RESEARCH ARTICLE

Isolation and Molecular Detection of *Enterococcus faecalis* from Cow Milk of Anand City with Special Reference to Biofilm Production and Multiple Drug Resistance

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Abstract

The present study was conducted to determine the drug resistance pattern and the occurrence of biofilm-producing *Enterococcus faecalis* from cow milk samples collected from in and around Anand city, Gujarat. A total of 100 samples were collected aseptically from the doorsteps of livestock farmers. Out of 100 samples, 73 (73%) samples were found to be positive on selective media, which was confirmed by polymerase chain reaction, which revealed that all the 73 isolates were of *Enterococcus faecalis*. The antibiotic sensitivity test showed that all the isolates were sensitive to ampicillin (100%) only, and resistance was observed against Trimethoprim (100%), Tigecycline (80.82%), Vancomycin (67.12%), Gentamicin (46.57%), Imipenem-cilastatin (28.76%), and Norfloxacin (10.95%). Out of all the positive isolates, 30.13% (22/73) were biofilm producers. So, it can be concluded that milk can be a possible intermediary vehicle for the spread of multidrug-resistant biofilm-producing enterococci strains to humans.

Keywords: Antimicrobial resistance, Biofilm, Cow milk, *ddl*E gene, *Enterococcus faecalis*, PCR, Vancomycin-resistant enterococci.

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Introduction

Enterococci are found all over the world. The natural habitat of these microorganisms is the digestive tract of humans and animals. Although more than a dozen different enterococcal species have been associated with human disease, *Enterococcus faecalis* is responsible for a number of human enterococcal infections (Abat *et al*., 2016). The potential of *E. faecalis* isolates to produce serious infections has been attributed to the inherent toughness of the bacterium, which allows it to survive in the hospital environment and many host defenses, enhanced by horizontal transfer of a number of variable virulence characteristics from other species (Weigel *et al*., 2003). Because *E. faecalis* survives heating to 60°C for 30 min in a neutral medium, in a wide range of pH (from 3.5 to 11) and has relatively high salt tolerance (6.5% NaCl), it can handle modest stress and withstand sloppy food preparation operations. Enterococcal infections are difficult to treat because the inherently antimicrobial-resistant *Enterococcus* has acquired high-level aminoglycoside resistance genes. The advent of antimicrobial-resistant animal enterococci poses a significant risk of transfer of these bacteria to humans (Hayes *et al*., 2003; Foulquié Moreno *et al*., 2006; Aarestrup *et al*., 2008; Olsen *et al*., 2012). Enterococci are ubiquitous bacteria normally occurring in foods, particularly those of animal origin, like meat and milk (Giraffa, 2002). Their presence in significant quantities in foods could suggest a lack of sanitation, and their propensity to function as a genetic reservoir of antibiotic resistance is a cause for concern (Jahan *et al*., 2015).

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Biofilm is a complex population of bacteria that can be found throughout the natural world. A biofilm is a brilliantly complex multispecies phenomenon in which ecological microniches are produced and occupied by certain organisms (Bryers, 2008). It is commonly resistant to antimicrobial medications, which complicates the treatment of illnesses (Stewart and Costerton, 2001; Stewart, 2002). Biofilm development is thought to be responsible for more than 60% of bacterial illnesses (Donlan and Costerton, 2002).

The production of bacterial biofilms is a key component of many diseases. Biofilm formation in root canals, biomaterials, and indwelling medical devices such as ureteral stents, intravascular catheters, and silicone gastrostomy devices have all been connected to enterococci. This study aimed to look at antibiotic sensitivity and resistance pattern of *Enterococcus faecalis* isolated from milk, as well as the ability of enterococci to build a biofilm.

Materials and Methods

Collection and Processing of Samples

Total of 100 cow milk samples were obtained from livestock farmers in and around Anand city (Gujarat, India). Samples were collected aseptically in pre-sterilized tubes. All the samples were collected with consent from the livestock owners in the presence of a qualified veterinarian.

Isolation and Identification of *Enterococcus faecalis* from Milk Samples

The milk samples were subjected to enrichment in 5 mL of *Enterococcus* selective broth individually in sterile test tubes for 18 h at 37°C. The samples in which the Enterococci were present turned blue. The loopful of enriched culture from the positive tubes was inoculated on Citrate Azide Tween Carbonate (CATC) media and incubated at 37°C for 18 h. Growth on CATC was observed, and the isolated bacteria were subjected to Gram's staining. Gram-positive diplococci were identified as *Enterococcus* spp. (Majhenic *et al*., 2005).

