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## **Detection of *Arcobacter butzleri* and *Arcobacter cryaerophilus* in Clinical Samples of Humans and Foods of Animal Origin by Cultural and Multiplex PCR Based Methods**

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### **ABSTRACT**

In recent years, the frequency of isolation and detection of *Arcobacter* organisms from animals and humans with enteritis and food samples, highlights the importance of arcobacters worldwide as emerging food-borne pathogens. Reports are very scanty regarding prevalence of arcobacters from India. Therefore, the present study aimed to know the prevalence of *Arcobacter* spp. (*Arcobacter butzleri* and *Arcobacter cryaerophilus*) in humans and foods of animal origin by employing cultural and multiplex PCR (mPCR) methods. A total number of 353 samples were collected from human hospitals, retail meat shops and milk suppliers [human stools (102), chicken meat (151), milk (100)] from in and around Bareilly region, Uttar Pradesh, India. By cultural method the overall prevalence rate of *Arcobacter* spp. was found to be 10.20% (36/353) while it was 18.13% (64/353) with mPCR which revealed mPCR to be a more efficient technique in detecting arcobacters. The highest prevalence rate was observed in chicken meat, followed by human stool and milk samples with *A. butzleri* having more prevalence. For simultaneous detection and differentiation of arcobacters at species level the cultural methods possess limitations while mPCR gave rapid and confirmatory detection of *A. butzleri* and *A. cryaerophilus* species. The results of the study add to the epidemiological data available for arcobacters. Extensive epidemiological studies employing the utility of mPCR are suggested for knowing the magnitude of *Arcobacter* infection animals, humans and various food sources in the country. This would help in designing appropriate prevention and control strategies for this important pathogen having public health concerns.

**Key words:** *Arcobacter butzleri*, *Arcobacter cryaerophilus*, prevalence, humans, stool, food, chicken meat, milk, isolation, multiplex PCR

### **INTRODUCTION**

Arcobacters have been implicated as emerging food-borne pathogens worldwide having zoonotic importance and are associated with enteritis and bacteraemia in animals and humans (Hsueh *et al.*, 1997; Engberg *et al.*, 2000; Patyal *et al.*, 2011; Merga *et al.*, 2013). The emerging era of antibiotic resistance and one world one health issues have highlighted the importance of checking important food-borne pathogens (*Campylobacter jejuni*, *Escherichia coli*, *Listeria monocytogenes* and *Arcobacter* spp.) and their related zoonosis; hence proper attention is mandatory for their early diagnosis, adopting appropriate prevention and control strategies so as to safeguard

health of animals and humans (Chakeri *et al.*, 2012; Dhama *et al.*, 2013a, b; Tiwari *et al.*, 2013). The genus *Arcobacter* was introduced in 1991 and belongs to the family Campylobacteraceae (Vandamme and De Ley, 1991; Vandamme *et al.*, 1991). Arcobacters have been reported to be isolated and detected from variety of foods like chicken meat, animal meat (pork, beef) and milk (Ridsdale *et al.*, 1998; Houf *et al.*, 2002a; Kabeya *et al.*, 2004; Amare *et al.*, 2011). *Arcobacter* can grow aerobically and microaerobically and has the ability to grow at 15°C which is the distinctive features that differentiates *Arcobacter* species from *Campylobacter* species (Vandamme *et al.*, 1992; Atabay *et al.*, 2006). In view of cultural difficulty and misidentification, nucleic acid based methods particularly the Polymerase Chain Reaction (PCR) and its versions are growingly being considered highly useful for detection, identification and monitoring of arcobacters in clinical samples of both animals and humans as well as foods of animal origin (Snelling *et al.*, 2006; Patyal *et al.*, 2011; Ferreira *et al.*, 2013). Both genus specific and species specific PCR have been developed for rapid, specific and confirmatory identification of *Arcobacter* spp. In addition to these, mPCR has been found to have wide practical applicability in detection and differentiation of arcobacters at species level (Gonzalez *et al.*, 2000; Houf *et al.*, 2000; Ramees *et al.*, 2014). mPCR enables detection of more than two *Arcobacter* species simultaneously and serves as a useful tool in screening of clinical samples and the food quality monitoring for arcobacters (Vytrasova *et al.*, 2003; Pentimalli *et al.*, 2009; Patyal *et al.*, 2011). Many reports are coming from worldwide countries regarding detection and prevalence of arcobacters, however from India such report are scanty. Therefore, the present study was designed with an aim to know the prevalence of *Arcobacter* spp. (*Arcobacter butzleri* and *Arcobacter cryaerophilus*) in clinical cases of humans and foods of animal origin by utilizing both conventional cultural and molecular tool of multiplex PCR (mPCR).

