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1 # Antibiogram analysis and characterization of bacterial pathogens
2 from Leucorrhea patients

3 ## Abstract

4 In recent research work bacteria were isolated from samples of leucorrhea patients admitted in
5 Lady Wellington hospital Lahore (gynae ward) and Basheer welfare hospital Shahdara Lahore.
6 The sampling was done from pregnant and non-pregnant leucorrhea patients aged 18 to 30 years
7 by using sterile culture sticks from vagina. The samples were spread on agar plates and incubated
8 for overnight, bacterial strains were isolated by streak plate method. The strains were named L1,
9 L2, L3 and L4. Identification was carried out by various morphological and biochemical tests.
10 Molecular characterization was also done to characterize bacteria up to species level. L1 strain
11 was identified as *Streptococcus pyogens*, L2 as *Staphylococcus aureus*, L3 as *Neisseria*
12 *gonorrheae* and L4 as *Escherchia coli*. Antibiotic resistance was analyzed by disc plate method.
13 L1, L2 and L3 strains showed maximum sensitivity with Cefepime antibiotics having values
14 17.74µg/ml, 13.63µg/ml and 12µg/ml respectively. L4 showed max sensitivity with
15 Azithromycin and Cloxacillin antibiotics *i.e.*, 6.25µg/ml and 6.4µg/ml respectively. Optimum pH
16 was 6.5 for L1 and L2, while 7 for L3 and L4. Optimum temperature was 37 for all strains.

17 ## Introduction

18 Leucorrhoea is a state characterized by grayish, yellowish, or white vaginal discharge, not related
19 to burning sensation, pain and uneasiness. In general vaginal discharge occurs at regular interval
20 and constancy during the rout of menstrual cycle. The amount of discharge is greater during
21 pregnancy and reduced after delivery, at the time of lactation and after menopause. Increase in
22 vaginal discharge may not necessitate treatment during normal physiological state. On the other
23 hand, pathological state concerning infection to *Trichomonas*, *Candida*, Gram positive and Gram
24 negative organisms may require its treatment (Canu *et al.*, 2002). Leucorrhoea is considered to
25 be influenced under the changes in vaginal epithelium, pH of vaginal secretion and variation in
26 normal bacterial flora. However, as it turns into pathological state it produce related problems
27 like itching burning sensation of valva. Low backache discomfort, poor appetite, pain in both
28 legs and general weakness. Fatigue, malnutrition, chronic illness, emotional disturbance,
29 improper diet, constipation and unhygienic conditions and chronic retroverted uterus are
30 responsible for leucorrhoea. Sometimes it is linked to contamination like *Candida albicans*,
31 *Trichomonas vaginalis* or various bacterial infections, monilial and gonococcal infection, lesions
32 of vaginal wall, and uterine cervix have been all related to leucorrhoea. Leucorrhoea discharge
33 can be increased by estrogen production or it may be natural defense sustaining the chemical
34 balance of vagina (Blackman, 2002). There are various reports that prove the presence of
35 organisms at vagina, uterus and fetus (Sweet and Gibbs, 2002). Numerous diseases are
36 associated with white discharge are clinical aspect. Pale vaginal discharge is unable for
37 reproduction (Diekema e*t al*., 2002). Irregular vaginal discharge is generally related to body
38 thirst and aches. White or reddish discharge having foul smell is also reported. All these
39 symptoms are because of the certain systemic deficiency disorders like calcium or vitamin
40 deficiency (Mandell *et al.,* 2000).Genital infections with *staphylococcus aureus* and *Escherichia*
41 *coli* are more frequent in nasal pathway of S. aureus or its risk factor can be increased in
42 condition of extended or repetitive hospitalization of patients. In gynecological or obstetrical