Biochemical Characterization of *Enterococcus* Species Isolates

The biochemical tests like Absence of catalase, Hydrolysis of bile esculin, Growth in the presence of 6.5% NaCl and Hydrolysis of PYR (L-pyrrolidonyl-ß-napthalamide) were performed as per standard procedures for further confirmation and biochemical characterization of *Enterococcus* species isolates as described by Manero and Blanch (1999).

Confirmation of the *Enterococcus faecalis* by PCR

Culturally and biochemically positive isolates of Enterococcus spp. were subjected to molecular characterization using PCR for confirmation by targeting ddlE gene. Preparation of template DNA from Enterococcus strains was carried out by boiling method. The PCR was carried out with primers targeting ddlE genes specific for Enterococci (Table 1). 25 µL of reaction mixture consisted of template DNA 5.0 µL, Primers 2.0 µL (10 pmol each primer), Mastermix (2X) 12.5 µL and nuclease free water 5.5 µL. The PCR amplification was conducted in an automated thermal cycler, as per protocol given in Table 2. The PCR products were analyzed by submarine electrophoresis in 1.5 % agarose gel in Tris-Borate-EDTA (TBE) buffer (1x). The gel was visualized under a UV transilluminator, and the images were documented in a gel documentation system.

I*n-Vitro* *Antibiogram Pattern of Bacterial Isolates*

The test was performed as per the method described by Kasimoglu-Dogru *et al*. (2010). The antibiotics used were Vancomycin (5 mcg), Norfloxacin (10 mcg), Gentamicin (30 mcg), Ampicillin (2 mcg), Trimethoprim (5 mcg), Tigecycline (15 mcg), and Imipenem-cilastatin (10 mcg). Zone of inhibition was interpreted as per CLSI guidelines.

Evaluation of Biofilm Production by *Enterococcus* Isolates

The isolates identified (Hassan *et al*., 2011) morphologically and molecularly by PCR were subjected to streaking on Congo Red Agar under aseptic conditions. The plates were incubated at 37°C for 18 h.

Results and Discussion

Seventy three out of 100 procured milk samples revealed the presence of *Enterococcus* spp. CATC agar test revealed the presence of Enterococci in all 73 samples (73%) and was confirmed that all the isolates obtained on CATC media were of *Enterococcus faecalis* by polymerase chain reaction on targeting *ddlE* gene (Fig. 1). Hosseini *et al*. (2016) however reported only 17.8% prevalence of Enterococci from raw milk samples.

All the 73 isolates showing typical colony characteristics of *Enterococcus* spp., were Gram-positive diplococci (Fig. 2). On Biochemical characterization all these isolates were found negative to catalase reaction, produced black colonies on bile esculin medium, PYR positive, and produced visible turbidity in BHI broth with 6.5 % NaCl, and were confirmed as typical *Enterococcus* spp.

Antibiotic disc diffusion assay for antibiotic sensitivity showed that all the 73 isolates were sensitive to ampicillin (100%) only; while varying resistance was observed against Trimethoprim (100%), Tigecycline (80.82%), Vancomycin (67.12%), Gentamicin (46.57%), Imipenem (28.76%), and Norfloxacin (10.95%). Thus hundred percent isolates showed

Table 1: Description of primer used for detection of *Enterococcus faecalis*

Target gene	*Primer sequence (5'-3')*	*Product size (Base pairs)*	*Reference*
ddlE	F: ATCAAGTACAGTTAGTCTT R: ACGATTCAAAGCTAACTG	941	Dutka-Malen *et al.* (1995)

Table 2: Master cycler conditions for *ddlE* gene

Cycling Conditions		*Temperature*	*Time*
Initial Denaturation		94°C	10 min
30 cycles	Denaturation	94°C	15 s
	Annealing	55°C	15 s
	Extension	72°C	45 s
Final Extension		72°C	5 min

Table 3: Detection of multidrug resistance isolates

Sr. no.	*Antibiotics*	*Sensitivity (%)*	*Intermediate (%)*	*Resistance (%)*
1.	Ampicillin	100	-	-
2.	Trimethoprim	-	-	100
3.	Tigecycline	19.17	-	80.82
4.	Vancomycin	6.84	26.02	67.12
5.	Gentamicin	12.32	41.09	46.57
6.	Imipenem-cilastatin	-	71.23	28.76
7.	Norfloxacin	17.80	71.23	10.95

