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

Ramees TP[1*], Rathore RS[1], Kumar A[2], Arun TR[3], Ravi Kumar GVPPS[4], Karthik K[3], Malik YS[5], Dhama K[6], Singh RK[7]

[1]Division of Veterinary Public Health, ICAR-Indian Veterinary Research Institute, Izatnagar, Bareilly (U.P.)
[2]Indian Council of Agricultural Research Krishi Bhavan, New Delhi-110001
[3]Division of Bacteriology & Mycology, ICAR-Indian Veterinary Research Institute, Izatnagar, Bareilly (U.P.)
[4]Animal Biotechnology Division, ICAR-Indian Veterinary Research Institute, Izatnagar, Bareilly (U.P.)
[5]Division of Biological Standardization, ICAR-Indian Veterinary Research Institute, Izatnagar, Bareilly (U.P.)
[6]Division of Pathology, ICAR-Indian Veterinary Research Institute, Izatnagar, Bareilly (U.P.) - 243122, India
[7]ICAR-Indian Veterinary Research Institute, Izatnagar, Bareilly (U.P.) - 243122, India

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.

* Corresponding author

E-mail: rameesvet@gmail.com (Dr. Ramees T P)

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 forsatisfactory 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-

RAPD (Atabay et al., 2002; Houf et al., 2002), and PCR-plus DNA sequencing (Karenlampi et al., 2004). The *16S rRNA* sequencing is more accurate which also indicates similarity of *Arcobacter* and the Epsilon proteobacteria genus (Gilbert et al., 2014).

It is assumed that *Arcobacter* contamination could be very common in fresh vegetables, as seen in meat samples. Perusal of literature reveals availability of limited knowledge on the detection of *Arcobacter* from vegetable sources and molecular characterization of the isolates. Hence, we investigated the *Arcobacter* contamination in fresh vegetable sources collected from retail shops in northern part of India. Subsequently these isolates were subjected to sequence analysis.

2 Materials and Methods

2.1 *Collection and processing of samples*

Total 204 fresh vegetable samples were collected from different retail shops of the Bareilly region, Uttar Pradesh, India, comprising of carrot (48), beet root (46), cabbage (38), tomato (29), coriander (21) and cucumber (22) (Table 1). From all the vegetables, 2 g of the samples or surface scrapings were put in 10 ml phosphate buffered saline (PBS, pH 7.2). One ml of these samples were inoculated in to 9 ml *Arcobacter* enrichment broth with 7% sheep blood and CAT (Cefoperazone, Amphotericin B, Teicoplanin) selective supplement and further these were incubated at 30° C for 48 hrs micro-aerobically. An amount of 2 ml of each enriched sample was centrifuged at 10,000 rpm for 15 min for pelleting and genomic DNA was extracted using CTAB method (Wilson, 1987). For the isolation purpose, all the 204 enriched samples were individually filtered through 0.45 μm pore size syringe filter on to the *Arcobacter* blood agar plates containing 7% blood and kept at 30°C for 48-72 hrs under micro-aerobic environment (Ramees et al., 2014b). Cultural plates

Table 1 Detection of *Arcobacter* species by multiplex PCR from vegetable samples

| Sl No. | Type of Samples | No. of samples | Samples detected Positive for *Arcobacter* spp. | | | |
			A. butzleri	*A. butzleri* And *A. skirrowii*	Total	Percentage
1	Carrot	48	11	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[T]) 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 MgCl$_2$, 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 *Arcobacter butzleri* strains used in phylogenetic analysis

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.	KF690259.1	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

