTI because their urethral opening is nearer to the vagina and anus (the common source of bacteria) and their urethra is shorter, which provides easier access to the bladders. 50-80% women experience UTI at least once or twice in their lives (Puri and Malhotra, 2009). Urinary tract infections (UTIs) are very common. They can be painful and uncomfortable, but they usually pass within a few days or can be easily treated with a course of antibiotics. UTI are more common in women than in men. It is estimated that half of all women in the UK will have a UTI at least once in their lives, 1 in 2000 healthy men may develop one each year. Children can also get UTIs, although this is less common. A UTI develops when part of the urinary tract becomes infected, usually by a group of bacteria. Bacteria can directly enter the urinary tract through the urethra or, more rarely, through the bloodstream. There is usually no obvious reason why the urinary tract gets infected, although some women find that they develop a UTI after having sex.

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It is well documented that UTIs are not sexually transmitted infections (STIs) but it is reported that some patients feel irritations/inflammation while having sex of unknown origins, but, in a few studies, it has been found that unhygienic conditions during sexual activities can sometimes triggers a UTI like infection and pelvic inflammatory disease UTIs in men are far less common than in women and need investigation to find an underlying cause. This cause may include narrowing of the urethra (a stricture), a previous STI, a bladder stone or any associated disease condition with the prostate gland. Enteric pathogens (e.g. E.coli.) are most commonly responsible, it is well established that for UTI, but Klebsiella sp., Enterobacter sp. and Pseudomonas aeruginosa, Proteus sp. are also responsible grams positive organisms including Enterococcus sp. Staphylococci and Streptococci have also been found to cause severe infections in human being (Farahtullah *et al*., 2009). A biochemical identification of isolated bacteria is performed in order to confirm the species of isolated organism.

MATERIALS AND METHODS

Most urine specimens were obtained from adult patients via the clean-catch midstream technique. This technique has the following advantages: it is neither invasive nor uncomfortable, it is simple and inexpensive, it can be performed in almost any clinical setting, there is no risk of introducing bacteria into the

bladder by catheterization, and there is no risk of complications. Colony counts from urine specimens collected by this method correlate reasonably well with those of specimens collected via suprapubic aspiration or straight catheterization.

Gram stain

The Gram stain has been in existence for more than 100 years, and remains a key starting point to identify microbial species. The stain makes use of the differing membrane structures between Gram positive (single cell membrane with a tough outer cell wall of peptidoglycan), and Gram negative organisms (have two layers of membranes, with a thin layer of peptidoglycan sandwiched between them).

Steps are as follows:

Heat fix: Light the Bunsen burner. Pass the slide (with the bacteria mounted on it) through the interface between the blue flame and the yellow flame - this is the hottest region of the flame - 5 times. The slide should sit in this region for no more than a second. Place slide on a slide holder or a rack. Flood (cover completely) the entire slide with crystal violet. Let the crystal violet stand for about 60 seconds. When the time has elapsed, wash slide for 5 seconds with the water bottle. The specimen should appear blue - violet when observed with the naked eye.

- Now, flood slide with the iodine solution. Let it stand about a minute as well. When time has expired, rinse the slide with water for 5 seconds and immediately proceed to step three. At this point, the specimen should still be blue -violet.

- This step involves addition of the decolorizer, ethanol. To be safe, add the ethanol dropwise until the blue□violet color is no longer emitted from your specimen. As in the previous steps, rinse with the water for 5 seconds.

- The final step involves applying the counterstain, saffranin. Flood the slide with the dye we did in steps 1 and 2. Let this stand for about a minute to allow the bacteria to incorporate the saffranin. Gram positive cells will incorporate little or no counterstain and will remain blue-violet in appearance. Gram negative bacteria, however, take on a pink color and are easily distinguishable from the Gram positives. Again, rinse with water for 5 seconds to remove any excess of dye.

EMB – Eosin Methylene Blue Agar is a selective and differential medium. Eosin differentiates between two major coliforms: E. coli (smaller, green-metallic sheen) and Enterobacter aerogenes (larger, rose color). Methylene blue selectively inhibits the growth of Gram+ bacteria. With this media we can also determine which bacteria are Gram-negative and which are Gram-positive, because only Gram-negative bacteria grow on this special media. The enhanced cell walls of Gram-negative bacteria protect these bacteria from the dye in the EMB plates. The dye is able to enter the cells of Gram positive bacteria and kill them.

