Antibiogram analysis and characterization of bacterial pathogens from Leucorrhea patients

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Abstract

In recent research work bacteria were isolated from samples of leucorrhea patients admitted in Lady Wellington hospital Lahore (gynae ward) and Basheer welfare hospital Shahdara Lahore. The sampling was done from pregnant and non-pregnant leucorrhea patients aged 18 to 30 years by using sterile culture sticks from vagina. The samples were spread on agar plates and incubated for overnight, bacterial strains were isolated by streak plate method. The strains were named L1, L2, L3 and L4. Identification was carried out by various morphological and biochemical tests. Molecular characterization was also done to characterize bacteria up to species level. L1 strain was identified as *Streptococcus pyogens*, L2 as *Staphylococcus aureus*, L3 as *Neisseria gonorrheae* and L4 as *Escherchia coli*. Antibiotic resistance was analyzed by disc plate method. L1, L2 and L3 strains showed maximum sensitivity with Cefepime antibiotics having values 17.74µg/ml, 13.63µg/ml and 12µg/ml respectively. L4 showed max sensitivity with Azithromycin and Cloxacillin antibiotics *i.e.*, 6.25µg/ml and 6.4µg/ml respectively. Optimum pH was 6.5 for L1 and L2, while 7 for L3 and L4. Optimum temperature was 37 for all strains.

Introduction

Leucorrhoea is a state characterized by grayish, yellowish, or white vaginal discharge, not related to burning sensation, pain and uneasiness. In general vaginal discharge occurs at regular interval and constancy during the rout of menstrual cycle. The amount of discharge is greater during pregnancy and reduced after delivery, at the time of lactation and after menopause. Increase in vaginal discharge may not necessitate treatment during normal physiological state. On the other hand, pathological state concerning infection to *Trichomonas*, *Candida*, Gram positive and Gram negative bacteria may require its treatment (Canu *et al.*, 2002). Leucorrhoea is considered to be influenced under the changes in vaginal epithelium, pH of vaginal secretion and variation in normal bacterial flora. However, as it turns into pathological state it produce related problems like itching burning sensation of valva. Low backache discomfort, poor appetite, pain in both legs and general weakness. Fatigue, malnutrition, chronic illness, emotional disturbance, improper diet, constipation and unhygienic conditions and chronic retroverted uterus are responsible for leucorrhoea. Sometimes it is linked to contamination like *Candida albicans*, *Trichomonas vaginalis* or various bacterial infections, monilial and gonococcal infection, lesions of vaginal wall, and uterine cervix have been all related to leucorrhoea. Leucorrhoea discharge can be increased by estrogen production or it may be natural defense sustaining the chemical balance of vagina (Blackman, 2002). There are various reports that prove the presence of organisms at vagina, uterus and fetus (Sweet and Gibbs, 2002). Numerous diseases are

associated with white discharge are clinical aspect. Pale vaginal discharge is unable for reproduction (Diekema e*t al*., 2002). Irregular vaginal discharge is generally related to body thirst and aches. White or reddish discharge having foul smell is also reported. All these symptoms are because of the certain systemic deficiency disorders like calcium or vitamin deficiency (Mandell *et al.,* 2000). Genital infections with *Staphylococcus aureus* and *Escherichia coli* are more frequent in nasal pathway of *S. aureus* or its risk factor can be increased in condition of extended or repetitive hospitalization of patients. In gynecological or obstetrical contagious pathology, *E. coli* can act as monoetiological pathogen that may cause chorioamniotis or urinary infections or can be separated in polymicrobial infections (*e.g*, wound infection postpartum endometritis and septic abortion). Both polymicrobial infections and monoetiological infections can be the source of septicemia (Forna and Gulmezoglu, 2003). The most common vaginal pathogens are *Staphylococcus aureus* and one of the remnant germ that mostly concerned with infection and whose occurrence increasing progressively. The aggregation of vaginal mucous membrane with this kind of germs can influence to toxicoseptic shock (Shah *et al.,* 2004). Lower female reproductive tract contain intricate normal flora but very little detail is known. Vaginal normal microflora mostly contain lactobacilli (90-95%), Gram negative, bacilli, Gram positive and Gram negative bacilli. Sometimes symbiotic relationship is established between host and her residential microflora. Because of best environment for the establishment in the vaginal mucous membrane, the inhabitant flora avoid the populating of vagina with other more violent species so, its basic role is antipathogenic.

For more than 60 years, devastating loss to the successful treatment of an ever rising variety of infections caused by microorganisms like bacteria, virus, fungi and parasite is antimicrobial reisistance. The result of antimicrobial resistance is to decrease the effectiveness of antibacterial, antiviral, antifungal and antiparasitic drugs, by making it difficult, costly or even impossible to treat the patient. The aims of present study were

1. Isolation/ screening of bacterial pathogen from leucorrhea infection.
2. Identification and characterization of bacterial isolates.
3. Ribotyping of bacterial isolates.
4. Antibiotic resistance of bacteria isolated from leucorrheal infection and detection of efficacy of a variety of antibiotics on resistant microbial agents.
5. To study optimum growth conditions (pH and temperature) of bacterial pathogens.
6. To evaluate the impact in diagnosis of female reproductive diseases (leucorrhea).

MATERIALS AND METHODS

In this study, the following procedure was performed to isolate the bacteria from leucorrhea sample from female patients suffering from leucorrhea infection. The steps for this procedure consist of:

Sample Collection

Total 30 leukorrheal samples were collected from outdoor and indoor patients. The vaginal smear was obtained from the vagina by means of sterile culture stick swab and the swab sticks were labeled accordingly (Fredrick *et al.,* 2005). The areas selected for sample collection were (i) Lady Wellington hospital Lahore (gynae ward) (ii) Basheer welfare hospital shahdara

Lahore). Leucorrheal samples were transferred to the microbiology laboratory of Zoology department GCU Lahore.

