



Study of Prevalence of *Arcobacter butzleri* in Chicken Meat, Mutton, Chevron, Carabeef and Milk using Conventional PCR

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Received: 27 October, 2015

Accepted: 04 January, 2016

ABSTRACT

The reports of prevalence of *Arcobacter butzleri* in mutton, chevon and cara beef are very rare in India. The foods of animal origin included in the present study are mutton, chevon and cara beef in addition to chicken meat and milk. A total of 154 samples viz. 23 for mutton, 20 for chevon and 10 for carabeef were collected from retail meat shops, Bareilly, U.P, India; 70 samples of chicken meat were collected from CARI, IVRI, Bareilly India and 30 milk samples were collected from various milk suppliers, vendors and dairy farms, Bareilly, India. Polymerase chain reaction (PCR) based screening was employed in this study to determine the prevalence of *Arcobacter butzleri* in the collected samples. PCR assays used in the present study were standardized as per the protocol of Houf *et al.* (2000). The optimised PCR assay gave an amplification product of 401 bp size in 18 samples out of 154 and it was specific only for *Arcobacter butzleri* and not for other related bacterial DNA's tested like that of *Arcobacter cryaerophilus*, *Arcobacter skirrow*, *C. jejuni*, *C. coli*, *Salmonella Typhimurium* and *E. coli*. While 12 of chicken meat, 03 mutton, 01 chevon, 01 carabeef, 01 milk samples were positive by PCR giving the respective prevalence of 17.14%, 13.04%, 5%, 10% and 3.22%. The overall prevalence of *Arcobacter butzleri* in the screened samples was found to be 11.69%.

Keywords: *Arcobacter butzleri*, PCR, prevalence, meat, milk

Arcobacter is included in *Epsilonproteobacteria* (rRNA superfamily VI of the *Proteobacteria*) and belongs to the family *Campylobacteriaceae*. The *Campylobacteriaceae* family also comprises of two other genera namely *Campylobacter*, *Sulfospirillum* in addition to *Arcobacter*. At first *Arcobacter* species were isolated from aborted foetuses of bovine and later from swine fetuses (Ellis *et al.* 1977, 1978). The genus *Arcobacter* comprises of fastidious Gram-negative, non-spore forming and spirally curved to 'S'-shaped rods showing a cork-screw or darting type of motility. They were formerly known as 'aerotolerant *Campylobacters*'. *Arcobacter* can grow microaerobically and aerobically and has the ability to grow at 15°C and 30°C, which is a distinctive feature that differentiates *Arcobacter* species from *Campylobacter* species (Vandamme *et al.* 1991 and Lehner *et al.* 2005).

Among the species of genus *Arcobacter*, *A. butzleri*, *A. cryaerophilus*, *A. skirrowii* and *A. cibarius* are considered as emerging food-borne pathogens (Collado *et al.* 2009; Amare *et al.* 2011). There is an increasing trend in number of reports on *Arcobacter* species during the last decade so researchers are now more focussed on *Arcobacter* species considering it as an emerging food-borne entero-pathogen. Out of all species of genus *Arcobacter*, *A. butzleri* is the most commonly associated with human disease, such as enteritis, severe diarrhea, bacteraemia and septicemia (Engberg *et al.* 2000; Lau *et al.* 2002).

Patients with *A. butzleri* infection usually suffer from diarrhoea associated with abdominal pain, with the occurrence of a variety of symptoms including nausea, vomiting and fever (Vandenberg *et al.* 2004). *A. butzleri* is the fourth most common *Campylobacter* like organism

isolated from stool samples of human patients in Belgium and France (Vandenberg *et al.* 2004, Prouzet-Mauleon *et al.* 2006).

Miller *et al.* (2007) recognized that the putative virulence genes in *A. butzleri* are homologous to those described for *Campylobacter* and concluded that *A. butzleri* can be considered as a free-living, water-borne organism that might be rightfully classified as an emerging pathogen.

Arcobacter species are commonly present in foods of animal origin. Recently, Collado *et al.* (2009) reported that the global prevalence of *Arcobacter* species in different food samples was 32%; it was highest in clams (100%) and chicken (64.3%) followed by pork (53.0%), mussels (41.1%), duck meat (40.0%), turkey meat and beef had a recovery rate of 33.3% & 31.3% respectively, and rabbit meat had the lowest rate (0.0%).

