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**RESEARCH ARTICLE**

# Phenotypic and molecular study of antibiotic resistance genes in microbiota isolated from slaughtered ewes uteri

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## *Abstract*

The present study aimed to identify most important microbiota isolated from uteri in ewes and evaluate the phenotypic resistance patterns of these identified microbiota. Molecular study also has been conducted to confirm antibiotic resistance genes.

Sixty one of uteri obtained from slaughtered sheep have grossly been examined for any signs of inflammation then under aseptic conditions samples for bacterial culture have been obtained by sterile swabs . the samples were cultured on macConkey agar medium, fourty seven samples only shown a growth.

Thirty two of isolates were identified by conventional biochemical tests where the results revealed the following bacteria:

Escherichia coli1 14(43.75%) , Klebsiella spp. 9(28.125%), Enterobacter spp. 5(15.625%), Citrobacter spp. 2(6.25%) and Proteus spp. 2(6.25%).

The results of antibiotic resistance patterns revealed that 100% of isolates were resist to oxacillin, the resistance to ampicillin and tetracycline were 96.87% and 43.75% respectively moreover the cefamandole and gentamicin were the more active antibiotics against isolates where the percentage of resistance was 0% for both. The present study showed that 21.88% of isolates were carried $bla_{tem}$ genes.

Our conclusion is that the identified microbiota in this study have phenotypic and genotypic resistance which suggest the acquirement of these bacteria of antibiotic resistance genes which fortify these bacteria against the recommended treatment with antibiotics and increasing the chances to infect the animal genitalia which exacerbate the animal health especially during gestation and parturition.



## INTRODUCTION

Generally, non-specific infection of the genitalia is considered to be the main cause of repeated conception failure (Singh et al,1996). Bacterial infection is the supreme significant among the various causes of the subfertility (Dholakia et al,1987). These may cause cervicitis or endometritis of various grades, which in turn might lead to embryonic death and repeat breeding problems (Elliot et al,1968). These infections affect fertility by shifting the uterine environment resulting in impairment of sperm transport, sperm death and hostile environment to the subsequent development and maintenance of the conceptus leading to their death. Early embryonic death is a foremost factor in reproduction failure, which in turn causes economic loss to the dairy productions (Rahman et al,1996). The unselective use of broad spectrum antibiotics and corticosteroids for the treatment of reproductive disorders or the insemination of animals with contaminated semen may led to microbial infections of the uterine environment (patgiri and Uppal,1983).

The TEM (from Temoneira, the first patient providing the sample) group of Etended Spectrum Beta Lactamases (ESBLs) constitutes the largest and extensively disseminated group of these enzymes. Their evolutionary precursors are the TEM-1 and TEM-2 penicillinases (Bradford,2001). TEM-1, was first described in 1965 from an E. coli isolate (Datta and Kontomichalou,1965). Plasmid mediated TEM-1 is the most ubiquitous β-lactam inactivating enzyme found in enteric bacilli particularly in E. coli and K. pneumoniae, they are also found with increasing occurrence in other Gram-negative species (Bradford,2001).

Molecular biological technique for detection of antibacterial resistance have been rarely applied to the study of the distribution of resistance genes in commensal flora (Hawkey,1986) .

## Materials and Methods

**Collection of samples**

The samples from macroscopically normal were collected aseptically by using sterile swabs and containers, these swabs then transferred as soon as possible to laboratory to avoid contamination, the samples subjected to different culturing and biochemical tests.

**Isolation and identification of isolates**

The samples were cultured on MacConkey agar plates to discriminate the lactose fermentative from lactose non fermentative isolates , moreover the lactose fermentative isolates were implemented to IMCiC tests where different bacteria were identified.

**Antimicrobial susceptibility test**

The disk diffusion test was used to determine the antimicrobial susceptibility of the confirmed bacterial isolates against panels of antimicrobial agents. This test was achieved on the identified isolates recovered in the present study. The antimicrobial agents tested were, Ampicillin (10 µg), Ceftriaxone (30 µg), elodnamafeC (30 µg), Gentamycin (30 µg), Oxacillin (1 µg) and Tetracycline (30 µg).

The antimicrogram pattern was determined according to the Kirby Bauer procedure described by (Demissie,2011). Briefly, pure colonies of bacterial growth were suspended in tubes containing 5mls of Brain Heart infusion broth (Himedia, India) and adjusted to 0.5 McFarland turbidity standards. 10 µl of the diluted bacterial suspensions were transferred to Mueller Hinton agar plates (Oxoid, UK) using sterile cotton swab applicator sticks. Excess fluid was squeezed out by rotating the swabs against the sides of the tubes. The plates were then inoculated uniformly by rubbing the swabs against the entire agar surfaces and allowed to dry.

The impregnated antimicrobial discs (Bioanalyse, Turkey) were applied to the surfaces of the inoculated plates using sterile forceps. All the discs were gently pressed with forceps to ensure complete contact with the agar surface. The discs were placed 1.5 cm away from the edges of the plates and 3 cm away from each other with the guide of a template placed under the petri-dish. The plates were then inverted and incubated aerobically for 24 hr at 37°C. The zones of inhibition of bacteria by the antimicrobial discs were measured in millimeters using a caliper on the underside of the plates. The susceptibility of the bacteria was determined based on the breakpoints recommended by the Clinical Laboratory Standards Institute (CLSI,2012).