Preparation and sterilization of medium

Two types of medium were basically used for the growth of bacteria.

i) Nutrient agar medium
ii) Nutrient broth medium

Nutrient agar medium:

Nutrient agar medium was prepared by dissolving 14 gm of dehydrated nutrient agar in 500 ml of distilled water. The medium was autoclaved at 121^0 C and 15lb pressure for 15 -20 minutes.

Nutrient broth medium:

Nutrient broth medium was prepared by dissolving 4gm of nutrient broth in 500ml of distilled water and autoclaved it at 121^0 C and 15lb pressure for 15-20 minutes.

Agar plate method for Spreading:

Agar plate method was used to spread the sample for isolation of bacteria. In laminar flow autoclaved nutrient agar medium was poured in the Petri plates, which were allowed to solidify. Then spread the samples on the solidified nutrient agar plate with the help of culture stick loop, and placed them in incubator at 37^0C for overnight incubation.

Isolation of the pure colonies:

Different types of bacterial colonies were formed on the Petri plates. Streak plate method was used to obtain pure colonies. Sterilized inoculating loop was used to pick up the single colony and streak it on the agar plate. Same method was conducted for other colonies to isolate them, and then the pates were again incubated at 37^0C for overnight (Figure 7).

Morphological Characterization:

The tests that were performed for the morphological characterization of unknown bacteria are as follows;

i) Motility test
ii) Gram's staining
i) Endospore staining
ii) Acid fast staining

Biochemical characterization:

Different biochemical tests were performed for the identification of unknown bacteria (Table1).

Determinations of conditions for optimum growth:

Two optimum bacterial growth conditions were checked that were pH and temperature.

Determination of Optimum pH:

For the bacterial growth prepare the nutrient broth medium by mixing 14 gm of nutrient broth in 1 liter of distilled water. For separate isolated bacterial strains take 5 sets of 100ml of conical flasks. By using pH meter, pH was maintained at 5, 6, 7, 8, 9 and 10 for each set and allowed it to autoclave. By using micropipette and autoclaved tips pour 10ml of bacterial broth culture into the conical flasks. Then, placed the flasks in shaking incubator at 37 ^{0}C temperature for overnight. The control that was without any bacterial strain was also run for pH. After the adjusted time growth of bacteria was checked and values of optical density was measured at wavelength of 600 nm in spectrophotometer. At last, plotted the growth curves for every isolated bacterial strain for all pH values.

Determination of Optimum Temperature:

Prepare the broth culture by mixing 14grams of nutrient broth in 1000 ml distilled water. Four sets of 100 ml conical flasks were prepared for the four isolated bacterial strains. In each flask add 50 ml nutrient broth then pour 10 ml of isolated bacterial broth culture aseptically. Place them in shaking incubator at different temperatures such as 20 ^{0}C, 25 ^{0}C, 30 ^{0}C, 37 ^{0}C, 40 ^{0}C, 45 ^{0}C and 50 ^{0}C for overnight. For each temperature range the control was also run that was without any bacterial strain. Next day growth of bacteria was observed and measured by taking values of optical density at wavelength of 600 nm in spectrophotometer. At the end, growth curve of temperature was plotted for every isolated bacterial strain of all the temperatures.

Measurement of Minimum Inhibitory Concentration (MIC):

Minimum inhibitory concentration is defined as the minimum concentration of antibiotics that can retard the visible growth of microorganisms. In order to perform this test nutrient broth culture for L1, L2, L3, and L4 were prepared and incubated at 37 ˚C for a night. Dilution of culture was made for the preparation of inoculums of about 105 to 106 colony forming units in each milliliter.

Method of dilutions of Antibiotics:

A significant amount of antibiotic is dissolved in relevant solvent to get stock solution. The method used to get different dilute concentrations of each antibiotic was twofold dilution method. Seventeen test tubes were taken out of which fifteen were labeled as 1 to 15. The 1st test tube was labeled as A.C (Antibiotic control) while the last test tube was labeled as G.C (Growth control). In each test tube poured 1 ml of nutrient broth and then mixed the 1 ml of antibiotic in all the test tubes except growth control tube. After mixing of both solutions 1 ml of mixture was taken from tube number 1 and transferred to tube number 2 by using micropipette having sterile tips. In the next step the same method was applied to transfer 1 ml media from next to next till 15th tube number. Always transfer the media by using new tip for every dilution. At the next step 1ml of mixture was taken from tube number 15 and discarded it. The growth control tube contained no antimicrobial agent. The 1st tube was considered as Antibiotic control because it contained no bacterial agent. Now, 1ml of broth culture of specific bacterial isolate was

inoculated in all test tubes except AC tube. In these tubes the final concentration of antibiotics was diluted to half of initial concentration because of equal volume of inoculums were mixed in broth. At the end all the test tubes were incubated at 37 ^{0}C temperature for overnight. At next morning, the turbidity observed in the test tubes in which bacterial growth was occurred, those test tubes that showed no visible growth of bacteria was considered as minimum inhibitory concentrations for that antibiotics.

Antibiotic resistance of microorganism assessment:

Assessment of antibiotic resistance to microorganism was checked against broad-spectrum antibiotics by performing Kirby-Bauer disc diffusion method. For this test, nutrient agar plates were prepared for different strains. Inoculate the plates by spreading plate method, under aseptic conditions. Placed antibiotics discs of known concentration on the plates with the help of sterilized forceps, and incubate them at 37 ^{0}C for 24 hours. Growth inhibiter zones appeared near the disc where microorganisms cannot grow. Measure the growth inhibitor zones from sides that indicate the resistance against that particular antibiotic. Clear zone indicate the sensitivity of tested bacterial strain against that antibiotic (Figure 8). Used antibiotics discs are as follows in Table 2.

RESULTS

In the study, 12 samples of vaginal secretions were collected from Basher welfare hospital Shahdara Lahore and Lady Wellington Lahore. 4 bacterial strains were isolated from these vaginal samples of leucorrhea infection. These bacterial strains were biochemically and molecularly characterized recognized by Ribotyping of 16S Ribosomal RNA. The growth curve of bacteria, pH effect, temperature effect, antibiotic resistance and minimum inhibitory concentration (MIC) were also checked by conducting experiments. The isolated strains were symbolically named as L1, L2, L3 and L4.

