



E-ISSN: 2320-7078

P-ISSN: 2349-6800

JEZS 2018; 6(1): 218-222

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Received: 17-11-2017

Accepted: 23-12-2017

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Isolation and identification of *Enterococcus faecalis* from cow milk samples and vaginal swab from human

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Abstract

The aim of the present study was to isolated the *Enterococcus spp.* from milk samples of cow and vaginal swabs from aborted women and patient women in Baghdad during September 2016 to april 2017. All 100 milk sample collecting was carried out on California Mastitis Test (CMT) and the positive Percentage of CMT reactions was 5% and the percentage of *Enterococcus* isolates from mastitic milk was 60% and 30% from nonmasitic milk. The prevalence of *Enterococcus spp.* was 31% of milk samples and the prevalence of *Enterococcus spp.* Isolates were 67.74% of the isolates of cow milk samples were *Enterococcus faecalis*, 25.80% was Group D and 6.45% was non groupable while *Enterococcus spp.* isolates from aborted women samples were 20% and all isolated was *Enterococcus faecalis*.

Enterococcus spp. was identified by Lancefield grouping test, biochemical tests. This was accomplished by the collection of 200 sample of bovine milk and vaginal swab of aborted women the samples growth in Todd Hewitt broth and incubated aerobically for 24 hours at 37C then cultured on azide blood agar by using selective and differential media like macConkey and tellurite along with Lancefield grouping kit.

Antibiotic sensitivity test has been done for some isolate which reflected high resistant to (vancomycin, pencillin, Ofloxacin, ciprofloxacin, nitrofurantoin, tetracycline and amikacin) the percentage of resistant to antibiotic was 100% in amikacin, nitrofurantoin and tetracycline for all isolated from aborted women and milk samples.

Keywords: isolation, milk samples, aborted women samples, *Enterococcus faecalis*

Introduction

Enterococci were described by The authors as "very hardy and tenacious of life" ^[1] studied the biochemical abilities of the *Enterococci* and manifests that this specific isolate was hemolytic, ^[2] also described organism isolated from fecal samples, that clotted milk and capable to ferment mannitol and lactose they called *s faecalis*, identical to that observed by MacCallum and Hastings. The scientest used the term faecalis to emphasize its intestinal origin ^[2,3]

In dairy cattle, mastitis in cattle and in sheep which cause reduction in milk production and made milk not suitable for processing and conception and cause economical loss ^[4], and may cause diarrhea in calves which has been reported ^[5, 6], in 2–20% of cases related to *Enterococci* etiological agent has been identified ^[7-9]. The means of spread of *Enterococci* in bovine mastitis is probably from the environment ^[10].

Enterococcus species normally found as a commensal bacteria in the digestive tract of man and Animal, insects, plants and birds also found in the environment in water, soil, food of animal origin like poultry beef and swine carcass ^[11-13].

Enterococcus species were thought not important as pathogen to human for many years and considered insignificant medically ^[14].

Currently *Enterococci* have turned out to be one of the most important nosocomial pathogen causing high mortality rate of up to 61% ^[15].

Enterococci colonize in the genitourinary tract, oral cavity and skin less often. while in hospitalized patients the gastrointestinal tract, delicate tissue wounds and ulcers are the major sites of colonization ^[16-18], so the aim of this study was the isolation of *Enterococcus spp.* from dairy milk cattle and from vagina of aborted woman and identification using the conventional method and molecular technique and determine the antibiotic sensitivity for *Enterococcus spp.* isolated from this study.

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Material and methods

Collection of bovine Milk sample

100 bovine milk sample were taken from different cows in alfadilia province, Baghdad Iraq from January to half of June 2017. the milk sample were collected in sterile containers the samples were tested for sub clinical mastitis With California mastitis test before culturing in Todd Hewitt broth.