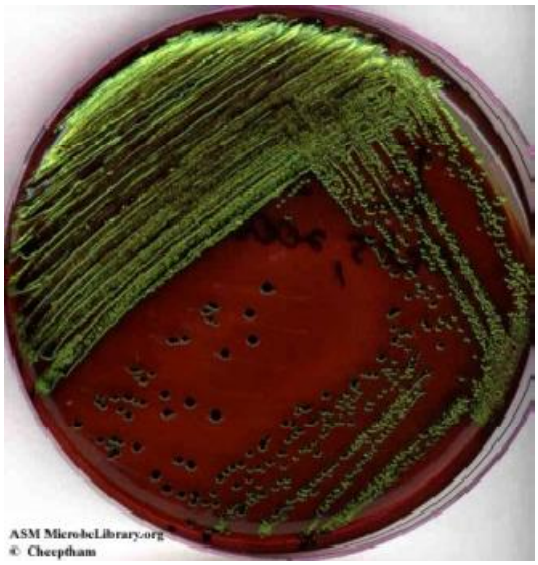


Fig. 1.

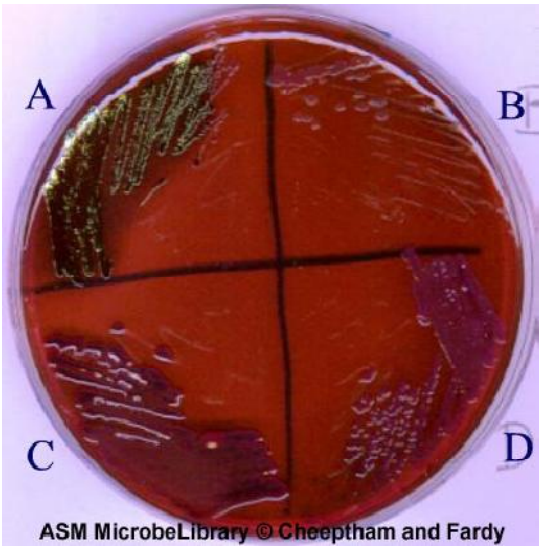


Fig. 2.

EMB: Eosin Methylene Blue
A=Escherichia coli
B=Pseudomonas
C=Klebsiella
D=Enterobacter aerogenes

MacConkey Agar

Demonstrates the ability of a gram negative bacterium to metabolize Lactose. MacConkey agar is both a selective and differential medium frequently used in culture testing. It contains crystal violet dye and bile salts, both of which inhibit the growth of most gram-positive bacteria. It contains lactose (a sugar) and neutral red indicator (a pH indicator which is yellow in a neutral solution, but turns pink to red in an acidic environment), which allow for differentiation. On MacConkey agar, Escherichia coli and Enterobacter aerogenes would ferment the lactose producing acid and would form colonies pink to red in color. On the same medium, Salmonella,

Shigella, and Pseudomonas species would not ferment the lactose and would form off-white colonies. The red colored colonies show that acid was produced from lactose, meaning the bacteria could utilize lactose as a carbon.

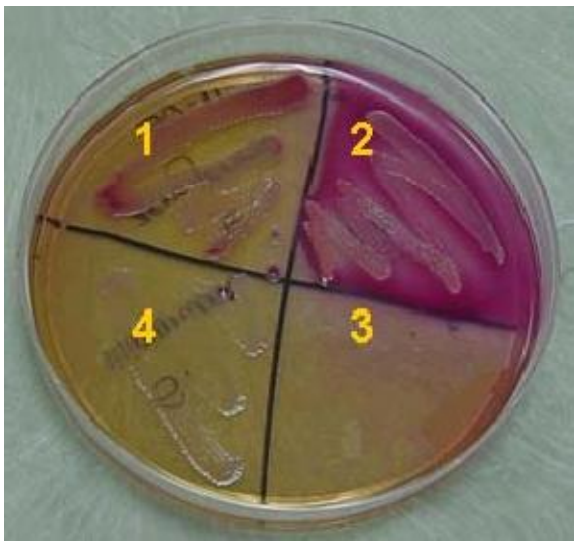


Fig. 3.

