j.Egypt.vet.med.Assoc 79, no 1. 61 – 74 (2019)

ISOLATION AND IDENTIFICATION OF *E. FAECALIS* FROM DIFFERENT SOURCES OF BACTERIAL CONTAMINATION IN LARGE ANIMAL SLAUGHTERHOUSE

By

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ABSTRACT

Three hundred forty-five swaps were collected from different sources of suspected bacterial contamination sites in cattle slaughter hall in El-Monieb slaughterhouse in Giza governorate, Egypt. Those samples were processed aiming for isolation and identification of *E. faecalis*. The results revealed that out of 345 samples, 122 *E. faecalis* samples were recorded representing 35.4% of the samples. The mean count *E. faecalis* among the different sources was 5.73 ± 1.7-log10 CFU/ cm^2. The sensitivity test for 16 different antimicrobials showed that all the 122 *E. faecalis* samples were multidrug resistance. The electrophoretic profile of PCR for *van*A gene was confirming vancomycin resistant *E. faecalis* isolates in 26 samples (21.3%). It was concluded that, they are more than one source of *Enterococci* contamination before, during and after slaughtering process that could act as a public health risk and play a potential role in food safety issue.

INTRODUCTION

Enterococci have over the years shifted from harmless commensals to opportunistic but important pathogen mainly causing nosocomial infections, **(Oskar, 2012).** *Enterococci* can persist for as long as 60 minutes after inoculation onto hands and as long as 4 months on inanimate surfaces. That is why where it can serve as a reservoir for ongoing transmission in the absence of regular decontamination, **(Michael *et al.*, 2014).** The modern classification techniques transferred some members of the genus *Streptococcus*, notably some of the Lancefield's Group D Streptococcus, to the new genus *Enterococcus*. *Enterococci* can grow and survive in harsh environments and can persist almost anywhere including soil, plants, water and food. It can also survive from 5 days to 4 months on dry inanimate surfaces. *Enterococci* are considered as indicators for fecal contamination. They have been implicated in outbreaks of foodborne illness and they have been ascribed a beneficial or

detrimental role in foods. In processed meats, *Enterococci* may survive heat processing and cause its spoilage. Meanwhile in certain types of cheeses its growth contributes to ripening and development of product flavor. Some Enterococci have become recognized as serious nosocomial pathogens causing bacteremia, endocarditis, urinary tract and other organs infection. This may explain the resistance of some of these bacteria to most antimicrobials that are currently in use including quinolones, macrolides, tetracyclines, streptogramins, and glycopeptides. Therefore, treatment of *Enterococci* infections may be difficult **(Franz *et al.*, 1999).** *Enterococci* also, are intrinsically resistant to a number of first line antimicrobial agents; they show low-level resistance to β-lactams, resistance to cephalosporins, and low-level resistance to aminoglycosides. Furthermore, *Enterococci* can acquire resistance to other antimicrobial agents and it is acquired by gene transfer systems, such as conjugative or non-conjugative plasmids or transposons. The virulence of *Enterococci* is not well understood but adhesions, hemolysis, hyaluronidase, aggregation substance and gelatinase are putative virulence factors. It appears that foods could be a source of vancomycin-resistant *Enterococci* **(Franz *et al.*,1999).** Over the past two decades, *E. faecalis* and *Enterococcus faecium* have become increasingly important pathogens worldwide, especially because of life-threatening infections related to the effluent systems, including bacteremia and infective endocarditis. *Enterococcal* bacteremia is associated with high mortality rates **(Hammerum, 2012).**

The present investigation aims to study the bacterial isolates of *E. faecalis* in suspected different sources of contamination in the large animal slaughterhouse by isolation, counting, characterization and identification of the microorganism that might be taken in consideration as a public health and food safety issue in the line of one health approach initiative.