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43 contagious pathology, *E. coli* can act as monoetiological pathogen that may cause chorioamniotis
44 or urinary infections or can be separated in polymicrobial infections (*e.g*, wound infection
45 postpartum endometritis and septic abortion). Both polymicrobial infections and monoetiological
46 infections can be the source of septicemia (Forna and Gulmezoglu, 2003). The most common
47 vaginal pathogens are *Staphylococcus aureus* and one of the remnant germ that mostly
48 concerned with infection and whose occurrence increasing progressively. The aggregation of
49 vaginal mucous membrane with this kind of germs can influence to toxicoseptic shock (Shah *et*
50 *al.,* 2004).Lower female reproductive tract contain intricate normal flora but very little detail is
51 known. Vaginal normal microflora mostly contain lactobacilli (90-95%), Gram negative, bacilli,
52 Gram positive and Gram negative bacilli. Sometimes symbiotic relationship is established
53 between host and her residential microflora. Because of best environment for the establishment
54 in the vaginal mucous membrane, the inhabitant flora avoid the populating of vagina with other
55 more violent species so, its basic role is antipathogenic.
56 For more than 60 years, devastating loss to the successful treatment of an ever rising variety of
57 infections caused by microorganisms like bacteria, virus, fungi and parasite is antimicrobial
58 reisistance. The result of antimicrobial resistance is to decrease the effectiveness of antibacterial,
59 antiviral, antifungal and antiparasitic drugs, by making it difficult, costly or even impossible to
60 treat the patient. The aims of present study were
61 1. Isolation/ screening of bacterial pathogen from leucorrhea infection.
62 2. Identification and characterization of bacterial isolates.
63 3. Ribotyping of bacterial isolates.
64 4. Antibiotic resistance of bacteria isolated from leucorrheal infection and detection of
65 efficacy of a variety of antibiotics on resistant microbial agents.
66 5. To study optimum growth conditions (pH and temperature) of bacterial pathogens.

67 6. To evaluate the impact in diagnosis of female reproductive diseases (leucorrhea).
68

69 ## MATERIALS AND METHODS

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

73 **Sample Collection**

74 Total 30 leukorrheal samples were collected from outdoor and indoor patients. The vaginal
75 smear was obtained from the vagina by means of sterile culture stick swab and the swab sticks
76 were labeled accordingly (Fredrick *et al.,* 2005). The areas selected for sample collection were
77 (i) Lady Wellington hospital Lahore (gynae ward) (ii) Basheer welfare hospital shahdara
78 Lahore). Leucorrheal samples were transferred to the microbiology laboratory of Zoology
79 department GCU Lahore.

80 ## Preparation and sterilization of medium

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

82 i) Nutrient agar medium
83 ii) Nutrient broth medium

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84 **Nutrient agar medium:**

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

87 **Nutrient broth medium:**

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

90 **Agar plate method for Spreading:**

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

95 **Isolation of the pure colonies:**

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

100 **Morphological Characterization:**

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

103 i) Motility test
104 ii) Gram's staining
105 i) Endospore staining
106 ii) Acid fast staining

107 **Biochemical characterization:**

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

110 **Determinations of conditions for optimum growth:**

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

112 **Determination of Optimum pH:**

113 For the bacterial growth prepare the nutrient broth medium by mixing 14 gm of nutrient
114 broth in 1 liter of distilled water. For separate isolated bacterial strains take 5 sets of 100ml of
115 conical flasks. By using pH meter, pH was maintained at 5, 6, 7, 8, 9 and 10 for each set and
116 allowed it to autoclave. By using micropipette and autoclaved tips pour 10ml of bacterial broth
117 culture into the conical flasks. Then, placed the flasks in shaking incubator at 37 $^0$C temperature
118 for overnight. The control that was without any bacterial strain was also run for pH. After the

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119 adjusted time growth of bacteria was checked and values of optical density was measured at
120 wavelength of 600 nm in spectrophotometer. At last, plotted the growth curves for every isolated
121 bacterial strain for all pH values.

122 **Determination of Optimum Temperature:**

123 Prepare the broth culture by mixing 14grams of nutrient broth in 1000 ml distilled water.
124 Four sets of 100 ml conical flasks were prepared for the four isolated bacterial strains. In each
125 flask add 50 ml nutrient broth then pour 10 ml of isolated bacterial broth culture aseptically.
126 Place them in shaking incubator at different temperatures such as 20 $^0$C, 25 $^0$C, 30 $^0$C, 37 $^0$C, 40
127 $^0$C, 45 $^0$C and 50 $^0$C for overnight. For each temperature range the control was also run that was
128 without any bacterial strain. Next day growth of bacteria was observed and measured by taking
129 values of optical density at wavelength of 600 nm in spectrophotometer. At the end, growth
130 curve of temperature was plotted for every isolated bacterial strain of all the temperatures.

