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# Determination of the prevalence, antibiotic resistance and virulence factors of *E.faecalis* isolated from different food samples

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**ABSTRACT:** Milk, juices, meat, etc., are the most perishable foods and they are consumed by most of the people. And most of the food borne diseases are due to microbial contamination and infections. But nowadays, these food items are being contaminated by microbes which are having "Multi-drug -resistance" property as their survival mechanism. Especially while talking about chicken meat, the microbial contamination by Enterococcus sp., is a worrisome factor because their resistance mechanism gets developed due to the addition of anti-microbial substances in the feed given to the chicken (brought up in the poultry environment). Hence, the ultimate aim of the present study is to check the prevalence of Enterococcus sp., that too E.feacalis in particular, from different food samples collected from places surrounding Namakkal region. Also, these isolates are subjected to several assays to determine their virulence and resistance and anlayse their bofilm forming property under this study.

**Keywords:** *Microbial prevalence, Food samples, Anti microbial resistance;*

## 1. INTRODUCTION

The term Food-borne diseases, acquired through consumption of uncleancontaminated food with microbes and chemicals. In addition, poisonous chemicals, or other harmful substances can cause foodborne diseases if they are present in food. More than 250 different foodborne diseases have been described. Most of these diseases are infections, caused by a variety of bacteria, viruses, and parasites that can be foodborne (www.cdc.gov/foodsafety/foodborne-germs.html). In 2013, Poonam was observed the coliform bacteria from various fruit and fruit juices at Vidarbha.Source of contamination of food was varying, mostly occurred by improper washing of fruits add these bacteria to extracts leading to contamination. In addition, use of unhygienic water for dilution, dressing with ice, prolonged preservation without refrigeration, unhygienic surroundings often with swarming houseflies and fruit flies and airborne dust can also act as sources of contamination. Such juices have shown to be potential sources of bacterial pathogens notably *E.coli, Salmonella, Shigella* and *Staphylococcus aureus,Klebsiella spp and Enterococcus spp* [1].

Meat is one of the most perishable foods, and its composition is ideal for the growth of a wide range of spoilage bacteria [2]. Food contamination with antibiotic-resistant bacteria can be a major threat to public health. The prevalence of antimicrobial resistance among food-borne pathogens has increased during recent decades. These antibiotic-resistant microbes causing infection was untreatable because its causative agent has been found to be resistant to cephalosporin as well as carbapenems due to extended spectrum β-lactamases (ESBL) mediated mechanism [3]. In addition, these bacteria are able to acquire resistance determinants through gene transfer mediated by plasmids and transposons. The use of antimicrobials in animal feed as growth promoters has created large reservoirs of transferable antibiotic resistance genes in several ecosystems, and consequently a possible route of transmission of resistant Enterococcus spp. via food chain is feasible [4]. One of the most important mechanisms responsible for antimicrobial resistance in organisms producing biofilms may be stuck penetration of the antimicrobial agents through the biofilm matrix, altered growth rate of biofilm organisms. Thus, the ability to form biofilm could be an effective strategy to enhance the survival and persistence under stressed conditions like host invasion or following antibiotic treatment. The present study aims to determine the prevalence, antibiotic resistance and virulence factors of *E.faecalis* isolated from different food samples.

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## 2. MATERIALS AND METHODS

### *2.1 Sample collection*

The samples were collected from approximately 10 g of meat and 100 ml of fruit juices and milk were obtained from the supermarkets and road side shops. All samples were stored at $4^0$C after sampling, until the analysis is conducted. Samples were collected within 12 hours post-slaughter and during early afternoons, in order to minimize the microbial changes due to environmental temperatures and post-slaughter timings.

### *2.2 Sample preparation*

Ten grams of collected meat samples were weighed and transferred to sterile flasks containing 10 ml of phosphate buffer saline (PBS) [5]. Samples were homogenized using a meat grinder under aseptic conditions and was inoculated in to different selective media such as Blood agar and Chromogenic media for *Enterococcus spp*. The plates were incubated for 48 hours at $37^0$C. Colony morphology on the plate was observed and Gram staining was conducted. Biochemical tests were performed to identify pathogenic bacteria related to food contamination. These tests included Oxidase, TSI, Urease, Motility, Catalase, Indole, Simmons citrate and Methyl red and veges proskauer.

### *2.3 Identification of food isolates*

Selected colonies from selective and differential media were subjected to macroscopy, microscopy and biochemical tests for identification. Characterization and identification of the isolates was done using the methods of Cowan (1985), Fawole and Oso's (1988) and Cheesbrough (2004).