L1 STRAIN:

Colonies of L1 bacterial isolates were grayish white, oval shaped having entire margins, small sized and in chains as represented in table 1. Gram staining properties showed that it is gram positive, no spore formation takes place for L1 strain and it is non motile. At genus level L1 strain was morphologically and biochemically identified as *Streptococcus* sp. certain biochemical reactions were negative for L1 bacterial isolate such as, oxidase, catalase, urease, hydrogen sulphide and mackonky agar test. Triple Sugar Iron test confirmed that L1 bacterial strain belonging to family *enterobacterioaceae.* Positive result was observed for Voges Proskauer test, gelatin test and it has enzyme for degradation of amino acids into indole. *Streptococcus* sp. also hemolyse the red blood cells by releasing hemolysin enzymes. L1 strain showed no effect at metabolism of glucose but metabolized lactose and sucrose into lactic acid (Table No. 1).

The strain of bacterial isolates was checked for optimum growth and was observed at a range of pH values from 4-9 pH. Broth cultures of bacterial isolates were checked at 600 nm for optical density at various pH levels. Optimum pH for L1 was in between 6-7, Optimum temperatures for

growth of L1 strain was ranged between 37 to 40°C while these bacteria were grew best at 37°C Tables 4&5).

L2 STRAIN:

Colonies of L2 were appeared as small, oval shaped, jet black colonies surrounded by white halo. Their colonial surface was in the form of irregular clusters (Table 1). Staining properties showed that these are gram positive cocci, no endospore formation takes place and these were motile bacteria.

At genus level bacterial isolates of strain L2 was characterized phenotypically and biochemically as *Staphylococcus* sp. The biochemical reactions that were positive for L2 bacterial isolate such as catalase, gelatin urease, citrate, triple sugar, litmus milk reactions and H_2S reactions. L2 bacterial isolate was oxidase and indole negative. In the present study, pathogenicity was also observed in L2 strain as positive. Carbohydrate fermentation reactions (i.e. glucose, sucrose and lactose) were also carried that gave positive result by acid/gas production. It was methyl red negative while Voges Proskauer test was positive.

The broth media containing L2 bacterial isolates showed higher growth at pH ranged 7-8 and the optical density showed that the optimum temperatures for L2 strain was in between 37 to 40°C but it grew best at 37°C as shown in table.

L3 STRAIN:

L3 bacterial colonies were coffee bean shaped diplococcic bacteria.Staining properties showed that L3 strain was gram negative, no spore formation takes place and these were motile bacteria.L3 bacterial isolate was clarified as *Neisseria* sp. This strain showed positive result for catalase, oxidase, nitrate reduction, urea utilization, gelatin hydrolysis, consumption of litmus milk and formation of indole. L3 also showed visible growth on blood agar and MacConky agar. Glucose fermentation indicated that it just metabolizes glucose into an acid and it was methyl red positive.

L4 STRAIN:

L4 colonies were metallic sheet, small and rounded, staining properties showed that this strain was gram positive, having no spore forming ability, and include motile bacteria.

L4 bacterial isolate was morphologically and biochemically identified as *E.coli.* Voges Proskauer, urease and citrate reactions showed negative result for L4 because bubble was not formed and no urea formation takes place. Blood agar test was also performed to check the pathogenicity that again examined as positive. *E.coli* was methyl red, catalase, and litmus milk test positive. Carbohydrate fermentation reactions were showed that L4 converted glucose, sucrose and lactose into lactic acid. It is positive for spot indole and kovacs indole, H_2S reaction. L4 strain showed optimum growth at pH ranged 6-8 and temperature 37 C^{o}.

Antibiotic sensitivity test:

Against antimicrobial drugs the resistance and vulnerability of isolated bacterial strain was analysed by disk diffusion method. Areas of inhibition for L1 (*Streptococcus* sp.), L2 (*Staphylococcus* sp.), L3 (*Neisseria gonorrhea*) and L4 (*E. coli*) bacterial strain were calculated. The area of inhibition of antibiotics against L1 bacterial isolate was measured, the largest zone of inhibition against L1 strain was 17.68±0.16 mm for Tetracycline (T30) and minimum zone of inhibition was 4.539±0.12 mm for Cefepime (FEP 30). L2 bacterial strain showed the zone of inhibition that 14.31±0.006 mm of Cefepime (FEP 30) and smallest zone was 4.5±0.15 mm of Penicillin (P 10) as recorded in table 4. L3 bacteria isolate showed the zone of bacterial inhibition ranged between 12± 0.02 mm for Cefepime to 20 ± 0.021 mm for penicillin. Ciproflaxime and Tetrcycline also have significant zones of inhibition i.e., 17.25mm and 16mm respectively. L4 bacteria isolate has maximum zone of inhibition 6.4± 0.004 mm for Cloxacillin and minimum zone of inhibition was 4.2 ± 0.14 mm for Oxacillin Tables 2&3).

The results of all the isolated strains L1, L2, L3 and L4 analyzed as follows, L1 and L2 strains ware sensitive to Azithromycine (AZM 15), Cephalexin (CL 30), Doxycyciline (DO 30), Cefepime (FEP 30), Tetracycline (T30), Teicoplanin (TEC 30µg) and resistant to penicillin (P10), Cloxacillin (OB 5), Amoxyllin (AMC 30), Oxacillin (OX 1), Ampicillin (AM 10) only with the exception against Penicillin means L2 strain is sensitive to it as shown in the table. L3 bacteria strain was sensitive against Azithromycine (AZM 15), Cephalexin (CL 30), Doxycyciline (DO 30), penicillin (P10), Cefepime (FEP 30), Tetracycline (T30), Amoxyllin (AMC 30), and Teicoplanin (TEC 30). L3 was resistant against Cloxacillin (OB 5), Oxacillin (OX 1), and Ampicillin (AM 10). L4 strain was resistant against Cephalexin (CL 30), Doxycyciline (DO 30) and Amoxyllin (AMC 30). This strain was sensitive to Azithromycine (AZM 15), Cloxacillin (OB 5), Oxacillin (OX 1), penicillin (P10), Ampicillin (AM 10) and Teicoplanin (TEC 30).

