European Journal of Molecular & Clinical Medicine
ISSN 2515-8260 Volume 7, Issue 09, 2020

# DETECTION of SOME VIRULENCE FACTORS and ANTIBIOTIC SENSITIVITY TEST of ENTEROCOCCUS FAECALIS ISOLATED FROM SHEEP by MULTIPLEX PCR

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***Abstract***:
**Enterococcus faecalis form an important population of commensal bacteria and have been reported to possess numerous virulence factors considered significantly important in exacerbating diseases caused by them.**
**Objectives: The present study was conducted to evaluates the presence of virulence factors and antibiotic susceptibility among Enterococcus faecalis isolated from sheep.**
**Methods: The study included the collection of 50 samples (25 Milk samples collected from the udder was washed and the teats were disinfected and dried using alcohol, the first milk drop removed. 5ml of milk collected on aseptic tube and 25 Feces samples collected from sheep diarrhea from rectal by aseptic gloves. (from October 2018 to March 2019 ) and transported to laboratory as soon as possible in sterile Brain heart infusion broth that incubated at 37 C for at least 24-28 hours to increasing chances of isolation. Enterococcus faecalis that were recognized by cultural characteristics, Gram stain, and biochemical reactions.**
**Results: The results of the laboratory cultural of 50 cotton swabs used s show that the isolation rate of Enterococcus spp. were 32% and 56% from milk and feaces respectively. the result of PCR test for detection of Enterococcus faecalis: show that the Enterococcus faecalis detected in rate of 66.6% from total Enterococcus spp. While the result of Enterococcus faecalis virulence factors showed that the Surface proteins, Gelatinase and Hemolysin were 75%, 33.3%, 25.5% respectively. Results of antibiotic sensitivity test showed the most bacterial isolated sensitive Nitrofurantoin , Imipenem and Nalidixic acid were 91.6%,83.3% and 58.3% % respectively**
**Conclusion: We report that our simple modification of the existing multiplex PCR had increased the detection of the enterococcal virulence genes. Predominance of virulence genes was in order of Surface proteins, Gelatinase and Hemolysin were 75%, 33.3%, 25.5%. This modified PCR protocol could be useful to resolve the problem of decreased detection of virulence determinants in enterococci.**

***Keywords: Detection, Virulence factors, antibiotic, Enterococcus faecalis, , multiplex PCR.***

## INTRODUCTION

Enterococcus is gram positive cocci arranged in pair or short chains, negative to catalase test, non motile, non spore forming, aerobic or Facultative anaerobic, growth in 10-45$^{O}$C. able to growth in media with 9.6pH, media with 40% bile salt, media with 6.5% Nacl [(1).]

It conceder as apart of normal flora in intestine. isolated from water, soil and animal product (milk and meat) [(2)]. They are 26 Enterococcus species but the most common are *Enterococcus faecalis* and *Enterococcus faecium*. [(3).]

It have many virulence factors like hemolysin, cytolysin, lipase , Haemagglutinins, Extracellular superoxide, Leucine – aminopeptidase, Pyrrolidonyl arylamidase, Protease . It causes many diseases in human like pericarditis, bacteremia, UTI, meningitis and wound infection[(4)].

It have many surface protein's material which play important role on attachment and invasion of host cells, also same strain have Adhesion collagen which help bacteria on attachment on collagen in the host cells. Hemolysin play important role in pathogenesis of bacteria by the Cytolytic effect on RBCs and WBCs [(5) (21) (22).]

## MATERIALS AND METHODS

Sample collected

a- Milk samples(25 samples) : The udder was washed and the teats were disinfected and dried using alcohol, the first milk drop removed. 5ml of milk collected on aseptic tube

b- Feces (25 samples from sheep diarrhea ): collected from rectal by aseptic gloves.

Bacterial isolation and identification

each sample culture on Azide blood agar, Azide bile esculin agar, MacConkey agar and cultivation on 37$^{O}$C for 48h. gram stain and groups of biochemical tests were applied according to [(6).]

