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ISOLATION AND MOLECULAR IDENTIFICATION OF PATHOGENIC BACTERIA AND FUNGI FROM CLINICAL SAMPLES: COMPARATIVE INHIBITION BY ANTIBIOTICS

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ABSTRACT

Hospital environment is the major cause of various infections including cut and burn wounds. Burn wounds are one of the most common and devastating forms of trauma, exposing the immune suppressed patients to early and serious infections. Faster detection of the causative Microbes and institution of proper therapy would help greatly in preventing septic complications. Recent advances in the field of Molecular Biology, including the amplification of genetic material by the Polymerase Chain Reaction (PCR) technologies have led to faster and more reliable microbial detection methods. Results would help in early detection of causative organisms and treatment regimes in patients with burn wounds.

KEYWORDS: Clinical Samples, PCR, Antibiotics, Klebesiella.

INTRODUCTION

Infection is an important cause of mortality in burns. It has been estimated that 75% of all deaths following thermal injuries are related to infections.[1-3] The rate of nosocomial infections are higher in burn patients due to various factors like nature of burn injury itself, immunocompromised status of the patient, invasive diagnostic and therapeutic procedures and prolonged ICU stay.[4,5] In addition, cross-infection results between different burn patients due to overcrowding in burn wards.[6] Complicating this high rate of infection is the fact that the spectrum of bacterial isolates varies with time and geographical area. In various countries, including India, the importance of Acinetobacter species, as a rapidly emerging nosocomial pathogen, has been documented and these bacteria are predominantly isolated from ICUs, burn units and surgical wards.[7-9] In addition, the problem of multi-drug resistance in gram-negative bacilli due to extended spectrum beta lactamases (ESBL) production is becoming a serious threat to the healthcare worker, who is likely to contract the infection, as the therapeutic options to these organisms are limited. This necessitates periodic review of the isolation pattern and antibiogram of the burn ward, which forms the basis for modification of drug regimen strategy.[8] Keeping this in mind, the present study was planned to determine the bacteriological profile and the resistance pattern from outer burn ward over a period of three years (June 2002 to May 2005) and we compared this data with the results obtained during the previous five years (June 1997-May 2002), to ascertain any change in the bacteriological profile and antimicrobial resistance pattern. Infectious diseases are common diseases all over the world.[10,11] Infectious diseases in non-industrialized countries caused 45% in all and 63% of death in early childhood. It is reported that infectious diseases are responsible for more than 17 million deaths worldwide each year, most of which are associated with bacterial infections.[12] The ability to control such bacterial infections is largely dependent on the ability to detect these etiological agents in the clinical microbiology laboratory. Diagnostic medical bacteriology consists of two main components namely identification and typing. Molecular biology has the potential to revolutionize the way in which diagnostic tests are delivered in order to optimize care of the infected patient, since the discovery of PCR in the late 1980s, there has been an enormous amount of research performed which has enabled the introduction of molecular tests to several areas of routine Clinical Microbiology.[13] Molecular biology techniques continue to evolve rapidly, so it has been problematic for many laboratories to decide upon which test to introduce before that technology becomes outdated.[14,15] Presently molecular biology offers a wide repertoire of techniques and permutations of these analytical tools, the last ten years of the twentieth century allowed for an exponential increase in the knowledge of techniques in molecular biology, following the cellular and protein era of the 1970s and 1980s. Molecular bacteriologists are now beginning to adopt general molecular biology techniques to support their particular area of interest.

MATERIALS AND METHODS

Samples Collection

Burn wound sample collected from hospital by wearing sterile gloves. The collected samples were immediately placed in sterile polythene bags, sealed and kept in a thermal cool box containing coolant packs. These samples were immediately brought to the lab for processing within hours of collection.

Morphological characterization

Grams staining, Endospore staining test, capsulated staining test, Motility test were carried out for the morphology of cell.

Biochemical characterization

Catalase, ONPG, Lysine decarboxylase, Ornithine, Urease, Phenyl alanine deamination, Nitrate reduction, H2S production, Citrate utilization, Voges proskaeurs, Methyl red, Indole and Malonate were suited for biochemical studies.

CARBOHYDRATE FERMENTATION

Rapid Biochemical Assay: The API-20E employs a plastic strip composed of 13 individual micro tubes, each containing a dehydrated medium in the bottom and upper cupules. The media become hydrated during inoculation of a suspension of the test organism, and the strip is then incubated in a plastic covered tray to prevent evaporation. In these manner 13 carbohydrates tests are performed. Following incubation, identification of the organism is made by using differential charts supplied by the manufacture or by means of a computer-assigned system called PRS.

