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Isolation, Biochemical and Virulence Characterisation of *Enterococcus faecalis* and *Enterococcus faecium* Isolated from Poultry Feces

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The present investigation was conducted to study the prevalence of two important *Enterococcus* species viz. *E. faecalis and E. faecium* and some of the selected phenotypic characters associated with virulence. A total of 50 fecal samples were collected from various poultry farms for isolation using a Pfizers Enterococcus agar. Preliminary identification was done by observing the colonial morphology, Grams staining and a set of 12 biochemical tests. Final confirmation was made by species-specific PCR. The biochemical characterization involved subjecting the isolates to a set of 18 different sugars to note their fermenting activity. From 50 samples, a total of 48 isolates were identified to belong to the genus *Enterococcus*. The species-specific PCR showed that out of 48 *Enterococcus* sp. isolates, 25 (52.08%) belonged to *E. faecalis*, 15 (31.25%) were identified as *E. faecium* while rest 8 isolates were considered as miscellaneous. Biochemical characterization of isolates showed atypical reaction in fermentation of some sugars *viz*. Adonitol, Arabitol, Dulcitol and inulin. Virulence characterization involved attributes viz. gelatinase-production, caseinase-activity and haemolysin-production. The study showed gelatinase, caseinase and haemolysin production to a level of 84%, 72% and 40% respectively by *E. faecalis* while 46.66%, 40% and 53.33% were seen by *E. faecium* isolates respectively. Except haemolysin production all properties were more evident in *E. faecalis* isolates as compared to that of the *E. faecium* isolates.

Key words: *Enterococcus faecalis*, *Enterococcus faecium*, Poultry, Virulence, Species-specific PCR.

Enterococci are the normal flora of the intestinal tract of humans and animals (Morrison *et al.*, 1997). They are Gram-positive cocci bacteria that occur singly, in pairs, or as short chains, facultative anaerobes, catalase negative, most thermo tolerant among non sporulating bacteria (Franz *et al.*, 1999), grow at wide range of temperature ranging 10°C to 45°C (optimum being 37°C), tolerates 6.5% NaCl, pH 9.6 and 40% bile and split esculin and hydrolyze L-pyrrolidonyl-^{2}-naphthylamide (Murray *et al.*, 1990). There are about 38 species within the *Enterococcus* genus with majority of them being *Enterococcus faecalis* and *Enterococcus faecium* (Murray *et al.*, 2009). Both of them are important human pathogens and considered as an indicator of contamination in some types of food products due to their resistance to freezing, low pH, food processing & heat treatment of food materials (Foulquie-Moreno *et al.*, 2006).

The identification and characterization of virulence factors of *Enterococcus* associated with disease severity has become an important subject of concern as they are important nosocomial pathogens and can affect humans via contaminated food products. The virulence of *Enterococcus* appears to be multifactorial which

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can define pathogenesis of bacterium in disease frequency and outcome (Klibi *et al.,* 2007). A number of studies have identified different virulence factors, most important among them being haemolysin, gelatinase, Enterococcal surface protein (Esp), aggregation substance (AS), MSCRAMM Ace (Microbial surface component recognizing adhesive matrix molecule adhesion of collagen from *Enterococci*), serine protease, capsule, cell wall polysaccharide and superoxide (Jett *et al.,* 1994). Thus, the present study was designed to screen the occurrence of *Enterococcus* species present in poultry feces and characterize them for various biochemical and phenotypic virulence associated attributes.

MATERIALS AND METHODS

Isolation and Identification

A total of 50 faecal samples were taken from healthy poultry faeces. After enrichment in buffered peptone water, were streaked onto selective Pfizer's enterococcus agar and incubated in micro-aerophilic conditions. Further, confirmation was done on the basis of Gram's staining, esculin hydrolysis, catalase test and growth in 6.5% NaCl. Standard biochemical identification was conducted using HiStrepTM commercial kit and HiCarbohydrate kits A and B (HiMedia, Mumbai).