### *2.4 Macroscopic observation*

Colony morphologyon agar surface aids to identify the bacterial isolates. Each and every individual species of colonies was characteristic to shape, size and appearance [6]. Characteristic features of the organism were observed by macroscopic observations. A loopful of culture from overnight grown broth was streaked on the surface of nutrient agar and as incubated at $37^0$C for 24 hours. Colony morphology, colour and consistancy were observed and tabulated.

### *2.5 Microscopic observations*

Microscopic observations like shape, grams nature and motility reveal the availability of different morphological characters among microorganisms. Simple staining, gram staining and hanging drop methods were done to look for their shape, grams nature and motility of the isolate respectively [7].

### *2.6 Determination of Antibacterial activity*

The disc diffusion method was followed to determine the anti bacterial activity [8]. Petriplates containing 20 ml of Mueller Hinton agar were seeded with 4 hours old fresh culture of clinical isolates and referral strains. By making use of template drawn discs weredispensed on the solidified Mueller Hinton agar with test organisms. This was incubated at $37^0$C for 24 hours in an incubator. The zone of inhibition was measured by making use of Antibiotic zone scale (Hi - media). The resistance patterns were interpreted as per CDC recommendations.

### *2.7 Determination of Multiple Antibiotic Resistance index (MAR)*

Multiple antibiotic resistance index (MAR) was determined using the formula MAR=x/y, where x was the number of antibiotics to which test isolate displayed resistance and y is the total number of antibiotics to which the test organism has been evaluated for sensitivity [9].

### *2.8 Biofilm formation*

The determination of the biofilm production was done on the basis of the adherence of the biofilm to borosilicate test tube as was done by Christensen *et al* procedure (1982). The suspicious isolates was inoculated with test tube containing trypticase soy broth and incubated for 24 hrs $37^0$C. The tubes were decanted and washed with PBS (pH7.2). Air dried the tubes and stained with 0.1% of crystal violet. Excess stain was removed and tubes were washed with deionized water, than tubes were dried and observed the result. The positive result was indicated as the presence of a layer of the stained material which adhered to the inner wall of the tubes. The exclusive observation of a stained ring at the liquid air interface was considered as negative.

### *2.9 Assay for beta lactamase production*

Beta lactamase production was assayed using the method of Lateef *et al*., 2004. Broth culture of the test organism was spot inoculated on to Mueller-Hinton agar and 1% starch and then incubated overnight at $37^0$C. The plates were then flooded with sterile phosphate buffered saline containing potassium iodide, iodine and penicillin. Beta lactamase production was assessed by the presence of clear colourless zones around the bacterial growth. All the bacterial isolates were tested for the production of beta lactamases.

### *2.10 Hemolytic assay*

The hemolytic activity of *E. faecalis* was assessed on blood agar plates prepared with Mueller-Hinton agar (MHA, Himedia, india) containing defibrinated sheep blood (final blood concentration, 5% v/v), by observation of the hemolysis zone around colonies after incubation for 24 h at 37ºC.

### *2.11 Amplification of cytolysin and surface protein (esp) from Enterococcus faecalis*

The PCR conditions and the primers used for the genotypic characterization of vancomycin resistant strains were as previously described. The following pairs of primers were used .

**Cytolysin primer (cylA) – 517BP**

F: 3' TGGATGATAGTGATAGGAAGT T 5'
R: 3'TCTACAGTAAATCTTTCGTCA 5'

**Esp primer- 932 BP**

F-3' TTG CTA ATG CTA GTC CAC GAC C A 5'
R-3' GCC TCA ACA CTT GCA TTG CCG A 5'

All PCR amplifications were performed in a final volume of 20 µl containing 50 pmol of each primer (1 µl), 1µl template DNA, 10 µl of master mix and 5 µl of molecular grade water. An initial cycle of denaturation (94˚C for 5 min) was followed by 30 cycles of denaturation (94˚C for 40 sec), annealing at 57˚C for 1 min and elongation 72˚C for 1 min and final elongation 72˚C for 10 min A. Thermal Cycler was used to carry out the PCR reactions. PCR products were analyzed by gel electrophoresis in 1.2% agarose stained with ethidium bromide (0.5 g·ml−1), and observed under UV transillumination and photographed.