MICs (Minimum inhibitory concentrations) of antibiotics:

By the observation of turbidity because of bacterial growth in the test tubes the minimum inhibitory concentration of different antibiotics was calculated (Table 6). Minimum inhibitory concentration (MIC) was the smallest antibiotic concentration at which bacteria showed no growth. The values of MIC for antibiotic Azithromycine of all isolated bacterial strains named as L1 (*Streptococcus pyogenes),* L2 (*Staphylococcus aureus),* L3 (*Neisseria gonorrhoeae)* and L4 (*Escherichia coli)* was 23.5µg/ml, 15µg/ml, 12.5µg/ml and 5µg/ml respectively. For Doxycycline the values of minimum inhibitory concentration (MIC) was 47.9µg/ml against *Streptococcus pyogenes* 29.3 µg/ml for *Staphylococcus aureus,* and 20µg/ml for *Neisseria gonorrhoeae*. Against *Streptococcus pyogenes, Staphylococcus aureus, Neisseria gonorrhoeae* and *Escherichia coli* the minimum inhibitory values were observed as 55.5 µg/ml, 46 µg/ml and 15µg/ml for Cephalexin. The minimum inhibitory concentration of tetracycline against isolated bacterial strains *Streptococcus pyogenes, Staphylococcus aureus, Neisseria gonorrhoeae* and *Escherichia coli* was observed as 46.5µg/ml, 95µg/ml, 30µg/ml and 23µg/ml respectively. Cefepime showed the value of MIC against bacterial isolates as *Streptococcus pyogenes, Staphylococcus aureus, Neisseria gonorrhoeae* and *Escherichia coli* were 10µg/ml, 7.92µg/ml, 2µg/ml and 32µg/ml. the minimum inhibitory concentration of Gentamicin was analyzed as 75.2 µg/ml, 38 µg/ml, 16 µg/ml, and 2 µg/ml for *Streptococcus pyogenes, Staphylococcus aureus, Neisseria gonorrhoeae* and *Escherichia coli* respectively. Amikacin has minimum inhibitory

concentration values as 12.2µg/ml for *Streptococcus pyogenes,* 23.1µg/ml for *Staphylococcus aureus,* 16µg/ml for *Neisseria gonorrhoeae* and 2µg/ml for *Escherichia coli* (Figures 3 to 6).

Molecular characterization of bacterial isolates:

The bacterial genomic DNAs of all biochemically analyzed bacterial isolates were isolated using phenol:chloroform extraction method and run Agarose gel using 1% agarose gel. 10kb plus bands were observed as shown in figure. PCR was performed and 16s rDNA specific sequences of bacterial DNA were amplified as shown in figures 1&2. The sequenced genes were analyzed at NCBI website and observed that L1, L2, L3 and L4 strains were molecularly identified as at species level (Table 7).

L1 (*Streptococcus pyogenes*)

GAGAGTTTGATCCTCCGCTCAGGACGAACGCTGGCGGCGTGCCTAATACATGCAAGT
AGAACGCTGAGAACTGGACTTGCACCGGTTCAAGGAGTTGCGAACGGGTGAGTAAC
GCGTAGGTAACCTACCTCATAACGGGGGATAACTATTGGAAACGATAGCTAATACC
GCATAAGAGAGACTAACGCATGTTAGTAATTATAAAAGGGGCAATTGCTCCACTAT
GAGATGGACCTGCGTTGTATTAGCTAGTTGGTGAGGTAAAGGCTCACCAAGGCGAC
GATACATAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCA
GACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGGGGCAACCCTGACCG
AGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGAGA
AGAATAGGTGGGAGTGGAAAATCCACCAAGTGACGGTAACTAACCAGAAAGGGAC
G

L2 (*Staphylococcus aureus*)

TTTATGGAGAGTTTGATCCTGGCTCAGGATGAACGCTGGCGGCGTGCCTAATACATG
CAAGTCGAGCGAACGGACGAGAGCTTGCTTCTATGATGTTAGCGGCGGACGGGTGA
GTAACACGTGGATAACCTACCTATAAGACTGGGATAACTTCGGGAACCGGAGCTAA
TACCGGATAATATTTTGAACCGCATGGTTCAAAAGTGAAAGACGGTCTTGCTGTCAC
TTATAGATGGATCCGCGCTGCATTAGCTAGTTGGTAAGGTAAGTTACCAAGGCAACG
ATGCATAGCCGACCTGAGAGGGTGATCGGCCACACTGGAACTGAGACACGGTCCAG
ACTCCTACGGGAGGCAGCAGTAGGGTCTTCCGCAATGGGCGAAAGCCTGACGGCCG
AGCAACGCCGCGTGAGTGATGAAGGTCTTCGGATCGTAAAACTCTGTTATTAGGGA
AGAACATATGTGTAAGTAACTGTGCACATCTCGCGGTACCTAATCAGAAAG

L3 (*Neisseria gonorrhoeae*)

TAGAAAGGAGGTGATCCAGCCGCAGGTTCCCCTACGGCTACCTTGTTACGACTTCAC
CCCAGTCATGAAGCATACCGTGAAGCGGACTCCTTGCGGTTACCCTACCTACTTCTG
GTATCCCCCACTCCCATGGTGTGACGGGCGGTGTGTACAAGACCCGGGAACGTATTC
ACCGCAGTATGCTGACCTGCGATTACCGCATTCCGACTTCATGCACTCGAGTTGCAG
AGTGCAATCCGGACTACGATCGGTTTTGTGAGATTGGCTCCGCCTCGCGGCTTGGCT
ACCCTCTGTACCGACCATTGTATGACGTGTGAAGCCCTGGTCATAAGGGCCATGAGG
ACTTGACGTCATCCCCACCTTCCTCCGGCTTGTCACCGGCAGTCTCATTAGAGTGGC

