A human–food web–animal interface on the prevalence of food-borne pathogens (Clostridia and Enterococcus) in mixed veterinary farms

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Abstract In the present work, we addressed the impact of a human–food web–animal interface on the prevalence of food-borne pathogens in mixed farms of Tamil Nadu, India. We have isolated and identified six strains of *Clostridium* sp. and five strains of *Enterococcus* sp. from food and animal sources disposed near to the veterinary and poultry farms. Phylogenetic relationships of these strains were inferred from their homologies in 16S rDNA sequences and rRNA secondary structures. The strain PCP07 was taxonomically equivalent to *C. botulinum* confirmed by neurotoxin-specific PCR primers, followed by mouse bioassay. Other Clostridial and Enterococcal isolates have shown a phylogenetic similarity to the *C. bifermentans* and *E. durans* isolated from veterinary farms, respectively. Results of our study revealed that a human–food web–animal interface has influenced the disease incidence and prevalence of these isolates in the poultry to veterinary farms, where human food acted as a likely transmittance vehicle for their infections.

Keywords Avian botulism · Poultry disease · Molecular diagnosis · Phylogeny · Clostridium · Enterococcus

Introduction

India is currently self-sufficient in livestock feeds and does not depend on imports. For cattle and poultry, nutritional standards and specifications have been prepared according to the Bureau of Indian Standards. Maize, sorghum, and millet are commonly used in animal feeds whereas soybean, groundnut, rapeseed, sesame, and sunflower meals are commonly used commodities in poultry feeds (<http://www.fao.org/docrep/ARTICLE/AGRIPPA/X9500E01.HTM>). Therefore, there is a change for the prevalence of food-borne pathogens between farm wastes and local food products in the local farmyards or mixed farms. Contaminated foodstuffs, soils, livestock products, animal feeds, poultry waste, and sediments are the primary environments for anaerobic bacteria, where an oxygen-deficient environment is created. Anaerobic bacteria are mobilized into a food web by surface water in heavy rain, or through dust carried away by wind and then subsequently enters into the gastrointestinal tract of veterinary animals and humans (Espelund and Klaveness, 2014). Consequently, a food web is being a global concern for transmitting anaerobic pathogens between species from human to animal or vice versa via ingestion of contaminated food (Allen, 2015; Dhama et al., 2013).

Most Clostridia and Enterococcus are harmless, others are opportunistic pathogens, and a few are quite dangerous. *Clostridium botulinum*, *C. perfringens*, *C. bifermentans*, *C. histolyticum*, *C. septicum* and *C. difficile* are a diverse group of Clostridial pathogens, which are causing difficult gastrointestinal disorders, gas gangrene, and botulism in

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veterinary animals (Daniel and Rapose, 2015; Sasaki et al., 2000). Clostridial infection is a global threat to community health in endemic areas, which has shown more disease prevalence in many mixed farms (Hogg et al., 1990; Liu et al., 2015; Souillard et al., 2015; Tavakoli et al., 2009). Various Enterococcal infections have been reported in a variety of avian species worldwide. *Enterococcus avium*, *E. durans*, *E. faecalis*, *E. cecorum*, *E. faecium*, and *E. hirae* are common pathogenic species of Enterococci genus causing acute septicemia, bacteremia, encephalomalacia, and tracheitis in affected birds and animals (Cho et al., 2013; Daniel and Rapose, 2015; Stalker et al., 2010). Antibiotic-resistant *E. faecium* in dogs are frequently associated with human infection. Hence, Enterococcal strains are commensals of the human and animal gastrointestinal tract where animals are served as a reservoir (Damborg et al., 2009).

The feeding of food scraps (leftover) to domesticated animals is the most common way of dealing with household food waste. Such waste discarded from the human food chain could be used to mix with a usual feed of veterinary animals and chickens despite discarded to garbage for a long time. Consequently, ruminants and chickens can consume those wastes either from commercial feed or environmental sites and then ingested in their intestinal tracts with food-borne pathogens. In this context, our study was intended to infer how a human–food web–animal interface impacted on the prevalence and emergence of food-borne pathogens (Clostridia and Enterococcus) in mixed farms. The 16S rDNA-based phylogeny is being of great interest in studying the emergence and transmission of epidemic healthcare-associated anaerobes (Cho et al., 2013; Liu et al., 2015). Besides, the RNA secondary structure comparison clearly provides homologous characters for alignment and reconstruction of a phylogenetic tree and taxonomic inferences (Keller et al., 2015; Razia et al., 2011). We also employed phylogenetic inference for identification and taxonomic classification of these bacterial isolates. This study could provide more realistic information for a better track of contamination for reducing the risk of food-borne illness in farmyards or mixed farms.