Hemolysin detection

(by use of blood base agar with 10% human blood).

DNA extraction from milk

1ml of milk mixed with 200 µL and 170 µL of SDS solution. The mixture incubated at 80$^{O}$C for 10 minutes. Then cooled on ice for 10 minutes. 20 micro litter of Proteinase K added to the mixture and incubated at 56$^{o}$C for 12 Hours. DNA templates isolated using standard protocol of Phenol-Chloroform-Isoamyl alcohol and according to [(7).]

DNA extraction from stool

2g of stool suspended on 15ml of lysis buffer with 0.9% SDS and 18 mM EDTA, and then use of phenol/ chloroform/isoamyl alcohol protocol according to [(8).] as in table (1)

Table (1) Compounds used in the preparation of Reaction Mixture

| Compounds used in preparation of Reaction Mixture | Volume (microliter ) | Reference and out product |
|---|---|---|
| Taq PCR Master Mix KIT (Qiagen, Germany) | 25 | (Jurkovič *et al*.,2006) Out product size:941bp |
| Primer A (ATCAAGTACAGTTAGTCTT) | 0.3 from 100pM Solution. | |
| Primer B(ACGATTCAAAGCTAACTG) | 0.3 from 100pM Solution. | |
| DNA Template | 3 | |
| DNA free water (Qiagen, Germany) | | |
| | | |

**Timer program of the thermocycler** as in table (2) according to [9]

Table (2) Timer program of the thermocycler

| Stage | Temperature(c°) | Time(minuts) | Cycles (numbers) |
|---|---|---|---|
| First Denaturation step | 94 | 2 minutes | 1 |
| Denaturation step | 94 | 1 minutes | 30 |
| Primer-annealing step | 57 | 1 minutes | |
| DNA extension step | 72 | 1 minutes | |
| Final DNA extension step | 72 | 10 minutes | 1 |
| End Temperature | 4 | —— — | - ———— |

Detection of virulence factors
According to [10] [20] by using of two primers
a-Surface proteins (*ef3314*): by use primer (F: AGA GGG ACG ATC AGA TGA AAA A. and R: ATT CCA ATT GAC GAT TCA CTTC ) with product size 566pb
b-Gelatinase (*gelE*): by use primer (F: ACC CCG TAT CAT TGG TTT . and R: CAG CAT TGC TTT TCC ATC ) with product size 405pb

## RESULTS

Bacterial isolation
From table (3) show that the isolation rate of *Enterococcus spp.* were 32% and 56% from milk and feaces respectively.

Table (3): isolation rate of *Enterococcus* from milk and feces of sheep

| Sample | NO. | NO. of positive sample | Rate of positive sample |
|---|---|---|---|
| Milk | 25 | 4 | 16% |
| Feces | 25 | 14 | 56% |
| Total | 50 | 18 | 36% |

The existing of Enterococci in milk may be due to contamination of milk and udder from farm. Also to its ability to grow at refrigeration temperatures in temperature (5-45° C) [10].

PCR test for detection of *Enterococcus faecalis*
From table (4) and figure(1)show that the *Enterococcus faecalis* detected in rate of 66.6% from total *Enterococcus* spp.

*Table (4):Result of PCR test specific for Enterococcus faecalis*

| Sample | NO. of *Enterococcus* spp. positive sample by culture methods | NO. of *Enterococcus faecalis*. positive sample by pcr | Ratio |
|---|---|---|---|
| Milk | 4 | 3 | %75 |
| Feaces | 14 | 9 | %64.2 |
| Total | 18 | 12 | 66.6% |