AACCGAATGATGGCAACTAATGACAAGGGTTGCGCTCGTTGCGGGACTTAACCCAA
CATCTCACGACACGAGCTGACGACAGCCATGCAGCACCTGTGTT

L4 (*Escherichia coli)*

AAATTGAAGAGTTTGATCATGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACAT
GCAAGTCGAGCGTAACAGGAAGAAGCTTGCTTCTTTGCTGACGAGTGGCGGACGGG
TGAGTAATGTCTGGGAAACTGCCTGATGGAGGGGGATAACTACTGGAAACGGTAGC
TAATACCGCATAACGTCGCAAGACCAAAGAGCCGGACCTTCGGGCCTCTTGCCATC
GGATGTGCCCAGATGGGATTAGCTAGTAGGTGGGGTAACGGCTCACCTAGGCGACG
ATCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGAACTGAGACACGGTCCAGA
CTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAG
ACTGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGGGGAGGA
AGGGTTGCAAGTTAATACCTTTGCTCATTGACGTTACGCGCAGAAGAAGCAC

DISCUSSION

Pathogenic bacteria can cause many diseases such as tuberculosis which is caused by *Mycobacterium tuberculosis.* This disease killed almost 2 million people per year in sub-saharan Africa. Certain globally important diseases also caused by pathogenic bacteria such as pneumonia which is caused by streptococcus and pseudomonas. Waterborne and foodborne diseases are caused by *salmonella, shigella and comylobacter*. Tetanus, Typhoid fever, diptheria, gastrointestinal infection, syphilis and leprosy etc are caused by pathogenic bacteria and these cause high mortality in infants mostly in developing countries. Another major bacterial infection in females especially is leuchorea. Almost all women in their lives suffer from this disease. This research work is performed for the purpose to emphasize the significance of antibiotic resistant isolates of bacteria that were identified by morphological, biochemical and molecular means. Different parameters were considered like effect of pH, effect of temperature on the growth of bacteria, minimum inhibitory concentration (MIC) of antibiotics for the present antibiotic resistant strains of bacterial isolates. In present research work the bacteria isolated as *Streptococcus pyogenes, Staphylococcus aureus, Neisseria gonorrhoeae* and *Escherichia coli* were taken from vaginal samples that cause diseases in reproductive tract. In most of the developing countries of world use of antibiotic is not regular; these are mostly over used or misused that become the reason of drug resistance. Resistance of antibiotics results in high rate of morbidity and mortality from infection causing diseases (Hart and Kariuki, 1998).

The temperature for the optimum growth of all the antibiotic resistant, isolated bacterial strains was ranged between 30^0C to 40^0C and optimum pH for maximum growth was ranged 6 to 8. In this research work the minimum inhibitory concentrations (MICs) of some antibiotics were observed, these antibiotics were azithromycin, cephalaxin, Dotxycyclin, Cefepime, Amikacin and Gentamicin. There concentration was increased as compared to the work done by Shadlia *et al*., 2008.

CONCLUSION:

The present study provides a precious data related to continuous increase in drug resistance against certain bacterial species. The misuse and overuse of antibiotics against infectious diseases results in the increase of drug resistance ability of microorganism.

CONSENT DISCLAIMER:

As per international standard or university standard patient consent has been collected and preserved by the author(s).

Table 1: Biochemical characterization of bacterial isolates

Biochemical Test	L1	L2	L3	L4
Cat Test	-tive	+tive	+tive	+tive
Urease Test	-tive	-tive	+tive	-tive
Gel Test	+tive	+tive	+tive	+tive
Lit milk Test	+tive	+tive	+tive	+tive
Tri sug Iron test	+tive	+tive	-tive	+tive
Cit Test	+tive	-tive	-tive	+tive
Oxi Test	-tive	-tive	+tive	-tive
Ind Test	+tive	+tive	+tive	+tive
Hyd Sul Test	-tive	-tive	-itve	-tive
Blood Agar Test	+tive	-tive	-tive	+tive
MAT	-tive	-tive	-tive	+tive

<table>
<tr><td rowspan="3">Car Fer Test</td><td>Glu</td><td>A (-tive)</td><td>A(+tive)</td><td>A/G (+tive)</td><td>A/G (+ve)</td></tr>
<tr><td>Suc</td><td>A/G (+tive)</td><td>A/G (+tive)</td><td>A(-tive)</td><td>A/G (+ve)</td></tr>
<tr><td>Lac</td><td>A (+tive)</td><td>A/G(+tive)</td><td>A(-tive)</td><td>A (+ve)</td></tr>
<tr><td rowspan="2">MRVP</td><td>MR</td><td>-tive</td><td>+tive</td><td>+tive</td><td>-tive</td></tr>
<tr><td>VP</td><td>-tive</td><td>-tive</td><td>-tive</td><td>-tive</td></tr>
<tr><td colspan="2">Bacterial Species</td><td>Streptococcus sp.</td><td>Staphlococcus sp.</td><td>N.gonorrhoeae</td><td>E. coli</td></tr>
</table>

Table 2: Antibiotic susceptibility test on isolated bacterial strain

Antibiotics	L1	L2	L3	L4
AZM15	S(14.52±0.142)	S(13.01±0.010)	S(3.13±0.152)	S(6.18±0.076)
CL30	S(7.70± 0.205)	S (4.72±0.047)	S(14.09±0.079)	R
DO30	S(10.63±0.060)	S (8.1± 0.010)	S(0.75± 0.132)	R
P10	R	S(5.3±0.100)	S(20.20±0.200)	S(14.16±0.208)
FEP30	S(17.65±0.055)	S(13.68±0.082)	S(12.23±0.252)	R
T30	S (4.97±0.066)	S(6.22±0.107)	S(1.61±0.036)	R
OB5	R	R	R	S(6.43±0.057)
AMC30	R	R	S(0.31± 0.76)	R
OX1	R	R	R	S (4.2±0.095)

AM10	R	R	R	S (4.73 ±0.115)
TEC30	R	S (7.97± 0.064)	S(10.23±0.252)	S(4.03±0.057)

All values representing mean ± SD.