131 **Measurement of Minimum Inhibitory Concentration (MIC):**

132 Minimum inhibitory concentration is defined as the minimum concentration of antibiotics
133 that can retard the visible growth of microorganisms. In order to perform this test nutrient broth
134 culture for L1, L2, L3, and L4 were prepared and incubated at 37 0C for a night. Dilution of
135 culture was made for the preparation of inoculums of about 105 to 106 colony forming units in
136 each milliliter.

137 **Method of dilutions of Antibiotics:**

138 A significant amount of antibiotic is dissolved in relevant solvent to get stock solution.
139 The method used to get different dilute concentrations of each antibiotic was twofold dilution
140 method. Seventeen test tubes were taken out of which fifteen were labeled as 1 to 15. The 1$^{st}$ test
141 tube was labeled as A.C (Antibiotic control) while the last test tube was labeled as G.C (Growth
142 control). In each test tube poured 1 ml of nutrient broth and then mixed the 1 ml of antibiotic in
143 all the test tubes except growth control tube. After mixing of both solutions 1 ml of mixture was
144 taken from tube number 1 and transferred to tube number 2 by using micropipette having sterile
145 tips. In the next step the same method was applied to transfer 1 ml media from next to next till
146 15$^{th}$ tube number. Always transfer the media by using new tip for every dilution. At the next step
147 1ml of mixture was taken from tube number 15 and discarded it. The growth control tube
148 contained no antimicrobial agent. The 1$^{st}$ tube was considered as Antibiotic control because it
149 contained no bacterial agent. Now, 1ml of broth culture of specific bacterial isolate was
150 inoculated in all test tubes except AC tube. In these tubes the final concentration of antibiotics
151 was diluted to half of initial concentration because of equal volume of inoculums were mixed in
152 broth. At the end all the test tubes were incubated at 37 $^0$C temperature for overnight. At next
153 morning, the turbidity observed in the test tubes in which bacterial growth was occurred, those
154 test tubes that showed no visible growth of bacteria was considered as minimum inhibitory
155 concentrations for that antibiotics.

156 **Antibiotic resistance of microorganism assessment:**

157 Assessment of antibiotic resistance to microorganism was checked against broad-
158 spectrum antibiotics by performing Kirby-Bauer disc diffusion method. For this test, nutrient
159 agar plates were prepared for different strains. Inoculate the plates by spreading plate method,

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160 under aseptic conditions. Placed antibiotics discs of known concentration on the plates with the
161 help of sterilized forceps, and incubate them at 37 C for 24 hours. Growth inhibiter zones
162 appeared near the disc where microorganisms cannot grow. Measure the growth inhibitor zones
163 from sides that indicate the resistance against that particular antibiotic. Clear zone indicate the
164 sensitivity of tested bacterial strain against that antibiotic (Figure 8). Used antibiotics discs are as
165 follows in Table 1.

## 166 RESULTS

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

### 174 L1 STRAIN:

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

186 The strain of bacterial isolates was checked for optimum growth and was observed at a range of
187 pH values from 4-9 pH. Broth cultures of bacterial isolates were checked at 600 nm for optical
188 density at various pH levels. Optimum pH for L1 was in between 6-7, Optimum temperatures for
189 growth of L1 strain was ranged between 37 to 40$^{o}$C while these bacteria were grew best at 37$^{o}$C
190 Tables 4&5).

### 191 L2 STRAIN:

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

196 At genus level bacterial isolates of strain L2 was characterized phenotypically and biochemically
197 as *Staphylococcus* sp. The biochemical reactions that were positive for L2 bacterial isolate such
198 as catalase, gelatin urease, citrate, triple sugar, litmus milk reactions and $H_2S$ reactions. L2
199 bacterial isolate was oxidase and indole negative. In the present study, pathogenicity was also

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200 observed in L2 strain as positive. Carbohydrate fermentation reactions (i.e. glucose, sucrose and
201 lactose) were also carried that gave positive result by acid/gas production. It was methyl red
202 negative while Voges Proskauer test was positive.