Materials and methods

Raw materials

Canned and packed foodstuffs (sauce, jam, pickles, syrups, and dried fishes and farm wastes including foods, poultry, and carcasses etc.) were collected from various locations in Namakkal (11°13'N 78°10'E), Tamil Nadu, South India. Namakkal is often referred to as “Egg City” and “Transport City”. Raw materials and experimental samples were

collected from the sites where human food wastes have been disposed as animal feeds to the interface of poultry and veterinary farms. Complete detail of food and farm samples including sample name, a manufacturing company, batch and food package material/originals used in this study is available in Online Appendix A. Briefly, a total of 52 samples was collected to examine the recovery of 11 food-borne anaerobic bacteria. The suspected sites for the prevalence of food-borne bacteria were identified from different parts of the farms, particularly feeding areas and the interface between farms and household foods. All materials were aseptically sampled in a properly sealed container without mixed together. After sampling, all materials were kept at 4 °C immediately and maintained as such until to transport to the laboratory.

Screening and isolation of food-borne pathogens

Solid samples (soil, hair, dung, flesh, sauce, jam, pickles, and dried fishes etc.) were transferred aseptically to a sterile mortar. These samples were mixed with 5 mL of sterile gel-phosphate buffer (Na₂HPO₄ 0.2 g; gelatine 0.4 g; distilled water 100 mL) and homogenised with a sterile pestle. We inoculated 5 mL of homogenized sample into 50 mL Tryptone–Peptone–Glucose–Yeast extract broth (TPGY; HiMedia Laboratories Pvt. Ltd., India). Five ml of liquid sample (honey, sauce, sewage, syrup etc.) was homogenized and then directly transferred into 50 mL TPGY broth. All samples were anaerobically incubated at 37 °C for 10–15 days for the enrichment of *Clostridium* sp. After that, 0.1 mL of enriched culture was spread on a TPGY agar plate and incubated for 24 h at 37 °C in an anaerobic jar (HiMedia Laboratories Pvt. Ltd., India). Similarly, we prepared the solid and liquid samples for the enrichment of *Enterococcus* sp. Each homogenized sample was diluted with bile esculin azide broth (HiMedia Laboratories Pvt. Ltd., India) and then spread on a bile esculin azide agar, followed by incubation at 37 °C and 48 h (Hayes et al., 2003). In the mean while, 5 g of each sample was analyzed to confirm the absence of natural contamination from small numbers of food-borne pathogens or mixed samples using enrichment cultures.

Biochemical characterization

We picked out typical Clostridial (milky white) and Enterococcal (black) colonies from TPGY agar and bile esculin azide agar, respectively. The pure colonies of each genus were streaked onto blood agar (HiMedia Laboratories Pvt. Ltd., India) prepared with 5% of defibrinated sheep blood and then incubated at 37 °C and 24 h. Identification of presumptive Clostridia was confirmed using the tests proposed by Oguma et al. (1986). Biochemical

classification of positive *Enterococcus* was carried out according to the methods described earlier (Facklam et al., 2007).

Bacterial genomic DNA isolation

After morphological and biochemical confirmation, the pure colonies of selected isolates were inoculated into TPGY broth and incubated for 18 h ($OD_{600} = 0.6$) at 37 °C. The culture was centrifuged at 14,000 rpm for 2 min at 4 °C using a cooling centrifuge (Eppendorf international, Germany). The cell pellet was washed twice with phosphate buffered saline (NaCl 7.65 g; Na_2HPO_4 0.72 g; KH_2PO_4 0.21 g; distilled water 1000 mL; pH 7.4). It was again centrifuged at 10,000 rpm for 5 min at 4 °C and re-suspended in 120 µL of phosphate buffered saline. Genomic DNA was isolated from cell pellet by using Bacterial DNA Purification Kit (Merck & Co., USA) according to the manufacturer's instruction. The quality and purity of isolated DNA were examined by agarose (1.2%, w/v) gel electrophoresis, which was carried out at 80 V.