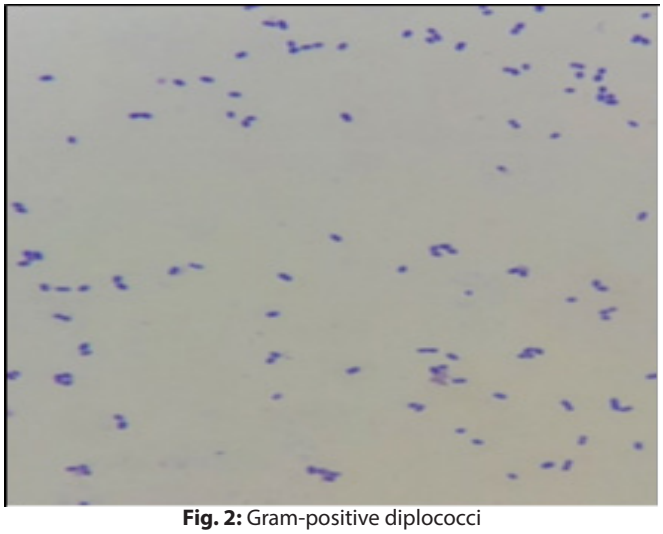

Fig. 2: Gram-positive diplococci

multiple drug (other than ampicillin) resistance phenotypes (Table 3, Fig. 3).

In contrast to the current study, Gousia *et al.* (2015) studied 500 raw beef, pork, and chicken meat samples and 100 pooled egg samples for the presence of vancomycin-resistant enterococci and observed that the majority of the Enterococcus species recovered were resistant to ampicillin (n = 93, 66%). The disc-diffusion approach revealed vancomycin resistance in 1.9% (n = 01) of the Enterococcus faecalis isolates (n = 53). *Stpień-Pyśniak et al.* (2016) isolated Enterococci from different poultry birds and found resistance and intermediate resistance to gentamicin up to 91.7, 82.4, and 68.2 % in broilers, layers, and turkeys, respectively. The gentamicin resistance was found in Enterococcus faecalis isolates from broilers (51.4 %). Guerrero-Ramos *et al.* (2016) studied the prevalence of vancomycin-resistant enterococci (VRE) in 160 samples of poultry (80), pork (40), and beef (40) preparations (red sausages, white sausages, hamburgers, meatballs, nuggets, minced meat, escalopes, and crepes), and found VRE in 38 (23.8%) of the total isolates. In addition to vancomycin. all strains showed resistance or intermediate susceptibility to three or more antimicrobials of clinical importance. Castaño-Arriba et al. (2020) subjected 200 isolates of Enterococci against multiple antibiotics, wherein 175 isolates (87.5%) showed multiple drug-

