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Antibiotic Resistance of *Arcobacter* spp. Isolated from Sewage, Meat Shop and Fish Market Environment of Bareilly City

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A B S T R A C T

Arcobacter genus is an emerging pathogen, with three main spp. are pathogenic (*A. butzleri*, *A. skirrowii* and *A. cryaerophilus*) in nature. In present study raw sewage water, chicken, mutton and fish market associated environmental samples were screened for presence of *Arcobacter* spp. from Bareilly city of India. Out of total 170 samples, 50 from sewage water, 40 each of meat shops (chicken, mutton, fish) associated environment such as swab samples from knife (10), chopper (10), wooden block (10) and utensils (10) from each meat shop respectively. 26/170 (15%) samples were positive for *Arcobacter* spp. by multiplex PCR detection and culture positive samples were 18/170 (10.58%). The 8/50 from sewage water, 8/40 from chicken shop, 4/40 mutton shop and 6/40 from fish market associated environment detected positive by m-PCR. Out of total 170 samples screened 18 were culture positive with isolation rate 6/50 (12%) from sewage water, 5/40 (12.5%) from chicken shop, 3/40 (7.5%) from mutton shop, 4/40 (10%) from fish market associated environments. Out of 18 Culture positive samples *Arcobacter* butzleri was predominant spp. isolated, than *A. skirrowii* and *A. cryaerophilus* and mix infection. The 18 positive strains were selected for disk diffusion, tested against 12 different types of antibiotic discs. High antibiotic resistance were seen as in cephalothin (17/18), co-trimoxazle (16/18), ampicillin (14/18), Vancomycin (14/18), tetracycline (14/18), kanamycin (12/18), Azitromycin (11/18) and other antibiotics shows greater sensitivity to *Arcobacter* butzleri with resistance pattern as 0/18 by nalidixic acid, Erytromycin (4/18), Ciprofloxacin (4/18), Gentamicin (5/18) and streptomycin (6/18). Multi drug resistance (MDR) was seen in this the study. Which may concerned to treatment failure in human and animal concerned to *Arcobacter* MDR strains. Sewage water may acts as reservoir of *Arcobacter* spp. as human, animal excreta are major part in and associated water. Most of the sewage water is recycled by municipal corporation. Hence water processing plant, purification methods must aware of probable public health risk related to sewage water. More cautions are recommended for personnel hygiene in processing, handling and awareness related to meat and meat products. The unnecessary use or misuse of antibiotics should be limited or avoided for public health safety.

Introduction

Though extensive study is not done on the epidemiology of *Arcobacter* species. The fact that they have many times isolated from poultry carcasses, sick animals and related to inflammation of intestine in humans strongly suggests that *Arcobacter* species may be key human bacterial pathogens (Philips, 2001).

In transmission of these pathogen water acts major source (Rice *et al.,* 1993), and enteritis and dysentery are associated with *Arcobacter* has been reported from drinking of contaminated water. Activated sludge and sewage water were reported for presence of *Arcobacter* spp. with varied range from 41%-80% (Stampi *et al.,* 1999) suggesting high implications for animal and human health.

Genus *Arcobacter* amongst most common bacterial genera (35.72%) seen in municipal sewage water (Gosh *et al.,* 2019). *Arcobacter* species are among the main bacterial pathogens associated with sewage water and well-known for pathogenic nature (Fresia *et al.,* 2019). Among the studies done at Study in the United States at west water treatment plant, McLellan *et al.,* (2010) reported *Arcobacter* at major proportion as compared to other bacteria confirmed by pyrosequencing. Miller and Raghavan (2017) reported the bacterial diversity in samples from west water processing plant and concluded that *Arcobacter* spp. contains MDR genes and are in greater percent in sewage microbiome of sewage. A study done by GhajuShrestha *et al.,* (2019) observed that that occurrence rate, detection of virulent genes (pldA and ciaB) of *Arcobacter* spp. by targeting 16S rRNA gene and detection 75% and 83% from effluent and influent plant respectively.