Quadrant 1: Growth on the plate indicates the organism, Enterobacter aerogenes, is not inhibited by bile salts and crystal violet and is a gram-negative bacterium. The pink color of the bacterial growth indicates E. aerogenes is able to ferment lactose.

Quadrant 2: Growth on the plate indicates the organism, Escherichia coli, is not inhibited by bile salts and crystal violet and is a gram-negative bacterium. The pink color of the bacterial growth indicates E. coli is able to ferment lactose.

Quadrant 3: Absence of growth indicates the organism, Staphylococcus epidermidis, is inhibited by bile salts and crystal violet and is a gram-positive bacterium.

Quadrant 4: Growth on the plate indicates the organism, Salmonella typhimurium, is not inhibited by bile salts and crystal violet and is a gram-negative bacterium. The absence of color in the bacterial growth indicates S. typhimurium is unable to ferment lactose.

Different test involved in the detection of uropathogens

INDOLE TEST (http://microbeonline.com/indole-test-principle-procedure-results)

Indole test is used to determine the ability of an organism to spilt amino acid tryptophan to form the compound indole.

Indole production is detected by Kovac's or Ehrlich's reagent which contains 4 (p)-dimethylamino benzaldehyde, this reacts with indole to produce a red coloured compound.

Indole test helps to differentiate Enterobacteriaceae and other genera.

Two methods are in used

1. a spot indole test, which detects rapid indole producing organisms and
2. a conventional tube method requiring overnight incubation, which identifies weak indole producing organisms.

Methods

a. Inoculate the tryptophan broth with broth culture or emulsify isolated colony of the test organism in tryptophan broth.
b. Incubate at 37°C for 24-28 hours in ambient air.
c. Add 0.5 ml of Kovac's reagent to the broth culture.

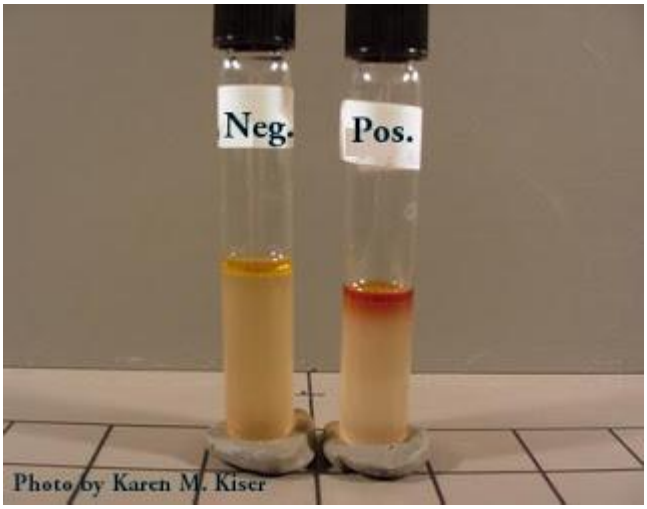


Fig. 4.

Reaction involved

Tryptophan ⟶ pyruvic acid + ammonia + Indole

Expected result

Positive: Pink colored rink after addition of appropriate reagent

Negative: No color change even after the addition of appropriate reagent. e.g. *Klebsiella Pneumoniae.*

Indole positive organisms

Most strains of *E.coli, P. vulgaris*, *M. morganii* and *Providenica* are indole positive. Indole test can also aid in species differentiation.

1. *Klebsiella* species: *Klebsiella oxytoca* is indole positive whereas *Klebsiella pneumoniae* is indole negative.
2. *Citrobacter* species: *Citrobacter Koseri* is indole positive where as *Citrobacter freundii* is indole negative
3. *Proteus* species: *Proteus Vulgaris* is indole positive whereas *Proteus mirabilis* is indole negative

METHYL RED TEST (http://web.clark.edu/tkibota/240/Unknowns/MethylRed.htm)

The Methyl-Red test for the ability to perform mixed-acid fermentation. MR-VP broth contains glucose, peptone, and a phosphate buffer. Organisms that perform mixed-acid fermentation produce enough acid to overcome the buffering capacity of the broth, so a decrease in pH results. Organisms that perform other kinds of fermentation cannot overcome the buffering capacity of the broth. After incubation, the pH indicator Methyl Red is added to the broth. Methyl Red is red at pH below 4.4 (this would be a positive result) and yellow at pH above 6.0. An orange color indicates an intermediate pH and would be considered a negative result. This test is among a suite of tests (Indole, Methyl-Red, Vogues-Proskauer, and Citrate) that are used to differentiate among the Gram-Negative bacilli in the family Enterobacteriaceae.