Molecular confirmation

Molecular confirmation of species was done using species-specific primer targeting sodA gene (Jackson *et al.,* 2004). Sequence of primers for *E. faecalis* is 5' ACTTATGTGACTAACTTAACC 3' and 5' TAATGGTGAATCTTGGTTTGG 3' while for *E. faecium* is 5'GAAAAAACAATAGAAGAATTAT 3' and 5' TGCTTTTTTGAATTCTTCTTTA 3'. The reaction mixture (total 25 ¼l) was prepared using Promega (USA) gene amplification kit by mixing 6 ¼l 5X assay buffer, 1 ¼l primer-1 (10 pM/¼l), 1 ¼l primer-2 (10 pM/¼l), 1 ¼l dNTP (10 mM), 3 ¼l Mgcl2 (1.5 mM/¼l), 0.3 ¼lTaq DNA polymerase (5 U/¼l), 9.7 ¼l deionised water and 3 ¼l DNA (25 ng/¼l). Following an initial denaturation at 95°C for 4 min, products were ampliûed by 35 cycles of denaturation at 95°C for 30 s, annealing at 55°C (*E. faecalis*) and 56°C (*E. faecium*) for 45 s and elongation at 72°C for30s. The PCR-amplified product was analysed on a 2% Agarose gel and photographed in UVP transilluminator.

Phenotypic analysis of virulence

Gelatinase production

Nutrient Gelatin plate method: In this method, a heavy inoculum was stab-inoculated onto Nutrient Gelatin plates (23 g/L Nutrient Agar; 8 g/L 275 bloom gelatin). Gelatin hydrolysis was indicated by clear zones around gelatinase-positive colonies (Catherine Hopper, University of Maine, pers. comm).

Caseinase production

Caseinase production was detected by caseinolysis using skim milk agar plates (Peter *et al.,* 2013).

Haemolysin production

Haemolysin production was detected by inoculating *Enterococci* on freshly- prepared beef heart infusion agar supplemented with seven per cent horse blood (Vergis *et al.,* 2003).

RESULTS AND DISCUSSION

Out of 50 faecal samples processed, a total of 48 were recovered as *enterococci*. Colonies obtained after 24-48 hours of partial anaerobic incubation on Pfizer's enterococcus agar were pin-point grayish, black centered with a halo around. The black discoloration of the colonies was obtained due to esculin hydrolysis. These findings were typical for *Enterococcus* genus. All tentatively identified *Enterococcus* species were catalase-negative and showed growth in a presence of salt.

As per the results obtained from commercial kits, all the *enterococcal* isolates fermented glucose, lactose, sucrose, Mannitol, maltose, fructose, dextrose, galactose and salicin. A total of 95.8% isolates were able to ferment trehalose, mannose and glycerol. A high proportion of the isolates also fermented adonitol (43.75%), Arabitol (29.16%), erythritol (22.91%) and Dulcitol (47.91%) and inulin (77.08%) which is not common is not reported earlier. The detailed results of various biochemical and sugar fermentation tests are shown in table 1 and 2.

All biochemical results except some sugar fermentation tests, obtained in this study were in concordance with the previous studies

conducted by Devrisse *et al*. (1987) who demonstrated that all isolates of both *E. faecalis* and *E. faecium* fermented lactose, trehalose, Mannitol (100% *E. faecalis*, 93.42% *E. faecium*), and sucrose(100% *E. faecalis*, 92.05% *E. faecium*). Similar results were also obtained by Manero & Blanch (1999). Previous studies suggested that none of the isolates of *E.faecalis* and *E. faecium* fermented Adonitol, Arabitol, Erythritol and Dulcitol (Manero & Blanch, 1999) and inulin (Devrisse *et al.*, 1987) which is in contrast to our studies. All the four sugars described above were reported not to be fermented by both *E. faecalis* and *E. faecium* but were fermented by other *enterococcal* species like *E. cecorum, E. columbae, E.flavescens, E. casseliflavus*, and *E. munditi* were capable of fermenting inulin (Manero & Blanch, 1999). Presently there seems to be no definite reason for the high variability in sugar fermentation tests but this observation can be due to plasmid-mediated acquisition of genes responsible for fermenting sugars in our isolates.