MATERIAL AND METHODS

The samples were collected and processed according to Sudhakar, 2009: Animal surface and hide (muffle area, under tail, back, fore limb and hind limb) before and after slaughtering process, Tools (Hooks and both sides of skinning and evisceration knives) before and immediately after operation, operators' hands (palms and knuckles of both hands) and cloths from back area (loading site) before and after operation, gall bladder, duodenum, ground, wall and washing water. A sterile cotton swab (3 cm long and 1 cm in diameter) on sticks swabbed an area of ten cm2 of the different surfaces. Each cotton swab was moistened with 1 ml of 0.1% peptone water prior to its use. The swabs were rubbed on sites continuously for 30

seconds and transferred to a sterile screw-capped test tube containing 1 ml of sterile maintenance medium peptone water. Ten ml of washing water was also collected in sterile screw-capped test tube. A complete gall bladder and part of duodenum were transferred to sterile caped container. All samples were brought to the laboratory in the icebox containing ice and processed immediately.

Preparation of samples:

One ml from each swab was separately transferred to a test tube containing nine ml of sterile peptone water (0.1%) to provide the original dilution (10^{-1}). From which further ten-fold. Serial dilutions were prepared up to (10^{-7}) **(Sudhakar, 2009).**

For duodenum and gall bladder, the organs were sliced into small pieces under complete aseptic condition then; 10 gm of the sliced tissues were added to 90 ml 0.1% peptone water to prepare a stock solution **(Sudhakar, 2009).**

Isolation and counting of *Enterococcal* species on KF media:

KF Streptococcus agars (Oxoid CM 0701) were prepared according to the manufacturer. Typical colonies (red to pink) were enumerated and recorded as presumptive Enterococcus count, **(MacFaddin *et al.,* 1985)**.

Counting of bacteria was done according to standard procedures for bacterial culture, **(Sudhakar,2009).** Petri dishes containing between 30 and 300 colonies were selected for bacterial counting. The number of counted colonies was expressed as colony forming units (CFU/cm2).

Identification of suspected *E. faecalis*:

Bile Aesculin Agar (Oxoid CM 1136) were prepared according to the manufacturer.

The presumptive Enterococcal colonies were picked up from KF Streptococcus agar plates for sub-culturing on Bile Aesculin Agar plate. The plates were incubated for 24 hours at 37ºC. Presumptive *E. faecalis* produce dark brown or black complex medium, **(Cain *et al,* 2015).** Sheep blood agar plates were prepared by preparation of Nutrient agar (Oxoid CM 0003) according to the manufacturer then enriching with up to 10% sheep blood then, sterilizing by autoclaving at 121ºC for 15 minutes.The plates were streaked aseptically with grown colonies then incubated for 24 hours at 37ºC to determine the grown non-hemolytic (gamma-hemolytic) colonies, **(Cain *et al.,* 2015).**

Microscopical Examination: Films were prepared from the pure culture of the isolated organisms, stained with Gram stain. The slide was examined under microscope at both

1000 x oil immersion to observe Gram-positive cocci in chains, **(Cain *et al,* 2015).**

Catalase test: Using a sterile loop, a small amount of colony growth was transferred on the surface of a clean, dry glass slide. A drop of 3% hydrogen peroxide (H2O2) was added on the glass slide to observe negative reaction, **(Cain *et al.,* 2015).**

Coagulase test, **(Tortora *et al.,*2013):** The tube test was done using citrated rabbit plasma that has been inoculated with a colony. The tube is then incubated at 37º C for 1.5 hours to observe negative reaction.

API 20-STREP biochemical identification system (BioMerieux, France), for differentiation between *E. faecalis* and *E. faecium*.

Determination of antimicrobial sensitivity for *E. faecalis* (CLSI, 2017):

The material used was Mueller Hinton Agar medium (Oxoid), antibacterial discs (Oxoid). The types of antimicrobials were selected as follows:

- AMC: amoxicillin/clavulanic acid (30 µg), AK: amikacin (30 µg), AMC: picillin/sulbactam (30 µg), FOX: cefoxitin (30 µg), VA: vancomycin (30 µg), AM: ampicillin (10 µg), GM: gentamicin (10 µg), CIP: ciprofloxacin (5 µg), RF: rifampicin (5 µg), E: erythromycin (15 µg), AZM: aztreonam (15 µg), CN: clindamycin (10 µg), CTX: cefotaxime (30 µg), CP: Cefepime (5 µg),CZ:ceftazidime (5 µg) STX:trimethoprim/sulfamethoxazole (1.25/23.75 µg) and E-test strips for oxacillin and vancomycin.McFarland nephelometer standards (0.5): McFarland nephelometer barium sulphate standard (0.5) was used for the determination of the approximate number of bacteria, in which McFarland 0.5 turbidity equals 1.5 X 10^8 organisms per ml. The disc diffusion technique was adapted according to [4]. The sensitivity was determined by measuring the diameter of a visible and clear zone of inhibition produced by diffusion of the antibacterial agent from the discs into the surrounding medium. Interpretation according to **(CLSI, 2017).**