In recent year, the importance of *Arcobacter* has been increased due to potential emergent zoonotic enteric pathogen (Ho *et al.,* 2006; Snelling *et al.,* 2006). The *Arcobacter* belongs within the epsilon class of proteobacteria in an atypical group and is recognized for a wide variety of hosts and habitats (Debruyne *et al.,* 2008; Wesley *et al.,* 2010). *Arcobacter* species are the pathogenic group merely associated with peritonitis, endocarditis and bacteremia and have been isolated as well as detected from stool irrespective of whether diarrheic or non diarrheaic (Abdelbaqi *et al.,* 2007; Ho *et al.,* 2006; Jiang *et al.,* 2010; Kopilovic *et al.,* 2008; Kownhar *et al.,* 2007; Lau *et al.,* 2002; Samie *et al.,* 2007; Woo *et al.,* 2001; vandamme *et al.,* 1992; Van Driessche *et al.,* 2003).

Arcobacter spp. have been associated with udder inflammation, abortions and GI disorders in the animals and also isolated from asymptomatic animals too (Vandamme *et al.,* 1992; Van Driesshe *et al.,* 2003).

As per ICMSF 2002, *Arcobacterbutzleri* is important zoonotic pathogen and may cause a serious health hazard in the humans and prevalent species from genus *Arcobacter* (Cardoen *et al.,* 2009).

*Arcobacter*s were isolated from different types of seafood irrespective of their sources such as fish landing centres, fish markets and water (Rathlavath *et al.,* 2017).

Materials and Methods

Sample collection and processing

Sample size and sources

A total 50 sewage liquid samples were collected from different sewage chokes and raw sewage pond in the Bareilly city. 120 samples were collected by the swabbing the sources as knife (10), chopper (10), wooden block (10) and utensils 10) each from retail

chicken shop, retail mutton shop and retail fish market in Bareilly city.

Sewage water

50 Sewage samples were collected from different Sewage choke in Bareilly city; 25 ml of sewage liquid samples were collected in pre-sterilized sample collection bottle.

Brought in bacterial zoonosis lab of division of Veterinary public health, IVRI, Bareilly and further processed by filtration using Mixed Cellulose Ester filters (0.22-µm pore size 47mm diameter, Sartorius, Germany) and inoculated in CAT broth subjected to incubation in microaerobic condition for 24 hrs (Fisher *et al.*, 2014, Newton *et al.*, 2013; Atabay and Corry, 1998)

Swab sampling

Sampling of different sources (knife, chopper, wooden block and utensils)

Before sampling, the sterile swabs were kept in 10 mL sterile peptone water in suitable conditions. After the sampling of the area, the swabs were placed aseptically back into peptone water. Immediately after sampling, the tubes containing the swabs were immediately carried to the lab in a mobile incubator and maintained at 4^0 C. When the samples arrived at the laboratory, each tube containing the swab was vortexed for 10 s to assure mixture of the sample and transferred to CAT broth and incubated at 37^0C in microaerobic condition (Kim and Yim, 2017 with some modification)

After enrichment, loopfull of enriched broth was taken and selective plating was done on selective agar medium *Arcobacter* agar with CAT supplement (CAT agar) and plates were incubated at 28^0C under aerobic condition for 48-72 hrs.

Conventional isolation method of *Arcobacter* spp.

Morphology and biochemical

Gram negative, comma or "S" shaped all samples gave positive result to catalase, oxidase, Nitrate reduction test, indoxyl acetate hydroxyl test, and showed negative result to urease test, Hippurate hydrolysis test and H_2S production on TSI. And shows growth at 25^0C and 37^0C and shows no growth at 42^0C.

Testing of samples for presence of *Arcobacter* spp.

DNA extraction and PCR assay

DNA was isolated by snap chill method (Heat-lysis method) Approximately 1.5 ml of the *Arcobacter* enriched broth was subjected to pellet formation by centrifugation for 5 min at 8000 rpm and then 100 µl volume of triple distilled water added in it.