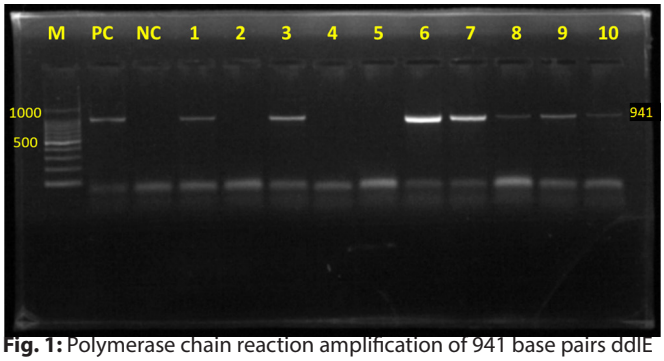

Fig. 1: Polymerase chain reaction amplification of 941 base pairs ddlE *gene*

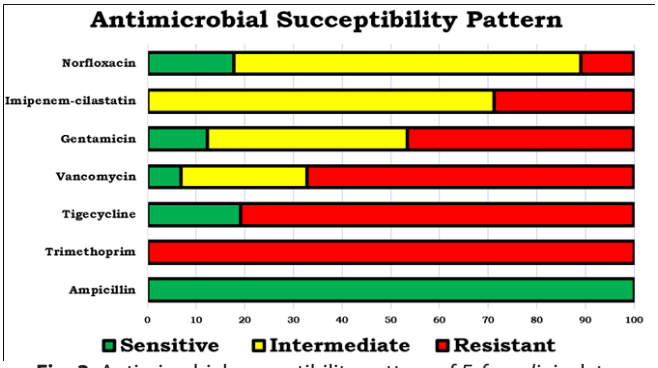

Fig. 3: Antimicrobial susceptibility pattern of *E. faecalis* isolates

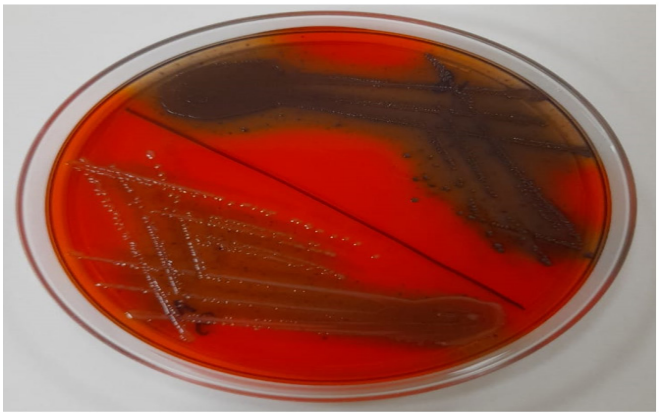

Fig. 4: Black coloured colonies on CRA showing biofilm production

phenotypes. The increased occurrence of vancomycin resistance in enterococci and high-level enterococcal resistance to penicillin and aminoglycosides poses a serious problem for clinicians treating patients with these organisms (Handwerger et al., 1993).

Based on streaking of the isolates on Congo Red Agar, and, the appearance of black color colonies on CRA 30.13% (22/73) isolates were found biofilm producers (Fig. 4). Our results are comparable with the reports of Esmaeili et al. (2018), who recorded 38% of isolates that developed biofilm. Fallah *et al.* (2017) isolated 57 enterococcal isolates from patients with urinary tract infections and tested them using conventional microbiological methods.

Conclusions

Identification of food isolates of enterococci could be a beneficial test for the dairy industry. Antibiotic resistance

can be used in conjunction with other food safety testing. Pathogenic and antibiotic-resistant microorganisms could be transmitted to people through eating of meat, milk, and related products if no restrictions are in place. Although there have been no reported cases of foodborne enterococcal infections, horizontal antimicrobial gene transfer is also a public health threat.

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References

Aarestrup, F.M., Wegener, H.C., & Collignon, P. (2008). Resistance in bacteria of the food chain: epidemiology and control strategies. *Expert Review of Anti-Infective Therapy, 6*(5), 733-750.

Abat, C., Huart, M., Garcia, V., Dubourg, G., & Raoult, D. (2016). *Enterococcus faecalis* urinary tract infections: Do they have a zoonotic origin?. *Journal of Infection*, *73*(4), 305-313.

Bryers, J.D. (2008). Medical biofilms. *Biotechnology and Bioengineering, 100*(1), 1-18.

Castaño-Arriba, A., González-Machado, C., Igrejas, G., Poeta, P., Alonso-Calleja, C., & Capita, R. (2020). Antibiotic resistance and biofilm-forming ability in enterococcal isolates from red meat and poultry preparations. *Pathogens*, *9*(12), 1-12.

Donlan, R.M., & Costerton, J.W. (2002). Biofilms: survival mechanisms of clinically relevant microorganisms. *Clinical Microbiology Reviews, 15*(2), 167-193).

Dutka-Malen, S., Evers, S., & Courvalin, P. (1995). Detection of glycopeptide resistance genotypes and identification to the species level of clinically relevant enterococci by PCR. *Journal of Clinical Microbiology*, *33*(1), 24-27.

Esmaeili, Z., Sadeghi, J., Razavi, S., Oshaghi, M., Sayyahfar, S., Rahbar, M., & Talebi, M. (2018). High level of biofilm formation and virulence factors in enterococci species isolated from clinical and normal flora samples. *Infectious Diseases in Clinical Practice*, *26*(1), 27-30.

Fallah, F., Yousefi, M., Pourmand, M.R., Hashemi, A., Nazari Alam, A., & Afshar, D. (2017). Phenotypic and genotypic study of biofilm formation in Enterococci isolated from urinary tract infections. *Microbial Pathogenesis*, *108*, 85-90.

Foulquié Moreno, M.R., Sarantinopoulos, P., Tsakalidou, E., & De Vuyst, L. (2006). The role and application of enterococci in food and health. *International Journal of Food Microbiology, 106* (l), 1-24.