## MATERIALS AND METHODS

**Sample collection and processing:** A total number of 353 samples were collected from human hospitals (human stools, 102), retail meat shops (chicken meat, 151) and milk suppliers, vendors and Indian Veterinary Research Institute (IVRI) Dairy Farm (milk of cow, 100) from in and around Bareilly region of Uttar Pradesh, India (Table 1).

The human stool samples (102) were collected from diarrhoeal cases of infants less than five years of age in swabs containing Cary-Blair transport (CBT) media without charcoal (HiMedia Pvt. Ltd., Mumbai). The samples were then transported in chilled conditions to the laboratory and kept in refrigerated conditions till processed. For processing, the stool samples were homogenized in phosphate buffer saline (PBS, pH 7.2) (1X) and then 1 mL of the faecal suspension was inoculated in to 9 mL *Arcobacter* enrichment broth in 1:9 ratio. Further incubation was performed for *Arcobacter* for enrichment at 30°C for 48 h under micro-aerophilic (5% O<sub>2</sub>, 10% CO<sub>2</sub> and 85% N<sub>2</sub>) conditions. For processing of food samples, 10 g of chicken meat samples were aseptically minced with scissors and suspended in 90 mL of PBS (pH 7.2). The mixtures were homogenized with stomacher for 1 min at 200 rpm. A 1 mL of the suspension was inoculated into 10 mL of CAT broth and incubated at 30°C under microaerophilic condition for 48 h for enrichment purposes. The 1 mL of milk sample was centrifuged at 12,000 rpm for 15 min and the sediment was used for enrichment as followed for processed stool and meat samples.

**Multiplex PCR detection of *Arcobacter* spp. in enriched samples:** The whole cell DNA was extracted from all the 353 enriched samples (human stools, chicken meat and cow milk) by heat lysis (snap chill) method. Briefly, 1.5 mL of the broth culture was pelleted (8,000 rpm, 5 min) in a microcentrifuge tube and re-suspended in 100 µL of sterile triple distilled water. It was then kept in a boiling water bath for 15 min and immediately transferred onto ice. The bacteria lysate was

centrifuged at 13,000 rpm for 5 min and the supernatant was used as DNA template for mPCR assay. For storage and further use of the bacterial lysate, the supernatant were diluted with Tris Borate (TE) buffer (1:10 v/v) and stored at -20°C.

The optimized protocols of multiplex specific PCR (mPCR) for *Arcobacter* spp. was attempted on the extracted DNAs of all the 353 samples. The detection of *Arcobacter* spp. (*A. butzleri* and *A. cryaerophilus*) was performed using primer sets BUTZ, ARCO, CRY-1 and CRY-2; which were designed from 16S rRNA and 23S rRNA genes by Houf *et al.* (2000) with slight modifications. Briefly, 50 µL reaction mixture was composed of 5 µL of 10×PCR buffer; 2.5 U of *Taq* DNA polymerase; 0.2 mM of each deoxyribo nucleotide triphosphate (dNTPs); 2.5 mM MgCl<sub>2</sub>; 30 pmol of the primers ARCO butZ, CRY-1 and CRY-2; 5 µL heat lyses DNA of the bacteria as template and the final volume was adjusted to 50 µL with nuclease free water (NFW). The mPCR involved an initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation (94°C for 30 sec.), primer annealing (51°C for 30 sec) and extension (72°C for 1.00 min) and final extension at 72°C for 10 min. The mPCR products were subjected to electrophoresis on 1.5% agarose gel and analyzed under UV trans-illuminator (Gel-Doc System-UVP Gel Seq software).