PCR identification of *E. faecalis van*A gene primers

The isolates were sent to animal health research institute for complete confirmed identification. DNA Extraction was applied by harvesting the Pure and young cultures from agar plates. Genomic DNA was extracted using (thermo scientific DNA purification kit) for all samples. According to **(Dutka-Malen *et al.,* 1990),** the *van*A gene was amplified with the primers *van*A F: 5-GGGAAAACGACAATTGC-3 and *van*A R: 5-GTACAATGCCGTTA-3 specific for the *van*A gene with Size of PCR 732 bp. The PCR amplification was carried out using 2x PCR master mix solution and a program consisted of an initial denaturation step at

94º C for 3 min. Then it was followed by 40 cycles of DNA denaturation at 94º C for 30 seconds, primer annealing at the appropriate temperature for each set of primers for 2 minutes and DNA extension at 72º C for 2 minutes. After the last cycle, the reaction was terminated by incubation at 72º C for 6 minutes, and the products were stored at 4ºC. The PCR products (5 ml) were analyzed by electrophoresis in TAE buffer (0.04 M Tris-acetate, 0.002 M EDTA [PH 8.5]) on 1% (wt./vole) agarose gels, and the gels were stained with ethidium bromide and a UV trans-illuminator.

Statistical analysis of results' data:

The data of the study were compiled in excel database, and organized for statistical analysis. The analysis was done using IBM SPSS version 21 **(Coakes, 2005),** a computer-based statistical software package. A statistical approach was used for comparing between means that was One Way ANOVA. The data was represented by mean of the samples collected per each suspected source of contamination.

RESULTS

The results analysis revealed that out of 345 samples from different sources of sampling sites in the slaughterhouse, 122 *E. faecalis* samples were recorded in prevalence 35.4%. The mean count among the mentioned sources was 5.73 ± 1.7-log10 CFU/ cm^2.

According to (Table 1-3), it was noticed that, the hind limb sites before and after slaughtering process were the highest sites had positive *E. faecalis* samples with percentage 93.3% per each from the collected samples from the floor sites and prevalence 4.1% from the grand total collected samples.

The highest *E. faecalis* load was recorded in floor sites with mean 7.18-± 0.86-log10 CFU/ cm^2.The minimum number of samples with positive isolates were found at abdomen after slaughtering process, neck at slaughter site tools before slaughtering process, butchers' hands, butchers' clothes and muffle area by prevalence 0.6%.

While, the lowest mean count of *E. faecalis* were 2.79 ± 0.55-log10 CFU/ cm2 at butchers' hands before slaughtering process.

There was no detection of *E. faecalis* in pharyngeal region after slaughtering, water samples, duodenum or gall bladder.

By classifying the different sampling sites according to the related category and time of sampling (before or after slaughtering) (Table 3), the sampling sites were categorized as

follow: Sampling sites from animals before slaughtering, sampling sites from animals after slaughtering, butchers (hands and clothes) and tools before slaughtering, butchers (hands and clothes) and tools after slaughtering and finally, sample sites from the slaughterhouse environment (floor and wall).

According to the samples category, the results showed that, the sampling sites from animals before slaughtering has the highest number of positive samples (42 positive samples) in prevalence 12.2% from the total collected samples, followed by butchers (hands and clothes) and tools after slaughtering (28 positive samples) representing prevalence 8.12% then, sampling sites from animals after slaughtering (22 positive samples) representing 6.4% and slaughterhouse environment (20 positive samples) representing 5.8%, and finally butchers and tools before slaughtering (10 positive samples) in prevalence 3%.