After keeping for 5 min in boiling water bath this pelleted volume containing bacterial lysate was then transferred directly to ice. Following bacterial lysate centrifugation at 13000 rpm for 5 min, supernatant was used as a DNA template for PCR analysis. The concentration of DNA isolated was estimated spectrophotometrically (Nanodrop®, US). The purity of DNA was checked as ratio of OD_{260} and $OD_{280.}$

Multiplex PCR protocol

m-PCR reaction mixture of 50 µl reaction volume was made containing following component. $MgCl_2$ (5 µl), dNTP 2mM (2 µl), primers ARCO, BUTZ, CRY-I, CRY-2 with 50 pmol volume (2 µl each) and SKIR 25 pmol (2 µl), 10X PCR Dream Taq buffer (5 µl), Taq Polymerase 5U (2 µl), Nuclease free water (22 µl) and DNA template (4 µl) (Primer details Table 1)

Cyclic conditions

Initial denaturation (94^0C for 5 min), Denaturation (94^0C for 30 sec), Annealing (52^0C for 30 sec), Extension (72^0C for 1 min) -30 cycles and final extension (72^0C for 10 min). In 1.5% agarose gel, the electrophoresis was done and thereafter analyzed by Gel Doc (UV trans-illuminator)

Primers and protocol was used as per Houf *et al.,* (2000) with some modification for optimization.

Antibiogram

CAMHA (cation adjusted Muller Hington Agar) used for disk diffusion. Antibiotic disk used in study are purchased from Hi-media laboratory, Bombay, India and BD Sensi-Disc. All isolates confirmed by PCR were subjected to disk diffusion method for antibiotic sensitivity for commonly used antibiotic as described by Bauer *et al.,* (1996).

Positive test culture was inoculated onto CAMHA plates by using sterile cotton swabs, plates are allowed to dry (10 min) then antibiotic discs were place with help of sterile foreceps. Further subjected to incubation for 48 hrs at 28^0C in microaerophilic condition.

Bacterial strain maintened in laboratory

Reference strain of *A. butzleri* (LMG10828T) maintained in Bacterial Zoonosis Lab, VPH was used as control positive DNA, whereas different positive strain of *C. coli, C. jejuni, Salmonella* and *E. coli,* used for differentiating from *Arcobacter* spp.

Microaerophilic Assembly

Microaerophilic jar, funnel filled water (60 ml), chemicals (0.6 gm sodium borohydrate, 0.6 gm sodium bicarbonate, 0.6 gm citric acid) and different ready to use gas packs. To maintain 5%O_2, 10% CO_2, 85% N_2 gaseous environment for selective growth of *Arcobacter* spp.

Results and Discussion

Out of total 170 samples screened sewage samples 16% (8/50) positive for *Arcobacter* out of 8 positive samples 6 were *A. butzleri*, one sample was a mixture of two strain (*A. cryaerophilus* and *A. butzleri*) and one sample was positive for *A. cryaerophilus* when detected by m-PCR.

Out of total 170 screened samples 26 were found positive for *Arcobacter* spp. with overall percentage 15.29 % detected by multiplex PCR (Fig. 5). Out of total 26 samples positive by PCR among them only 18 samples were culture positive (Fig. 1 and 2) and subjected to antibiotic sensitivity assay by using Disc diffusion methodology (Table 3, Fig. 3 and 4).

26 samples were found positive out of total 170 screened samples of *Arcobacter* spp. with overall percentage 15.29 % detected by multiplex PCR. Out of total 26 samples positive by PCR among them only 18 (10.58%) samples were cultures positive and subjected to antibiotic sensitivity assay by using Disc diffusion methodology (Bauer *et al.,* 1996). *Arcobacter* isolation rate was much lower by culture isolation and biochemical tests as compared to the detection by multiplex PCR amplification assay. These results imply the high accuracy of *Arcobacter* detection level by multiplex PCR over conventional culture method. In 2007, similar results were reported from waste water samples and chicken samples by Gonzalez and colleagues during the direct detection of *Arcobacter* by PCR and conventional culture method. The m-PCR assay which can detect the species of *Arcobacter* specifically and

rapidly than conventional biochemical identification method from the contaminated wastewater samples and chicken samples (Fig. 5).