Isolation and rapid amplification of DNA

DNA was isolated from overnight grown culture and dissolved in TE buffer (100 mM Tris hydrochloride,1 mM EDTA,P^H 8.0.DNA concentration was estimated spectrophotometrically at 260 nm. DNA purity was checked by scanning he absorbance of DNA samples between 200 and 400 nm and monitoring the absorbance ratios at 260/280 nm and at 260/230 nm.DNA preparations were also subjected to electrophoresis in 1% agarose gels to check for shearing and degradation.

FORWARD PRIMER

5’-CTGGCGGGAATTACAGTGTT-3’

REVERSE PRIMER

5’-TGGTGGATTCATGGGGTACT-3’

PCR Mixture

The PCR mixtures were prepared with H_2O (Mili-Q grade), 2 µl of 20 pmol of both forward and reverse primers, 1µl of 10 mM dNTP, 5 µl of 1U Taq DNA polymerase, 5 µl of 10X PCR buffer, 4.0 µl of 25 mM $MgCl_2$, 1µl DNA Sample. Water was added to adjust the final reaction volume to 50µl.PCR Products were analyzed with 2% agarose gel electrophoresis.

DISC DIFFUSION FOR ANTIBITICS COMPARITIVE INHIBITION

The disk-diffusion method (Kirby-Bauer) is more suitable for routine testing in a clinical laboratory where a large number of isolates are tested for susceptibility to numerous antibiotics. An agar plate is uniformly inoculated with the test organism and a paper disk impregnated with a fixed concentration of an antibiotic is placed on the agar surface. Growth of the organism and diffusion of the antibiotic commence simultaneously resulting in a circular zone of inhibition in which the amount of antibiotic exceeds inhibitory concentrations. The diameter of the inhibition zone is a function of the amount of drug in the disk and susceptibility of the microorganism.

This test must be rigorously standardized since zone size is also dependent on inoculums size, medium composition, and temperature of incubation, excess moisture and thickness of the agar. If these conditions are uniform, reproducible tests can be obtained and zone diameter is only a function of the susceptibility of the test organism.

Zone diameter can be correlated with susceptibility as measured by the dilution method. Further correlations using zone diameter allow the designation of an organism as "susceptible", "intermediate", or "resistant" to concentrations of an antibiotic which can be attained in the blood or other body fluids of patients requiring chemotherapy.

RESULTS AND DISCUSSION

STREAK PLATE TECHNIQUE

Observation

Color less colonies were observed over the medium (Fig 1).

Fig 1: Growth of colonies on Agar Medium.

GRAM STAINING

Observation

On Gram staining pink color rods were observed. Hence it is a Gram negative bacterium (Fig 2).

Result
From above observation it is said that is a Gram negative bacteria.

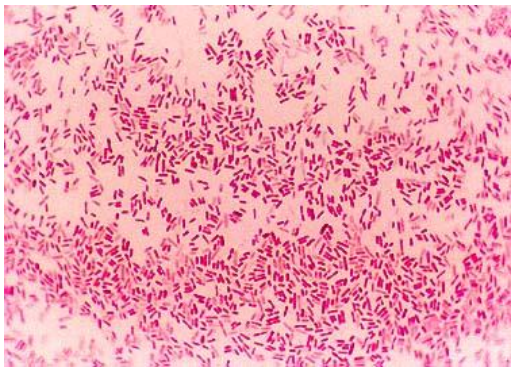


Fig 2: Gram staining pink color rods.

NEGATIVE STAINING
Observation
On negative staining spherical cells occurring in clusters appear transparent (colorless) against a blue-black ground (Fig 3).

Result
From above observation it is said that this is capsulated organism.