Note: 'R' stands for Resistant, while S' stands for Sensitive AZM (Azithromycin), OB (Cloxacillin), DO (Doxycycline), CL (Cephalexin), OX (Oxacillin), AM (Ampicillin), FEP (Cefepime), T (Tetracycline), P (Penicillin).

Table 3: Antibiogram of antibiotics was shown against particular bacterial isolates

Isolated Bacterial Strains	Antibiogram	
	Sensitive	Resistant
L1	AZM^{S}, T^{S}, CL^{S}, DO^{S} , FEP^{S},	AMC^{R}, OX^{R}, AM^{R}, P^{R} , OB^{R}
L2	AZM^{S}, T^{S},CL^{S},DO^{S},FEP^{S},P^{S},TEC^{S}	AMC^{R}, OX^{R}, AM^{R} , OB^{R}
L3	CL^{S}, DO^{S},FEP^{S},P^{S} ,TEC^{S},T^{S},AMC^{S}	OX^{R}, AZM^{R}, OB^{R} AM^{R}
L4	AZM^{S}, OB^{S}, TEC^{S}, T^{S}	CL^{R}, DO^{R}, AMC^{R}, OX^{R},AM^{R}

Note: 'R' stands for Resistant, while S' stands for Sensitive AZM (Azithromycin), OB (Cloxacillin), DO (Doxycycline), CL (Cephalexin), OX (Oxacillin), AM (Ampicillin), FEP (Cefepime), T (Tetracycline), P (Penicillin).

Table 4: Effect of pH on the growth of bacteria isolated from vaginal secretion of patient suffering from Leucorrhea

pH	Bacterial isolates				
	Control	L1	L2	L3	L4
5	0.000	0.112±0.12	0.130±0.006	0.036±0.004	0.194±0.002
6	0.000	0.420±0.010	0.440±0.125	0.248±0.100	0.357±0.016
7	0.000	0.446±0.007	0.461±0.011	1.09 ±0.055	0.535±0.031
8	0.000	0.346±0.002	0.354±0.100	0.280±0.004	0.338±0.014
9	0.000	0.176±0.002	0.23±0.020	0.116±0.004	0.045±0.002
10	0.000	0.129±0.151	0.154±0.025	0.079±0.006	0.014±0.015

Note:

- All values in bacterial isolated strains (L1, L2, L3 and L4) represented optical density Mean ± SD. n=3

Table 5: Effect of temperature on Growth of Isolated Bacterial strains by comparison of values of mean optical density

Temperature	Cont	L1	L2	L3	L4
20	0.000	0.185±0.006	0.286±0.031	0.233±0.021	0.215±0.005
25	0.000	0.404±0.005	0.426±0.038	0.407±0.005	0.318±0.015
30	0.000	0.565±0.101	0.503±0.006	0.525±0.031	0.443±0.010
37	0.000	0.615±0.004	0.604±0.003	0.584±0.013	0.584±0.009

40	0.000	0.551±0.016	0.525±0.020	0.504±0.006	0.468±0.015
45	0.000	0.333±0.015	0.441±0.005	0.254±0.043	0.326±0.039
50	0.00	0.157±0.016	0.317±0.015	0.110±0.010	0.048±0.006

Note:

- All readings in bacteria isolates (L1, L2, L3 and L4) represent Mean± SD; n=3.

Table 6: Minimum Inhibitory Concentration (MIC) of sensitive antimicrobial agents against bacterial isolates

Antibiotics®	**MIC Values for Bacterial strains**			
	L1	**L2**	**L3**	**L4**
Azithromycine	23.5	15	12.5	5
Doxycycline	47.9	29.3	20	-
Cephalexin	55.5	46	15	-
Cefepime	10	7.92	2	32
Tetracycline	46.5	95	30	23
Gentamicin	75.2	38	6	2
Amikacin	12.2	23.1	16	2

Note:

- ®Refer to the table (3), for explanation of Antibiotics
- All MICs values of antibiotics against each bacterial isolate were in *µg/ml*.

Table 7: Molecular Characterization of Bacterial isolated strain

Isolated Bacterial Strain	Description	Max. Query	Max. Index	Source
L1	*Streptococcus pyogenes*	505 BP	98 %	Leucorrhea fluid
L2	*Staphylococcus aureus*	502 BP	97 %	Leucorrhea fluid
L3	*Neisseria gonorrhoeae*	499BP	99%	Leucorrhea fluid
L4	*Escherichia coli*	500 BP	98%	Leucorrhea fluid