Fig. 5.

RESULTS

+ = Positive (Red)

- = Negative (Orange to Yellow)

V = Variable

Methods

1. Obtain two MR-VP broths from the back shelf.
2. Inoculate one broth using aseptic technique. Leave the other broth uninoculated (this will be a control).
3. Incubate at appropriate temperature (whatever temperature your organism grows well at). Incubate for two to five days.
4. Obtain your broths from the incubator.
5. Add a dropperful of Methyl Red to each broth. Methyl Red is found in small dropper bottles on the back shelf.
6. Observe the color (which should develop within a few minutes).

VOGES–PROSKAUER TEST

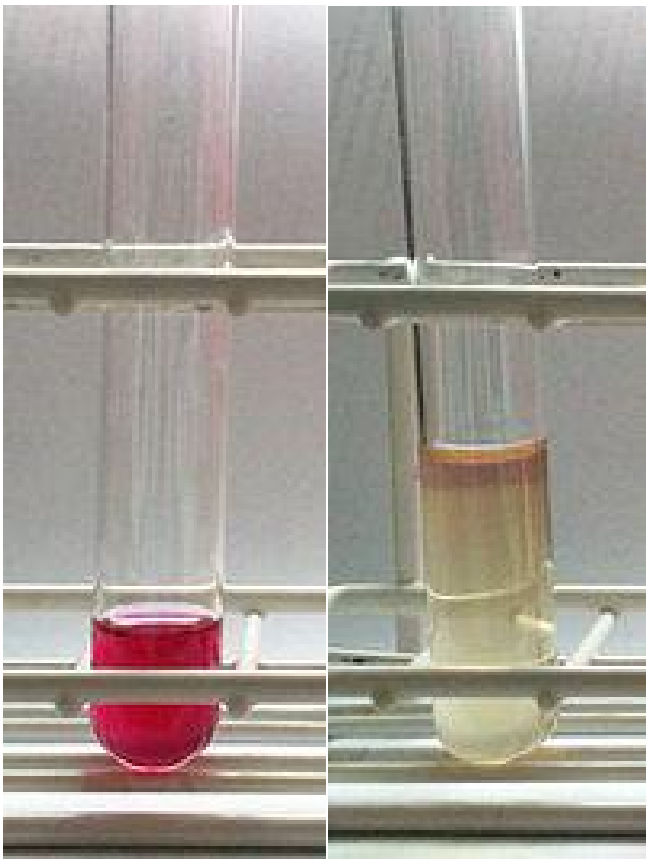


Fig. 6.

Enterobacter cloacae ***Proteus myxofaciens***

Voges–Proskauer /ˈfoʊgəs ˈprɒskaʊ.ər/ or **VP** is a test used to detect acetoin in a bacterial broth culture. The test is performed by adding alpha-naphthol and potassium hydroxide to the Voges-Proskauer broth which has been inoculated with bacteria. A cherry red color indicates a positive result, while a yellow-brown color indicates a negative result.

Procedure: First, add the alpha-naphthol; then, add the potassium hydroxide. A reversal in the order of the reagents being added may result in a weak-positive or false-negative reaction.

VP is one of the four tests of the IMViC series, which tests for evidence of an enteric bacterium.

Reaction involved

2 pyruvate + NADH2CO_2 + 2,3-butanediol.

