PHYLOGENETIC ANALYSIS OF *Arcobacter butzleri* AND *Arcobacter skirrowii* ISOLATES AND THEIR DETECTION FROM CONTAMINATED VEGETABLES BY MULTIPLEX PCR

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ABSTRACT

*Arcobacter* is an identified emerging food-borne bacterial pathogen. In the current study, 204 different vegetable samples were collected from retail shops or local vendors from northern part (Uttar Pradesh) of India and screened for *Arcobacter* spp. The samples were enriched in *Arcobacter* enrichment broth followed by multiplex PCR based detection of the *Arcobacter* species. *Arcobacter butzleri* and *Arcobacter skirrowii* were detected in 13.73% (28/204); 23 were positive for *A. butzleri*, while 5 showed mixed contamination of *A. butzleri* and *A. skirrowii*. The specific PCR amplicons from positive samples were purified and sequenced for further analysis. Sequence analysis of *Arcobacter* spp. showed a significant genetic similarity irrespective of country and source of origin. *A. skirrowii* isolation is reported for the first time from a vegetable source. The higher incidences of enteric infections in human in resource poor settings, particularly developing countries, could be due to high frequency of *Arcobacter* contaminations in vegetables. Further epidemiological studies are warranted to probe the role of vegetable contamination in transmission of this important pathogen of global public health concern.

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1 Introduction

The raw fruits and vegetables have been known to serve as vehicles of various food-borne infections throughout the world and are more frequently noted in developing countries (Greene et al., 2008; Berger et al., 2010; Ferreira et al., 2015). Poor quality water irrigation system is one of the major factors behind contamination of vegetables at the pre-harvest step with food-borne pathogens. A number of outbreaks of human gastroenteritis have been reported by the intake of contaminated fresh vegetables and fruits (Gonzalez & Ferrus, 2011; Lee & Choi, 2013; Ferreira et al., 2015). Enterohemorrhagic Escherichia coli, Salmonella, Shigella flexneri, Bacillus cereus, Aeromonas hydrophila and Aeromonas sobria have been isolated from various vegetable sources (Beuchet, 1996; Franz & van Bruggen, 2008; Park et al., 2012). In one of our previous study, we isolated Campylobacter jejuni from fresh vegetables collected from local market in Bareilly, Uttar Pradesh state of India (Kumar et al., 2001).

Arcobacter is a Gram-negative organism, categorized under the Campylobacteraceae family. Arcobacters have been detected from animal-origin foods such as chicken meat, pork, sea foods, beef and milk (Rivas et al., 2004; Patyal et al., 2011; Dhama et al., 2013; Ramees et al., 2014a; Ramees et al., 2014b). For specific detection of C. jejuni and A. butzleri in food products, multiplex PCR assay (mPCR) was suggested by Winters & Slavik (2000). Using a variable 16S rRNA and 23S rRNA region, Houf et al. (2000) established a species-specific multiplex PCR assay for the concurrent detection and identification of three important Arcobacter spp. (A. butzleri, A. skirrowii and A. cryaerophilus). Gonzalez et al. (2000) developed a newer method of PCR-culture technique for the quick detection of Arcobacter spp. in chicken meat after a short selective enrichment of samples. Gonzalez et al. (2010) developed a real-time PCR for the detection of Arcobacter spp. in fresh lettuce samples, where A. butzleri being the only species detected by mPCR. Lately, the presences of Arcobacter strains have been shown in a carrot-processing plant (Hausdorf et al., 2011) from lettuce (González & Ferrús, 2011). A. butzleri was isolated from water body in Germany, a drinking water pool (Jacob et al., 1998) and in the USA from well-water (Rice et al., 1999), signifying that A. butzleri can survive in water sources and may even spread through drinking water (Ramees et al., 2017).

Numerous methods are in use for the differentiation of Arcobacter strains including RAPD-PCR, ERIC-PCR, AFLP and PFGE (Hume et al., 2001; Houf et al., 2002; On et al., 2003). Among available detection methods, ERIC-PCR and RAPD-PCR are the more commonly used techniques employed on A. butzleri, A. cryaerophilus, and A. skirrowii isolates for satisfactory typing. Various other nucleic acid-based methods have been used for detection, identification and monitoring of Arcobacters in food including PCR-plus-RFLP (Neubauer et al., 2003), PCR-plus-