PCR amplification and sequencing

Genes coding for the 16S rDNA gene fragment, botulinum neurotoxin (*bont/C*) and cytotoxins (C2I catalytic subunit, C2II binding subunit, and C3 exotoxin) were amplified from the bacterial genomic DNA using a conventional PCR technique. PCR primers and conditions used in this study were described in Table 1 (Bagge et al., 2010; Dmochewitz et al., 2013; Kimura et al., 1998; Nobuhiro et al., 1996; Williamson et al., 1999). PCR was conducted with a 100 µL reaction mixture containing reverse primer 4 µL (10 pmol), forward primer 4 µL (10 pmol), Taq 2X PCR master mix (Amplicon, Denmark) 50 µL, template DNA 8 µL (20–50 ng) and deionized water 34 µL. PCR amplification was carried out by a Thermal Cycler (Nexus X1, Eppendorf international, Germany). PCR products were confirmed by agarose (1.2%, w/v) gel electrophoresis and visualized as a single compact band of the expected size by gel documentation system (BioRad, USA). The amplified PCR product was purified by using a Gel extraction kit (Merck & Co., USA) according to the manufacturer's protocol. Forward and reverse DNA sequencing reactions of PCR amplicons were carried out with corresponding primers using BigDye® Terminator v3.1 Cycle sequencing kit following manufacturer's instruction on ABI 3730xl Genetic Analyzer (Applied Biosystems, USA).

Phylogenetic analysis

The 16S rDNA gene sequences of the PCR products were served as templates to retrieve the similarity sequences of

closely related species isolated from food and animal sources of different geographic regions by using the NCBI-BLASTn tool (Altschul et al., 1997). We collected detailed information on accession, strain details, isolation source, country, and relevant literature for phylogenetic analyses (Online Appendix A). All the sequences were aligned together by ClustalX 2.0.11 software (Thompson et al., 1997). Multiple substitutions were corrected in the aligned sequences and then manually inspected for proper alignment. The phylogenetic tree was constructed from the aligned sequences by MEGA 6.0 software (Tamura et al., 2013) using a neighbor-joining algorithm and 1000 bootstrap replicates. Gamma distribution (0.5) with invariant sites was used to calculate the evolution rate among sites. The Kimura-2 parameter was employed as a model to estimate the evolutionary distance across the closely related species.

Prediction of rRNA secondary structure

We used the Greedy algorithm for the prediction of RNA secondary structures and loop energy conservation from the selected sequence. The greedy algorithm takes into account data on the conservative and compensational substitutions at different positions of aligned sequences. The energy of both conservative and compensational pairing accounts on the conditional energy for complete secondary structure. From aligned sequences, optimal RNA secondary structure was built with conditional structural energy. In this study, the rRNA secondary structure was predicted from aligned sequences by GeneBee-NET program (Brodsikii et al., 1995) using a greedy algorithm. We calculated free energies of loop structures from the sequence of our isolates and compared with those structures computed from closely related strains. Conservative and compensation substitutions in the predicted loop structures were accounted for the similarity analysis between strains.

Mouse bioassay

The biological activity of BoNT/C was measured in NMRI mice weighing 18–25 g. A portion of untreated bacterial culture was diluted to 1:10, 1:100 and 1:1000 in the gel-phosphate buffer. For each test, 3 pairs of mice were injected intraperitoneally with 0.5 mL of untreated undiluted culture and 0.5 mL of each dilution of untreated culture. Animals were observed for symptoms of botulism (ruffling of fur, difficulty in breathing and weakness of limbs) or death due to respiratory failure over a period of 48 h.