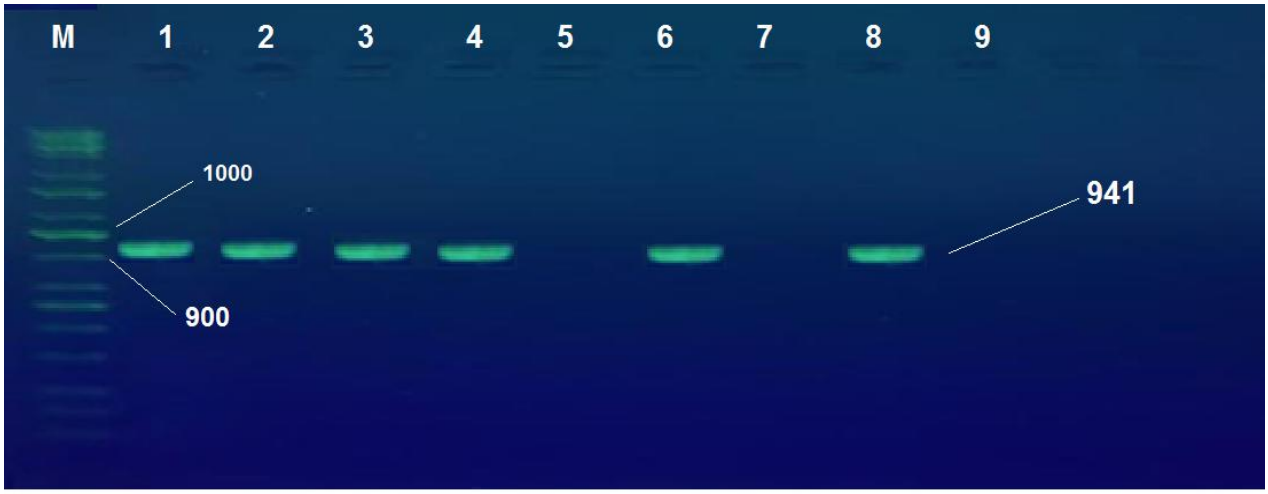


Figure (1): Electrophoresis on 2 % a garose gel and ethidium bromide staining, showing the results of PCR procedures. M: DNA marker wells 1,2,3,4,6 and 8) positive samples of *Enterococcus faecalis* which showed band in size 941 bp

The results of current study showed dominant of *Enterococcus faecalis* in compare with other *Enterococcus* spp. this result agreed with result of [11,12]. That's due to its resistant to antibiotic and environmental condition or its consider as apart of normal flora.

Detection of *Enterococcus faecalis* virulence factors

The result showed that the Surface proteins, Gelatinase and Hehemolysin were 75%, 33.3%, 25.5% respectively. As in table (2) and figure (2.3 and 4)

Table (5): *Enterococcus faecalis* virulence factors

| Sample | NO. of *E. faecalis*. isolates | No.& rate of isolates carry the virulence factors | | |
|---|---|---|---|---|
| | | Surface proteins (*ef3314* | Gelatinase (*gelE*) | Hemolysin |
| Milk | 3 | 66.6% (2:3) | 33.3% (1:3) | %33.3 (1:3) |
| Feaces | 9 | %77.7 (7:9) | 33.3% (3:9) | %22.2 (2:9) |
| Total | 12 | 75% (9:12) | 33.3% (4:12) | 25.5% (2:12) |