Routine detection of *Arcobacter* species is mainly based on isolation on selective media followed by phenotypic identification. Cultural detection of *Arcobacter* species is generally performed by an enrichment step under aerobic conditions at 25°C and takes on average of about 4-5 days until the identification of a suspected *Arcobacter* species colony is completed. However, for the specific isolation of *Arcobacter* species none of the presently available selective supplements allows the growth of all *Arcobacter* spp. and at the same time fully suppresses the accompanying flora present in biological samples (Houf *et al.* 2001). Currently there are no standardized methods for isolation and identification of *Arcobacter* species by conventional techniques (Hamill *et al.* 2008).

Like *Campylobacter*, routine identification of *Arcobacter* species is difficult because these organisms have fastidious growth requirements and are relatively biochemically inert (Kiehlbauch *et al.* 1991). They are morphologically similar to *Campylobacter*. These factors that may contribute to incorrect detection and identification of *Arcobacter* species when relying on agar plating or phenotypic tests. So, conventional culture and phenotypic protocols may provide recovery and differentiation of *Arcobacter* species from related organisms, but these techniques are cumbersome to perform, time-consuming, and highly limited in specificity (Prouzet-Mauleon *et al.* 2006). These drawbacks emphasize the need for rapid, reliable and sensitive techniques for the specific detection of *Arcobacter* species in food products.

In view of culture failure and misidentification, nucleic acid approaches, particularly PCR-based methods, are increasingly being considered for detection, identification, and monitoring of *Arcobacter* species in foods of animal origin, clinical samples of both animals and humans. DNA based assays were established for rapid and specific identification of *Arcobacter* species. Both genus specific and species specific PCR have been developed for rapid detection of *Arcobacter* species.

MATERIALS AND METHODS

A total of 154 samples viz. 23 for mutton, 20 for chevon and 10 for carabeef were collected from retail meat shops, Bareilly, U.P, India; 70 samples of chicken meat were collected from CARI, IVRI, Bareilly India and 30 milk samples were collected from various milk suppliers, vendors and dairy farms, Bareilly and carried to laboratory in Cary-Blair Transport (CBT) media without charcoal (HiMedia Pvt lmt Mumbai). The samples were then transported in chilled conditions to laboratory and kept in refrigerated conditions till processed.

Enrichment of *Arcobacter butzleri* from Foods of Animal Origin

Meat and Milk Samples

10 gm of chicken, meat, chevon, carabeef and samples were aseptically minced with scissors and suspended in 90 ml of PBS. The mixtures were homogenized with stomacher for 1 min at 200 rpm. 1ml of suspension was inoculated into 10 ml of CAT broth and incubated at 30°C under microaerophilic (5% oxygen, 10%CO₂ and 85%N₂) condition for 48 hrs. One ml of milk sample was centrifuged at around 12000 RPM for 15 min and the sediment was used for enrichment in the same way as for meat samples.

DNA extraction by Whole Cell Heat-Lysis method (Snap chill method)

After enrichment, whole cell DNA was extracted from all the samples by heat lysis (snap chill) method and were subjected to species specific PCR for *Arcobacter butzleri*, targeting 16S rRNA gene (Houf *et al.* 2000; Ramees *et al.* 2014) of *Arcobacter butzleri*. About 1.5

Table 1: List of Primers used in the PCR assays

Primers	Genes	Position	Nucleotide sequence (5'-3')	References
BUTZ	16S rRNA	959-983	CCT GGA CTT GAC ATA GTA AGA ATGA	Houf <i>et al.</i> 2000
ARCO	16S rRNA	1357-1338	CGT ATT CAC CGT AGC ATA GC	Houf <i>et al.</i> 2000

ml of the broth culture was pelleted (8000 rpm, 5 min) 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 PCR assay. Purity and concentration of DNA were detected by UV Spectroscopy after agarose gel electrophoresis and using spectrophotometer by reading the optical density (OD) at 260 nm and 280 nm. The concentrations of the DNA were calculated using the fact that one absorbance unit equates to 50 µg DNA/cm³. Concentration of DNA sample (µg cm⁻³) = 50 × A₂₆₀ which yield 5 to 20 µg DNA / ml. And a ratio of absorbance at 260 nm to that at 280 nm were approximately 1.8 which indicates the extracted DNA is free from protein contamination.

Optimization of conventional PCR for *Arcobacter butzleri*

PCR assays used in the present study were standardized following the original author's protocols (Houf *et al.* 2000) with slight modification and by optimizing the concentrations of the components of the reaction mixture in the PCR assay and by varying the annealing temperature and cycling conditions for individual target gene using the Corbette® thermocycler. The published primers are listed in Table 1.