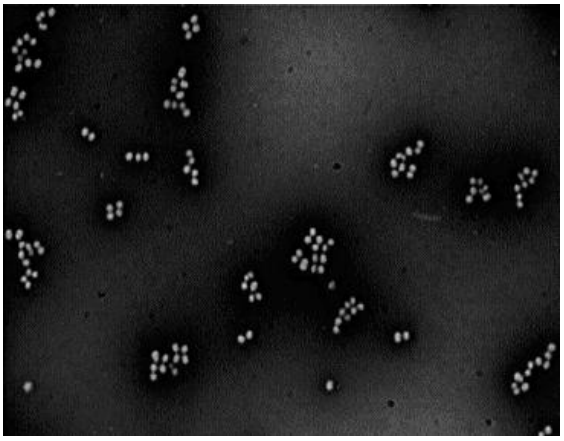
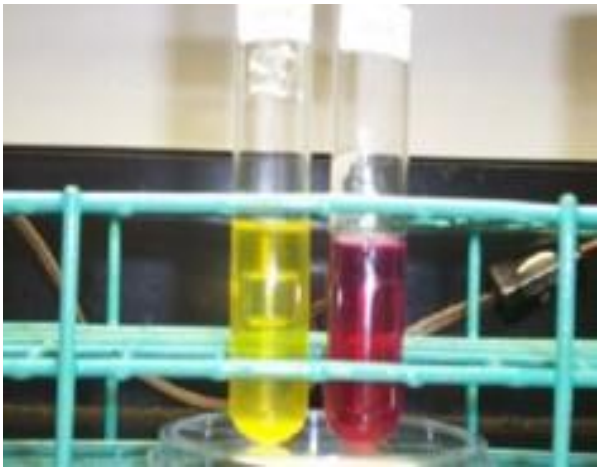


Fig 3: Negative staining spherical cells.

FERMENTATION OF CARBOHYDRATES
Observation
After 48 hrs of incubation it was observed that sugars that are glucose, sucrose and lactose were utilized by *organism* acid was produced in glucose, lactose and sucrose (Fig 4).

Result
Organism utilized all the three sugars and produced to the acid so it is positive.



A B

A: negative
B: Positive

Fig 4: Fermentation of carbohydrates test.

CATALASE ACTIVITY
Observation
After 48 hours of incubation when four drops of hydrogen peroxide was added to the slants slow appearance of gas bubbles was observed (Fig 5).

Result
After the addition of hydrogen peroxide gas bubbles were observed which indication of positive test.

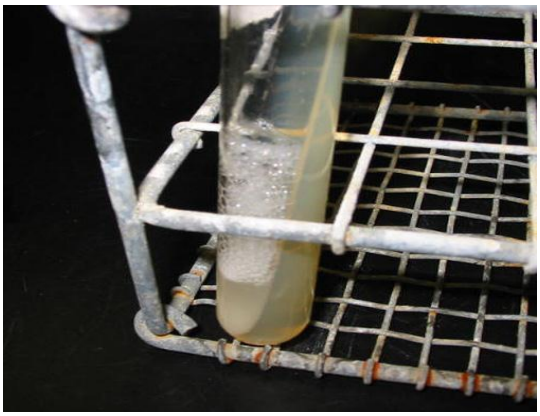
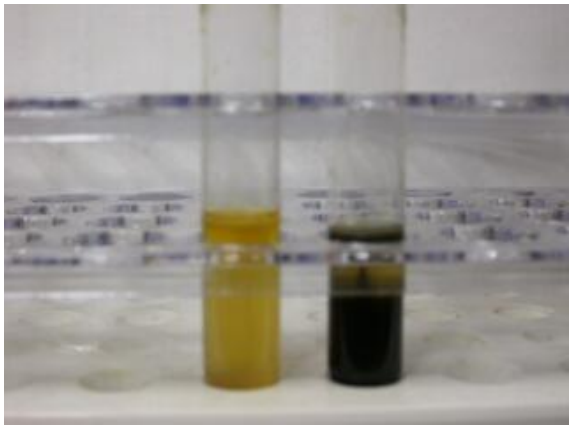


Fig 5: Catalase activity.

HYDROGEN SULPHIDE PRODUCTION TEST
Observation
No black coloration along the line of stab inoculation was observed (Fig 6).

Result
Black coloration along the line of stab inoculation was not observed. Hence the organism may be H_2S negative.



A B

A. Left side negative test
B. Right side positive test

Fig 6: Hydrogen sulphide production test.

