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Prevalence of Campylobacter spp. in marine fishes, crustaceans and molluscs in Kozhikode district, Kerala#

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Abstract

Campylobacteriosis is one of the leading causes of bacterial gastroenteritis world over, with a self-limiting nature. Nevertheless some rare sequelae like Guillain-Barre syndrome, Reiter syndrome and reactive arthritis are evident. With poultry as a major reservoir, the organism is ubiquitous in nature and has been isolated from various sources like animals and the environment. The present study was undertaken to evaluate the occurrence of Campylobacter spp. in marine fishes (n=48), crustaceans (n=35) and molluscs (n=50) from Puthiyappa, a fish catchment area of coastal Kerala (India) using multiplex polymerase chain reaction (mPCR) in combination with conventional plating technique using Blood-free campylobacter broth and modified Charcoal Cefoperazone Deoxycholate (mCCD) agar. Campylobacter spp. was found to be present in marine fishes, crustaceans and molluscs at a level of 6.3, 8.6 and 28.0 per cent, respectively. The predominant species was Campylobacter jejuni in fishes, while Campylobacter coli was more prevalent in molluscs. Of the seasonally available fishes, the organism was found to be present in ribbon fish, pink perch, pony fish, while among molluscs, it was present in mussel, squid and clam. Only 8.3 and 6.0 per cent of Campylobacter spp. were recoverable from fishes and molluscs, respectively, on mCCDA plates. Campylobacter spp. in these marine fishes and shellfishes indicate that these can act as possible sources for the transmission of food-borne campylobacteriosis.

Keywords: *Campylobacter, fish, shellfish, PCR*

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An uprise in the number of foodborne illness caused by *Campylobacter* spp. has been evidenced in both developed and developing countries of the world, even in high-income countries (Kaakoush *et al.*, 2015). Being one among the four leading causes of bacterial gastroenteritis, the organism usually causes a self-limiting disease in humans. With a wide range of animals being identified as reservoirs, birds remain the major reservoir (WHO, 2020). The organism is maintained in the environment and a number of sources, including water (Jones *et al.,* 2001). Though a commensal bacterial organism of the gastrointestinal tract of wild animals, birds, farm animals and companion animals, it has a zoonotic potential. Poultry meat has been reported to account for 38-77 per cent of campylobacteriosis (Skarp *et al.,* 2016). Other foods including meat, milk and water are also associated with the transmission of the infection in fishes available in Kozhikode, which is a northern district in the southern-most coastal state of Kerala, India. Other foods including meats and water are also responsible for transmission of the infection. Fishes and shellfishes have also been incriminated as sources of infection, though limited studies have been documented from different parts of the world (Costa *et al.,* 2016; Rince *et al.,* 2018) in this regard. Infection is acquired by the faecal-oral route of transmission by ingestion of contaminated food and water. Usual symptoms are diarrhoea (watery to severe inflammatory) with fever along with abdominal pain, which subsequently may lead to serious consequences. The main complications include Guillain-Barre Syndrome, irritable bowel syndrome and Reactive Arthritis (Facciola *et al.,* 2017).

India is the second largest producer of fish (Annual Report 2018-19, Department of Animal Husbandry, Dairying and Fisheries, GoI) and Kerala, with over 7 per cent of the water bodies in India, was ranked 10th among the fish producing States in India (2017-18) and the second largest state in marine fish production. Fish and fish products have also been incriminated in foodborne outbreaks during 2010-2017 in the European Union (EFSA, 2019), though rare, with 0.4 per cent of the total attributed source of the outbreaks to be fish, and based on very limited studies. Being a fish-consuming state, the microbiological evaluation of fish is necessary to determine the safety of fish intended for domestic consumption/on sale in retail markets. Poor hygiene during the skinning and gutting of fish or during meal preparation, may aid in the spread of pathogens affecting food safety, giving rise to grave public health concerns. Documented studies on the prevalence of *Campylobacter* spp. in fishes of Kerala are scarce and have not been undertaken in many types of fishes. Prevalence of human campylobacteriosis and it's occurrence in animals and birds has been reported in India and Kerala (Hariharan *et al.,* 1996; Vaishnavi *et al.*, 2015; Pillai *et al.*, 2018; Suseela and Varma, 2018). A global surveillance of campylobacteriosis would help to procure data from different parts of the world, including case notifications, to help in data typing and source attribution of strains from human and other sources, including food and probable contamination sources. Cross contamination from containers and water, which may be frequented by wild birds and animals, and the ubiquitous nature of the organism, make campylobacteriosis a difficult disease to prevent, and epidemiological trend of infection remains high throughout the world. Therefore, the present study was taken up to bring forth the public health significance of Campylobacter in fishes available in the northern district of the southern-most coastal state of Kerala, India, Kozhikode.

