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Detection of Beta-Lactam Resistance in *Arcobacter* Species of Animal and Human Origin

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ABSTRACT

A set of 41 Arcobacter isolates (A. butzleri, 16; A. cryaerophilus, 13; A. skirrowii, 12) isolated from diverse sources like faecal swabs of livestock (21), raw foods of animal origin (13) and human stool samples (7) were screened for beta-lactam resistance by disc diffusion method and PCR targeting bla_{TEM}, bla_{SHV}, bla_{OXA}, bla_{AmpC}, bla_{CTX-M} group-1 and 2 beta-lactamase genes. Resistance to aztreonam (65.8%), cefotaxime (63.4%), ceftazidime (58.5%) and ceftriaxone (53.6%) was detected, with an overall frequency of 80.4% (33/41) beta-lactam resistance. Extended Spectrum Beta-lactamase (ESBL) phenotype was confirmed in a total of 15 (36.5%) Arcobacter isolates. Beta-lactamase genes were detected in 63.4% of Arcobacter isolates, with bla_{TEM} being the predominant gene detected (51.2%, 21/41) followed by bla_{CTX-M} group-1 (36.5%, 15/41), bla_{AmpC} (29.2%, 12/41), bla_{OXA} (29.2%, 12/41), bla_{SHV} (14.6%, 6/41) and bla_{CTX-M} group-2 (14.6%, 6/41) genes. CTX-M beta-lactamase was found to be the most frequent mechanism of ESBL resistance in Arcobacter isolates. The results highlighted the beta-lactam resistance in Arcobacter species, with special emphasis on ESBL phenotype, which is of grave concern to animal and human health in this region.

Key words: *Arcobacter, beta-lactam resistance, beta-lactamase genes, ESBL.*

INTRODUCTION

Arcobacter is an emerging foodborne pathogen under the family *Campylobacteraceae*[1]. Beta-lactamase is a broader term given to bacterial enzymes that hydrolyze the beta-lactam ring, inactivating various beta-lactam antibiotics [2]. Extended Spectrum Beta-Lactamases (ESBLs) are variants of beta-lactamases that hydrolyze penicillins, first, second and third generation cephalosporins as well as monobactams and are inhibited by beta-lactamase inhibitors[3]. Based on general prevalence, ESBLs are broadly grouped into major and minor ESBLs. Major ESBLs include TEM (Temoneira), SHV (sulfhydryl variable) and CTX-M (cefotaximase-Munich)[3].

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Minor ESBLs include OXA (oxacillinases), PER (*Pseudomonas* extended-resistant) etc[3]. The AmpC beta-lactamases were encoded mainly in the chromosomes of many Gram-negative bacteria[4]. Genes encoding three putative beta-lactamases (*lrgAB* operon AB1486, AB1306 and AB0578) have been identified in *A. butzleri* RM4018 genome and are likely to result in beta-lactam resistance[5].

Studies on beta-lactam resistance of *Arcobacter* species are lacking in India, although some studies have been done on antimicrobial sensitivity of *Arcobacter* species against few beta-lactam antibiotics in other countries[6,7]. Hence, the present study aimed at the detection of beta-lactam resistance with special emphasis on ESBL phenotype in *Arcobacter* species from different sources (livestock, foods of animal origin and humans) in Andhra Pradesh, India.

MATERIALS AND METHODS

Reference strains: The reference strain of *A. butzleri* (ATCC 49616) as well as positive DNA of *A. cryaerophilus* and *A. skirrowii* were obtained from Division of Veterinary Public Health, Indian Veterinary Research Institute, Izatnagar.

Bacterial isolates: A set of 41 *Arcobacter* isolates recovered from diverse sources like faecal swabs of livestock (21), raw foods of animal origin (13) and human stool samples (7) were used in this study. The identification of each isolate was carried out as per the methods of Sekhar *et al.* [8]. Further, all the 41 isolates were confirmed at genus level as *Arcobacter* by genus specific PCR targeting 16S rRNA gene[9] and at species level as *A. butzleri* (16), *A. cryaerophilus* (13) and *A. skirrowii* (12) by multiplex PCR targeting 16S and 23S rDNA[10]. *Arcobacter* isolates from faecal swabs of livestock include those from pigs (8), chicken (6), turkey (2), cattle (2), sheep (2) and duck (1). *Arcobacter* isolates from raw foods of animal origin include those from chicken (5), pork (4), milk (2) and mutton (2). *Arcobacter* isolates from human stool samples include those from pig/poultry farm workers (3), veterinary students (2) and diarrhoeic humans (2). Whole cell DNA was extracted by boiling and snap chilling method[8].