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

206 **L3 STRAIN:**

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

214 **L4 STRAIN:**

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

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

224 **Antibiotic sensitivity test:**

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

237 The results of all the isolated strains L1, L2, L3 and L4 analyzed as follows, L1 and L2
238 strains ware sensitive to Azithromycine (AZM 15), Cephalexin (CL 30), Doxycyciline (DO 30),
239 Cefepime (FEP 30), Tetracycline (T30), Teicoplanin (TEC 30µg) and resistant to penicillin

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(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)* was23.5µg/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 and run at Agarose gel for further studies like Ribotyping as shown in figure. After this, 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

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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

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

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322 antibiotic resistant strains of bacterial isolates. In present research work the bacteria isolated as
323 *Streptococcus pyogenes, Staphylococcus aureus, Neisseria gonorrhoeae* and *Escherichia coli*
324 were taken from vaginal samples that cause diseases in reproductive tract. In most of the
325 developing countries of world use of antibiotic is not regular; these are mostly over used or
326 misused that become the reason of drug resistance. Resistance of antibiotics results in high rate
327 of morbidity and mortality from infection causing diseases (Hart and Kariuki, 1998).

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

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

337

338 **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 |

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| | | | | | |
|---|---|---|---|---|---|
| MAT | | -tive | -tive | -tive | +tive |
| Car Fer Test | Glu | A (-tive) | A(+tive) | A/G (+tive) | A/G (+ve) |
| | Suc | A/G (+tive) | A/G (+tive) | A(-tive) | A/G (+ve) |
| | Lac | A (+tive) | A/G(+tive) | A(-tive) | A (+ve) |
| MRVP | MR | -tive | +tive | +tive | -tive |
| | VP | -tive | -tive | -tive | -tive |
| Bacterial Species | | *Streptococcus sp.* | *Staphlococcus sp.* | *N.gonorrhoeae* | *E. coli* |

**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) |

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| 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) |

341 All values representing mean ± SD.

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

345

346 **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** | $AZM^S$, $OB^S$, $TEC^S$, $T^S$ | $CL^R$, $DO^R$, $AMC^R$, $OX^R$,$AM^R$ |

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

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351 **Table 4: Effect of pH on the growth of bacteria isolated from vaginal secretion of patient**
352 **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 |

353 **Note:**

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

356 **Table 5: Effect of temperature on Growth of Isolated Bacterial strains by comparison of**
357 **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 |

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| 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 |

358 **Note:**

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

360 **Table 6: Minimum Inhibitory Concentration (MIC) of sensitive antimicrobial agents**
361 **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 |

362 **Note:**

363 - ®Refer to the table (3.4), for explanation of Antibiotics
364 - All MICs values of antibiotics against each bacterial isolate were in *μg/ml*.

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372 **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 |

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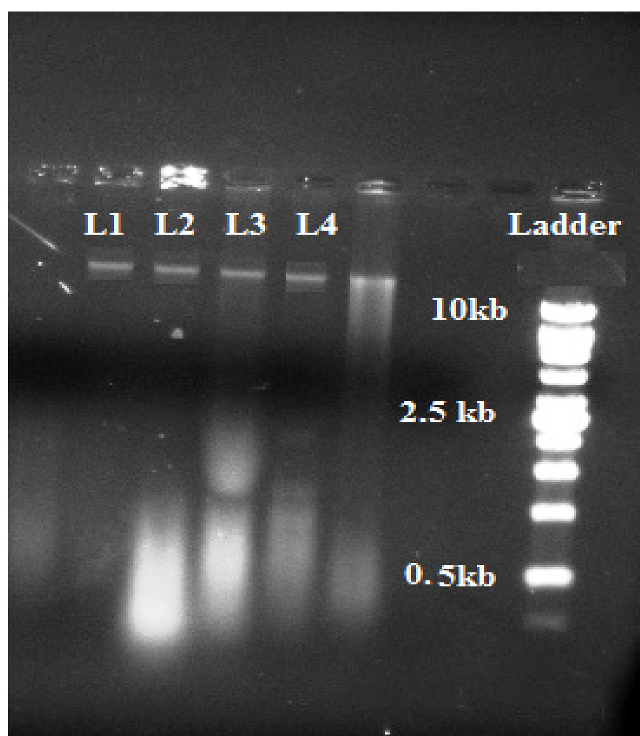


413 **Figure 1: Agarose gel (0.8%) indicates the genomic DNA of bacterial isolates**

