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Research article

ISOLATION AND MOLECULAR IDENTIFICATION OF PATHOGENIC BACTERIA FROM URINE SAMPLE

Suhad A. Ibrahim

Department of Chemistry, College of Science, Al-Nahrain University, Baghdad.Iraq

ABSTRACT: Hospital environment is the major cause of various infections including cut and burn wounds. Urine Samples are one of the most common and devastating forms of trauma, exposing the immunosuppressed 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.

Key words: Molecular Identification, PCR, Primer, Urine sample

INTRODUCTION

Infection remains the leading cause of death among patients who are hospitalized for burns. Rapidly emerging nosocomial pathogens and the resistant microbes remain the main cause for the isolation of the microorganisms [Abraham, E., and A. A. Freitas. 1989 and Acikel, C et al 2003]. The risk of infection is related directly to the extent of the burn and is also related to the failure of the body's immune system due to the disruption of the cell's integrity [Agnihotri, N et al 2004 and Alexander, J. W**.** 1990].Burn injury causes mechanical disruption to the skin, which allows opportunistic pathogens to invade deeper tissues [Altman, L. C et al 1977]. The usual skin barrier is replaced by a moist layer that helps microbial growth [Altoparlak, U et al 2004].The burn wound surface is sterile immediately following injury; however, it is repopulated quickly with gram-positive organisms from hair follicles, skin appendages, and the environment during the first 48 hours [American Burn Association**.** 2000]. More virulent gram-negative organisms replace the gram-positive organisms after 5-7 days [Amshel, C. E et al 2000]. Gram-negative organisms have greater motility, possess many antibiotic resistance mechanisms, and have the ability to secrete collagenases, proteases, lipases, and elastases, enabling them to proliferate and penetrate into the cells. If host defenses are inadequate, invasion of viable tissue occurs. Normally thermal injury has a severe impact on the host's cellular immune systems [Appelgren, P et al 2002 and Arons, M. S**.** 1965]. The degree of immune suppression is proportional to the duration and temperature of thermal exposure [Atiyeh, B. S et al 2003 and Atiyeh, B. S et al 2002].

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. The rate of nosocomial infections are higher in burn patients due to various factors like nature of burn injury itself, immune compromised status of the patient, invasive diagnostic and therapeutic procedures and prolonged ICU stay. In addition, cross-infection results between different burn patients due to overcrowding in burn wards [Atiyeh, B. S et al 2003]. 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 [Atiyeh, B. S et al 2005]. 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 [Atiyeh, B. **S** et al 2002 and Avdakoff, V**.** 1876].

This necessitates periodic review of the isolation pattern and antibiogram of the burn ward, which forms the basis for modification of drug regimen strategy [Backstein, R et al 1993]. 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 and we compared this data with the results obtained during the previous five years, to ascertain any change in the bacteriological profile and antimicrobial resistance pattern [Baddley, J. W., and S. A. Moser. 2004 **and** Baker, C. C et al 1979].

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METHODOLOGY

Samples Collection:

Urine 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 Ma lonate 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 an upper cupule. 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 this 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 scaning 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 . Test Primer xyn s gene (endo β 1,4 D-xylanase) from bacillus sp. to detect xylanase gene in Arthrobacter .

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.

RESULTS AND DISCUSSION

Streak plate technique

Observation

Colour less colonies were observed over the medium

Figure 1: Colonies of *Staphylococcus aureus* on Nutrient agar medium

This *Staphylococcus aureus* was grown on Nutrient Agar medium by Streak plate technique.

GRAM STAINING

Observation

On Gram staining blue colored cocci were observed. Hence it is a Gram positive Bacterium.

RESULTS

From above observation it is said that is a Gram-positive bacterium

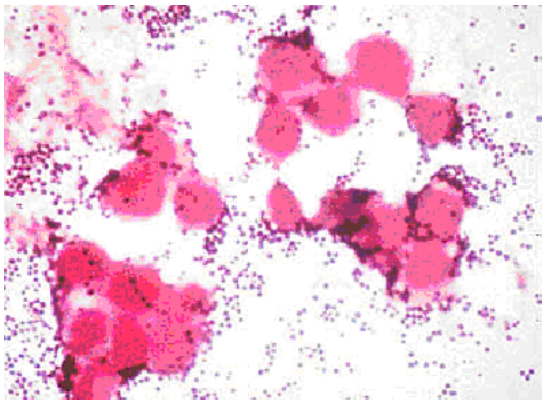


Figure 2: Gram stain of *Staphylococcus aureus*

NEGATIVE STAINING

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

Result: From above observation it is said that may be *Staphylococcus*.