The 50 µl reaction mixture was composed of 5 µl of 10x PCR buffer; 2.5 U of *Taq* DNA polymerase; 0.2 mM of each deoxyribo nucleotide triphosphate; 2.5 mM MgCl₂; 30 pmol of the primers ARCO and BUTZ; 5 µl heat lyses DNA of the bacteria as template and the final volume was adjusted to 50 µl with Nuclease free water.

The PCR 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 chain extension (72°C for 1 min) and final extension at 72°C for 10 min. The DNA extracted from all the samples by snap

chill method was subjected to optimized conventional PCR. The PCR product was subjected to electrophoresis on 1.5% agarose gel and analyzed by using UV transilluminator (Gel-Doc system-UVP Gel Seq Software).

Agarose Gel Electrophoresis

Agarose gel (1.5%) was prepared by boiling agarose in an appropriate volume of 1x TAE buffer. After cooling for about 5 min, ethidium bromide was added to the agarose solution to a final concentration of 0.5µg/ml. The comb was fitted into the slots on the tray. Then molten agarose was poured into the tray. The tray was kept undisturbed till the gel solidified. The tray containing the gel was then placed in a submarine horizontal electrophoresis unit filled with 1x TAE buffer up to a level of 1 mm above the gel surface and the comb was then taken out carefully.

Table 2: Prevalence of *Arcobacter butzleri* by using PCR assay

Type of Animal Food Samples	No. of Samples	No. of samples detected positive	Prevalence
Chicken meat	70	12	17.14%
Milk	31	01	3.22%
Mutton	23	03	13.04%
Chevon	20	01	5%
Cara beef	10	01	10%
Total	154	18	11.69%

About 10 µl of each PCR product was mixed with 2 µl of bromophenol blue loading dye and loaded into each well. Electrophoresis was performed at 5 V/cm and the mobility was monitored by the migration of the dye. After sufficient migration, the gel was observed under UV transilluminator to visualize the bands. The PCR product size was determined by comparing with a standard molecular weight marker (DNA ladder) and was photographed by the gel documentation system.

Analytical Specificity of the PCR assay

Specificity of optimized PCR assay and primers were checked with other bacterial cultures (*Arcobacter cryaerophilus*, *Arcobacter skirrowii*, *Campylobacter coli*, *Campylobacter jejuni*, *Salmonella Typhimurium* and *E. coli*) by preparing templates using whole cell heat lysis method and subjecting to PCR assay together with the known positive and negative controls. PCR-products were electrophorised on agarose gel, photographed and analyzed under UV transilluminator (Gel-doc system).

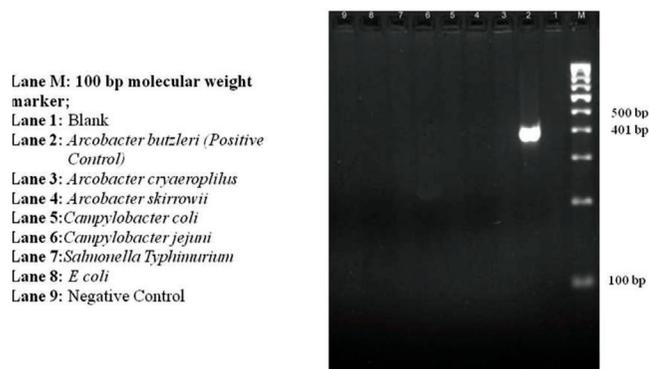


Fig. 1: Gel electrophoresis of analytical specificity of conventional PCR

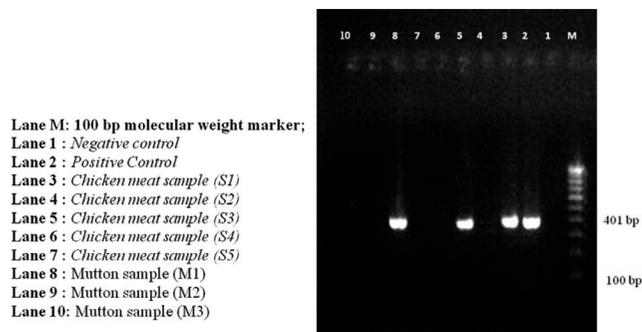


Fig. 2: Gel electrophoresis of a batch of samples

RESULTS

The optimised PCR assay gave an amplified product of 401 bp size only with the DNA extracted by snap chill method from the standard reference culture of *Arcobacter butzleri* (positive control). Thus, PCR was specific only for *Arcobacter butzleri* and not for other related bacterial DNAs tested like that of *Arcobacter cryaerophilus*, *Arcobacter skirrowii*, *C. jejuni*, *C. coli*, *Salmonella Typhimurium* and *E. coli* (Fig. 1). The purity and concentration of extracted