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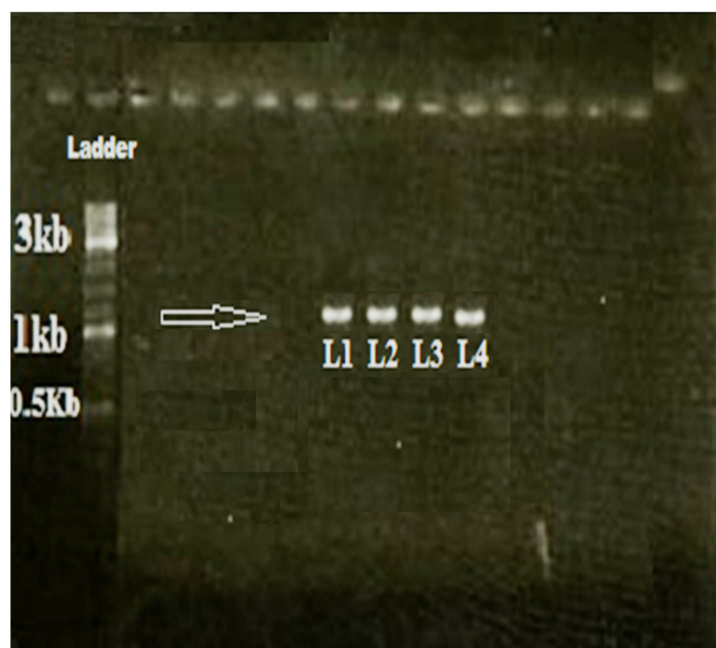


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417 **Figure 2: PCR products of 16S rDNA of bacterial isolates**

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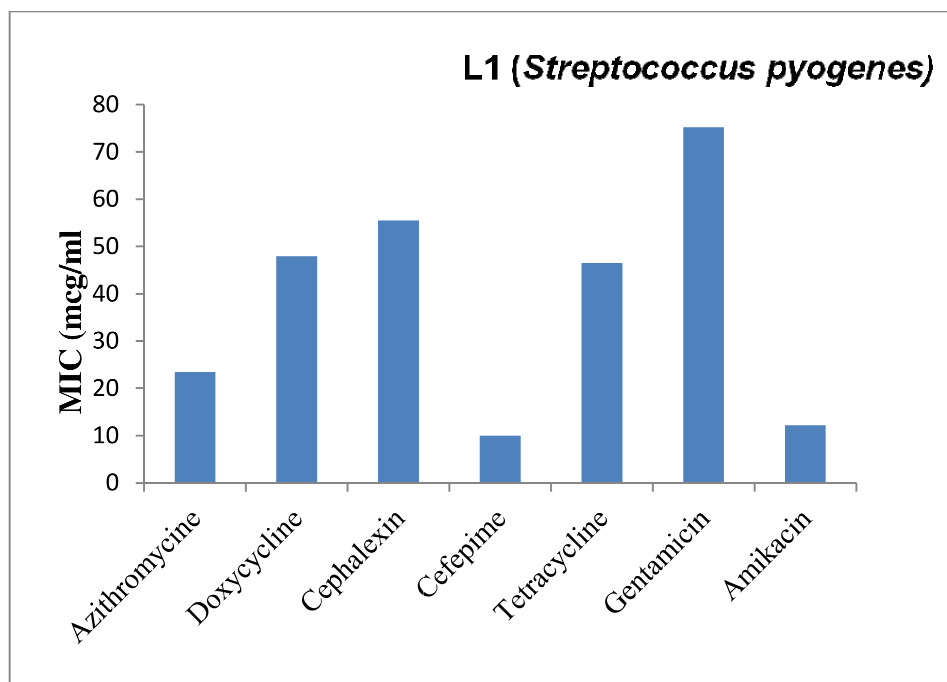