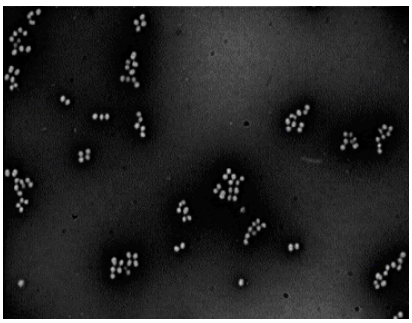


Figure 3: Negative stain of *Staphylococcus aureus*

FERMENTATION OF CARBOHYDRATES

Observation: After 48 hrs of incubation it was observed that sugars that are glucose, sucrose and lactose were utilized by *Staphylococcus aureus* acid was produced in glucose, lactose and sucrose (Figure 4).

Result: As *Staphylococcus aureus* utilized all the three sugars and produced to the acid so it is positive. Where as *P.vulgarius* did not utilized the any sugars so it is negative.

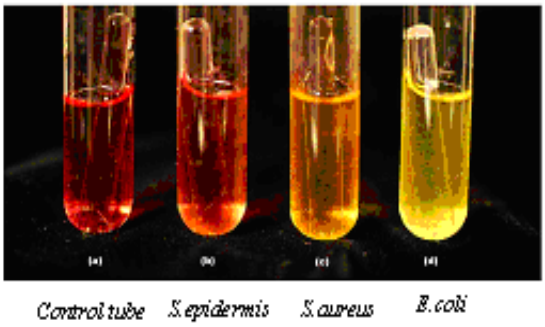


Figure 4: Fermentation of carbohydrates

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 (Figure 5).

Result: After the addition of hydrogen peroxide gas bubbles were observed which is the indication of positive test. Hence *Staphylococcus aureus* is positive for catalase

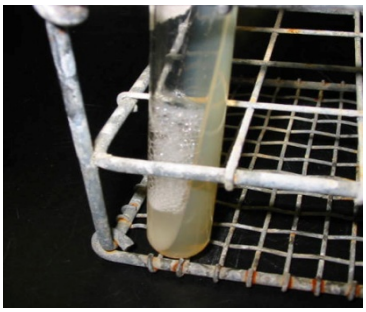


Figure 5: Catalase activity: – *S.aureus*

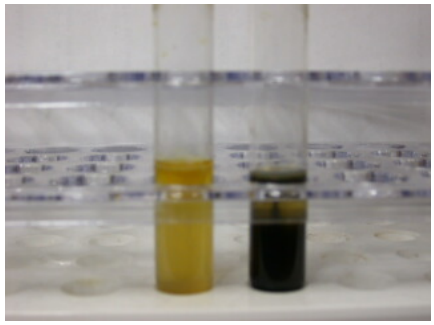
HYDROGEN SULPHIDE PRODUCTION TEST

Observation

No black coloration along the line of stab inoculation was observed (Figure 6).

Result

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



A B

Figure 6: Hydrogen sulphide production test

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

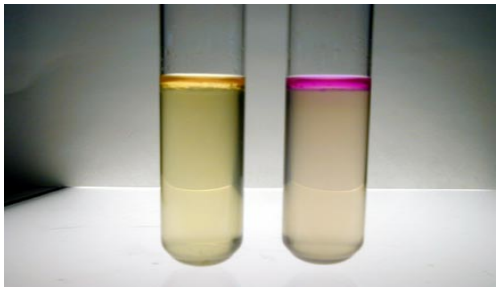
INDOLE PRODUCTION TEST

Observation

Development of cherry (deep) red color in the top layer of the tube is not observed. Hence, *Staphylococcus aureus* an indole – negative bacterium (Figure 7).