**Cultural isolation and identification of arcobacters:** All the 353 enriched positive samples were filtered separately using 0.45 µm Pore Size Polyethersulfone (PES) syringe filter directly on to *Arcobacter* blood agar plates (7% blood) and incubated under aerobic conditions at 30°C for 48-72 h. Cultural plates showing characteristic translucent to whitish, 2-4 mm, round and convex bacterial colonies were selected as suspected *Arcobacter* spp. colonies and tested for motility using wet mount method and for typical morphology by Gram's staining. Those organisms which gave Gram's negative staining, showed spirally curved rod or short "S" shape morphology with rapid corkscrew-like motility and catalase and oxidase positive reaction (colonies taken from agar plates), were considered as suspected *Arcobacter* species. These were then streaked on *Arcobacter* blood agar plates with selective supplements for further biochemical testing and molecular studies.

**Multiplex PCR detection of *Arcobacter* spp. from cultural colonies:** The genomic DNA was extracted from all the *Arcobacter* positive colonies (n = 36) by the whole cell heat lysis (snap chill) method. Briefly, loopful (3-5 suspected colonies) of 48 h growth culture of the test organism was suspended in 150 µL of NFW in a 0.5 mL centrifuge tube. After mixing properly, the tubes were heated in 100°C water bath for 15 min and immediately placed on ice (-20°C). After 20 min, the bacterial lysate was centrifuged at 13,000 rpm for 5 min and the supernatant was used as DNA template for mPCR assay. The DNA was also extracted from all the *Arcobacter* positive colonies by using Dneasy Blood and Tissue Kit (QIAGEN, USA) as per manufacturers protocol.

## RESULTS

**Multiplex PCR detection of *Arcobacter* spp. in enriched samples:** Out of 353 enriched samples, 64 (18.13%) showed positive results with multiplex PCR and gave an amplification product of 401 bp and 257 bp sizes specific for *Arcobacter butzleri* and *Arcobacter cryaerophilus*, respectively. Within different kind of samples, chicken meat revealed higher positivity of 32.45% (49/151), followed by human stool samples of 11.76% (12/102) and cow milk of 3% (3/100) for presence of arcobacters (Table 1, Fig. 1).

**Cultural isolation and identification of arcobacters:** Cultural isolation of arcobacters showed 36 (10.20%) samples out of a total of 353 to have the presence of Arcobacters. Culturally Arcobacters were identified on basis of specific characteristics viz., translucent to whitish, 2-4 mm,

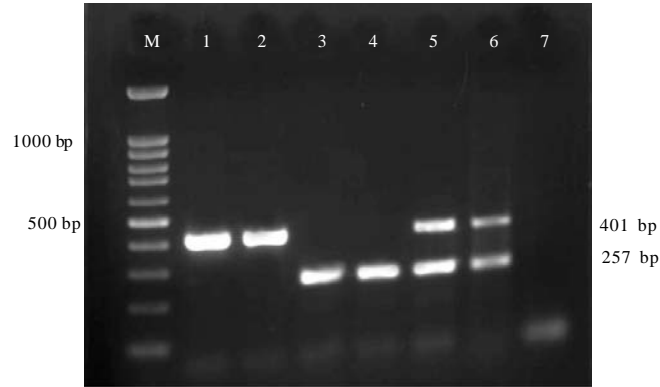


Fig. 1: Multiplex PCR detection of *Arcobacter butzleri* and *Arcobacter cryaerophilus* in chicken meat samples in agarose gel electrophoresis, Lane M: Molecular weight marker, 100 bp, Lane 1, 2: *Arcobacter butzleri* (401 bp), Lane 3, 4: *Arcobacter cryaerophilus* (257 bp), Lane 5, 6: Mixed infection of *Arcobacter butzleri* (401 bp) and *Arcobacter cryaerophilus* (257 bp), Lane 7: Negative control

Table 1: Comparison of conventional cultural and multiplex PCR methods for detection of *Arcobacter* in clinical and food samples

Type of samples	No. of samples	No. of samples detected positive	
		Cultural method	Multiplex PCR method
<b>Clinical</b>			
Human stool	102	02 (1.96%)	12 (11.76%)
<b>Foods</b>			
Chicken meat	151	33 (21.85%)	49 (32.45%)
Raw milk of cow	100	1 (1.00%)	3 (3.00%)
<b>Total</b>	<b>353</b>	<b>36 (10.20%)</b>	<b>64 (18.13%)</b>

round, motile and convex bacterial colonies; tested as Gram's negative, showing spirally curved rod or short "S" shape morphology, rapid corkscrew-like motility, catalase and oxidase positive reactions. Within different kind of samples, chicken meat revealed higher positivity of 21.85% (33/151), followed by human stool samples of 1.96% (02/102) and cow milk of 1.0% (01/100) for presence of arcobacters (Table 1).