Out of 48 isolates, 25 samples showed an amplicon size of 360 bp which is typical for *E. faecalis* (Fig.1.) while as rest 15 isolates revealed

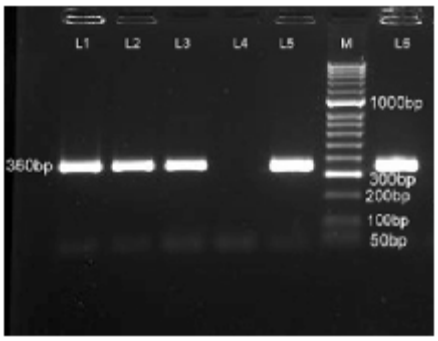

Fig. 1. PCR amplified product of *Enterococcus faecalis* (360 bp). L1 to L3, L5 and L6: positive samples, L4: negative control.

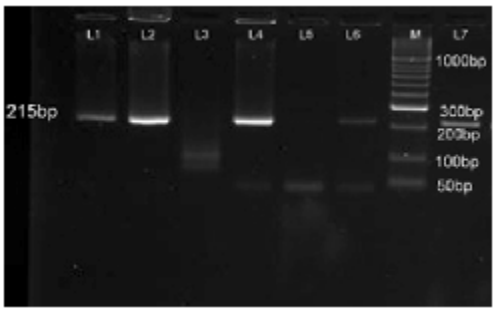

Fig. 2. PCR amplified product of *Enterococcus faecium* (215 bp). L1, L2, L4, L6 and L7: positive samples, L3: negative sample and L5: negative sample.

Table 1. Results obtained from HiStrep™ kit

S. No	Test	% of Isolates Positive	% of Isolates Negative
1.	Voges Proskauer's	93.75%	6.25%
2.	Esculin	100%	-
3.	PYR	97.91%	2.08%
4.	ONPG	22.91%	77.08%
5.	Arginine utilization	100%	-
6.	Glucose	100%	-
7.	Lactose	100%	-
8.	Arabinose	33.33%	66.67%
9.	Sucrose	100%	-
10.	Sorbitol	64.58%	35.41%
11.	Mannitol	100%	-
12.	Raffinose	72.91%	27.08%

Table 2. Results of various sugar fermentation tests as obtained from HiCarbohydrate™ A and B Kits

S. No.	Sugar	% of Isolates Positive	% of Isolates Negative
1.	Xylose	81.25%	18.75%
2.	Maltose	100%	-
3.	Fructose	100%	-
4.	Dextrose	100%	-
5.	Galactose	100%	-
6.	Trehalose	95.83%	4.16%
7.	Melibiose	89.58%	10.41%
8.	Mannose	95.83%	4.16%
9.	Inulin	77.08%	22.91%
10.	Sodium gluconate	89.580%	10.41%
11.	Glycerol	89.58%	10.41%
12.	Salicin	100%	-
13.	Dulcitol	47.91%	52.08%
14.	Inositol	41.66%	58.33%
15.	Adonitol	43.75%	56.25%
16.	Arabitol	29.16%	70.83%
17.	Erythritol	22.91%	77.08%
18.	α-CH_3-D-glucosidase	29.16%	70.83%

an amplicon size of 215 bp corresponding to *E. faecium* (Fig.2).

The predominance of *E. faecalis* (52.08%) followed by *E. faecium* (31.25%) in this study from poultry faeces is similar to that reported earlier (Hassan *et al.,* 2008). During their study, the prevalence of *E. faecalis* and *E. faecium* was 48% and 25.7% respectively from broilers. Various other studies from poultry production environments also demonstrated the predominance of *E. faecalis* followed by *E. faecium* (Tejedor *et al.,* 2005; Poeta *et al.,* 2006). Study conducted by Ruzauskas *et al.,* (2009) in Lithuanian poultry farms in contrast, suggested that *E. faecium* was the dominant enterococcal species of poultry faecal flora whereas a Belgian study conducted by Devriese *et al.,* (1991) demonstrated a predominance of *E. cecorum* in older chickens.