Giraffa, G. (2002). Enterococci from foods. *FEMS Microbiology Reviews*, *26*(2), 163-171.

Gousia, P., Economou, V., Bozidis, P., & Papadopoulou, C. (2015). Vancomycin-resistance phenotypes, vancomycin-resistance genes, and resistance to antibiotics of enterococci isolated from food of animal origin. *Foodborne Pathogens and Disease*, *12*(3), 214-220.

Guerrero-Ramos, E., Cordero, J., Molina-González, D., Poeta, P., Igrejas, G., Alonso-Calleja, C., & Capita, R. (2016). Antimicrobial resistance and virulence genes in enterococci from wild game meat in Spain. *Food Microbiology*, *53*, 156-164.

Handwerger, S., Raucher, B., Altarac, D., Monka, J., Marchione, S., Singh, K. V., Murray, B. E., Wolff, J., & Walters, B. (1993). Nosocomial outbreak due to *Enterococcus faecium* highly resistant to vancomycin, penicillin, and gentamicin. *Clinical Infectious Diseases*, *16*(6), 750-755.

Hassan, A., Usman, J., Kaleem, F., Omair, M., Khalid, A., & Iqbal, M. (2011). Evaluation of different detection methods of biofilm formation in the clinical isolates. *Brazilian journal of infectious diseases*, *15*(4), 305-311.

Hayes, J.R., English, L.L., Carter, P.J., Proescholdt, T., Lee, K.Y., Wagner, D.D., & White, D.G. (2003). Prevalence and Antimicrobial Resistance of *Enterococcus* Species Isolated from Retail Meats. *Applied and Environmental Microbiology*, *69*(12), 7153-7160.

Hosseini, S.M., Zeyni, B., Rastyani, S., Jafari, R., Shamloo, F., Tabar, Z.K., & Arabestani, M.R. (2016). Presence of virulence factors and antibiotic resistances in *Enterococcus* sp collected from dairy products and meat. *Der Pharmacia Lettre*, *8*(4), 138-145. http://eprints.umsha.ac.ir/2255/

Jahan, M., Zhanel, G.G., Sparling, R., & Holley, R.A. (2015). Horizontal transfer of antibiotic resistance from Enterococcus faecium of fermented meat origin to clinical isolates of *E. faecium* and *Enterococcus faecalis*. *International Journal of Food Microbiology*, *199*, 78-85.

Kasimoglu-Dogru, A., Gencay, Y. E., & Ayaz, N. D. (2010). Prevalence and antibiotic resistance profiles of Enterococcus species in chicken at slaughter level; absence of vanA and vanB genes in *E. faecalis* and *E. faecium*. *Research in veterinary science*, *89*(2), 153-158.

Majhenič, A. Č., Rogelj, I., & Perko, B. (2005). Enterococci from Tolminc cheese: population structure, antibiotic susceptibility and incidence of virulence determinants. *International Journal of Food Microbiology*, *102*(2), 239-244.

Manero, A., & Blanch, A. R. (1999). Identification of Enterococcus spp. with a biochemical key. *Applied and environmental microbiology*, *65*(10), 4425-4430.

Olsen, R.H., Schønheyder, H.C., Christensen, H., & Bisgaard, M. (2012). *Enterococcus faecalis* of Human and Poultry Origin Share Virulence Genes Supporting the Zoonotic Potential of *E. faecalis*. *Zoonoses and Public Health*, *59*(4), 256-263.

Stępień-Pyśniak, D., Marek, A., Banach, T., Adaszek, Ł., Pyzik, E., Wilczyński, J., & Winiarczyk, S. (2016). Prevalence and antibiotic resistance of *Enterococcus* strains isolated from poultry. *Acta Veterinaria Hungarica*, *64*(2), 148-163.

Stewart, P.S. (2002). Mechanisms of antibiotic resistance in bacterial biofilms. *International Journal of Medical Microbiology*, *292*(2), 107-113.

Stewart, P.S., & Costerton, J.W. (2001). Antibiotic resistance of bacteria in biofilms. *Lancet, 358* (9276), 135-138.

Weigel, L.M., Clewell, D.B., Gill, S.R., Clark, N.C., McDougal, L.K., Flannagan, S.E., Kolonay, J.F., Shetty, J., Killgore, G.E., & Tenover, F.C. (2003). Genetic Analysis of a High-Level Vancomycin-Resistant Isolate of *Staphylococcus aureus*. *Science*, *302*(5650), 1569-1571.