DNA was confirmed by UV Spectroscopy as mentioned in the material and methods

A total of 154 samples were processed, out of which 18 showed positive results with conventional PCR and gave an amplification product of 401 bp size which is specific only for *Arcobacter butzleri*. While 12 of chicken meat, 03 mutton, 01 chevon, 01 carabeef, 01 milk samples were positive by PCR giving the respective prevalence of 17.14%, 13.04%, 5%, 10% and 3.22%. The overall prevalence of *Arcobacter butzleri* in the screened samples was found to be 11.69%. (Table 2). The gel electrophoresis of a batch of 8 samples (5 chicken meat and 3 mutton) is shown in figure 2. In this batch of samples 2 chicken meat samples (S1 and S3) and 1 mutton sample (M1) are positive for *Arcobacter butzleri*.

DISCUSSION

Food-borne zoonotic pathogens are of great significance as far as health and protection of consumer is concerned. In recent years *Arcobacters* are considered as potential emerging food and water-borne pathogens. They are increasingly being isolated from a wide range of food products all over the world (Vandenberg *et al.* 2004; Prouzet-Mauleon *et al.* 2006). There are increasing evidences that *Arcobacter* is an emerging human pathogen (Ho *et al.* 2006, Snelling *et al.* 2006). However, the incidence of *Arcobacter* species is considered to be underestimated mainly due to limitations in current detection and identification methods (Vandenberg *et al.* 2004). The recent past has seen many studies from different countries on the isolation and identification of *Arcobacter* spp. with variable prevalence rates reported from animals (cattle, pigs, chickens, sheep, dogs), from meat (poultry, pork, lamb, beef, sea foods) and from cases of human enteritis and bacteraemia (Bagalakote *et al.* 2013; Ramees *et al.* 2014). The presence of *Arcobacter* spp. in healthy animals indicates the importance of foods of animal origin as potential sources of *Arcobacter* infection in humans. In human beings, the most probable sources of infection are consumption of raw meat, cross-contamination of undercooked meat products (Lehner *et al.* 2005).

The conventional cultural isolation methods require several (5-6) days to obtain confirmatory results. Therefore, in the present study, molecular technique like Polymerase chain reaction (PCR) has been used as an important tool

for rapid detection of *Arcobacter butzleri* from various samples. In the present study, 154 samples were screened using conventional PCR which was optimized using the protocol of Houf *et al.* (2000). 12 samples of chicken meat, 3 samples of mutton, 1 sample each of chevon, cara beef and milk were found positive for *Arcobacter butzleri*. The prevalence of *Arcobacter butzleri* in different sources of samples (chicken meat, mutton, chevon, cara beef and milk) was obtained as 17.14%, 13.04%, 5%, 10% and 3.22 % respectively. The overall prevalence of *Arcobacter butzleri* as obtained by using conventional PCR in all the samples was found to be 11.69 %. From this study it was concluded that the *Arcobacter butzleri* which is a potent emerging zoonotic pathogen was present in foods of animal origin (chicken, mutton, chevon, cara beef and milk which may play role in the contamination of the environment and human food chain. High prevalence rate of *Arcobacter butzleri* was found in chicken meat and mutton samples and this could be of significant human health risk, as these form the bulk of non-vegetarian diet. The results of present study revealed that *Arcobacter butzleri* deserves more attention as a food-borne pathogen like *Campylobacter* spp. To implement effective preventive measures against emerging pathogens like *Arcobacter butzleri* equivocal diagnostic methods and concrete epidemiological data are essential. Molecular techniques like PCR assay used in this study have distinct advantage over conventional cultural methods for detection and confirmation of *Arcobacter* spp. in different type of samples. The standardized PCR assay offers the advantages of specificity, sensitivity, rapidity and the capacity to detect small amounts of target nucleic acid in a given sample.

ACKNOWLEDGEMENTS

Authors are thankful to ICAR and Director I.V.R.I for providing technical and financial assistance for this study.

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