Table 3 *Arcobacter skirrowii* strains used in phylogenetic analysis

SI no	NCBI	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

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. and 11 of which 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.
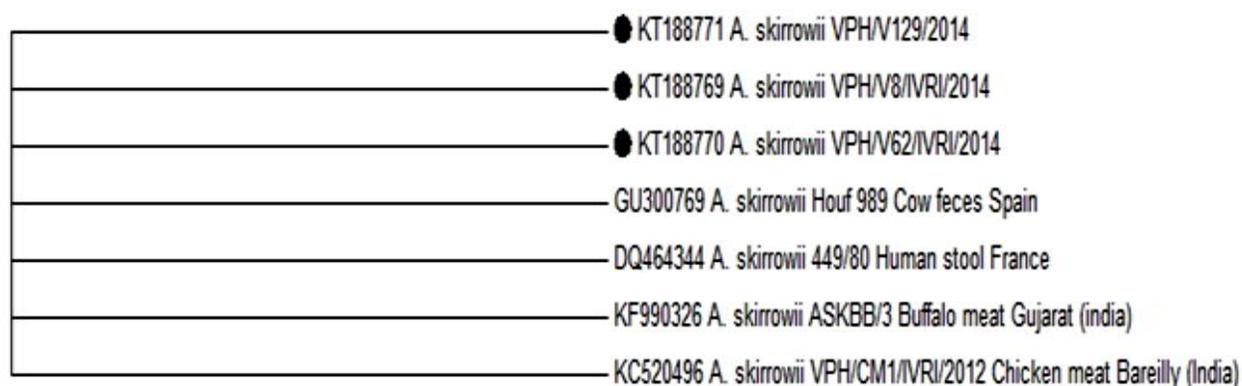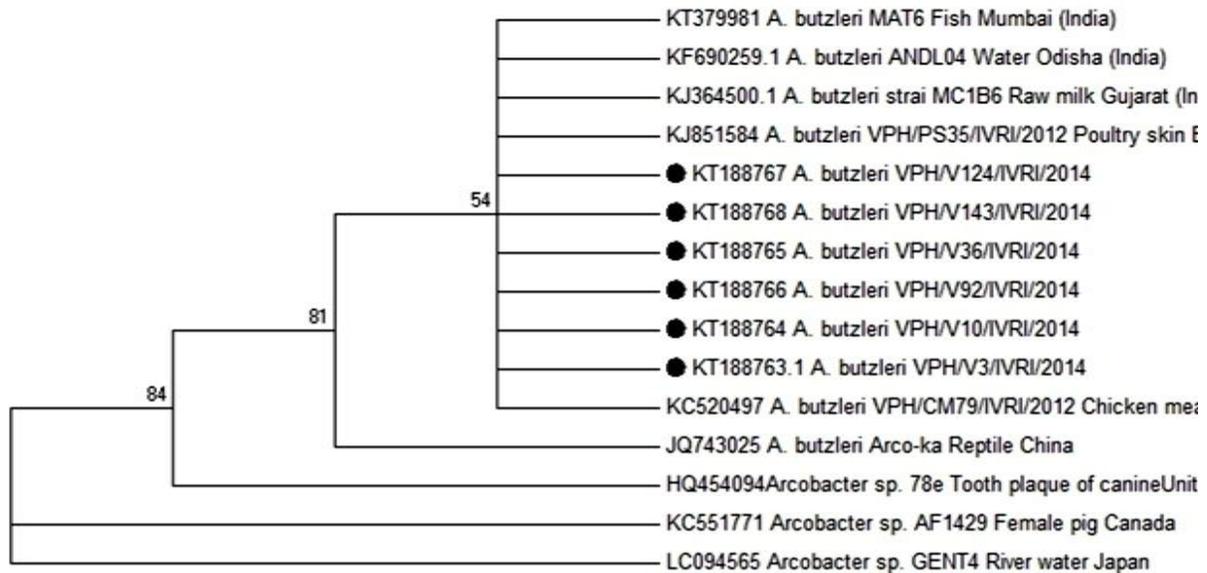

Figure 1 Multiplex PCR detection of *Arcobacter butzleri* and *Arcobacter skirrowii* from vegetable samples in agarose gel electrophoresis
Lane M: Molecular weight marker, 100 bp plus
Lane 1, 2: *Arcobacter butzleri* (401 bp)
Lane 3: Mixed infection of *Arcobacter butzleri* (401 bp) and *Arcobacter skirrowii*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

Figure 2 Phylogenetic tree of *Arcobacter butzleri* isolates. Phylogenetic relationship were inferred by MEGA6 software using the maximum likelihood method model and the evolutionary distances were computed by Kimura 2-parameter with gamma distribution for *A. butzleri* sequences. Isolate of present study are marked with black spot.
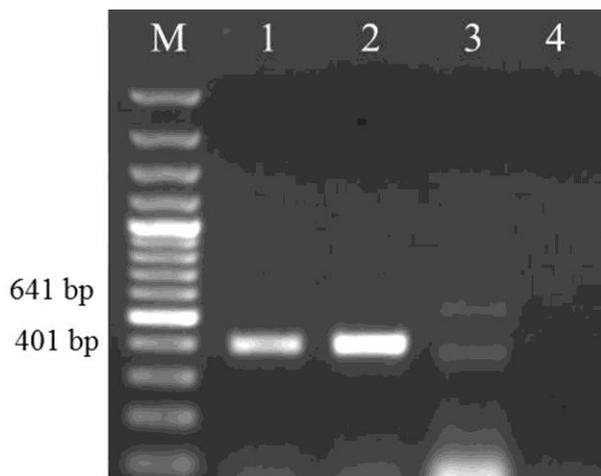
Figure 3 Phylogenetic tree of *Arcobacter skirrowii* isolates. Maximum likelihood tree was constructed using Jukes-Cantor algorithm. Isolate of present study are marked with black spot.

4 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 (1spinach 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 phylogenic 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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