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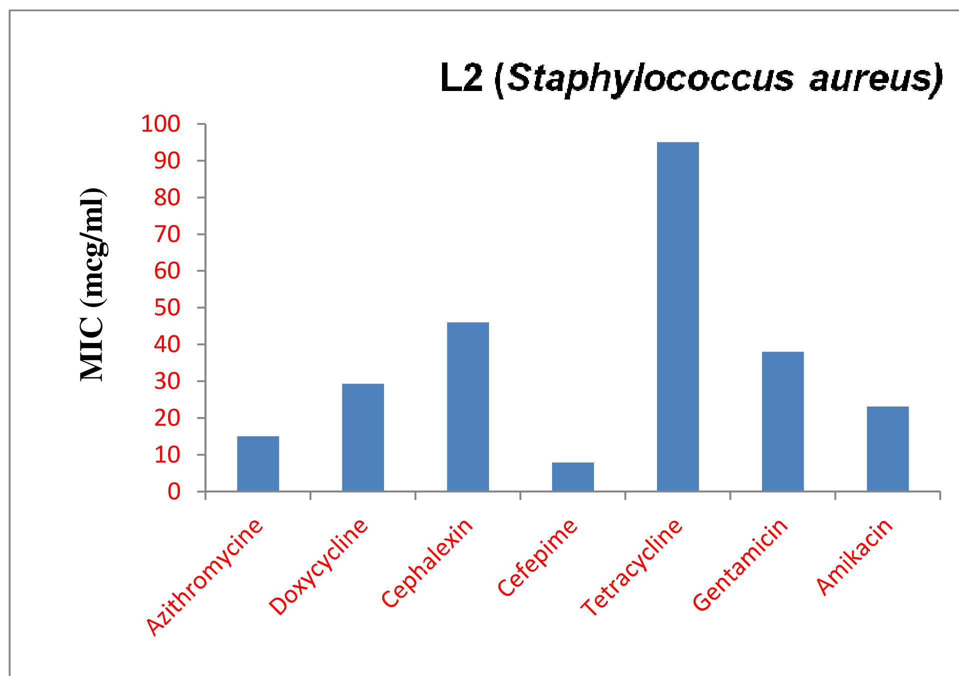
419 **Figure 3: MICs of various antibiotics against L1 Bacterial isolate**

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421 **Figure 4 : MICs of various antibiotics against L2 Bacterial isolate**

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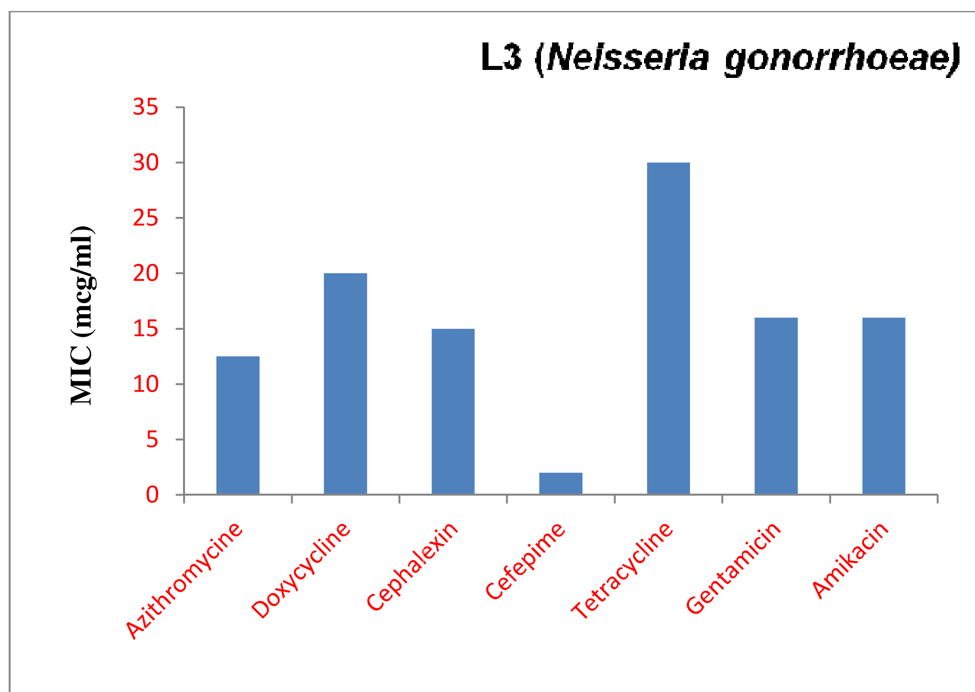