Materials and methods

The distribution of *Campylobacter* spp. in marine fishes, crustaceans and molluscs from fish catchment areas in Kozhikode district was studied.

Sampling

A total of 133 marine samples (48 fishes, 35 crustaceans and 50 molluscs) were collected from Puthiyappa fishing harbour along the coastal stretch of Kozhikode district. While *Sardinella gibbosa* (sardine), *Rastrelliger kanagurta* (mackerel), *Stolephorus indicus* (Indian anchovy), *Trichiurus lepturus* (ribbon fish), *Nemipterus japonicus* (pink perch), *Leiognathus equulus* (pony fish), *Pampus argenteus* (pomfret), *Sphyraena*

jello (barracuda), Cynoglossus acaudatus (tonguefish), *Comberomorus guttatus* (king/ seer fish), *Cynoglossus semifasciatus* (soulfish) and assorted small fishes constituted the different varieties of marine fishes, crustaceans comprised of shrimp and crab, and molluscs consisted of squid, clam, mussel, octopus and cuttlefish. Almost all samples were collected directly from fishing boats/fish vendors and from coastal fish markets. Individual samples were collected in individual sample polythene bags, sealed and immediately transported under chilled conditions in thermocol containers with ice packs to the laboratory to be processed within 4 h or limited possible time.

Processing of samples

Samples collected were subjected to isolation and identification of *Campylobacter* spp. (OIE, 2017). Selective enrichment of samples was performed in Blood-Free Campylobacter broth (CCD broth- HiMedia, India) with CCDA selective supplement (FD-135) under microaerophilic conditions at 42°C for 48 h. A 25 gram portion of each sample (comprising of the parts of gills, intestines and flesh) was aseptically transferred to 225 ml of modified CCD broth in a sterile stomacher bag and homogenized using a smasher at high speed for 2 min. This was later transferred to a conical flask to be incubated under microaerophilic conditions at 42°C for 48 h.

On completion of the incubation period, 1.5ml of the broth was transferred into an Eppendorf tube and subjected to DNA isolation by the snap-chill method. The DNA derived from the broth was then screened for *Campylobacter* spp. by multiplex polymerase chain reaction (mPCR). Multiplex-PCR was used to confirm and to identify the isolates to the species level for the two thermophilic species, *Campylobacter jejuni* (*C. jejuni*) and *Campylobacter coli* (*C. coli).*

On completion of 48 h incubation, a loopful of the samples enriched in mCCD broth was selectively plated onto the Blood-Free Campylobacter Selectivity agar (modified Charcoal Cefoperazone Deoxycholate, mCCDA) media supplemented with CAT selective supplement (FD-145), Campylobacter supplement V (FD-067) and Polymyxin B selective supplement (FD-003) as per the procedure described by Chon *et al*., (2012). The plates were then incubated under microaerophilic condition (10 per cent CO_2) at 42°C for 48 h. Colonies on mCCDA plates with characteristic greyish, flat, spreading type, shiny, mucoid and moistened surface having a tendency to spread, with or without metallic sheen and with a morphology suggestive of *Campylobacter* spp. on gram staining were selected (OIE, 2017).

Isolates were subjected to further identification using biochemical tests: oxidase, catalase, H_2S production in triple sugar iron agar, susceptibility to nalidixic acid and cephalotin, hippurate hydrolysis, nitrate reduction and indoxyl acetate hydrolysis. All the positive isolates were subjected to mPCR for the confirmation of *Campylobacter* spp.