**Multiplex PCR detection and differentiation of Arcobacters at species level:** Using the multiplex-PCR assay, two different species of *Arcobacter* genus were detected in 64/353 (18.13%) samples found positive for *Arcobacter* DNA. The comparative detection of *Arcobacter butzleri* and *Arcobacter cryaerophilus* species in broth (before cultural isolation) and colony (after cultural isolation) testing revealed are presented in Table 2. The 64 mPCR positive broth samples showed 33 to be having the presence of *A. butzleri*, 19 with *A. cryaerophilus* and 12 having mixed presence of both the spp. (*A. butzleri* and *A. cryaerophilus*). Out of a total of 36 arcobacters positive colonies, mPCR revealed 19 to be of *A. butzleri*, 11 of *A. cryaerophilus* and 6 having mixed presence of both the spp. (*A. butzleri* and *A. cryaerophilus*). The mixed presence of the two *Arcobacter* spp. was particularly not observed in case of cow milk samples tested. Within different kind of samples, *A. butzleri* species was found to be having more prevalence as compared to *A. cryaerophilus* and with decreasing proportions in chicken meat, followed by human stool samples and cow milk; details of which are presented Table 2.

Table 2: Comparative detection of *Arcobacter* species by multiplex PCR from cultural broth and colonies

Type of samples	Total No. of samples detected positive for <i>Arcobacter</i> spp.		Species wise detection					
			<i>A. butzleri</i>		<i>A. cryaerophilus</i>		<i>A. butzleri</i> and <i>A. cryaerophilus</i>	
	Broth	Colony	Broth	Colony	Broth	Colony	Broth	Colony
<b>Clinical</b>								
Human Stool	12	2	7	1	4	1	1	-
<b>Foods</b>								
Chicken meat	49	33	24	17	14	10	11	6
Raw milk of cow	3	1	2	1	1	-	-	-
Total	64	36	33	19	19	11	12	6

## DISCUSSION

Arcobacters cause enteritis and bacteraemia in animals and humans, are emerging food-borne pathogens and have public health concerns worldwide (Snelling *et al.*, 2006; De Smet *et al.*, 2011; Patyal *et al.*, 2011; Merga *et al.*, 2013). These bacteria have been found to be associated with causing mastitis in cattle, gastric ulcers in swine and reproductive disorders in animal species (Logan *et al.*, 1982; Suarez *et al.*, 1997; On *et al.*, 2002). Arcobacters have been especially detected/recovered more from chicken and chicken products (De Boer *et al.*, 1996; Rivas *et al.*, 2004; Son *et al.*, 2006). Only very scarce reports are available regarding occurrence and prevalence of arcobacters in animals, humans and/or food sources from India (Kownhar *et al.*, 2007; Jiang *et al.*, 2010; Patyal *et al.*, 2011). In view of this context, the present study reports the prevalence of two *Arcobacter* spp. (*A. butzleri* and *A. cryaerophilus*) in humans with diarrhea and foods of animal origin employing conventional cultural as wells as molecular technique of multiplex PCR (mPCR).

In the present study, multiplex PCR screening of the 353 samples including of human stools, chicken meat and cow milk obtained from in and around Bareilly region of Uttar Pradesh, India revealed an overall prevalence rate of 18.13%. The chicken meat showed higher prevalence rate (32.45%) for arcobacters followed by human stools (11.76%) and cow milk (3%). Several species of arcobacters have been isolated, identified and detected from animals (cattle, pig, fish) and humans (particularly faeces/stool samples) and from foods of various origin viz., chicken meat, animal meat (pork, beef), fish and sea foods, milk and others as detected by different isolation methods and molecular techniques (Houf *et al.*, 2002b; Van Driessche *et al.*, 2003; Patyal *et al.*, 2011).