The slaughterhouse environment (floor and wall) had the highest mean count for *E. faecalis* 6.8 ± 0.9 log10 CFU/ cm2, while The butchers (hands and clothes) and tools before slaughtering is the lowest category had *E. faecalis* mean count (mean count: 4.2 ± 1.8 log10 CFU/ cm2).

Analysis of variance between the different sources of contamination in the slaughterhouse reveled a significant difference ($P<0.01$) in mean *E. faecalis* count with F value 5.12.

Table (1): Number and Percentage of positive *E. faecalis* samples isolated from different sampling sites in the slaughterhouse.

Source of Sampling	Number of samples	Number of Positive	Percentage of Positive
Abdomen after slaughter	15	2	13.3%
Back after slaughter	15	4	26.7%
Back before slaughter	15	5	33.3%
Floor	15	13	86.7%
Fore limb before slaughter	15	9	60%
Hind limb after slaughter	15	14	93.3%
Hind limb before slaughter	15	14	93.3%
Muffle before slaughter	15	2	13.3%
Neck after slaughter	15	2	13.3%
Pharyngeal region	15	0	0%
Hand after slaughter	15	7	46.7%
Hand before slaughter	15	2	13.3%
clothes after slaughter	15	8	53.3%
clothes before slaughter	15	2	13.3%
Tool 1 after slaughter	15	6	40%
Tool 1 before slaughter	15	4	26.7%
Tool 2 after slaughter	15	7	46.7%
Tool 2 before slaughter	15	2	13.3%
Under tail before slaughter	15	12	80%
Wall	15	7	46.7%
Water sample	15	0	0%
Duodenum	15	0	0%
Gall bladder	15	0	0%
Total	345	122	35.4%

Table (2): Mean counts (log10 CFU/ cm^2 ± Standard Deviation), 95% confidence interval of mean and % prevalence of positive *E. faecalis* in the different sources of sampling in the slaughterhouse.

Source of Sampling	Mean log^{10} CFU/ cm^2	Std. Deviation	Prevalence
Abdomen after slaughter	**6.55**	**0.53**	**0.6%**
Back after slaughter	**4.97**	**0.66**	**1.2%**
Back before slaughter	**4.78**	**0.74**	**1.5%**
Floor	**7.18**	**0.86**	**3.8%**
Fore limb before slaughter	**5.67**	**1.62**	**2.6%**
Hind limb after slaughter	**7.09**	**1.36**	**4.1%**
Hind limb before slaughter	**6.61**	**1.59**	**4.1%**
Muffle before slaughter	**3.08**	**0.64**	**0.6%**
Neck after slaughter	**3.25**	**1.07**	**0.6%**
Hand after slaughter	**4.94**	**1.58**	**2%**
Hand before slaughter	**2.79**	**0.55**	**0.6%**
clothes after slaughter	**4.92**	**1.80**	**2.3%**
clothes before slaughter	**3.04**	**0.72**	**0.6%**
Tool 1 after slaughter	**4.66**	**1.55**	**1.7%**
Tool 1 before slaughter	**4.68**	**1.97**	**1.2%**
Tool 2 after slaughter	**4.51**	**1.22**	**2%**
Tool 2 before slaughter	**6.18**	**1.46**	**0.6%**
Under tail before slaughter	**6.36**	**1.38**	**3.5%**
Wall	**5.78**	**0.28**	**2%**
Total	**5.73**	**1.71**	**35.4%**

Table (3): Total Mean counts (log10 CFU/ cm^2 ± Standard Deviation), 95% confidence interval of mean and percentage of prevalence of positive *E. faecalis* in the different sources of sampling before and after slaughtering process.