Molecular detection

Multiplex PCR was performed to detect the presence of the organism as per the procedure described by Denis *et al.* (1999) with slight modifications. The isolated DNA, both from broth and colonies on mCCDA plates were screened for the presence of *Campylobacter* Spp., targeting genus-specific 16S*r*RNA gene (816 bp). Species level identification was conducted by the detection of *C*. *jejuni* specific *map*A gene (589 bp) and *C*. *coli* specific *ceu*E gene (462 bp). All the *Campylobacter* isolates were also screened for the presence of the conserved virulence gene, *cad*F (416 bp). The PCR amplicons were visualized in 2 per cent agarose gel using SYBR (Synergy Brands Inc.) safe DNA gel stain solution (Invitrogen).

Results and discussion

In the state of Kerala, where fish form a major part of the common man's diet, the microbiological examination of fishes, including assessing the presence of food pathogens is a matter of high priority, especially with recent consumer concerns on food safety. In this context, and with the persistence of this organism in water bodies, along with the stringent growth conditions and cumbersome isolation procedure for this microaerophilic

organism, evaluating the presence of this organism is vital. Fish samples comprising of marine fish, crustaceans and molluscs from the coastal area of Kozhikode were examined for *Campylobacter* spp. The survival of the organism, especially outside the gastrointestinal tract *ie.,* in the environment, is relatively less. Under unfavorable conditions, the organism enters the viable but non-culturable (VBNC) state (Altekruse *et al.*, 1999). Conventional methods in culturing this microaerophilic organism, are difficult and tedious, which necessitated the use of an alternative method, using polymerase chain reaction (Tan *et al.,* 2008). Hence, the enriched broth samples were also subjected to the molecular method of detection using mPCR.

Molecular detection of Campylobacter spp. in enriched broth

Molecular methods of identification for *Campylobacter* spp. provide more dependable identification (Rawat *et al.,* 2018). The organism was detected in 6.3 per cent of 48 fish, 8.6 per cent of 35 crustacean and 28.0 per cent of the 50 mollusc broth enriched samples, by mPCR. *Campylobacter s*pp. could be detected in the various types of samples, as depicted in Table 1 and the molecular detection is represented in Fig. 1.

The isolates from the mCCDA plates after biochemical confirmation were subjected to mPCR for further confirmation. Results revealed that *C. jejuni* isolates were obtained from ribbonfish, pink perch and pony fish among the fishes, and squids among the molluscs and *C. coli* was isolated from squids and mussels among the molluscs. *Campylobacter* species, being one of the leading causes of bacterial foodborne and waterborne infections (Igwaran and Okoh, 2019) in recent years, is an organism to be monitored among the foodborne pathogens, so as to bring to light the prevalence of these pathogens in different regions of the world. The present study revealed that, though *Campylobacter* species was detected in 6.3 per cent of the 48 broth enriched fish samples by mPCR, only 4.1 per cent isolates from the marine fish samples were culturable by the conventional method. Similarly, in the case of crustaceans, of the 8.6 per cent PCR broth enriched positive samples examined, none were positive by the conventional technique, while of the 28.0 per cent broth enriched PCR

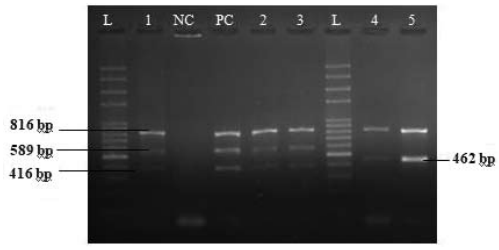

Fig. 1. Multiplex PCR profile of *Campylobacter* spp. using 16S *r*RNA, *map* A, *ceu* E and *cad* F genes

L- Molecular Ladder (100bp), PC- Positive control (*C. Jejuni* NCTC 11168), NC- Negative control, Lane 1-5- Positive samples, L- Molecular ladder (100bp)

Table 1. Distribution of *Campylobacter* spp. identified from mCCD broth by multiplex PCR in various types of fishes, crustaceans and molluscs from Kozhikode district