Collection of Human sample

One hundred swab was taken from vagina of aborted woman at aleilwia hospital, Baghdad, Iraq. From December 2016 to April 2017. Using sterile swab with transport media (amies) to keep it moist until taking to the laboratory.

Isolation and identification were done in the college of veterinary medicine in the zoonotic unit laboratory.

Culturing of samples

All samples was inoculated into Todd Hewitt broth and incubated aerobically at 37 °C for 24 hours after that culture the broth on azide blood agar ^[19] and incubate aerobically at 37 °C for 24 hours the growing colonies examined physically by naked eye according to color, size and shape etc...

Biochemical tests

Biochemical tests were conducted according to ^[19] such as catalase, oxidase and sugar fermentation.

Lancefield grouping test

Using a bacteriological loop, make a light suspension of the culture in a tube of the enzyme solution. A single sweep of growth should be sufficient: it is frequently possible to obtain result by picking as few as 5 large colonies to emulsify in the enzyme, incubate the suspension at 37 °C in a water bath for a minimum of 10 minutes then one drop (20µl) of each latex suspension onto a separate circle on Reaction Card after that place one drop (40 ul) of extract in each of the six circles on the reaction card then mix the contents in each circle in turn with a mixing stick and rock the card gently for a maximum of one minute. The patterns obtained are clear cut and can be recognized easily under all normal lighting condition, a positive result is indicated by the development of an agglutinated pattern showing clearly visible clumping of the latex particle while in a negative result the latex does not agglutinate and the milky appearance remains substantially unchanged throughout the one-minute test^[19].

Antibiotic susceptibility test

The Antibiotic susceptibility test was performed by Kirby Bauer disc diffusion method on Mueller Hinton agar plate media MHA. according to the recommendations of the National Committee for Clinical Laboratory Standards (National Committee for Clinical Laboratory Standards ^[20]).

Commercially prepared antimicrobial sensitivity discs (Becton-Dickinson) were used to evaluate the susceptibility of *Enterococcus* spp. The antibiotic disc were used are Tetracycline, penicillin, vancomycin, Amicacin, Ciprofloxacin and Ofloxacin. Isolates were assorted as susceptible, intermediate and resistant according to the break point developed by the National Committee for Clinical Laboratory Standards NCCLS ^[20]. Test results were accepted only when the zone of inhibition for the fell within the acceptable ranges.

Results

All 100milk sample collecting was carried out on California Mastitis Test (CMT) and the score represented two scales: 0, negative or (+) positive the results appear that from 100 milk samples 5 samples have positive CMT as represented in Table 1 and Fig. 1

Table 1: Percentage distributions of milk CMT reactions represented two scales 0, negative or (+) positive test

Milk number	CMT reactions	Percentage of CMT reactions	Number of <i>Enterococcus</i> spp.	Percentage of bacterial isolates
5	+	5%	3	60%
95	0	95%	29	30%



Fig 1: CMT positive result appear solid gel forms (mastitis milk)

Isolation of *Enterococcus* spp. from Dairy milk samples and aborted women.

Out of 100 Dairy milk sample isolation of *Enterococcus* spp was 31 isolates (31%) and 20 isolates (20%) out from 100 genital swabs from aborted women samples as shown in Table 2.

Table 2: The number and percentage of *Enterococcus* spp isolated from different clinical samples

Specimen	Number of specimen tested	Number of specimen positive for <i>Enterococcus</i> spp.	Isolation percentage
Dairy milk	100	31	31%
Genital swabs of aborted women	100	20	20%

Species distribution of *Enterococcus* spp isolated from clinical specimens is illustrated in Table 3.