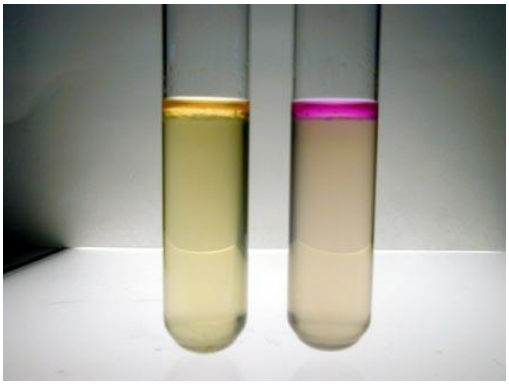
INDOLE PRODUCTION TEST

Observation

Development of cherry (deep) red color in the top layer of the tube is not observed (Fig 7).

Result

As development of cherry red color is not observed in the top layer of the tube so it is negative test.



A B

A– negative B – positive

Fig 7: Indole production test.

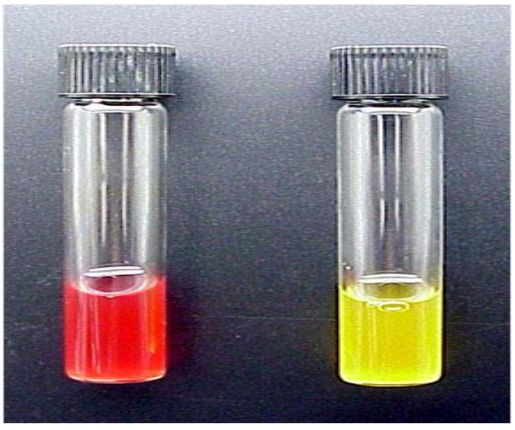
METHYL-RED AND VOGES-PROSKAUER TESTS

Observation

The tubes in which methyl red was added no red color was observed in the V-P test tubes when V-P reagents I & II were added red color was observed (Fig 8 & 9).

Result

As in the methyl red test red color is observed hence, it is Negative test.



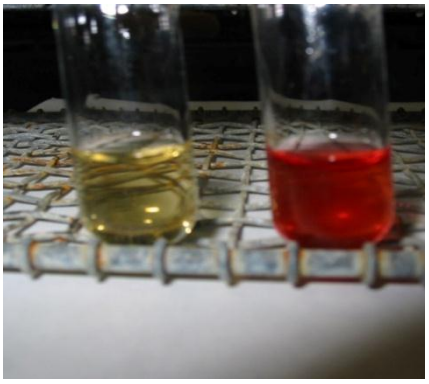
A **B**

A-positive B- negative

Fig 8: Methyl-red test.

VP TEST RESULT

In the VP test, red color is observed hence, it is positive test.



A B

A- Negative B- Positive

Fig 9: Voges-Proskeur Test.

CITRATE UTILIZATION TEST

Observation

After 48 hours of incubation it was observed that there is change in the medium color (Fig 10).

Result

From the above observation it is positive to this test.

A B

A – Positive; B – Negative

Figure 10: Citrate utilization test.

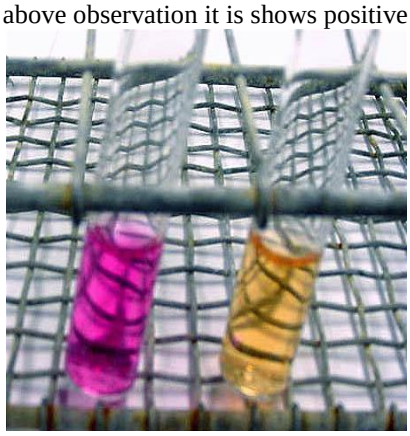
UREASE TEST

Observation

After 48 hours of incubation it was observed that there is change in the medium (Fig 11).

Result

From the above observation it is shows positive test.



A B

A – Positive; B – Negative

Figure 11: Urease test.

Isolation of DNA and PCR – RESULTS

Following are the pictures taken of the PCR sample upon running the gel, PCR cycler (instrument used) and picture of me working in the lab. Based on the amplification result the organism was identified as Klebsella Pneumonia and Fungi Aspergillus.

GENOMIC DNA

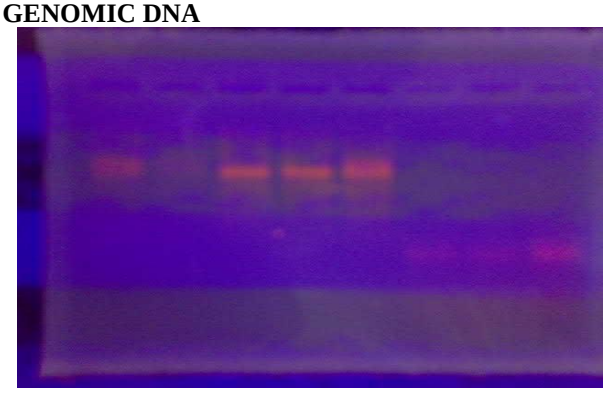


Fig 12: Isolation of genomic DNA.