### 2.12 Biotyping of the E.faecalis

All isolates of *E.faecalis* were biotyped with using 6 types of parameter such as biofilm, betalactamase, surface protein (esp) cytolysin, hemolysis and Vancomycin resistance, this typing was carryout with previous studies of Vijayalakshmi *et al*., 2013.

## 3. RESULTS AND DISCUSSION:

Since Enterococci are pervasive and predominant inhabitants of the gastrointestinal tract of humans and animals, they are found in soil, water and food. For many years, they were considered as normal flora and unharmful to man. This study aims to prove their evloutionary state of resistance as well as their virulence factor. As a report, in this study, a total of about 19 isolates of Enterococcus faecalis were observed from 33 samples of meat, milk and fruit juices samples. Among them, highest prevalence was observed in chicken meat and goat meat (71.4%). The high occurrence of enterococci in meat products can be attributed to the natural presence of this microorganism in the gastrointestinal tract of animals and the microorganism's ability to adapt and develop in unfavorable environmental conditions.

Serious worry is about the bacterial antimicrobial resistance from food-borne pathogens. Both the World Health Organization (WHO) and the Food and Agriculture Organization of the United Nations (FAO) and the World Organization for Animal Health (OIE) consider resistance in zoonotic bacteria as a public health threat and recognize that resistance may be the consequence of the use of antimicrobials in food animals and may be transmitted to humans (European Food Safety Authority (EFSA), Parma, Italy).

Enterococcus, particularly E. faecalis and E.faecium, showed intrinsic resistance to several antimicrobial drugs, including aminoglycosides, β-lactams and quinolones. In addition, these microorganisms can acquire and transfer genetic elements that confer resistance to other classes of antibiotics, especially glycopeptides such as vancomycin and teicoplanin [10]. Antibiotic susceptibility tests showed that in the present study, the VRE isolates were resistant to at least four antibiotics including gentamicin, ciprofloxacin, erythromycin and ampicillin. This has been confirmed by other studies which have found the prevalence of antibiotic-resistant enterococci in farm animals and their meat to be higher than 60% [11]. Here we determined that resistance to vancomycin was very high among the isolates from food sources and our isolates showed resistance against Nalidixic acid (79%), Gentamycin - 63.1% which is frequently being used for treating infections (which is worrisome). Totally 46.3% of antibiotic resistance was recorded from isolates of Enterococcus faecalis (Figure 1,2). Thus, the percentage (63.1 %) of strains found in our study is worrisome. Differently from our results, Fracalanzza*et al*.,(2007) have detected the occurrence of gentamycin resistance in 10.6% of the strains isolated from chicken samples.

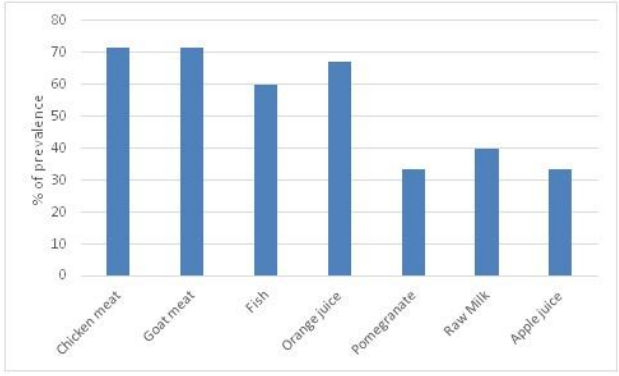


**Fig.1 Prevalence of *Enterococcus faecalis***

β-lactamases are a major antibiotic resistance mechanism against the widely used β-lactam antibiotics, which target penicillin-binding proteins (PBPs) involved in bacterial cell wall synthesis. β-lactamase enzymes inactivate β-lactam antibiotics, by hydrolyzing their β-lactam ring essential to antibiotic function. Notably, many of the 'ESKAPE' pathogens (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumonia, A cinetobacterbaumanni, P seudomonas aeruginosa and Enterobacter* species) produces enzymes.