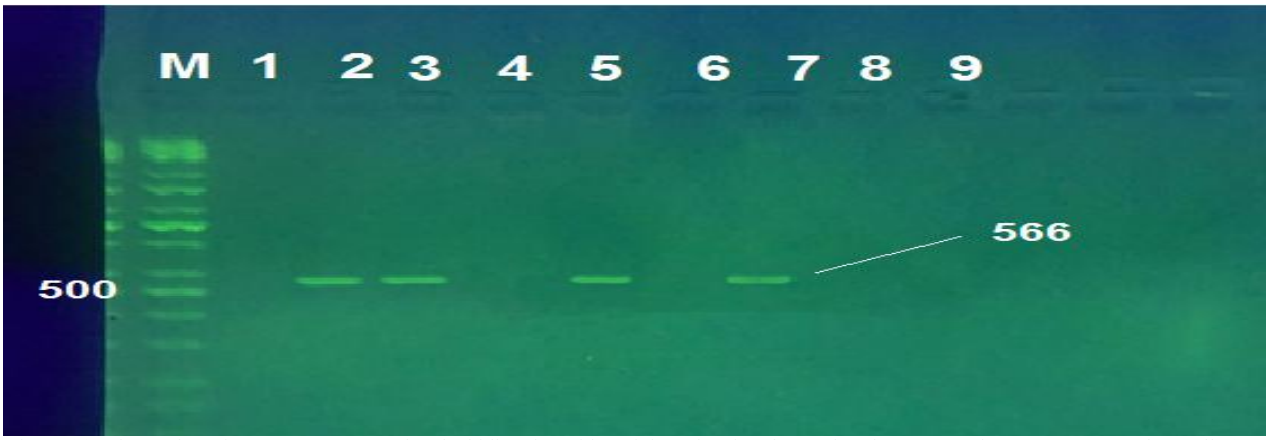


Figure (2): Electrophoresis on 2 % a garose gel and ethidium bromide staining, showing the results of PCR procedures. M: DNA marker wells (2,3,5 and 7) positive samples Surface proteins (*ef3314*) of *Enterococcus faecalis* which showed band in size 566 bp

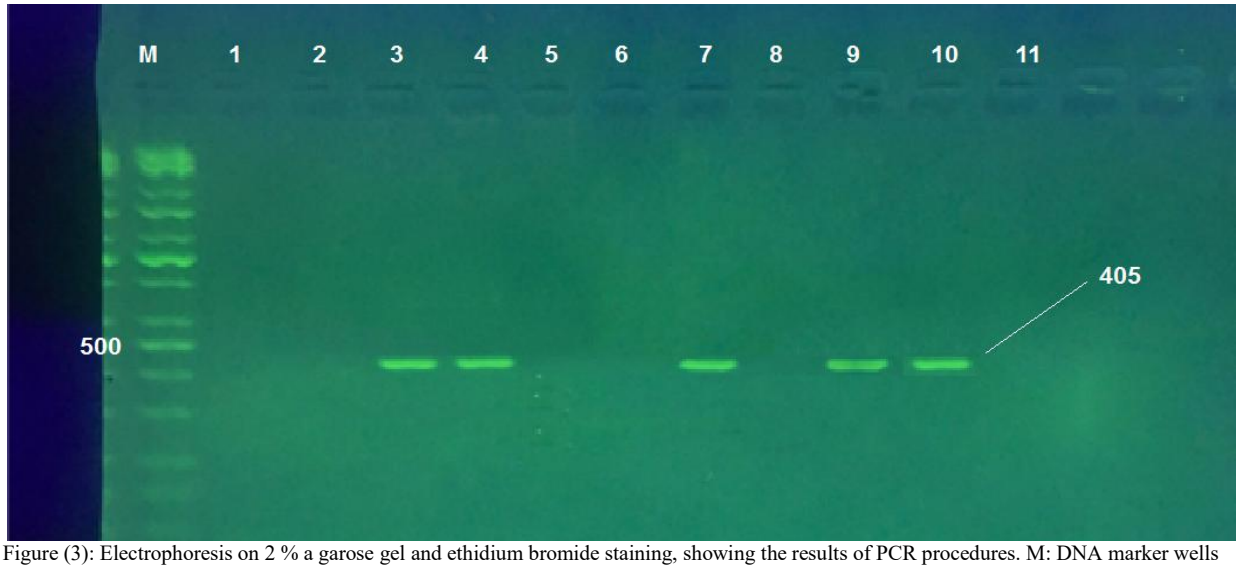


Figure (3): Electrophoresis on 2 % a garose gel and ethidium bromide staining, showing the results of PCR procedures. M: DNA marker wells (3,4,7,9 and 10) positive samples Gelatinase (*gelE*) of *Enterococcus faecalis* which showed band in size 405 bp