PCR

Observation: After running the PCR, we got the product was obtained near 200bp region. That is, the fragment size after amplification is found to be 200bp. Since, this is a partial clone; efforts are underway to pull out the full length clone.

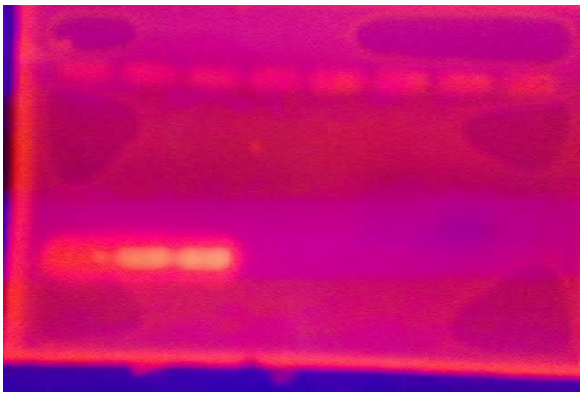


Fig 13: Amplification of DNA using PCR.

COMPARITIVE ANTIBIOTICS INHIBITION

Result

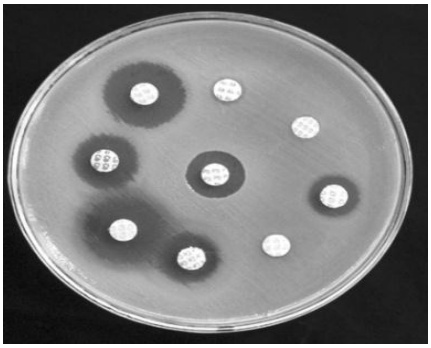


Fig 14: Staphylococcus aureus.

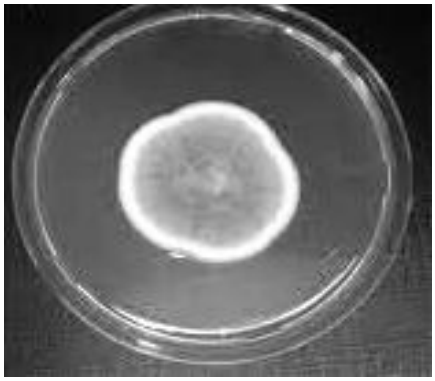


Fig 15: Aspergillus niger.

This type of antibiotic sensitivity is observed.

Table 1: Antibiotic sensitivity test.

Number of samples	Action of micro organism	SLB	SF	LM	CFP	AN
1	KLEBESIELLA	resistant	Sensitive	Sensitive	Sensitive	Sensitive

SF = Sparfloxacin, AN = Amikacin, SLB = Sulbactum, CFP = Cefoperazone, LM = Lomefloxacin,

CONCLUSION

The present study suggests that wound sample contains *Klebsiella pneumonia*, *Aspergillus*, are involved in the biodegradation process of 2-picoline, it converts the carcinogenic harmful substance (2-picoline) into harmless and ecofriendly products such as ammonia, and

CO_2. So these compounds are either useful to the organism as its carbon and nitrogen sources. So in this way, the organism will also survive**.**

We know that the generation time of *Klebsiella pneumonia* is more than *E.coli*. So in the present work, the comparitive inhibition studies were taken to study the effect of different antibiotics on the growth of both pathogenic bacteria and fungi. Only few antibiotics have resistant, and most of the antibiotics are suspetable to these bacteria and fungi. From the PCR amplification I t was identified that the pathogenic bacteria was klebsiella and fungi was Aspergillus. Plasmid curing technique helped to understand the importance of plasmid DNA containing dioxygenase in the organism which is involved in the process of bioremediation. So in order to transfer the plasmid into the host organism effectively, competent *E.coli DH5α* cells were taken. Which can accept the plasmid (foreign) DNA more effectively as these cells are well known as versatile strain that can be used in cloning applications. Moreover intact plasmid can be transferred into these cells. So in this present work dioxygenases's role in bioremediation was successfully studied and this gene was transferred into suitable host to attain best result in shorter time.

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