Table 1 PCR primers and conditions used in this study

Gene	Primers used	PCR conditions			References	
		Initial denaturation	No. of cycles	Cycling Condition		
16S rRNA	FP: AGAGTTGATCTGGCTCAG RP: GGTACCTTGTTACGACTT	94 °C for 2 min	35	94 °C for 1 min 55 °C for 1 min 72 °C for 1 min	72 °C for 5 min	Bagge et al. (2010)
	FP:AAACCTCCTCGAGTTACAAGCCC RP: GAAAATCTACCCCTCTCCTACATCA	95 °C for 1 min	30	95 °C for 1 min 55 °C for 1 min 75 °C for 2 min	72 °C for 10 min	Williamson et al. (1999)
C2I	FP:AAGGAAGATAAAACAAAAAT RP:CCTAATGATACAAATGAAAA	95 °C for 3 min	35	95 °C for 45 s 45 °C for 2 min 72 °C for 1 min	72 °C for 5 min	Nobuhiro et al. (1996)
C2II	FP:GCAGAAGTTTCAGGTAGTTACCAAC RP:CGCATTCTATAACGACCTTCTGGA	94 °C for 3 min	40	94 °C for 45 s 53 °C for 1 min 72 °C for 1 min	72 °C for 5 min	Kimura et al. (1998)
C3botI	FP:GGAATTCATCGAAGTCGTGCTTATTCAAATACTTACCAGGAG RP:GGAATTCCTTATTAGGATTGATAGCTGTGCC	94 °C for 60 s	30	94 °C for 45 s 50 °C for 60 s 68 °C for 120 s	68 °C for 120 s	Dmochewitz et al. (2013)

GenBank accession

The partial 16S rDNA sequences determined from this study have been deposited in the GenBank of NCBI with accession numbers KT367811, KT598250–KT598254, and KU158754–KU158758.

Results and discussion

Clostridial and enterococcal strains are more common food-borne anaerobes, which may cause disease incidence and prevalence in many mixed farms (Cho et al., 2013; Hogg et al., 1990; Liu et al., 2015; Souillard et al., 2015). A PCR-based diagnosis assists in targeting control measures of them in mixed farms. In this study, 40 anaerobic bacteria were isolated from human foodstuffs such as sauce, jam, honey, pickle, and dried fish discarded around mixed farms. Food samples were highly contaminated with Gram-negative, non-spore forming bacteria compared to farm waste. *Clostridium* sp. and *Enterococcus* sp. found as more representative anaerobes present in the food and farm wastes. Both organisms were more abundant in farm waste (60%) than those isolated from food origins (40%). Fifty-two experimental samples were collected from food (69%) and animal sources (31%) out of which 40 samples (77%) have shown positive colonies on TPGY agar. We isolated 6 Gram-positive, spore-forming rods (PCP02, PCP06–09, and PCP12) and 5 non-spore forming cocci (PCP03–04, PCP10–11, and PCP13) (Table 2). As shown in Fig. 1, dense and distinct bands observed in agarose gel for all PCR products. The length of the PCR products (16SrDNA gene) was ranged from 416 to 1082 bp and the average length was 671 bp. We used the sequences of the PCR products of each bacterial isolate for further identification and taxonomic classification. The predicted rRNA secondary structures of each isolate were compared with homologous structures of closely related strains (Online Appendix A).

We found the biochemical characteristics of strain PCP07 showing as represented for *C. botulinum*/*C. sporogenes*. Sequence similarity analysis indicated that strain PCP07 has shown a maximum sequence similarity (97%) with *C. botulinum*. Phylogenetic analysis of this study revealed that strain PCP07 was grouped with a cluster comprising of non-proteolytic *C. botulinum* type E and proteolytic group-III strains (type C, D and C/D) (Fig. 2). Non-proteolytic *C. botulinum* types B and G were grouped separately in the tree in which type B strain closely related to *C. sporogenes*. Strong conservation between *C. botulinum* and *C. sporogenes* has been restricted its taxonomic classification at the species-level (Olsen et al., 1999). Therefore, we used the toxin gene-specific PCR markers for taxonomic classification of this isolate. It

implied that strain PCP07 encompassed genes coding for botulinum toxin (*bont/C*) and cytotoxins (C2 endotoxin and C3 exoenzyme). The biological activity of botulinum toxin (BoNT/C) produced by strain PCP07 was also confirmed by mouse bioassay using toxin injected mice (Online Appendix B).