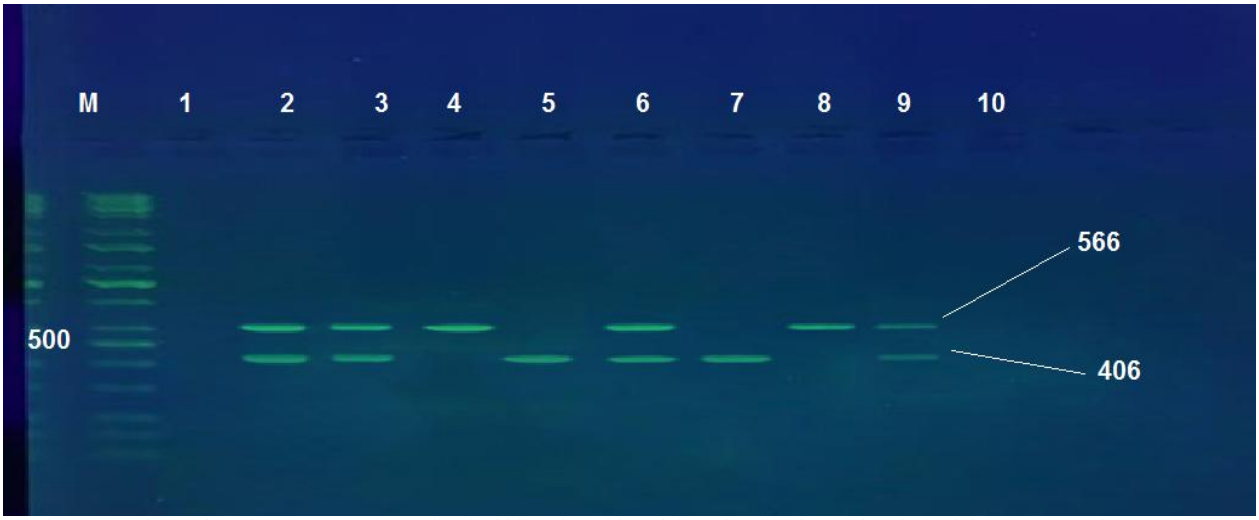


Figure (4): Electrophoresis on 2 % a garose gel and ethidium bromide staining, showing the results of multiplex PCR procedures. M: DNA marker

- wells (4 and 8) positive samples Surface proteins (*ef3314*) of *Enterococcus faecalis* which showed band in size 566 bp
- wells (5 and 7) positive samples of Surface proteins (*ef3314*) Gelatinase (*gelE*) of *Enterococcus faecalis* which showed band in size 405 bp
- wells (2,3 and 9) positive samples for Surface proteins (*ef3314*)and Gelatinase (*gelE*) of *Enterococcus faecalis*

The result showed that high percentage of isolate have surface protein. That supported what referred by (Joyanes *et al*.,2000)[13] about the ability of *Enterococcus faecalis* to attachment on epithelial cell. and role of surface protein in this attachment [(14).]The gelatenase appear in rate of 33.3% this result is coincide with result of (Seno *et al*.,2005)[15]. The presence of this gene is associated with bacteria isolate from clinical infection cases and isolates that have beta hemolysin feature is more virulence, in other isolates may be appeared Silent hemolysin genes [(16,17).]

Results of antibiotic sensitivity test: from table (6) showed that the antibiotic sensitivity test for twelve *Enterococcus faecalis* isolates.

Table (6): antibiotic sensitivity test for *Enterococcus faecalis*

| Antibiotic type | Number of sensitive isolate | Rate of sensitive isolate |
|---|---|---|
| Amikacin | 2 | 16.6% |
| Cephalexin | 1 | 8.3% |
| Cefotaxime | 1 | 8.3% |
| Ceftriaxone | 2 | 16.6% |
| Nitrofurantoin | 11 | 91.6% |
| Imipenem | 10 | 83.3% |
| Nalidixic acid | 7 | 58.3% |
| Erythromycin | 5 | 41.6% |

In compare between this result and other results [18,19] showed different in rate of sensitivity, that's maybe due to different in the sources of isolates , number of isolate, date and geographic location of study.

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