Table 1 Detection of Arcobacter species by multiplex PCR from vegetable sources

| SL No. | Type of Samples | No. of Samples | Samples detected | Positive for Arcobacter spp. | A. butzleri | A. skirrowii | Total | Percentage |
|--------|-----------------|----------------|-----------------|-----------------------------|-------------|-------------|-------|------------|
| 1      | Carrot          | 48             | 11              | A. butzleri                 | 3           | 14          | 29.17 |            |
| 2      | Beet root       | 46             | 8               | 2                            | 10          |             | 21.74 |            |
| 3      | Cabbage         | 38             | 4               | 0                            | 4           |             | 10.53 |            |
| 4      | Tomato          | 29             | 0               | 0                            | 0           |             | 0     |            |
| 5      | Coriander       | 21             | 0               | 0                            | 0           |             | 0     |            |
| 6      | Cucumber        | 22             | 0               | 0                            | 0           |             | 0     |            |
| Total  |                 | 204            | 23              | 5                            | 28          |             | 13.73 |            |
showing characteristic morphology of *Arcobacter* were confirmed by biochemical testing and multiplex PCR.

### 2.2 Multiplex PCR for detection of Arcobacter spp.

The optimized multiplex PCR (mPCR) assay was used to screen all the 204 vegetable samples and simultaneous detection of *A. butzleri*, *A. cryaerophilus*, and *A. skirrowii*. The published primer pairs (BUTZ, ARCO, SKIR, CRY-1, and CRY-2) from 16S rRNA and 23S rRNA genes were used in the study (Houf et al., 2000; Patyal et al., 2011). For the positive DNA control, *A. butzleri* (LMG 10828′) was used. The PCR reaction was carried out in 25 µl reaction buffer taking 2.5 µl of 10X PCR buffer; 1 IU of Taq DNA polymerase; 0.2 mM of each deoxyribonucleotide triphosphate (dNTPs), 2.5 mM MgCl₂, 15 pmol of the primers ARCO, BUTZ, CRY-1, CRY-2 and 7.5 pmol of primer SKIR; 2 µl of template DNA and to make the final volume nuclease free water was added. The multiplex PCR cycles involved the single first step of initial denaturation at 94°C for 5 min, subsequently 30 thermal cycles of denaturation (94°C for 30 sec.), primer annealing (51°C for 30 sec) and chain extension (72°C for 1.00 min), and single step of final stage extension at 72°C for 10 min. On completion of PCR cycles, amplification was observed by running the PCR products on 1.5% agarose gel and visualizing using UV trans-illuminator (Gel-Doc system).

### 2.3 Sequencing and phylogenetic analysis

The extraction of DNA from the positive sample clones was carried out using DNeasy Blood & Tissue Kit (QIAGEN). The samples were confirmed by mPCR and the specific PCR amplicons were subjected for purification using QIAquick Gel Extraction Kit (QIAGEN). The purified PCR products were sequenced (Eurofins, Bangalore, India) by Sanger sequencing method. Sequences were edited through MegAlign and EditSeq programme from DNA star and were submitted to GenBank database. A data set of partial 16S rRNA gene sequences of different isolates of *A. butzleri* (Table 2) and *A. skirrowii* (Table 3) were retrieved from GenBank (http://www.ncbi.nlm.nih.gov/GenBank/) for phylogenetic reconstruction. Phylogenetic tree for the 16S rRNA gene sequences were constructed in MEGA6 software (Tamura et al., 2013) using the maximum likelihood method model and the evolutionary distances were computed by Kimura 2-parameter with gamma distribution for *A. butzleri* sequences. For the phylogenetic relationship study of *A. skirrowii*, maximum likelihood tree was derived using Jukes-Cantor algorithm (Jukes & Cantor, 1969). The phylogenetic distances between different sequences were calculated by using MegAlign programme.

### 3 Results

#### 3.1 Arcobacter detection

Out of the 204 fresh vegetable samples, 28 samples (13.73%) were found positive for *Arcobacter* species by mPCR with expected size amplification of 401 bp and 641 bp products, specific for *Arcobacter butzleri* and *Arcobacter skirrowii*, Table 2