Campylobacter species	Types of marine fishes	No. of positive marine fish samples	Types of crustaceans	No. of positive marine crustacean samples	Types of molluscs	No. of positive marine mollusc samples
C. jejuni	ribbon fish, pink perch, pony fish	1 1 1	-	0	musssel squid clam	1 4 1
C. coli	-	0	-	0	mussel squid clam cuttlefish	3 3 1 1
Mixed cultures of *C. jejuni* and *C. coli*	-	0	-	0	-	0
Other *Campylobacter* spp.	-	0	-	0	-	0

positive mollusc samples, only 8.0 per cent was culturable. This low occurrence was in accordance with the results of Novotny *et al.* (2004) who also reported a low incidence of *Campylobacter* spp. in fish products (2.3 per cent). **T**he present study was contrary to the findings of many authors, who revealed that the organism was not detectable in marine or freshwater fish samples or products, as reported by Ha and Pham (2006) in Vietnam, Monika *et al.* (2016) in Uttarakhand (India), Ozbey *et al.* (2017) in Turkey and Raeisi *et al.* (2017) in Iran. This may be on account of the methods of detection employed by various researchers, since in most studies the conventional method was used, but this study relied both on the conventional and PCR techniques for the detection of the organism. On the contrary, Tan *et al.* (2008) in Malaysia, reported the average prevalence of *Campylobacter* spp. in retailed sushi (uncooked crab egg and salmon as well as cooked octopus, eel and omelet) as 26.6 per cent.

The organism was absent in marine crustaceans in this study. A higher prevalence of 15 per cent and 5.8 per cent of *C. jejuni* and *C. coli*, respectively, was reported in crabs by Reinhard *et al.* (1996) in Virginia. The prevalence of 28.0 per cent in molluscs reported in the present study was in agreement with the 26.6 per cent prevalence in shellfish reported by Rince *et al.* (2018) in France, whereas this was lower than the 47.9 per cent prevalence in shellfish observed by Gourmelon *et al.* (2015) in France.

Presence of the organism in fishes and molluscs can be attributed to the poor hygienic practices followed during the skinning/ deshelling and gutting of the fishes and shellfishes that were observed and practised by some fish vendors including the repeated use of the same cutting block and repeatedly used water. Differences in campylobacter prevalence in seafoods are most likely associated with the difference in the type of fishes examined and also the differences in the environmental conditions between countries, favouring the survival of the organism, together with differences in the study protocols used, including the media and the incubation conditions and number of samples under study.

Results showed that, of the total fish, crustacean and mollusc samples examined, while 12.03 per cent of broth enriched samples were campylobacter-specific PCR positive, only 4.5 per cent could be successfully recovered on mCCDA plates. This was almost in consonance with the study on fishes in Tamil Nadu (India) where 2 of the 3 Campylobacter specific PCR positive samples were isolated by culture method (Srigowthami, 2013). In the present study, *C. jejuni* was found to be the predominant species in fishes, whereas *C. coli* was predominant in molluscs. Boats and harbour floors could have been likely sources of contamination since some of the catches were stored in baskets/ containers, while some were unloaded onto the floor of the harbour. Since the availability of fish varieties differ from place to place and this study was not focused on any particular type, a more elaborate study with a larger sample size is pertinent in these areas. This was in tune with the observation by Chai *et al.* (2007), who demonstrated that since campylobacter has the tendency to exist in a non-active coccoidal/ VBNC form, it may be impossible to isolate it from PCR positive samples and also as a result of the lack of appropriate methods for campylobacter recovery. Molecular methods are faster compared to conventional methods, considering the difficulty in culturing this fastidious bacterium, with its stringent growth conditions (microaerophilic) and selective media used.

Conclusion

In the food production chain, a number of preventive measures like health monitoring and surveillance, vaccination of birds and food hygiene practices, help to limit the disease transmission and outcomes. This, in turn can check the contamination of water bodies, which are inevitably associated with fish and shellfish production/aquaculture. Measures to improve the hygienic practices in fish harbours include use of treated water, proper cleaning / washing of fish handling areas, routine floor washing practices at intervals during loading and unloading the catch. Campylobacteriosis remains to be a bacterial foodborne disease that poses a challenge to global health in the years to come. Thus, it is a high research priority

to improve the management strategies in fish harbours as well as to prevent the disease.

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Conflicts of interest

The authors declare that they have no competing interests.

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