Result

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



A B

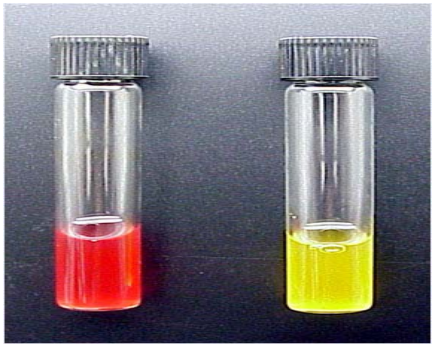
Figure 7: Indole production test

A– negative B – positive

METHYL-RED AND VOGES-PROSKAUER TESTS

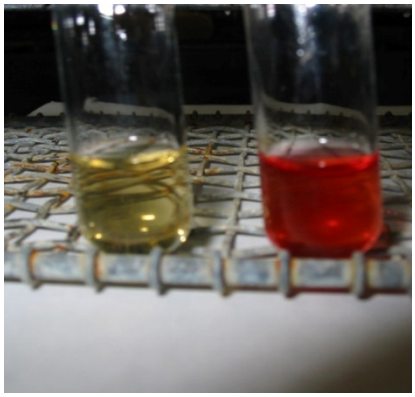
Observation: The tubes in which methyl red was added no red color was observed in the V-P test tubes when V-P reagents I & II were added no red color was observed (Figure 14 & 15).

Result: As in the methyl red test red color is observed hence, it is positive test. In the VP test, red color is not observed hence, it is negative test.



A B

Figure 8: M.R and V.P test A.*positive* B-*negative*



A B

Figure 9: Voges-Proskeur Test A- *negative* B- *positive*

CITRATE UTILIZATION TEST

Observation: After 48 hours of incubation it was observed that there is no change in the medium colour.

Result: From the above observation it is said that *staphylococcus aureus* is negative to this test.

A B

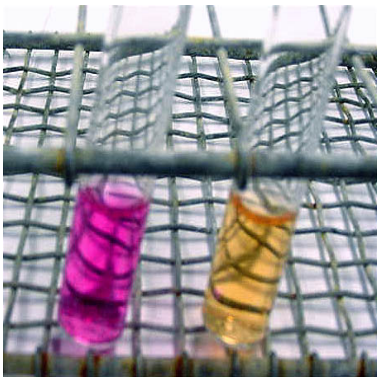
Figure 10: Citrate utilization test

A – *positive* B – *negative*

UREASE TEST

Observation: After 48 hours of incubation it was observed that there is no change in the medium.

Result: From the above observation it is said that *staphylococcus aureus* shows positive test.



A B

Figure 11: Urease test A – *Positive* B – *negative*

DISC DIFFUSSION METHOD

Result

Table 1: Type of antibiotic sensitivity is observed.

Number of samples	Action of micro organism	SF	AN	CR	CFP	CIP	ACX	S	P
1	Sensitive	Sen	Sen	Sen	Sen	Sen	Sen	Res	Res

SF = Sparfloxacin, AN = Amikacin , CR = Cefuroxime , CFP = Cefoperazone , CIP = Ciprofloxacin , ACX = Ampiclox, P. = penicillin S. = streptomycin

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 amphilification of DNA that organism is known as Staphylococcus areus.