VP positive organisms include *Enterobacter*, *Klebsiella*, *Serratia marcescens*, *Hafnia alvei*, *Vibrio damsela*, and *Vibrio alginolyticus*. (Farahtullah *et al.,* 2009)

VP negative organisms include *Citrobacter freundii*, *Shigella*, *Yersinia*, *Edwardsiella*, *Salmonella*, *Citrobacter*, *Vibrio furnissii*, *Vibrio fluvialis*, *Vibrio vulnificus*, and *Vibrio parahaemolyticus*

OXIDASE TEST (http://howmed.net/microbiology/ oxidase-test-principle-procedure-and-interpretations)

This test depends on the presence of cytochrome oxidase in bacteria that will catalyze the transport of electrons between electron donors and redox dye. Tetramethyl-p-phenylene diamine dihydrochloride in the reagent is reduced to deep purple color. This test is used for the screening of Pseudomonas, Vibrio, Neisseria, Brucella and Pasteurella, which give positive test. Enterobacteriaceae are oxidase negative.

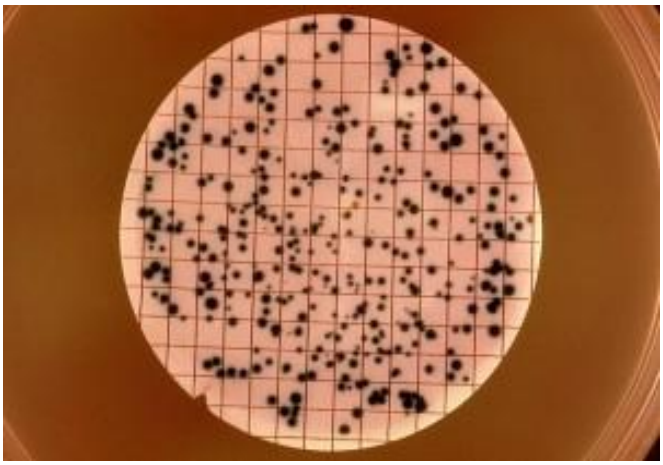


Fig. 7.

Oxidase reagent is specially prepared as 10g/l or 1% solution of tetramethyl-p-phenylene diamine dihydrochloride.

Procedure

Filter Paper Method: Place a piece of filter paper in petri dish and add 3 drops of freshly prepared oxidase reagent. Using a sterile glass rod, remove a colony of test organisms from a culture plate and smear it on the filter paper.

Interpretation: Oxidase positive organisms give blue color within 5-10 seconds, and in oxidase negative organisms, color does not change.

Reaction involved

$4\ Fe^{2+}$-cytochrome $c_{red} + 8H^{+}_{in} + O_2 \rightarrow 4\ Fe^{3+}$-cytochrome $c_{ox} + 2H_2O + 4H^{+}_{out}$

CATALASE TEST (http://microbeonline.com/catalase-test-principle-uses-procedure-results)

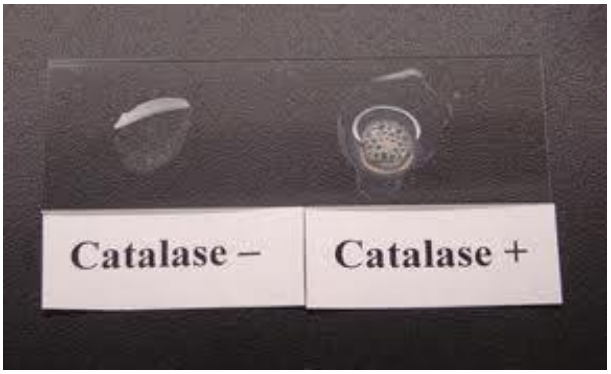


Fig. 8.

Catalase is an enzyme, which is produced by microorganisms that live in oxygenated environments to neutralize toxic forms of oxygen metabolites; H_2O_2. The catalase enzyme neutralizes the bactericidal effects of hydrogen peroxide and protects them. Anaerobes generally lack the catalase enzyme. Catalase mediates the breakdown of hydrogen peroxide H_2O_2 into oxygen and water. To find out if a particular bacterial isolate is able to produce catalase enzyme, small inoculums of bacterial isolate is mixed into hydrogen peroxide solution (3%) and the rapid elaboration of oxygen bubbles occurs. The lack of catalase is evident by a lack of or weak bubble production. Catalase-positive bacteria include strict aerobes as well as facultative anaerobes. They all have the ability to respire using oxygen as a terminal electron acceptor. Catalase-negative bacteria may be anaerobes, or they may be facultative anaerobes that only ferment and do not respire using oxygen as a terminal electron acceptor (ie. Streptococci).