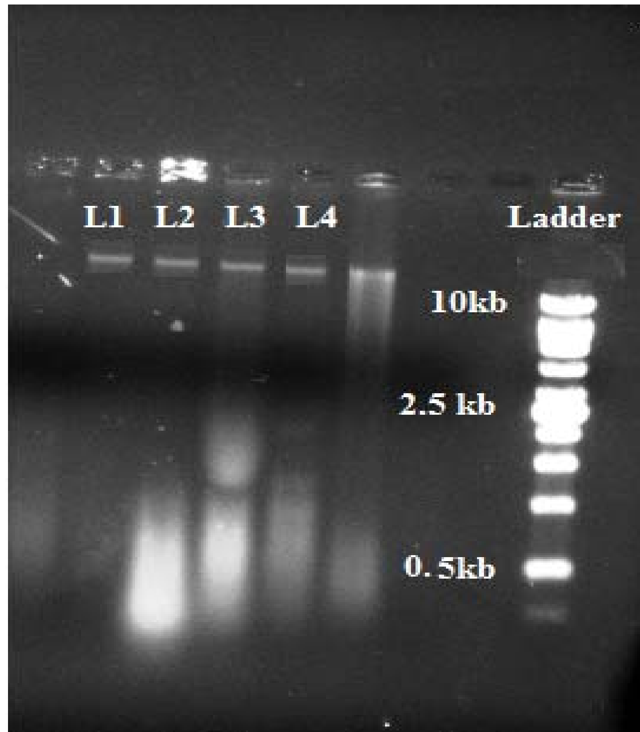

Figure 1: Agarose gel (0.8%) indicates the genomic DNA of bacterial isolates

Figure 2: PCR products of 16S rDNA of bacterial isolates

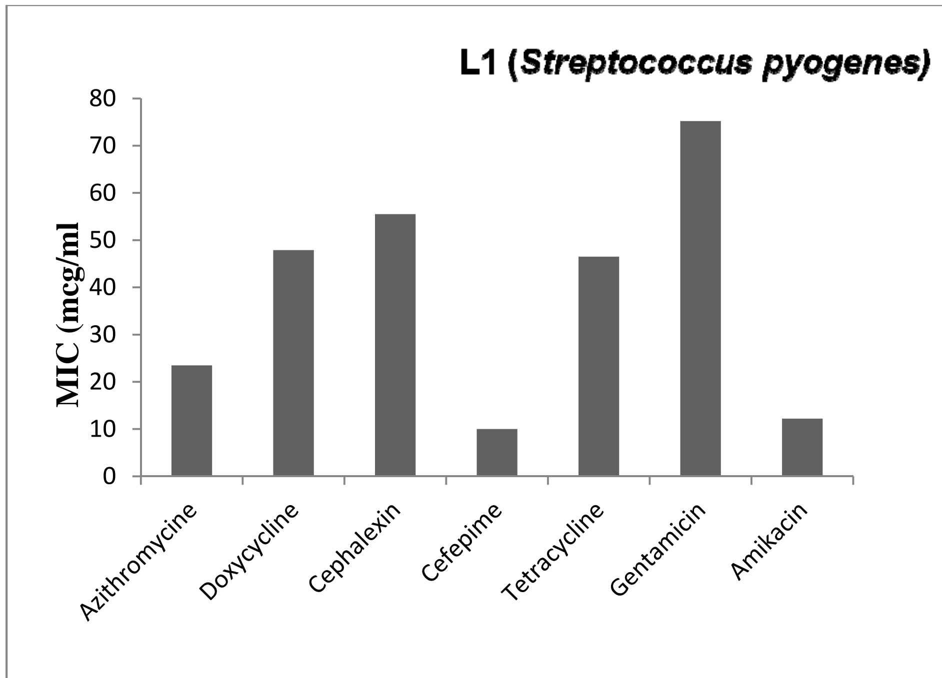

Figure 3: MICs of various antibiotics against L1 Bacterial isolate

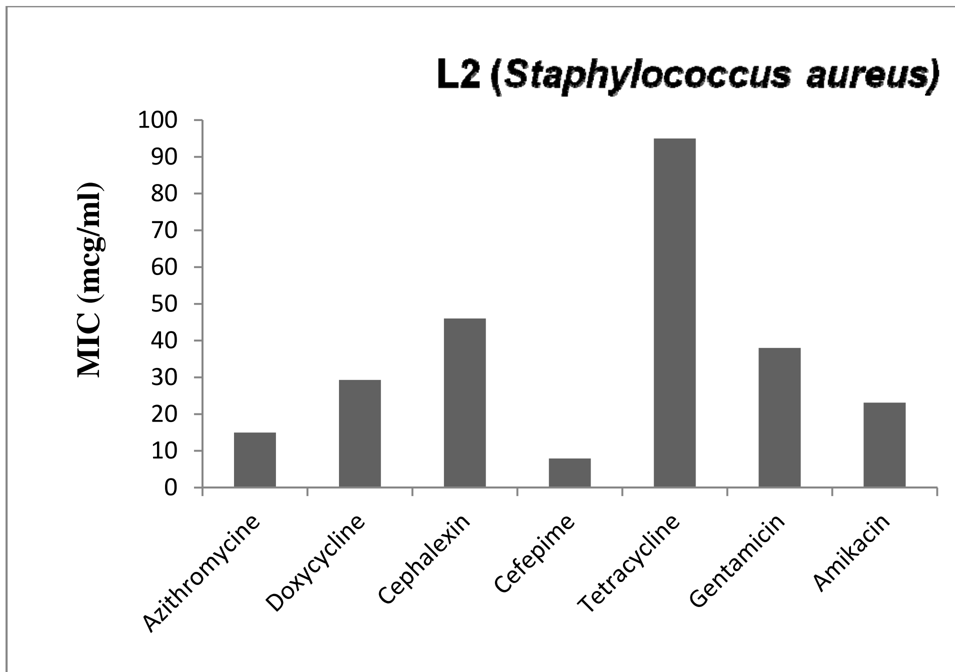

Figure 4 : MICs of various antibiotics against L2 Bacterial isolate

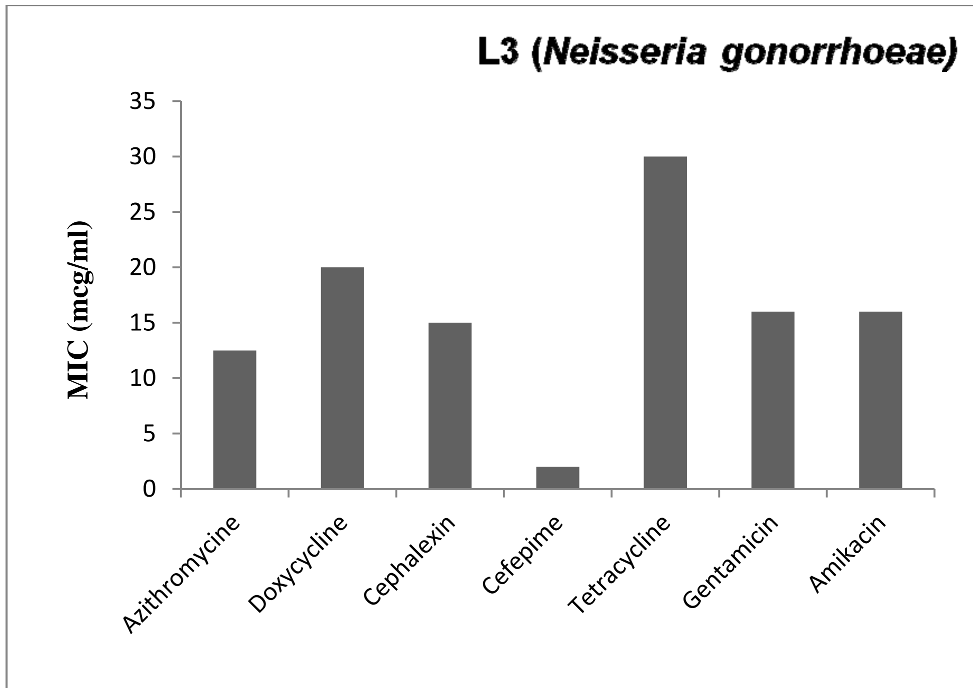