Approximately 200 closely related species are found within the *Clostridium* genus, which limits to differentiate them from each other and for accurate identification of individual strains (Chellapandi and Prisilla, 2018; Lindstrom and Korkeala, 2006). We observed phylogenetic proximity between strain PCP07 and *C. botulinum* type C isolated from the geographic regions of Georgia and Sweden. Toxin gene-specific PCR markers and mouse bioassay further confirmed its biological activity. As shown by our analysis, strain PCP07 was taxonomically equivalent to *C. botulinum* serotype C in accordance with the previous phylogenetic conclusions (Lawson and Rainey 2016; Olsen et al., 1999). *C. botulinum* spores from contaminated food or animal sources are mobilized to the poultry and veterinary farms via air dispersal or surface water (Bano et al., 2013; Espelund and Klaveness, 2014). Cattle farms and their environments contaminated via poultry botulism that can be an intermittent carrier of *C. botulinum* type C/D after poultry botulism outbreaks in mixed farms (Bano et al., 2013; Hogg et al., 1990; Souillard et al., 2015). In this study, mango pickle was a primary source of strain PCP07 disposed of as a food additive along with animal feeds in veterinary farms. It clearly implied that botulism outbreak may be associated with closely sited veterinary and poultry farms through human food contaminated with its spores as an intermittent carrier/vehicle.

Maximum sequence identity of other clostridial strains was observed to be 96–99% with *C. bifermentans*. The nucleotide sequence of strain PCP08 exhibited a low sequence similarity (87%) with *C. bifermentans*. In the phylogenetic tree, three major clusters were separately formed in which our strains (PCP02, PCP06–09, PCP12) grouped with different strains of *C. bifermentans* isolated from the samples of sewage sludge, cured duck fat liver, and cattle manure (Fig. 3). Our isolates were closely related to those similarly isolated from cattle manure of Canada, France, and the UK. It is a hyper-ammonia producing organism associated with gas gangrene, Clostridial endocarditis, enterotoxaemia and bacteremia in poultry and veterinary animals (Haines et al., 2013). It can also reduce sperm motility of male broiler breeder and Beltsville Small White Turkey upon its exposure (Triplett et al., 2016). It was identified from various food items and herbage on livestock farms in Nigeria and reported that animal feeds as a likely vehicle of its infections (Princewill et al., 1985). As similar to that, our *C. bifermentans* strains could be associated with Clostridial diseases in mixed farms, where

Table 2 Details of Gram-positive anaerobic food-borne pathogens isolated from our study

Strain ID	Isolation source	Organism	Accession	Sequence identity (%)	Free energy of rRNA secondary structure (kcal/mol)
PCP02	Poultry waste	<i>C. bifementans</i>	KT598250	99	− 96.0
PCP03	Animal carcass	<i>E. durans</i>	KU158754	98	− 146.3
PCP04	Poultry waste	<i>E. durans</i>	KU158755	94	− 131.4
PCP06	Poultry farm sludge	<i>C. bifementans</i>	KT598251	99	− 119.4
PCP07	Mango pickle	<i>C. botulinum</i>	KT367811	97	− 105.6
PCP08	Food waste	<i>C. bifementans</i>	KT598252	87	− 81.7
PCP09	Mixed vegetable pickle	<i>C. bifementans</i>	KU158756	96	− 80.7
PCP10	Dried fish	<i>E. durans</i>	KU158757	85	− 129.3
PCP11	Honey	<i>E. durans</i>	KU158758	98	− 155.7
PCP12	Date syrup	<i>C. bifementans</i>	KT598254	97	− 207.9
PCP13	Canteen waste	<i>E. durans</i>	KT598253	98	− 160.0

Fig. 1 Showing bands on an agarose gel for the PCR products of 16S rDNA genes from the genomic DNA of our bacterial isolates. (Lane M-100 bp Marker, Lane 1-PCP02, Lane 2-PCP03, Lane 3-PCP04, Lane 4-PCP06, Lane 5-PCP07, Lane 6-PCP08, Lane 7-PCP09, Lane 8-PCP10, Lane 9-PCP11, Lane 10-PCP12, Lane 11-PCP13)

cattle manure, poultry farm sludge, and food waste act as intermittent vehicles.