| SI no | NCBI       | Strain       | Source     | Country     |
|-------|------------|--------------|------------|-------------|
| 1.    | KT188763.1 | VPH/V3/IVRI/2014 | Vegetable  | India (Present study) |
| 2.    | KT188764   | VPH/V10/IVRI/2014 | Vegetable  | India (Present study) |
| 3.    | KT188765   | VPH/V36/IVRI/2014 | Vegetable  | India (Present study) |
| 4.    | KT188766   | VPH/V92/IVRI/2014 | Vegetable  | India (Present study) |
| 5.    | KT188767   | VPH/V124/IVRI/2014 | Vegetable  | India(Present study) |
| 6.    | KT188768   | VPH/143/IVRI/2014 | Vegetable  | India (Present study) |
| 7.    | KJ851584   | VPH/PS35/IVRI/2012 | Poultry skin  | Bareilly (India) |
| 8.    | KC520497   | VPH/CM79/IVRI/2012 | Chicken meat | Bareilly (India) |
| 9.    | KJ364500.1 | MC1B6 | Raw milk | Gujarat (India) |
| 10.   | KT379981   | MAT6 | Fish | Mumbai (India) |
| 11.   | KP690259.1 | ANDL04 | Water | Odisha (India) |
| 12.   | JQ743025   | Arco-ka | Reptile | China |
| 13.   | KC551771   | AF1429 | Female pig | Canada |
| 14.   | LC094565   | GENT4 | River water | Japan |
| 15.   | HQ454094   | 78e | Tooth plaque of canine | The United States |
Phylogenetic analysis of *Arcobacter butzleri* and *Arcobacter skirrowii* isolates by multiplex PCR

respectively (Figure 1). Out of 28 positive samples, 23 samples were positive for *A. butzleri*, 5 showed mixed infections of *A. butzleri* and *A. skirrowii*. Of the note all the samples were found negative for *A. cryaerophilus* (Table 1). Out of 48 fresh carrot samples, 14 were positive for *Arcobacter* spp., of which 8 were positive for *A. butzleri* and three revealed mixed infections of *A. butzleri* and *A. skirrowii* with prevalence rate of 29.17%. Out of 46 fresh beet root samples, 10 (21.74%) were detected positive for *Arcobacter* spp., of which 8 were positive for *A. butzleri* and 2 showed mixed infection of the two *Arcobacter* spp. Out of 38 fresh cabbage samples, 4 (10.53%) samples were positive for *A. butzleri*. The samples from tomato (29), coriander (21) and cucumber (22) were found negative for *Arcobacter* spp. tested (Table 1). Cultural isolation of samples showed 6 *Arcobacter* positive colonies from carrot, which were further confirmed by both the biochemical tests (indoxyl acetate hydrolysis test and nitrate reduction test) and mPCR assay, 3 samples were positive for *A. butzleri* and 3 for *A. skirrowii*.

### 3.2 Phylogenetic analysis

The phyloanalysis using the maximum likelihood method for *A. butzleri* sequences showed that all the six *A. butzleri* isolates of the current study were clustering together with other Indian isolates forming a distinct clade (Figure 2). However, the low bootstrap values indicate that the Indian isolates are not diverse from the isolates of other parts of the world. Indian isolates were found more phylogenetically related with Chinese sequence from reptiles (JQ743025 strain Arco-ka). Also, no significant diversity between *A. butzleri* isolates of vegetable, environmental or animal origin was observed. Similarly, the maximum likely hood tree obtained using the Jukes-Cantor method for *A. skirrowii* sequences did not reveal any significant diversity according to the geographical location or the source of isolation (Figure 3). Analysis of phylogenetic distance by MegAlign programme showed 94.9 to 100% identity between *A. butzleri* isolates and 99.2 - 100% between *A. skirrowii* isolates.

| SI no | NCBI Accession No | Strain          | Source     | Country           |
|-------|-------------------|-----------------|------------|-------------------|
| 1     | KT188769          | VPH/V8/IVRI/2014| Vegetable  | India (Present study) |
| 2     | KT188770          | VPH/V62/IVRI/2014| Vegetable  | India (Present study) |
| 3     | KT188771          | VPH/V129/2014   | Vegetable  | India (Present study) |
| 4     | KC520496          | VPH/CM1/IVRI/2012| Chicken meat | Bareilly (India) |
| 5     | KF990326          | ASKBB/3         | Buffalo meat | Gujarat (India) |
| 6     | GU300769          | Houf 989       | Cow faeces | Spain |
| 7     | DQ464344          | 449/80          | Human stool | France |

Figure 1 Multiplex PCR detection of *Arcobacter butzleri* and *Arcobacter skirrowii* from vegetable samples in agarose gel electrophoresis
Lane M: Molecular weight marker, 100 bp plus
Lane 1, 2: *Arcobacter butzleri* (401 bp)
Lane 3: Mixed infection of *Arcobacter butzleri* (401 bp) and *Arcobacter skirrowii* (641 bp)
Lane 4: Negative control

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Discussion

The timely identification of the main source and cause of a disease outbreak helps in adopting measures to reduce the disease associated hazards in the future. Numerous disease incidences and outbreaks are reported in humans due to the ingestion of uncooked vegetables and fruits and their number has amplified in the recent years (Buck et al., 2003; Rahal et al., 2014). Major source of pre-harvest contamination of vegetables is irrigation water and post-harvest sources of bacterial contamination include human handling, harvesting equipment, transport containers, wild and domestic animals and transport vehicles (Burnett & Beuchat, 2001).