Out of total 170 samples screened 18 were culture positive with isolation rate 6/50 (12%) from sewage water, 5/40 (12.5%) from chicken shop, 3/40 (7.5%) from mutton shop, 4/40 (10%) from fish market associated environments respectively. In this study 8/50 (16%) sewage water sample were detected positive by mPCR amongst these six were *Arcobacter butzleri*. one sample had mixed infection (*A. cryaerophilus* and *A. butzleri*) and one *Arcobacter skirrowii* strain. While only six samples were found culture positive of them five were *Arcobacter butzleri* and one was identified as *Arcobacter skirrowii* isolate. In present study the *Arcobacter* incidence observed in water samples associated with fish market utensil was similar to those reported by Rathlavath *et al.*, (2017).

The 18 positive strains were selected and tested for antibiotic susceptibility by disk diffusion method and tested against 12 different types of antibiotic discs. High antibiotic resistance were reported in cephalothin (17/18), co-trimoxazle (16/18), ampicillin (14/18), Vancomycin (14/18), tetracycline (14/18), kanamycin (12/18), Azitromycin (11/18) and other antibiotics shows greater sensitivity to *Arcobacter butzleri* with resistance pattern as 0/18 by nalidixic acid, Erytromycin (4/18), Ciprofloxacin (4/18), Gentamicin (5/18) and streptomycin (6/18) respectively.

Antibiotic susceptibility pattern of *Arcobacter spp.* isolates from sewage water and meat shops (chicken, mutton and fish) associated environment such as swab samples from knife, chopper, wooden block and utensils from each meat shop and seafood market were also done (Table 3).

Resistance spectrum of *Arcobacter* spp. for 12 antibiotics tested in present study descending order was seen for cephalothin, co-trimoxazol, ampicillin, vancomycin, tetracycline, kanamycin, azithromycin, streptomycin, gentamicin, ciprofloxacin, erythromycin and nalidixic acid (Table 3).

Table.1 Details of primers used in present study

Sr. no.	Primer	Gene	Primer sequence (5'-3')	Position	Product size
1	BUTZ (Forword)	16S rRNA	CCTGGACTTGACATAGTAAGAATGA	959-983	401 bp
2	ARCO (Reverse)	16S rRNA	CGTATTCACCGTAGCATAGC	1357-1338	
3	SKIR (Forword)	16S rRNA	GGCGATTTACTGGAACACA	705-723	641 bp
4	ARCO (Reverse)	16S rRNA	CGTATTCACCGTAGCATAGC	1357-1338	
5	CRY-I (Forword)	23S rRNA	TGCTGGAGCGGATAGAAGTA	105-124	257 bp
6	CRY-2 (Reverse)	23S rRNA	AACAACCTACGTCCTTCGAC	359-340	

Table.2 Distribution of *Arcobacter spp.* in various samples isolated from sewage water, chicken, mutton and fish market associated environment of Bareilly city

Sr no.	Source	*Arcobacter* spp.	*Arcobacter butzleri*	*Arcobacter cryaerophilus*	*Arcobacter skirrowii*	Mix of *A. butzleri* and *A. cryaerophilus*
1	Sewage (50)	8	6	0	1	1
2	Sample from chicken shop	8				
	i) knife (10)		1	0	0	0
	ii) chopper (10)		2	0	0	0
	iii) wodden block (10)		3	0	0	0
	iv) utensils (10)		2	0	0	0
3	Samples from mutton shop	4				
	i) knife (10)		0	0	0	0
	ii) chopper (10)		1	0	0	0
	iii) wodden block (10)		2	0	0	0
	iv) utensils (10)		1	0	0	0
4	Samples from fish markets	6				
	I) knife (10)		0	0	0	0
	ii) chopper (10)		1	0	0	0
	iii) wooden block (10)		2	0	0	0
	iv) utensils (10)		3	0	0	0

Fig.1 Gram's staining of *Arcobacter* spp.