Compared to the present study, earlier studies regarding screening of chicken meat by mPCR reported a low prevalence rate of 24% in Netherlands (De Boer *et al.*, 1996) and 23% in Japan in retail chicken meat (Kabeya *et al.*, 2004). A higher prevalence rate of 73% in chicken has been reported from Australia (Rivas *et al.*, 2004). *Arcobacter* spp have also been reported to be common contaminants of retail raw meats (62% in poultry meat, 35% in pork) in Northern Ireland (Scullion *et al.*, 2006). Even up to 85.7% prevalence of *Arcobacter* spp has been reported in retail chicken meat by Pentimalli *et al.* (2009). In another study, sea food (clams) revealed 100%, chicken (64.3%), pork (53.0%) and mussels (41.1%) prevalence of arcobacters (Collado *et al.*, 2009). In poultry abattoirs, *A. butzleri* has been commonly found and thus poultry carcasses may be contaminated while processing (Houf *et al.*, 2003; Gude *et al.*, 2005; Son *et al.*, 2007). The slaughterhouse environment, including of equipments and water used while processing, might serve as an important source of *Arcobacter* contamination of animal carcasses (Atabay and Corry, 1997; Gude *et al.*, 2005; Van Driessche and Houf, 2007). Recently, PCR screening showed an overall prevalence of *Arcobacter* spp. as 12% in chicken meat and 4.0% in human stools from India (Patyal *et al.*, 2011). More recently, *Arcobacters* have been reported to be present in skin with a

prevalence rate of 22.88% (35/153) (Ramees *et al.*, 2014). Hence, proper biosecurity, sanitary, hygienic, disinfection and biosafety practices need to be followed so as to reduce the chances of food-borne zoonotic infections including of arcobacters.

The samples of cow milk as tested in the present study revealed the least percentage of only 3% (3/100) to be positive for arcobacters by mPCR. In earlier studies, 5.8% prevalence of *Arcobacter* species was reported from cow milk; with *A. butzleri* as the dominant species (60%), followed by *A. cryaerophilus* (40%) from Malaysia (Shah *et al.*, 2012). A 6% positivity of *Arcobacter* has been documented from raw milk sample in Turkey (Ertas *et al.*, 2010).

The human stool samples revealed the presence of arcobacters in 12 out of 102 samples (11.76% prevalence rate) by mPCR; these samples were collected from diarrhoeal cases of infants less than five years. In earlier studies, 1.4% positivity of *Arcobacter* has been documented from human stool samples in Belgium (Houf and Stephan, 2007); 8% to be associated with traveler's diarrhea in persons from Mexico and India (Jiang *et al.*, 2010) and 10% from India recently (Bagalakote *et al.*, 2013).

In the present study, cultural isolation and identification of arcobacters showed 10.20% prevalence rate. A total number of 36 colonies were identified as *Arcobacter* isolates based on the specific characteristics and properties of the bacterium, as has been documented like Gram's negative staining, rapid corkscrew-like motility, spirally curved rods ("S" shape) and biochemical reactions (catalase and oxidase positive) (Snelling *et al.*, 2006; Engberg *et al.*, 2000; Patyal *et al.*, 2011). Highest recovery/isolation rates of 21.85% (33/151) for arcobacters were observed with chicken meat while human stool samples revealed only 1.96% (02/102) and the cow milk the least of 1.0% (01/100). Recently, Patyal *et al.* (2011) reported isolation of 63 *Arcobacter* spp. out of 600 samples (10.50%) inclusive of pig faeces, sea foods, poultry faeces, pork, chicken meat and human stools. On account of a lack of full proof standardized methodology, membrane filtration onto blood agar plate was used in the present study for isolation and recovery of arcobacters from different kind of samples which is considered as reliable isolation method (Gonzalez *et al.*, 2000; Patyal *et al.*, 2011).