Source of Sampling	Mean log^{10} CFU/ cm^2	Std. Deviatio	Prevalence
Slaughterhouse environment	**6.8**	**0.9**	**5.8%**
Sampling sites from animals before	**6**	**1.6**	**12.2%**
Butchers and tools before slaughtering	**4.2**	**1.8**	**3%**
Sampling sites from animals after	**6.3**	**1.7**	**6.4%**
Butchers and tools after slaughtering	**4.8**	**1.5**	**8.12%**
Total	**5.73**	**1.71**	**35.4%**

The results of antimicrobial sensitivity revealed that all the 122 positive *E. faecalis* samples determined as multidrug resistance against the 16 antimicrobials tested for the sensitivity, (Table 4). Out of 16 different antimicrobials, Vancomycin (30 µg), Cefoxitin (30 µg), Cefotaxime (30 µg) and Cefepime (5 µg) showed an antibacterial action on *E. faecalis* in percentage (66%), (29%), (90%) and (86%) from the total 122 positive *E. faecalis* samples respectively. While, the other antimicrobials were mostly resisted.

Table (4): The interpretation pattern of antimicrobial sensitivity testing for *E. faecalis* against 16 antimicrobials.

	Resistant	Intermediate	sensitive
Amoxicillin/ Clavulanic acid	**122**	**0**	**0**
Ampicillin	**122**	**0**	**0**
Amikacin	**122**	**0**	**0**
Ampicillin/ Sulbactam	**122**	**0**	**0**
Vancomycin	**20**	**22**	**80**
Aztreonam	**122**	**0**	**0**
Ciprofloxacin	**122**	**0**	**0**
Cefoxitin	**70**	**17**	**35**
Gentamycin	**122**	**0**	**0**
Sulpha/ Trimethoprim	**122**	**0**	**0**
Rifampicin	**122**	**0**	**0**
Clindamycin	**122**	**0**	**0**
Erythromycin	**122**	**0**	**0**
Ceftazidime	**122**	**0**	**0**
Cefotaxime	**3**	**9**	**110**
Cefepime	**17**	**0**	**105**

The electrophoretic profile of PCR for *vanA* gene was confirming Vancomycin Resistant *E. faecalis* isolates, Fig. (3). 26 samples were confirmed as Vancomycin Resistant *E. faecalis* (VRE) in percentage 21.3% from the total 122 *E. faecalis* samples and prevalence 7.5% from the total 345 samples collected from the different sites of sampling in the slaughterhouse, (Table 5).

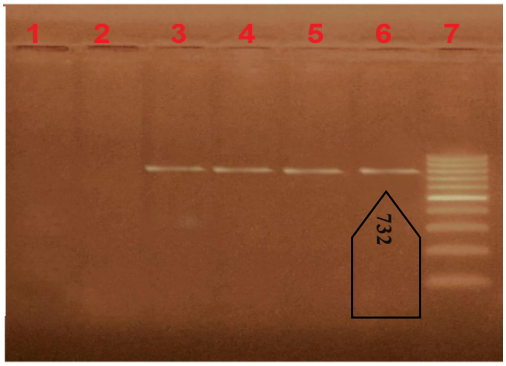

Fig.(3): PCR for *vanA* gene Lane 1 Control-ve Lane 2, 3, 4 and 5 samples Lane 6 Control +ve (732 bp) Lane 7 marker (NEB 100bp).

Table (5): Numbers and percentage of Vancomycin Resistant *E. faecalis* (VRE) isolates recovered from different sources in the slaughterhouse.

Source of samples	# of isolated *E. faecalis*	# of positive *van*A gene
Abdomen after slaughter	**2**	**1**
Back after slaughter	**4**	**1**
Back before slaughter	**5**	**1**
Floor	**13**	**3**
Fore limb before slaughter	**9**	**-**
Hind limb after slaughter	**14**	**1**
Hind limb before slaughter	**14**	**2**
Muffle before slaughter	**2**	**-**
Neck after slaughter	**2**	**-**
Hand after slaughter	**7**	**1**
Hand before slaughter	**2**	**2**
clothes after slaughter	**8**	**-**
clothes before slaughter	**2**	**-**
Tool 1 after slaughter	**6**	**3**
Tool 1 before slaughter	**4**	**2**
Tool 2 after slaughter	**7**	**3**
Tool 2 before slaughter	**2**	**1**
Under tail before slaughter	**12**	**4**
Wall	**7**	**1**
Total	**122**	**26**

DISCUSSION

The analysis of the results revealed that 122 *E. faecalis* samples were recorded in prevalence 35.4%. This is mean that *E. faecalis* has considered probability for contaminating the slaughterhouse environment and infection of their workers. From another aspect the studies of **(Joshua *et al.,* 2003),(Sudhakar, 2009), (Hams,2013) and (Bakhtiary *et al.,* 2016)** recorded prevalence 29 %, 50 %, 15.5% and 18 % respectively that considered a significant percentage might cause a hazard in the slaughterhouse environment.