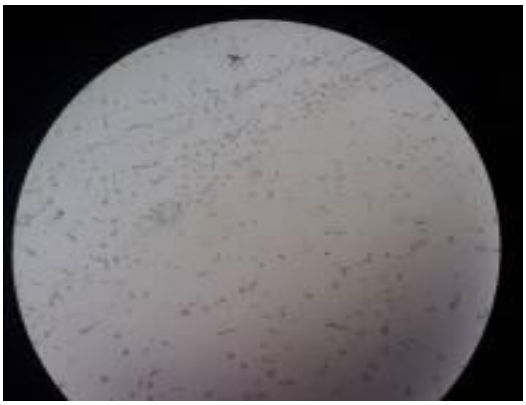


Table.3 Antibiotic susceptibility pattern of 18 selected strains of *Arcobacter spp.*

Sr No.	Types of Antibiotics	Concentration (mcg/disc)	Sewage	Chicken shop				Mutton shop				Fish market			
			(6)	(5)				(3)				(4)			
				i	ii	iii	iv	i	ii	iii	iv	I	ii	iii	iv
					(-1)	(-2)	(-2)		(-1)	(-1)	(-1)		(-1)	(-2)	(-1)
1	Tetracycline	30 mcg/disc	(4+)(2-)	*	+	+ +	+ +	*	+	-	-	*	+	+ +	+
2	Gentamicin	10 mcg/disc	(1+)(5-)	*	-	- +	- -	*	-	-	+	*	-	+ +	-
3	Vancomycin	5 mcg/disc	(5+)(1-)	*	+	- +	+ +	*	+	+	-	*	-	+ +	+
4	Ampicillin	10 mcg/disc	(5+)(1-)	*	+	+ +	- -	*	-	+	+	*	+	+ +	+
5	Erytromycin	15 mcg/disc	(2+)(4-)	*	-	- -	+ +	*	-	-	-	*	-	- -	-
6	Nalidixic acid	30 mcg/disc	(6-)	*	-	- -	- -	*	-	-	-	*	-	- -	-
7	Azitromycin	15 mcg/disc	(4+)(2-)	*	-	+ -	+ +	*	+	-	+	*	+	+ -	-
8	Kanamycin	30 mcg/disc	(4+)(2-)	*	-	+ +	+ -	*	+	+	-	*	-	+ +	+
9	Cephalothin	30 mcg/disc	(6+)	*	+	+ +	+ +	*	+	+	-	*	+	+ +	+
10	Streptomycin	10 mcg/disc	(3+)(3-)	*	-	- -	- -	*	+	-	-	*	-	+ -	+
11	Ciprofloxacin	5 mcg/disc	(1+)(5-)	*	-	- -	- +	*	-	+	-	*	-	- +	-
12	Co-trimoxazle	25 mcg/disc	(5+)(1-)	*	+	+ +	+ +	*	+	-	+	*	+	+ +	+

Remark: i- sample from knife, ii-sample from chopper, iii-sample from wooden block, iv- sample from utensils
Sign + : Antibiotic Resistant isolate; - : Antibiotic Sensitive isolate * : culture negative /not tested