As appeared from various previous studies (Turkyilmaz *et al.,* 2010; Silva *et al.,* 2011), *E. faecalis* may not be a predominant species in sources other than poultry. Phenotypic and molecular analysis revealed that *E. faecium* as the predominant species followed by *E. faecalis* except in poultry and clinical cases. Predominance of these two species is not only reported in a faecal flora of animals but humans too (Silva *et al.,* 2011). Both species have now emerged as important pathogens and the third leading cause of nosocomial infections. *E. faecalis* alone accounts for 80-90% of enterococcal isolates of clinical origin, with E. faecium the second most prevalent *enterococcal species* (Gordon *et al.,* 1992). This is congruent with isolation of these species as predominant species from various clinical settings from both human and animal specimens as described in various previous studies conducted by Palanisamy *et al.,* (2013), Shafiyabi *et al.,* (2013). The degree to which *enterococcal* populations from the food animal production environment enter the human microbiota is likely heavily influenced by the degree of contamination of the foods consumed.

This study revealed that 84% of the *E. faecalis* and 46.6% of *E. faecium* were positive for the gelatinase production which is almost similar to the previous studies conducted on clinical and non-clinical enterococci. Poeta *et al.,* (2006) demonstrated that 88% of the *E. faecalis* of poultry origin showed gelatinase production while only 3.03% of *E. faecium* showed this activity. The importance of gelatinase as a virulence factor has been associated with initiation and propagation of inflammatory processes (Waters *et al.,* 2003) and a role in biofilm formation (Dworniczek *et al.,* 2012). This study showed that only 46.6% of *E. faecium* isolates were positive for the gelatinase production as compared to 88% of positive *E. faecalis*. Similar pattern was shown by *E. faecium* isolates of poultry origin in a study conducted by Poeta *et al.,* (2006). An earlier investigator also stated that none of the *E. faecium* strains showed the gelatinase production (Peter *et al.,* 2012).

In the present study, caseinase production was seen in 72% of *E. faecalis* isolates and 40% of *E. faecium* isolates which is not in agreement with non-clinical isolates obtained from human and chickens, among which none of the *E. faecalis* were positive for caseinase production and 5.6% of *E. faecium* of human origin and 6.15% of *E. faecium* of chicken origin produced only casein in a study conducted by Peter *et al.,* (2013). Clinical isolates of *E. faecalis* were have been demonstrated to be Caseinase positive in various studies (Patidar *et al.,* 2013; Furumura *et al.,* 2006).

E. faecium is often ±-hemolytic which is due to the production of peroxidases while *E. faecalis* is usually non-hemolytic but may be ²-hemolytic due to the production of plasmid transmitted haemolysin, which can be used as a differentiating feature (Colman *et al.,* 1944). In this study, *Enterococcus* species of poultry origin showed hemolytic activity on horse blood incorporated in BHI agar and did not lyse sheep blood. The pattern of haemolysis obtained in the present study showed that 40% of *E. faecalis* showed ²-haemolysis and rest were negative. The haemolysis shown by *E. faecium* was partial and 53.33% of the isolates were positive. It is worth to mention here that we obtained high percentage of haemolysis in non-clinical strains, contrary to other reporters have demonstrated high degree of haemolysis in clinical isolates (Furumura *et al.,* 2006; Palanisamy *et al.,* 2013).

On analyzing the virulence properties of both species of *Enterococcus sp.,* it was found that except haemolysin production, rest all studied

virulence attributes were more evident in *E. faecalis* as compared to *E. faecium* isolates. One of the interesting observations of the study was of this study showed the virulence properties like gelatinase, caseinase and haemolysin in non-clinical *enterococcal* strains which indicate the pathogenic potential of these isolates. Although *enterococci* from the nonclinical sources are not conclusively linked as direct causes of clinical infections, the potential virulence factors in them is increasingly an issue of public concern. *Enterococci* have the spoilage potential since many of the strains possess protease and lipase. Transfer of *Enterococcus* with the virulent properties from environmental compartments to humans can occur through surface water or through food. The present study points to the necessity to control the spread of this potentially virulent bacterium capable of producing nosocomial infection.

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