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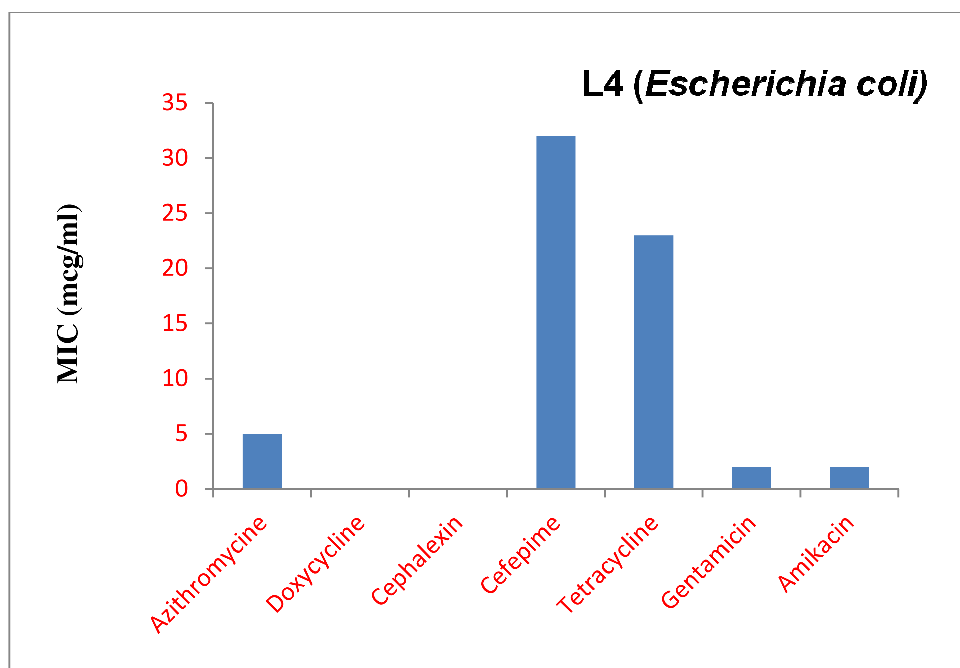
423 **Figure 5: MICs of various antibiotics against L3 Bacterial isolate**

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425 **Figure 6: MICs of various antibiotics against L4 Bacterial isolate**

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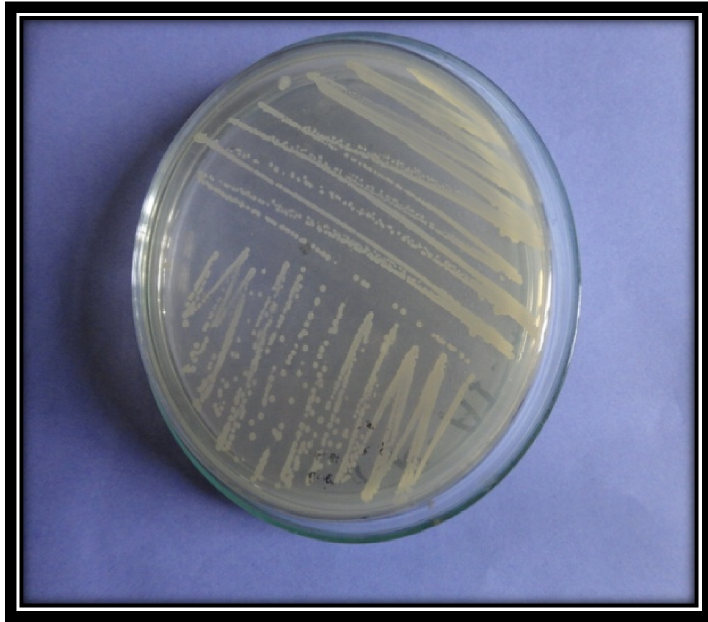
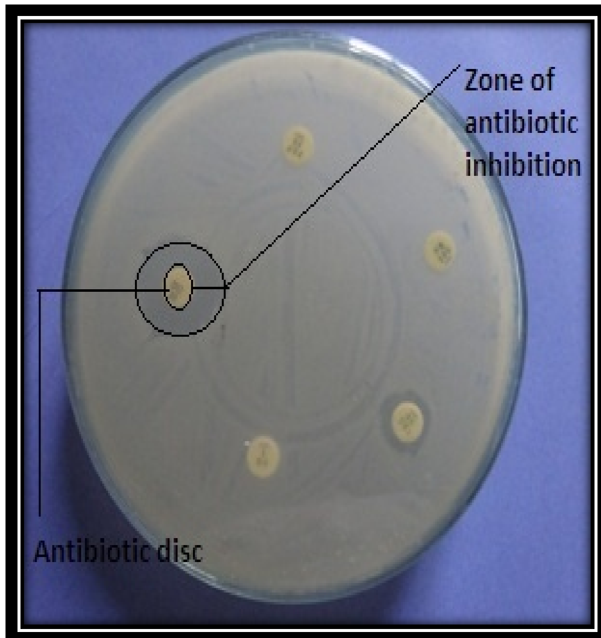
427 

428 **Figure 7: Isolation of bacteria by streaking plate method**

429 


430 **Figure 8: Antibiotic resistance of bacteria**

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