Fig.2 Typical *Arcobacter* colony on CAT agar

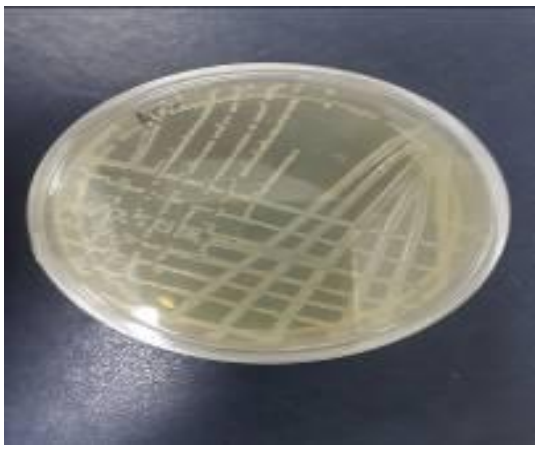


Fig.3 Antibiotic Assay (Disc diffusion) showing resistance and sensitivity

Fig.4 Disc diffusion showing sensitivity to *Arcobacter*

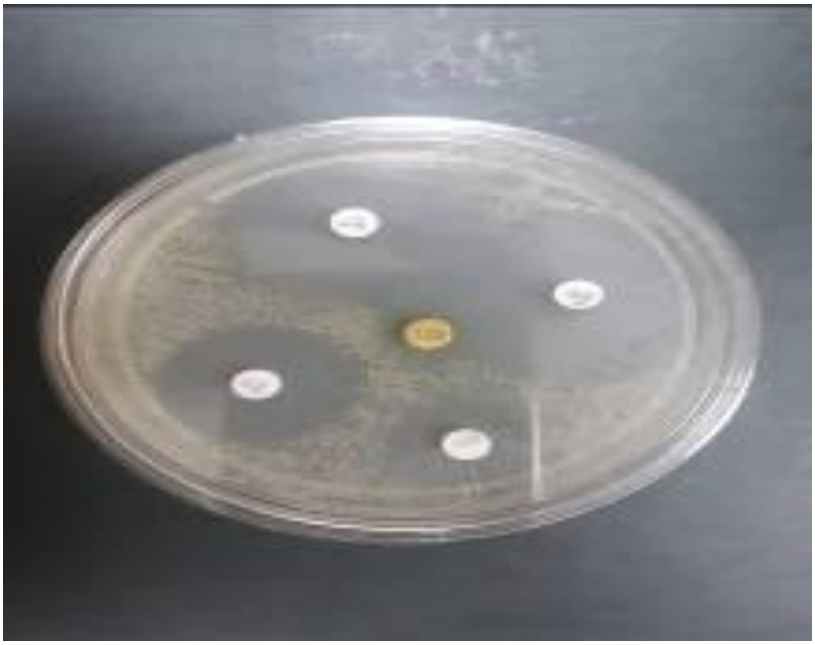
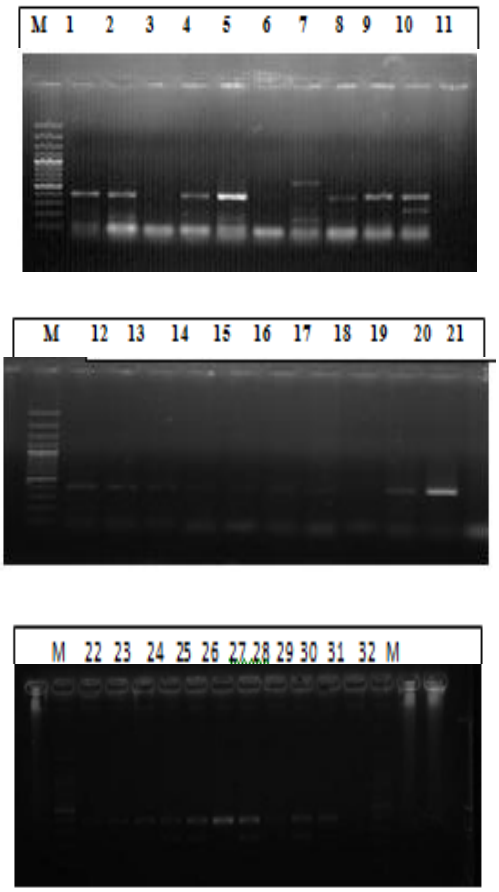


Fig.5 Identification of *Arcobacter* spp. amplified from enriched culture broth by multiples PCR



Lane M	:	100 bp ladder
Lane 1, 2, 4, 5, 8, 9	:	*Arcobacter butzleri* (401 bp)
Lane 3, 6, 11, 19, 32	:	Negative samples
Lane 7	:	*Arcobacter skirrowii* (641 bp)
Lane 10,25,26,28,30	:	Mix (*A. butzleri* 401 bp and *A. cryaerophilus* 257 bp)
Lane 12-18, 20, 21,22	:	*Arcobacter butzleri* (401 bp)
Lane 22-24,27,29,31	:	*Arcobacter butzleri* (401 bp)

Due to the major contribution of fecal contamination in sewage water, we also agreeing the hypothesis that these samples were more rich and diverse in antibiotic resistance mechanisms and can generate the MDR bacterial strains as compared to strains detected from municipal water samples as observed by Fresia *et al.*, (2019).

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