The mean count among the mentioned sources was 5.73 ± 1.7 log10 CFU/ cm^2 and this is higher than **(Sudhakar, 2009)** who recorded 3.82 ± 0.3 7 log10 CFU/ cm^2 the highest *E. faecalis* load was recorded in floor sites with mean 7.18 ± 0.86 log10 CFU/ cm2. This result act as an infective dose and probable risk for human as mentioned by **(Keith, 2012).**Qualitatively, this result is matching with **(Sudhakar, 2009),** but quantitatively the load in **(Sudhakar, 2009)** is lower than the present study in record 4.5 ± 0.38 log10 CFU/ cm2. There was no detection of *E. faecalis* in duodenum or gall bladder keeping up the result of **(Dias *et al.,* 2014).** The sampling sites from animals before slaughtering has the highest prevalence 12.2% from the total collected samples, followed by butchers (hands and clothes) and tools after slaughtering 8.12% then, sampling sites from animals after slaughtering 6.4%, slaughterhouse environment 5.8% and finally butchers and tools before slaughtering 3%. This is indicating that, the animal hides and tools are playing a major role in the contamination process with *E. faecalis* and this is the same conclusion of **(Bakhtiary *et al.,* 2016).** Analysis of variance between the different sources of contamination in the slaughterhouse interpreting that there is a continuous cross contamination between the mentioned sources of contamination in the slaughterhouse and this matching with study of **(Sudhakar, 2009).** The results of antimicrobial sensitivity revealed that, the 122 positive *E. faecalis* samples determined as multidrug resistance against the 16 antimicrobials that were tested for the sensitivity out of 16 different antimicrobials. Vancomycin (30 µg), Cefoxitin (30 µg), Cefotaxime (30 µg) and Cefepime (5 µg) showed an antibacterial action on *E. faecalis* in percentage (66%), (29%),(90%) and (86%) from the total 122 positive *E. faecalis* samples respectively. While, the other antimicrobials were mostly resisted.

These results are matching with those recorded by **Miroslav *et al.,* (2007)** and in controversy to those recorded by **Joshua *et al.,* (2003)** as they recorded 0% resistance. The electrophoretic profile of PCR for *vanA* gene was confirming Vancomycin Resistant *E. faecalis* isolates in prevalence 7.5% from the total 345 samples collected from the different sites of sampling in the slaughterhouse indicating significant role of animal contaminated hides, slaughterhouse ground, workers and tools in spreading and contamination of Vancomycin Resistant *E. faecalis* directly and indirectly. The same was reported by **(Biswas *et al.,* 2011) and (Miroslav *et al.,* 2007)** who concluded that, the contamination could come from the animals via the contaminating microorganisms from their intestinal or respiratory tracts as well as large numbers of microorganisms contaminating hides, hooves and hair.

CONCLUSION

The presence of multidrug resistant *E. faecalis*, in particular VRE, in different sources in bovine slaughterhouse, has an epidemiological significance. As it represents a potential threat for the spread of this pathogen in the community through cross contamination of meat and/or contamination of utensils, workers' hands and cloths and environment of the slaughterhouse as well as possibility for directing infection of the works who could play a significant role in public and animal health. The contamination is mainly due to bad hygiene and improper practices in the slaughterhouse that ensure a continuous contamination. There is a need to study the antigenic relation of VRE inside slaughterhouse to ensure the main genetic source to know whether it of animal or human origin for better understanding of the main reservoir of the microorganism. A quantitative risk assessment study with genetic epidemiological investigation have to be applied for better understanding of the way and source of spreading of VRE before introducing animals to slaughter house. It is recommended to follow the proper hygienic measures before, during and after slaughtering process and to use the appropriate disinfectants to ensure decreasing the *Enterococcal* load in the slaughterhouse environment.

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