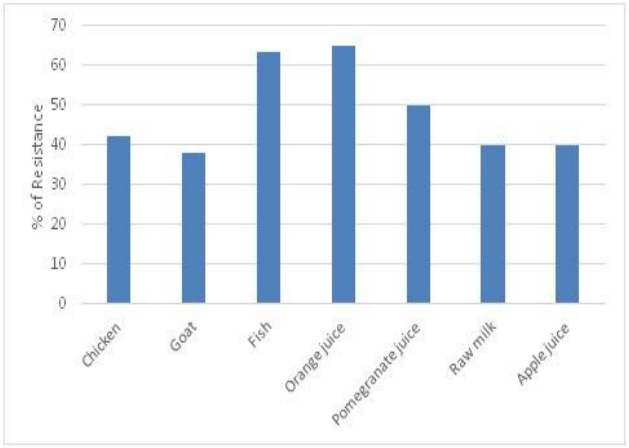


**Fig.2 Prevalence of antibiotic resistance of *E.faecalis* on food samples**

Furthermore, Antibiotic resistance is due to the production of biofilm by microorganisms. This biofilm formation enhanced resistance to antibiotics and other environmental stresses [12]. In this study, 42.1% isolates were in vitro positive for the biofilm production. We conclude that biofilm production in food isolates of E. faecalis are an important pathogenic factor. Also, this result was correlated to VRE result, most of the VRE isolates as biofilm producers compared than VSE isolates (Table 1).

**Table 1: Relationship between virulence factors and vancomycin resistance Among *Enterococcus faecalis***

| S.No | Samples | Virulence factors (n=19) | | |
|---|---|---|---|---|
| | | Biofilm positive | Betalactamase | Hemolysis |
| 1. | VRE | 7 (44%) | 9(56.2%) | 5 (31.2%) |
| 2. | VSE | 1(33.3%) | 1(33.3%) | 0 (0%) |

In our study, the expression of three virulence factors was evaluated (Figure 3). They were hemolysis, betalactamase production and biofilm formation. In addition, an attempt was made to detect the presence of the "esp" gene in some isolates of Enterococcus. Enterococci also secrete cytolysin (Cyl), a bacterial toxin that shows haemolytic activity against erythrocytes of human, rabbit and horses [13]. In this study, all isolates were subjected to multiplex PCR analysis for amplification of cytolysin and esp gene. Among them, percentage of esp gene (58%) was high, compare than cytolysin gene (31.5%). That is most of the biofilm positive isolates harbor the 'esp' gene. According to a study of Toledo-Arana *et al*., (2001) the biofilm-forming capability of enterococcal strains is confined to strains possessing the "esp" gene. Similar results were observed in current investigation.

The molecular detection of Cytolysin gene was most predominant in hemolytic isolates. Previous investigations reported, the presence of the cylA gene did not correlate completely with its phenotypic expression [14].

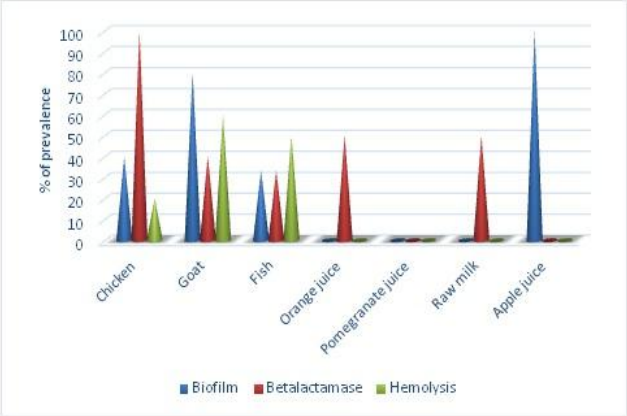


**Fig.3 Prevalence of virulence factors on *E.faecalis***

Typing of bacteria is a necessary procedure for monitoring the transmission of the organism among carriers and in epidemiological follow-up which may enhance effective eradication [15].

Biotyping is an easily performed technique that can be practiced without a need for expensive equipment. Other investigators have employed biotyping as an effective means to the investigation and surveillance of S. aureus infection [16-17]. In the present study, all isolates were characterized by biotyping method. In this study, isolates were divided into 3 groups (A-C). Type ability by biotyping was found to be 37% of isolates. In this study each group had 2 isolates and 63% of the isolates could not be categorized into any of the above mentioned groups and hence were called non-type able group. Among the 3 groups, category A and C had highly antimicrobial resistance and also, a number of virulence factors were observed from A group.

## 4. CONCLUSION

The results of this study revealed that enterococci are common contaminants in meat, milk and juices were purchased in retail stores and road side shop in Namakkal area. The data according to the study says that the occurance of enterococcus in the food source is high and that their resistance to antibiotics is worrisome. In addition, the utilization of new antimicrobials should be done in a very rational way, both in human as well as in animal therapy. Monitoring of antimicrobial resistance is essential since constant surveillance can halt the dissemination of Enterococcus clones resistant to several drugs, as well as the emergence of new resistance mechanisms.

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