The conventional cultural isolation methods require several (5-6) days to obtain confirmatory results and are also laborious and tedious to perform, have limitations and difficulties. Therefore, in the present study, molecular technique of multiplex PCR was used as an important tool for rapid detection, confirmation and characterization of *Arcobacter* spp. from various types of samples. PCR and its various versions (nested PCR, multiplex PCR, real time PCR and others) have high utility for rapid and confirmatory detection and screening of arcobacters in clinical samples and foods of animal origin (Snelling *et al.*, 2006; Patyal *et al.*, 2011; Ferreira *et al.*, 2013). In the present study, mPCR was found to be useful in rapid screening of all the 353 samples for the confirmatory presence of arcobacters. mPCR was found more efficient with over all detection level of 18.13%, highly specific, sensitive and time saving for detection and confirmation of *Arcobacter* spp. as compared to conventional cultural methods which revealed over all detection level only 10.20% and which were also more time consuming and laborious. Many workers have demonstrated and supported the superior efficacy of mPCR in rapidly detecting the *Arcobacter* spp. as compared to conventional cultural and biochemical identification methods needing at least 96 h, whereas PCR requires only few hours (Gonzalez *et al.*, 2000; Vytrasova *et al.*, 2003; Bagalakote *et al.*, 2013).

Apart from screening of the samples in the present study, mPCR also differentiated arcobacters at species level, revealing the presence of two species (*Arcobacter butzleri* and *Arcobacter cryaerophilus*). Multiplex PCR testing revealed specific amplicons of 401 and 257 bp sizes for *A. butzleri* and *A. cryaerophilus*, respectively which is in accordance to (Houf *et al.*, 2000). mPCR analysis revealed that 33 out of 64 positive broth samples were having the presence of *A. butzleri*

while only 19 were *A. cryaerophilus* and 12 were having both the species (*A. butzleri* and *A. cryaerophilus*). mPCR testing of the 36 cultural isolates/colonies revealed *A. butzleri* in 19, *A. cryaerophilus* in 11 mixed presence of both the spp. (*A. butzleri* and *A. cryaerophilus*) in 6 *Arcobacter* isolates. Particularly, the mixed presence of *A. butzleri* and *A. cryaerophilus* was not found in cow milk samples compared to stool and meat samples examined. Overall sample analysis revealed that *A. butzleri* species showed more prevalence when compared to *A. cryaerophilus* with a decreasing trend observed in chicken meat, followed by stool samples of humans and milk of cow. mPCR has been employed by several workers to detect and differentiate more than two *Arcobacter* species simultaneously and is a potent tool for testing clinical samples as well as food samples (food safety monitoring) for arcobacters (Amare *et al.*, 2011). Utilizing mPCR, 03 different *Arcobacter* species have been detected (*A. butzleri*, *A. cryaerophilus* and *A. skirrowii*) from human stool samples and foods of animal origin with mixed infections being more predominant (Patyal *et al.*, 2011).

The rationale for differences in detection and isolation/recovery rates of *Arcobacter* spp. from different animals, humans and various kinds of samples as reported in several studies might be due to various factors. These include multiple factors including of geographic region, seasonal variations, hygienic/sanitary conditions of production and processing of animal meat and its products; variations in sample size, differences in sensitivity and specificity of isolation and detection methods employed (Collins *et al.*, 1996; Gonzalez *et al.*, 2000; Atabay *et al.*, 2003; Patyal *et al.*, 2011). These reasons might have attributed for the differences in prevalence rates observed in the present study as compared to the earlier ones.

## CONCLUSION

The study reports the detection and prevalence of *Arcobacter* spp. (*A. butzleri* and *A. cryaerophilus*) from humans and foods (chicken meat, milk) of animal origin employing traditional cultural isolation/identification and molecular test of multiplex PCR. With mPCR a higher prevalence rate of 18.13% was recorded as compared to 10.20% with cultural methods; the mPCR test results were rapid and less laborious and enabled differentiation of arcobacters at species level. Altogether, these finding supports that mPCR is a highly sensitive, specific and superior test for screening of various kinds of samples for arcobacters. Since reports on arcobacters from India are very few/scanty, therefore the results of the present prevalence study adds to the data available for this important animal pathogen having zoonotic concerns and supports the significance of arcobacters as emerging food-borne pathogens. Further explorative studies are suggested regarding detailed epidemiological surveys in animals and various food sources, experimental pathogenicity and molecular characterization of the Indian isolates of arcobacters, antibiogram patterns and revealing the zoonotic aspects associated.

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