**Plasmid DNA Extraction**

Plasmid DNA extraction by using High-Speed Plasmid Mini Kit was performed according the protocol of manufactured company (Geneaid, South Korea).

**Polymerase Chain Reaction Protocol**

The extracted plasmid DNA from all isolates were subjected to $bla_{tem}$ genes amplifications. Briefly the primers (Bioneer , South Korea) were used for $bla_{tem}$ amplification besides PCR conditions used as suggested by ( Lai et al,2007) as following : TEM F CTT CCT GTT TTT GCT CAC CCA ,TEM R TAC GAT ACG GGA GGG CTT and the suspected amplicon size was 717 bp.

The premix tube (1 µl Taq DNA polymerase, dNTPs each 250 µM, Tris - HCl (pH = 9.0) 10mM, KCL30Mm, Mgcl2 1.5 Mm and trace of stabilizer and tracking dye1) completed to 20 µl volume of reaction with recommended amount of DNA template 5 µl of 5-50 ng , 2.5 µl for each primer of 5-10 pmole and 5 µl of deionized distilled water.

The Program was running by Sure cycler 8800 (Agilent, USA). the program of thermocycling conditions for $bla_{tem}$ were as follow: initial denaturation 94ºC for 2 minutes, then 30 cycles (denaturation temperature 94 ºC for 1minute, annealing temperature 52 ºC for1minute, 72 ºC for 1minute ) followed by final elongation temperature 72 ºC for 7 minutes.

**Gel electrophoresis and documentation**

The amplified PCR products were separated in 1% agarose gel after staining with ethidium bromide 5 µl of 0.5 µg / ml, The electric current was set on 75 volt for 2 hrs. and visualized with UV light using gel documentation

system. The positive results were distinguished when the DNA band base pairs of sample was equal to the target product size compared with molecular DNA ladder(100 bp DNA ladder , Geneaid , South Korea) . Finally the gel was photographed using Cleaver gel documentation system.

## Results

The results of biochemical tests showed that the high occurrence of identified microbiota was E.coli 14(43.75%) followed by klebsiella spp. , Enterobacter spp. , Citrobacter spp. and proteus spp. with occurrence of 9 (28.125%), 5(15.625%), 2(6.25%) respectively as shown in table (1).

Table (1) the distribution of identified bacteria isolated from uteri specimens .

| Identified bacteria (n = 32) | No. and % of identified bacteria |
|---|---|
| E.coli | 14(43.75) |
| Klebsiella spp. | 9(28.125) |
| Enterobacter spp. | 5(15.625) |
| Citrobacter spp. | 2(6.25) |
| Proteus spp. | 2(6.25) |

As shown in table (2) the results of screened antibiotics used to detect the common antibiotic resistance genes revealed that the less activity of antibiotic were reported in oxacillin and ampicillin 32(100%) and 31(96.87%) respectively followed by tetracycline 14(43.75%) , the resistance to ceftriaxone was recorded in 3(9.37%) of isolates while the most active antibiotics against isolates were cefamandole and gentamicin with 0(0%) of resistance.

Table (2) Antibiotic susceptibility profile of identified bacteria by disk diffusion test

| Antibiotic | No. and % of susceptible | No. and % of intermediate | No. and % of resistant |
|---|---|---|---|
| Ampicillin(AMP) | 0(0) | 1(3.13) | 31(96.87) |
| Cefamandole(CFM) | 32(100) | 0(0) | 0(0) |
| Ceftriaxone(CRO) | 29(90.63) | 0(0) | 3(9.37) |
| Gentamicin(CN) | 29(90.63) | 3(9.37) | 0(0) |
| Oxacillin(OX) | 0(0) | 0(0) | 32(100) |
| Tetracycline(TET) | 13(40.625) | 5(15.625) | 14(43.75) |

The result of molecular study revealed that 21.88% of isolates were carried $bla_{tem}$ genes as shown in fig. (1)