Arcobacter is an important food-borne pathogen causing numerous outbreaks worldwide and cause diarrhoeal symptoms in humans and animals (Ramees et al., 2017). Arcobacters isolation has been successfully achieved from different food sources including meat (chicken meat, pork, beef, chevon, mutton and shellfish), meat products, water, milk, milk products, and vegetables (Winters & Slavik, 2000; Rivas et al., 2004; Kabeya et al., 2004; Morita et al., 2004; Patyal et al., 2011; Ramees et al., 2014c; Ramees et al., 2014d). Hitherto reports confirm that meat serves as an important cause of human infections (Houf et al., 2003; Collado & Figueras, 2011; Ramees et al., 2017).

In the present study, a prevalence rate of 13.73% for Arcobacters from raw vegetables was noted, which could be a potential source of infection from vegetables to humans. A. butzleri was the predominant species in vegetable samples followed by A. skirrowii. Reports regarding detection of Arcobacter spp. from vegetable sources are there from worldwide, and the present report establishes the finding of detecting Arcobacters from vegetable sources from India. Furthermore, we could detect A. skirrowii from a vegetable source, which has not been shown to be found in vegetable sources. Arcobacter spp. have been detected from vegetables (broccoli, carrot, celery, cauliflower, cantaloupe, lettuce, mushrooms, and tomato) and fruits (apple, grapes, kiwi,
pine apple, strawberries, and watermelon) (Winters & Slavik, 2000). *Arcobacter* spp. was identified in 20% of the samples from fresh lettuces by real-time PCR where *A. butzleri* predominated (Gonzalez & Ferrus, 2011). Similarly, *Campylobacter* has been isolated from India in vegetables where 56 samples of different vegetables were analysed and 2 samples (spinsinach and 1 fenugreek) showed the presence of *Campylobacter jejuni* (Kumar et al., 2001). Cultural isolation of *Arcobacter* positive colonies and biochemical testing results also confirmed the presence of Arcobacters in vegetable samples.

All the six *A. butzleri* isolates in the current study were phylogenetically related to other Indian isolates irrespective of the source of isolation and revealed a common phylogenetic origin. The 16S rRNA gene sequences are conservative in *Arcobacter* spp without major mutation. Nayak et al. (2014) studied 16S rRNA gene sequence analysis of *A. butzleri* and reported a high similarity between isolates with minimum divergence. Indian *A. butzleri* isolates were phylogenetically related with Chinese isolates (of non-food origin) indicating a geographical relationship between the isolates from neighbouring countries without any significance with reference to source. *A. skirrowii* sequences did not reveal any geographical diversity and were not grouped in a different clade. The sequence analysis of 16S rRNA from a wide number of undefined bacterial isolates from environment or clinical cases showed a significant genetic similarity between *A. skirrowii* (Drancourt et al., 2000). The *Arcobacter* species are genetically similar irrespective of source and countries of origin. These findings show that Arcobacters are stable organism without significant genetic modification/mutation. There are limited studies on epidemiological aspect of *Arcobacter* spp., which calls for extensive epidemiological and explorative research to know the real magnitude and role of vegetables in acting as potential source of Arcobacters and spreading the infection/disease to humans and their companion animals.

**Conclusion**

The study confirms that a high frequency of contamination of vegetable sources with *Arcobacter* species. Arcobacters contamination was detected in carrot, beet root and cabbage that are the vegetables usually consumed fresh in salads. Hence, there may be a chance of *Arcobacter* infection to humans from vegetable sources consumed without proper cooking and getting infection from eating of raw vegetables. This is the first report regarding the detection of Arcobacters from vegetable sources from India, and detection of *A. skirrowii* also from a vegetable source. The sequence analysis showed a significant genetic similarity among isolates from India and abroad. To decipher more knowledge on the magnitude of prevalence of Arcobacters in vegetable sources, their role as source of infection to human population, and to design suitable preventive measures to counter this pathogen of public health importance, additional intensive epidemiological studies are warranted in near future, particularly in resource poor countries.

**Conflict of interest**

There is no conflict of interest among all or any of the authors and also with the funding agencies.

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