Enterococcal strains (PCP03-04, PCP11, and PCP13) isolated in this study have shown a 94–98% sequence identity with *E. durans*. All of these strains were grouped together and formed three major clusters consisting of different species of this genus in the tree (Fig. 4). The first cluster has comprised different species of this genus isolated from animal sources. In the second cluster, our bacterial isolates exhibited phylogenetic proximity to the *E. durans* isolated from the dried milk. Enterococcal strains isolated from water and environmental sources were closely related together in the third cluster. The most of the species of this genus were isolated from food and animal sources of Japan. *E. durans* strains isolated from an animal carcass, poultry waste, dried fish, honey and chicken pickle

were related to the same species isolated from Germany and Japan. *E. durans* strains isolated from feces of chicken in intensive poultry farms, ostriches, feedlot steers, cattle, and piggery farms have shown wide dissemination of antibiotic resistance in the farm environment (Bekele and Ashenafi, 2010). Multi-resistant antibiotic *E. durans* strains from broiler and veterinary animals could be transmitted to humans through the food chain (Daniel et al., 2017; Iweriebo et al., 2015), and also associated with human infection in small animal hospitals (Damborg et al., 2009).

The results of RNA secondary structural homologies show that strain PCP07 has a stable and conserved structure with the significant free energy of loop conformation (− 105 kcal/mol) similar to that predicted for type C (105.6 kcal/mol) (Online Appendix A). It also observed that strains PCP02 (− 111.9 kcal/mol) and PCP12

Fig. 2 16S rDNA gene-based phylogeny of *Clostridium botulinum* strain PCP07 constructed by a neighbor-joining algorithm using closely related species isolated from food and animal sources of different geographic origins. *C. drakei* was chosen as an out-group organism for placing *C. botulinum* in the phylogenetic tree, which was previously proposed to restrict the genus *Clostridium* to *C. butyricum* and related species (Lawson and Rainey, 2016; Olsen et al., 1999)

(− 243.2 kcal/mol) were equivalent to strain ATCC 638 whereas strains PCP06 (− 148.5 kcal/mol) and PCP08 (− 143.6 kcal/mol) related with strain DSM 10716.

Fig. 3 16S rDNA gene-based phylogeny of *Clostridium* sp. constructed by a neighbor-joining algorithm using closely related species isolated from food and animal sources of different geographic origins. *C. phytofermentans* strain ISDg and *Alkaliphilus crotonoxidans* acted served as out-group organisms (Kjeldsen et al., 2007)

Maximum free energy of loop conformation (− 97 to 170.3 kcal/mol) was calculated between our Enterococcal strains and *E. durans* strain NBRC 100479. Results of this study found all of our strains closely related to the free energy of structures of the known bacterial strains.

We conclude that these isolates are closely related to the different strains of *C. botulinum*, *C. bifermentans* and *E. durans* isolated from food and animal sources of different geographic origins. As a result of this study, a human–food web–animal interface is associated with the prevalence of Clostridial and Enterococcal diseases in mixed farms of Southern India. However, a large-scale sampling data is required to reveal the statistical significance of a human–food web–animal interface on their prevalence and disease incidence in mixed farms. Interestingly, this is a first report on the identification of *C. botulinum* type C strain from mango pickle. This work presents a new clue for the prevalence of avian botulism via the disposal of contaminated human food near to the poultry farm site. Control measure is that contaminated food or dead/infected poultry or animals should be recycled to farms or disposed to the natural environment. Thus, a quick, cost-effective and safe recycling procedure is essential in order to reduce the risk of food-borne diseases in connecting with farms. Antitoxin administration and supportive therapies are currently used to treat veterinary animals and broilers (Prisilla and Chellapandi, 2019). The preventive measures of these organisms can increase the efficient production of livestock and poultry products, which in turn in resolving the problems of international trade.

Fig. 4 16S rDNA gene-based phylogeny of *Enterococcus* sp. constructed by a neighbor-joining algorithm using closely related species isolated from food and animal sources of different geographic origins. *Lactococcus lactis* subsp. *cremoris* SR11 served as an out-group organism (Repizo et al., 2014)

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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