Uses of catalase test

1. The catalase test is primarily used to distinguish among Gram-positive cocci: Member of the genus *Staphylococcus* are catalase-positive, and members of the genera *Streptococcus* and *Enterococcus* are catalase-negative.
2. Catalase test is used to differentiate aerotolerant strains of *Clostridium*, which are catalase negative, from *Bacillus* species, which are positive.
3. Semiquantitative catalase test is used for the identification of *Mycobacterium tuberculosis*

Catalase test can be used as an aid to the identification of Enterobacteriaceae. Members of Enterobacteriaceae family are Catalase positive.

Procedure of catalase test

1. Transfer a small amount of bacterial colony to a surface of clean, dry glass slide using a loop or sterile wooden stick
2. Place a drop of 3% H_2O_2 on to the slide and mix.
3. A positive result is the rapid evolution of oxygen (within 5-10 sec.) as evidenced by bubbling.
4. A negative result is no bubbles or only a few scattered bubbles.

5. Dispose of your slide in the biohazard glass disposal container.

Reaction involved

$2\ H_2O_2 \rightarrow 2\ H_2O + O_2$

Precautions while performing catalase test

1. Do not use a metal loop or needle with H_2O_2; it will give a false positive and degrade the metal.
2. If using colonies from a blood agar plate, be very careful not to scrape up any of the blood agar as blood cells are catalase positive and any contaminating agar could give a false positive.

CITRATE UTILIZATION TEST (http://microbeonline.com/citrate-utilization- test-principle-procedure-expected-results-and-positive-organisms)

Principle: Citrate utilization test is commonly employed as part of a group of tests, the IMViC (Indole, Methyl Red, VP and Citrate) tests, that distinguish between members of the Enterobacteriaceae family based on their metabolic by-products. Citrate utlilization test is used to determine the ability of bacteria to utilize sodium citrate as its only carbon source and inorganic ($NH_4H_2PO_4$) is the sole fixed nitrogen source. When an organic acid such as citrate (Remember Krebs cycle) is used as a carbon and energy source, alkaline carbonates and bicarbonates are produced ultimately. In addition, ammonium hydroxide is produced when the ammonium salts in the medium are used as the sole nitrogen source. The color change of the indicator is due to alkali production by the test organism as it grows on the medium. Growth usually results in the bromothymol blue indicator, turning from green to blue. The bromothymol blue pH indicator is a deep forest green at neutral pH. With an increase in medium pH to above 7.6, bromothymol blue changes to blue.

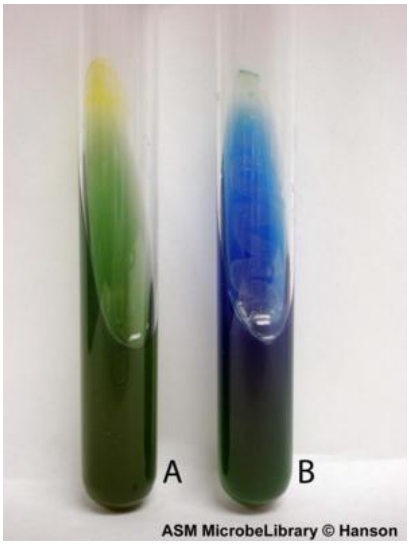


Fig. 9.

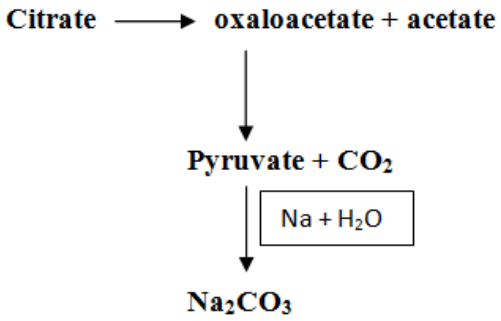
Citrate Utilization Test

A: Negative B: Positive

Procedure of citrate utilization test

1. Inoculate Simmons Citrate Agar lightly on the slant by touching the tip of a needle to a colony that is 18 to 24 hours old.
2. Incubate at 35°C to 37°C for 18 to 24 hours. Some organisms may require up to 7 days of incubation due to their limited rate of growth on citrate medium.
3. Observe the development of blue color; denoting alkalinization.