Figure 5: MICs of various antibiotics against L3 Bacterial isolate

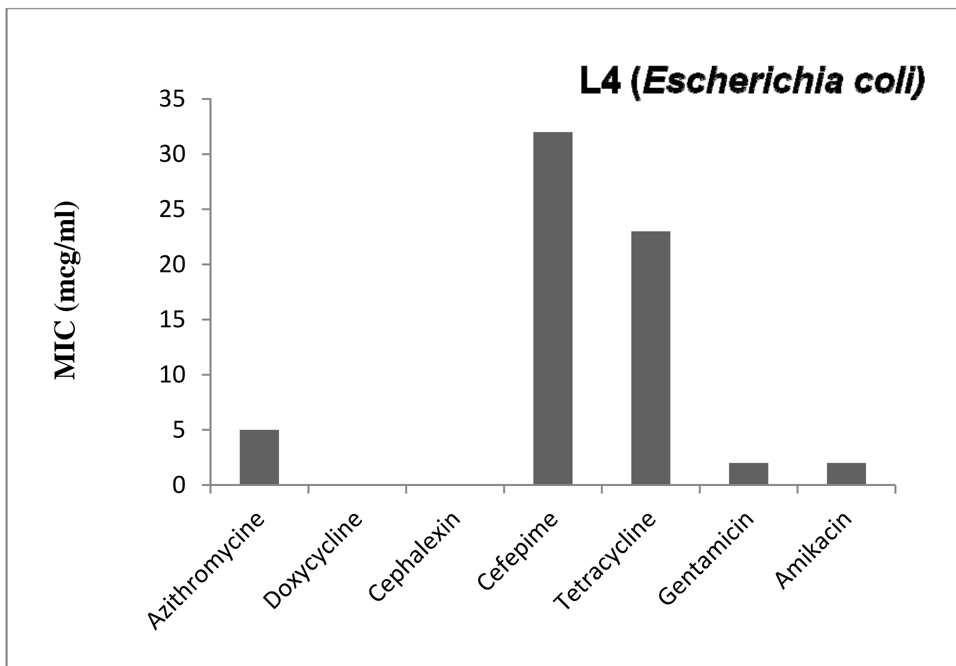

Figure 6: MICs of various antibiotics against L4 Bacterial isolate

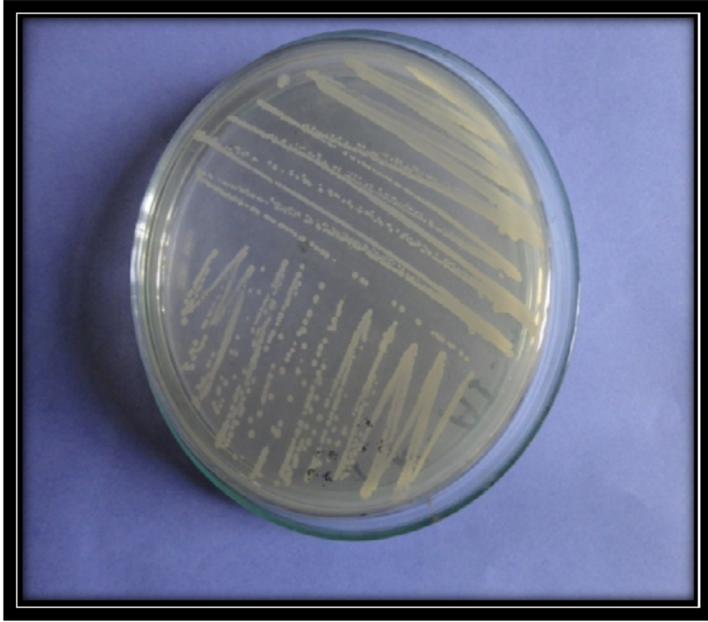

Figure 7: Isolation of bacteria by streaking plate method

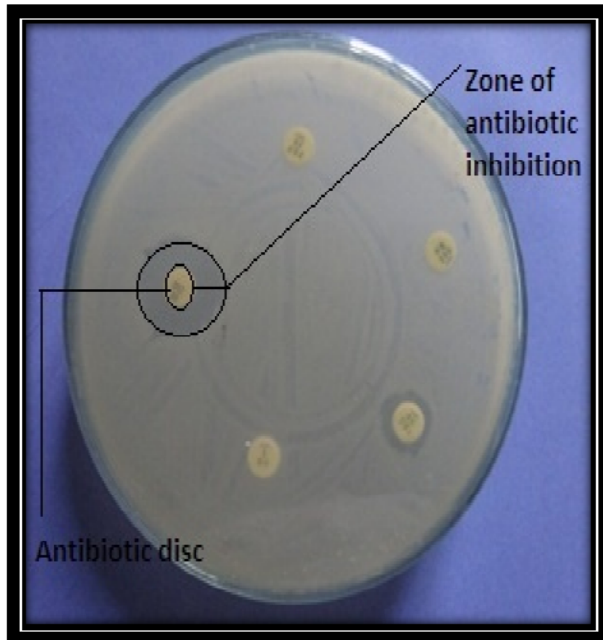

Figure 8: Antibiotic resistance of bacteria

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