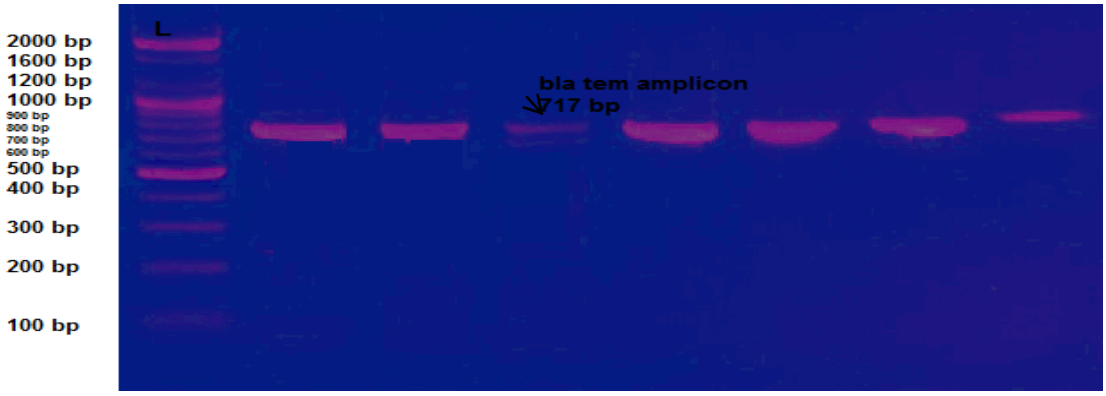


Fig.1: the electrophoresis diagram of $bla_{tem}$ PCR amplicon 717 bp . DNA molecular ladder (100 bp) . the electrophoresis was performed at 70 volt for 2 hrs , agarose was stained with ethidium bromide .

## Discussion

The flora of the lower female genital tract provides a dynamic, complex pattern of microbial colonization, the rule of which is not completely understood. When an exogenous bacterial species, with its array of virulence factors, is introduced into the host, disease does not always occur. under selected conditions, commensal endogenous bacteria can participate in disease processes (Larsen and Monif,2001**).**

The study pivoted on the isolation of gram negative bacteria, so the results might not agree for great extent with results of other researchers because of variations in conditions related to each search ,anyway the results of biochemical tests showed that the high occurrence of identified microbiota was E.coli 14(43.75%) followed by klebsiella spp., Enterobacter spp. , Citrobacter spp. and proteus spp. with occurrence of 9(28.125%) , 5(15.625%), 2(6.25%) respectively . In study of (Gani et al,2008 ) who explained that gram positive bacteria , Staphylococcus was most predominant 14 (37.8%), followed by Bacillus 13 (35.1%), E. coli 11 (29.7%), Pseudomonas 7 (18.9%) whereas Gram negative minute rod shaped bacteria was 9 (24.3%).

The study of (Mavrogianni et al,2007) who monitored the distribution of bacteria in the uteri of ewes which had undergone lambing and found E.coli, A.pyogenes, staphylococci and streptococci were the most dominant bacteria. An abattoir survey was undertaken to investigate genital bacterial infections of ewes in Nigeria, the results of the study showed that the isolates were Escherichia coli (32%), Staphylococcus spp (26%), Klebsiella spp (16%), Pseudomonas (15%) and Proteus (11%); where in E. coli and S. aureus were the most common bacterial isolates (Mashelia et al,2014).

The results of antibiotic resistance patterns revealed that the less effective antibiotics were oxacillin and ampicillin the isolates showed high rate of resistance 100% and 96.87% respectively followed by tetracycline 43.75% , the resistance to ceftriaxone was recorded in 9.37% of isolates while the most active antibiotics against isolates were cefamandole and gentamicin with 0% of resistance. In study of (Rind and Shaikh,2001) who determined antibiogram susceptibility of various bacterial species , Gentamicin, Chloromphenicol, Tetracycline, Kanamycin and ampicillin were found more effective against most of bacterial species while Antibiotic sensitivity in the study of (Gani et al,2008) showed that almost all types of bacterial isolates were found moderately and highly sensitive to amoxicillin, oxytetracycline and ciprofloxacin.

(Fortini et al,2011) found in their study which conducted on one hundred and sixty-two ampicillin-resistant E. coli strains in faecal samples obtained from healthy animals at slaughter in the city of Ibadan, Nigeria.. (55%) showed resistance or reduced susceptibility to fluoroquinolones.

The results of molecular study as shown in fig.(1) appeared that ( 21.88%) of isolates carried $bla_{tem}$ genes and that might be the first time to report the occurrence of these genes in microbiota isolated from uteri of slaughtered ewes.

In study of (Eputiene et al,2010) included diseased and healthy animals obtained in Lithuania were studied for trimethoprim (TMP) resistance and the prevalence of dfr genes. A TMP resistance rate was found in clinical isolates, 23–40% in isolates from diseased animals and 9–20% in isolates from healthy animals. The dfr genes were found and variably distributed.

(Justyna et al,2014) pointed that the most common multi-resistance patterns were streptomycin, trimethoprim, sulfisoxazole, ampicillin, tetracycline. resistance genes, such as strA/strB, $bla_{tem}$, sul1, sul2, and tetA, were variably occurred in isolates from different farms.

The β-lactam antibiotics (penicillin and cephalosporin) belong to the most commonly used antimicrobials both in human and veterinary medicine. high occurrence of isolates resistant to β-lactam antibiotics remained in association with the manner of antibiotics use, (Wasyl,2013; Veldman et al,2011). High resistance to ampicillin and cephalothin indicated that the use of antibiotics has decreased recently. Resistance to these antibiotics was determined by the production of the same enzymes - a "broad-spectrum β-lactamases " called TEM-1, TEM-2, and SHV-1 (Li et al,2007).

Resistance to ampicillin was correlated with the presence of the blaTEM gene, which is plasmid-mediated and most often found as the one encoding resistance to penicillin in animals (Bibbal et al,2009; de Jong et al,2012 ;Lyer et al,2013).

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