Reaction involved



1. Citrobacter spp
2. *Klebsiella pneumoniae*
3. *Enterobacter aerogenes*

Members of enterobacteriaceae such as *Escherichia coli, Proteus mirabilis etc* gives negative citrate utilization test.

VII. TRIPLE SUGAR IRON AGAR (http://www.austincc.edu/microbugz/triple_sugar_iron_agar.php)

Triple sugar iron agar (TSI) is a differential medium that contains lactose, sucrose, a small amount of glucose (dextrose), ferrous sulfate, and the pH indicator phenol red. It is used to differentiate enterics based on the ability to reduce sulfur and ferment carbohydrates. As with the phenol red fermentation broths, if an organism can ferment any of the three sugars present in the medium, the medium will turn yellow. If an organism can only ferment dextrose, the small amount of dextrose in the medium is used by the organism within the first ten hours of incubation. After that time, the reaction that produced acid reverts in the aerobic areas of the slant, and the medium in those areas turns red, indicating alkaline conditions. The anaerobic areas of the slant, such as the butt, will not revert to an alkaline state, and they will remain yellow. This happens with *Salmonella* and *Shigella*.

Reaction involved

H_2S+ iron salts ⟶ black precipitate (ferric sulfide)

NOTE

SIM medium should be read after an incubation of only 24 hours because a longer incubation time can cause a false negative. Vigorous fermenters such as *Escherichia coli* and E*ntrobacter cloacae* will ferment all the available sugars and then begin using the amino acids. This will produce amine groups and cause the medium to turn alkaline. If an organism can reduce sulfur, the hydrogen sulfide gas which is produced

will react with the iron to form iron sulfide, which appears as a black precipitate. If the precipitate is formed, it can mask any acid/alkaline results. Sulfur reduction requires an acidic environment, so if the black precipitate is present, some fermentation took place. If the butt of the slant is obscured by the precipitate, look at the top of the slant to determine if the organism could ferment only dextrose (red), or if it could ferment either lactose and/or sucrose (yellow). If the fermentation produced gas, you may see fissures in the medium, or the entire slant may be raised above the bottom of the test tube.

UREASE TEST (http://www.austincc.edu/microbugz/urease_test.php)

Urease broth is a differential medium that tests the ability of an organism to produce an exoenzyme, called urease, which hydrolyzes urea to ammonia and carbon dioxide. The broth contains two pH buffers, urea, a very small amount of nutrients for the bacteria, and the pH indicator phenol red. Phenol red turns yellow in an acidic environment and fuchsia in an alkaline environment. If the urea in the broth is degraded and ammonia is produced, an alkaline environment is created, and the media turns pink.

Fig. 10
Staphylococcus aureus
Exhibits acidic fermentation

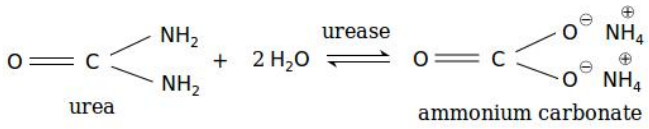
Fig. 11
Salmonella typhimurium
ferments glucose & reduces sulfur

Fig. 12
***Micrococcus luteus* uses the amino acids and does not grow in the butt of the slant**

Fig. 13.
Bacillus megaterium **fermented sugars but didn't grow in the anaerobic area of the butt**

Fig.14.
Enterobacter aerogenes **fermented the sugars but turned to the amino acids**

Reaction involved



Proteus mirabilis **is rapid urease positive as evidenced by the pink color of the media.**

Escherichia coli **on the right is negative.**

Many enterics can hydrolyze urea; however, only a few can degrade urea rapidly. These are known as "rapid urease-positive" organisms. Members of the genus *Proteus* are included among these organisms. Urea broth is formulated to test for rapid urease-positive organisms. The restrictive amount of nutrients coupled with the use of pH buffers prevent all but rapid urease-positive organisms from producing enough ammonia to turn the phenol red pink. Urease broth can be used to differentiate members of the genus *Proteus* (as well as those of *Morganella* and *Providencia*, but we don't use those in our lab) from other enterics.