Table 3: Species isolated of *Enterococcus spp* from clinical specimens

<i>Enterococcus spp</i>	Dairy milk	Percentage of isolates	Genital swabs of aborted women	Percentage of isolates
<i>Enterococcus faecalis</i>	21 isolate (+)	67.74%	20 isolate (+)	100%
Group D <i>Enterococci</i>	8 isolate (+)	25.80%	0	0
Non groupable	2 isolate (+)	6.45%	0	0
Total number	31 isolates		20 isolates	

The most common species isolated was *Enterococcus faecalis* followed by Group D *Enterococci* and Non groupable *Enterococcus spp*, 68% of the isolates from cow milk samples were *Enterococcus faecalis* and 26% was Group D *Enterococci*. Genital swabs of aborted women samples

yielded 100% of *Enterococcus faecalis* only.

The antimicrobial sensitivity test of the *Enterococcus spp*. isolates from clinical specimens of human and animal in this study was summarized in Table 4.

Table 4: Antibiotic sensitive results of *Enterococcus spp*. isolated from the dairy milk samples and genital aborted women.

<i>Enterococcus spp.</i> number isolates	Specimen	Antibiotic type by Disc diffusion						
		Ak.*	NT.*	Van.*	P.*	Cip.*	T.*	Ofx.*
<i>Enterococcus faecalis</i>	Genital women swabs	0%	25%	0%	37%	0%	0%	25%
<i>Enterococcus faecalis</i>	Dairy milk	0%	0%	0%	17%	0%	0%	11%
<i>Enterococcus faecium</i>	Dairy milk	0%	0%	33%	66%	0%	0%	0%
<i>Enterococcus gallinarum</i>	Dairy milk	0%	0%	0%	50%	0%	0%	0%
<i>Enterococcus durans</i>	Dairy milk	0%	0%	0%	0%	100%	0%	0%

Discussion

All the isolate were cultured on selective media such as Azide blood agar, which is selective for *Enterococcus spp*. Isolation, due to presence of sodium azide which inhibits growth of gram negative bacteria. While Black zone around colonies are formed on kanamycin esculin azide agar, Kanamycin sulphate and sodium azide are the selective inhibitory components [21].

Based on the result of biochemical tests, Tellurite tolerance, mannitol fermentation, and tetrazolium reduction and inability to ferment arabinose, 21 *E. faecalis* strain were identified from milk and 20 *E. faecalis* strain identified from human [22-25].

Conventional methods for routine species identification are still based on physiological characteristics [26, 27]. However, no commercial kit include the whole set of tests needed for the complete identification of *Enterococcus spp*. The advantage of these tests is the easy use in diagnostic labs where a high number of isolates are examined, for rapid biochemical identification.

Raw milk consumption could present a potential risk for public health due to the presence of food borne pathogens and spoilage bacteria from the raw milk samples.

The percentage of isolates were; *E. faecium* (10%), *E. durans* (3%), *E. gallinarum* (12%), *E. faecalis* (72%) which accounted for most of the isolates from raw milk samples, is higher than a survey of raw and pasteurized milk samples in which *E. faecalis* (46.1%) and *E. faecium* (29.0%) were the most predominant enterococcal isolates. Other *Enterococci* species identified *E. durans* (2.1%) *E. gallinarum* (7.4%). that was reported by [28].

In another study [29], *E. faecalis* (54.2%), other enterococcal isolates *E. durans* (6.2%), *E. gallinarum* (3.0%).

In most studies *Enterococcus faecalis* were the dominant species and the ratio of *E. faecium* *E. durans* and *E. gallinarum* are variable.

In this study the percentage of *E. faecalis* is lower than that 87.0% which was isolated from raw milk by [30]. *E. faecalis* ratio nearly close to (65.0%) *E. faecalis* that was isolated from raw milk by [31]. Also *E. faecalis* was predominant species (64.7%), followed by *E. faecium* (18.8%), *E. gallinarum* (5.9%) and *E. durans* (4.7%) that reported by [32].

These results are proportional to 71% *E. faecalis*, *E. faecium*

19%, *E. durans* and *E. gallinarum* 4%. Reported by [33].

In other study by [34], reported that *E. faecalis* was the predominant *Enterococcus* in Indian dairy products.