Genomic DNA

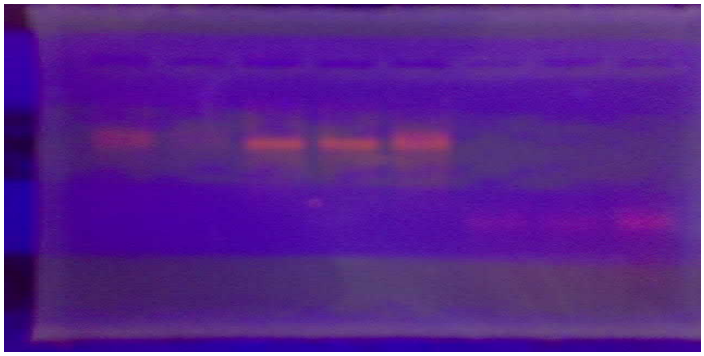


Fig 12: Isolation of Genomic DNA

PCR

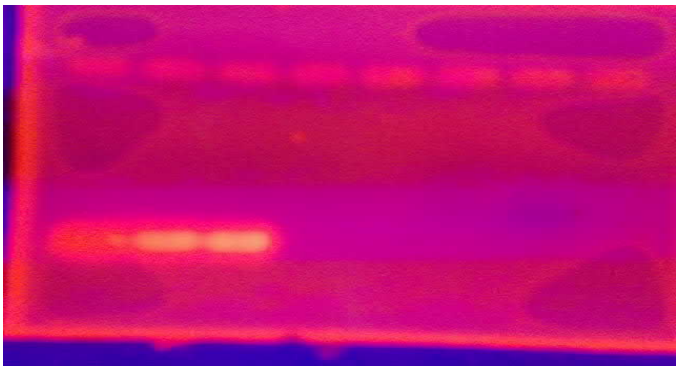
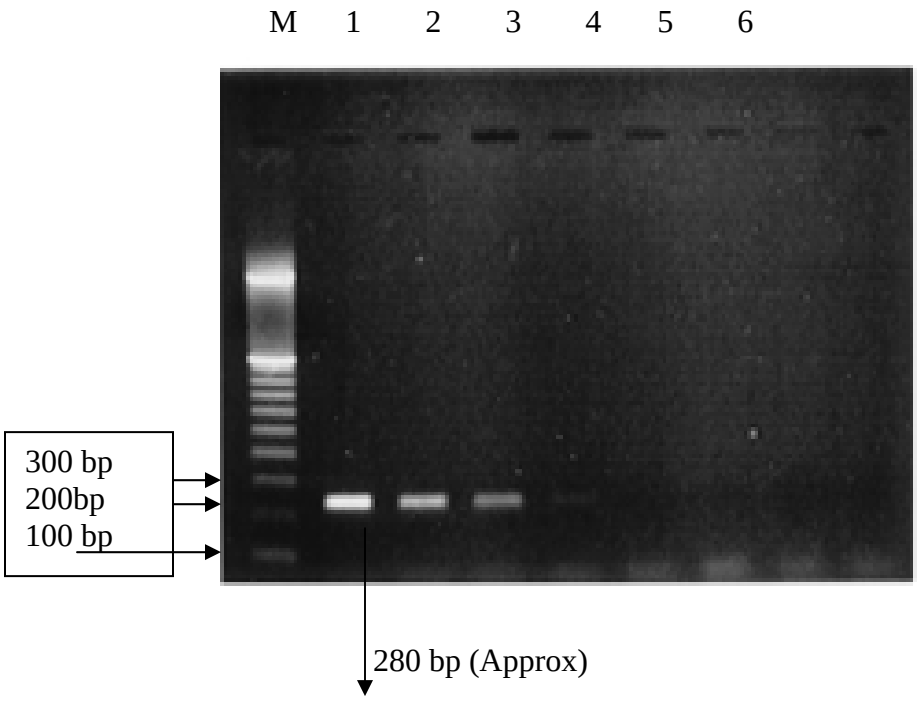


Fig 13: Amplification of DNA

PCR Detection CDO genes in biodegradation of 2-picoline

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.



Transformed competent *E.coli* DH5α cells:

Plasmid of *Bacillus cereus* was taken and transformation of the competent E.coli DH5α cells was made. And then these cells were made to grow on to the nutritive medium containing 2-picoline. Normal cells i.e., non-transformed cells will die as normal E.coli cells are not resistant to 2-Picoline as it has no dioxygenase gene in it. Where as the transformed cells have dioxygenase gene in it, so they survive on 2-picoline medium. The transformed cells were cultured for 2-3 generations in order to check the stability of the transformation. And the cells were found to be stable. This is the photograph of the cell culture in 3rd generation.

Fig-14: Transformed cells

CONCLUSION

The present study suggests that urine sample contains *Bacillus cereus,* is 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 u seful 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 *Bacillus cereus* is more than *E.coli*. So in the present work, the dioxygenase gene was transferred into the *E.coli* cells, through plasmid curing techniques. So that the process of bioremediation is made faster. 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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