Germ Tube Test (http://www.austincc.edu/microbugz/urease_test.php)

Principle

The Germ Tube Test is a screening procedure used to differentiate Candida albicans from other yeast. Approximately 95 - 97% of Candida albicans isolated develop germ tubes when incubated in a proteinaceous media. Germ tubes are short non-septate germinating hyphae. They are ½ the width and 3 - 4 times the length of the cell from which they arise. The junction of the germ tube and cell is not constricted. Buds and pseudo-hyphae can be distinguished from germ tubes by the constricted attachment.

Reagents

Sheep serum (Colorado serum #CS1342)

Procedure

1. Put 0.5 ml (12 drops) of sheep serum in a 10 x 75 mm tube.

2. Make a light suspension of the suspect yeast colonies by touching 1-2 large colonies or 3-4 smaller colonies with a sterile wooden applicator stick and then inoculating the sheep serum with the applicator stick.

Note: Too large of an inoculums will inhibit germ tube formation.

3. Incubate the tube for 2-3 hours in a 35 - 37°C incubator.

Warning: *Do not over-incubate the tube. Candida tropicalis may produce pseudo-germ tubes after 3 hours of incubation.*

4. Place a drop of the suspension on a slide using a Pasteur pipette and cover slip.

5. Examine the wet mount microscopically for production of germ tubes (long tube-like projections extending out from the yeast cells).

RESULTS

Out of 175 urine samples collected for the study (30.23%) showed the significant bacteriuria. The mean age in years was 35.6 (0-93years). Male were 93 and female 79. The main aim of the study was identification and characterization of microbial flora of UTI patients through biochemical test.
The Table 1.

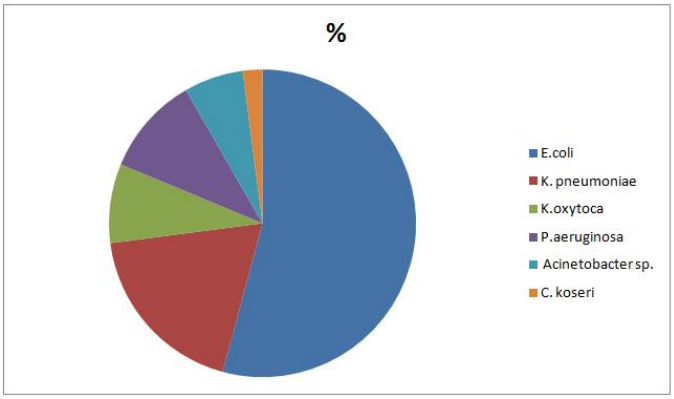


Fig. 15.
Pie chart depicting the percentage (%) of different uropathogens

study shows 50% E.coli, 17.30% K. pneumonae, 7.69% K.oxytoca, 9.61% P. aeruginosa, 5.76% Acetinobacter sp., 1.92% C. koseri, 1.92% P.vulgaris, 5.76% Candida non albicans.

Conclusion

UTI is very common in patients; so, regular urine routine examination and culture is suggested to diagnose UTI. After diagnosis of UTI, active treatment should be started to prevent morbidity and mortality in them. Empirical treatment with an antibiotic should be started and urine culture including all positive confirmed cases i.e. Biochemical Test of UTI either for *E. coli, K.pneumoniae*, or other uropathogens performed to guide the choice of antibiotics. Biochemical test are very important for the identification and confirmation of species of the uropathogens.
This study will further help researcher in the molecular characterization of the bacteria and development of site specific antibiotic having potent action with minimal side effects.

UROPATHOGENS	Indole	Methyl red	vogespr oskauer	Citrate utilization	Urease	Oxidase	Catalase	Triple sugar iron agar	Germ tube test
E.coli	+	+	-	-	-	-	+	A/A with gas	
K.pneumoniae	-	-	+	+	+	-	+	A/A with gas	
K.oxytoca	+	-	+	+	+	-	+	A/A with gas	
P. aeruginosa	-	-	-	+	-	+	+	K/K	
Acinetobacter sp.	-	-	-	+	-	-	+	K/K	
Citrobacter koseri	+	+	-	+	-	-	+	K/A	
Proteus vulgaris	+	+	-	-	+	-	+	A/A	
Cadida non. albicans									-

BIOCHEMICAL TEST

Where:
A= acidic
K= alkaline

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