There is a lack of studies performed in raw milk from healthy cows, whereas antimicrobial resistance has been surveyed in milk from mastitic cows. Little information is available on *Enterococci* as pathogens isolated from milk samples, and most studies focus on the two major species *E. faecalis* and *E. faecium*. Data are sparse; the incidence of *Enterococci* as etiological agents of bovine mastitis according to different sources varies from 0 to 21.2% [35, 36].

Casari [37] concluded that it is important to culture the vaginal discharge for *Enterococcus agalactiae*, *Enterobacteriaceae* and *Enterococci*. He has also indicated that the prevalence of some microorganisms (*Gardnerella vaginalis*, *Enterobacteriaceae* and *Enterococci*, *Enterococcus agalactiae*) in the population of asymptomatic women with infertility problems need to be better analyzed.

Enterococci isolated from vaginal specimens in our study probably do not reflect the true incidence of infections caused by this organism but definitely suggest the increased frequency of their isolation.

In Belgium cultured vaginal and rectal specimens from pregnant women, showed *Enterococci* rate of 4.6% which is less than that found by our study [38]. Whereas in other studies, *E. faecalis* was 85% (the most prevalent species), followed by *E. faecium*. [39, 40]. In a study the predominant species was respectively *E. faecalis* (89.8%), *E. faecium* (6.1%) reported by [41].

E. faecalis among other isolates of *Enterococci*, was the dominant species of *Enterococcus* in hospitalized patients in Hilla. Iraq. [42].

In a study 40% of *E. faecalis* isolates were resistant to penicillin and ciprofloxacin [43]. It is nearly the same as 31% percentage as *E. faecalis* in this study and lowers than percentage of ciprofloxacin.

The same finding for *E. faecalis* and *E. faecium* as [44]. reported that *E. faecium* isolates exhibited a higher percentage of resistance to penicillin while *E. faecalis* isolates were more resistant to tetracycline and penicillin.

Tetracycline resistance observed in our study is nearly the same as 73% reported from studies on mastitis in Finland [35],

and Uruguay ^[45], and proportional to that reported by ^[46] tetracycline (64.3%), but higher than that of penicillin (6.0%) and ciprofloxacin (3.6%).

Resistance to Nitrofurantoin was 31% in milk the same as 30% reported by ^[47].

E. faecalis can get adaptation to different condition: it become less susceptible to ordinary lethal levels of sodium dodecyl sulfate, hydrogen peroxide, bile salts, hyper osmolarity, ethanol, acidity and alkalinity, heat, freezing temperature, also these organisms are involved in outbreaks of food poisoning ^[48]. Multi drug resistant are abundant in animal's food and also food of animal origin ^[49, 50], so it can be transferred to human by eating food or through the environment. And it may spread thorough the agri- food chain ^[51].

These results demonstrated a high resistance rate which could explained by the massive use of antibiotic in treatment of infection coupled in most times by abuse, the genes of antibiotic resistance in *Enterococci* are usually carried on transposon that can easily be transferred to other bacteria. In the current study most of the *Enterococci spp* isolated were multi drug resistant, the presence of VRE in food must be regarded as an important risk factor for potential human VRE infection in combination with other hospital-associated risk factors inappropriate use of cephalosporin as well as poor hospital infection control measures. Consequently, preventing VRE contamination of food must be an integral part of the worldwide strategy to prevent human VRE infections.

Conclusion

These study conclude that Five milk samples appeared to be mastitic by California mastitis test and *E. gallinarum* were isolated from 3 of mastitic milk sample and different *Enterococcus spp.* was isolated from milk and the predominant strain was *E. faecalis* otherwise all isolated colonies from genital swab of aborted women were *E. faecalis*. *E. faecalis* isolated from aborted woman vagina showed high resistant to (vancomycin, ciprofloxacin, Ofloxacin, nitrofurintion, pencillin, amekacin).

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