Phenotypic screening test for ESBL production: *Arcobacter* isolates were screened for resistance against four indiacator beta-lactam antibiotics: cefotaxime (CTX, 30 µg), ceftazidime (CAZ, 30 µg), ceftriaxone (CTR, 30 µg) and aztreonam (AT, 30 µg) by disc diffusion method [11] on Mueller Hinton (MH) agar supplemented with 5% defibrinized sheep blood by incubating at 30°C for 48 h under micro-aerophilic conditions. Sensitivity and resistance patterns were interpreted as per Clinical and Laboratory Standards Institute (CLSI) guidelines [12]. Resistance to at least one of the four indicator antibiotics used was considered as 'positive' screening test for possible ESBL production[12,13].

Phenotypic confirmatory test for ESBL production: All the isolates that were found to be positive in screening test were subjected to phenotypic 'confirmatory test' by combination disc method using three pairs of antibiotic discs (i.e., with and without beta-lactamase inhibitor) were placed: ceftazidime (CAZ, 30 µg), ceftazidime plus clavulanic acid (CAC, 30/10 µg), cefotaxime (CTX, 30 µg), cefotaxime plus clavulanic acid (CEC, 30/10 µg) and ceftriaxone (CTR, 30 µg), ceftriaxone plus sulbactam (CIS, 30/10 µg). ESBL production was confirmed if the zone size was expanded by a minimum of 5 mm in presence of beta-lactamase inhibitor[12,13].

PCR for the detection of beta-lactamase genes: All the *Arcobacter* isolates were subjected to two multiplex PCR assays [14] and a single uniplex PCR [15] for the detection of beta-lactamase genes. Multiplex PCR I was carried out for the amplification of bla_{TEM} (800 bp), bla_{SHV} (713 bp) and bla_{OXA} (564 bp) genes using oligonucleotide primers (bla_{TEM} F: 5'-CAT TTC CGT GTC GCC CTT ATT C-3', R: 5'-CGT TCA TCC ATA GTT GCC TGA C-3', bla_{SHV} F: 5'- AGC CGC TTG AGC AAA TTA AAC-3', R: 5'- ATC CCG CAG ATA AAT CAC CAC-3' and bla_{OXA} F: 5'- GGC ACC AGA TTC AAC TTT CAA G-3', R: 5'-GAC CCC AAG TTT CCT GTA AGT G-3') in an optimized 25 µl reaction mixture

containing 2 µl of DNA template; *Taq* buffer (10x) – 3.5 µl; dNTP mix (10mM) - 1 µl; $MgCl_2$ (25mM) - 1.0 µl; three forward primers (10 pmol/µl) - each 0.5 µl; three reverse primers (10 pmol/µl) - each 0.5 µl; *Taq* DNA polymerase (1 U/µl) - 1 µl and nuclease free water - 13.5 µl.

Multiplex PCR II was carried out for the amplification of $bla_{\text{CTX-M}}$ Group 1 (688 bp) and Group 2 (404 bp) genes using primers (bla_{CTX-M} group 1 F: 5'-TTA GGA AAT GTG CCG CTG TA-3', bla_{CTX-M} group 2 F: 5'- CGT TAA CGG CAC GAT GAC-3' and bla_{CTX-M} group 1 and 2 R: 5'- CGA TAT CGT TGG TGG TAC CAT-3') in an optimized 25 µl reaction mixture containing 1.5 µl of DNA template prepared from each isolate; *Taq* buffer (10x) – 2.75 µl; dNTP mix (10mM) – 0.5 µl; $MgCl_2$ (25mM) - 1 µl; two forward primers (10 pmol/µl) - each 0.75 µl; two reverse primers (10 pmol/µl) - each 0.75 µl; *Taq* DNA polymerase (1 U/µl) - 1 µl and nuclease free water – 15.25 µl. The two multiplex PCR assays were carried out in an Eppendorf thermal cycler (USA) under the following standardized cycling conditions - initial denaturation at 94ºC for 10 min, 30 cycles of denaturation at 94ºC for 40 sec, annealing at 60ºC for 40 sec, elongation at 72ºC for 1 min, final elongation at 72 ºC for 7 min and hold at 4ºC.

An uniplex PCR assay was carried out for the amplification of bla_{AmpC} gene (631 bp) using oligonucleotide primers (bla_{AmpC} F: 5'-CCC CGC TTA TAG AGC AAC AA-3' and R: 5'- TCA ATG GTC GAC TTC ACA CC-3') in an optimized 25 µl reaction mixture containing 1 µl of DNA template; *Taq* buffer (10x) – 2.5 µl; dNTP mix (10mM) – 0.5 µl; $MgCl_2$ (25mM) - 1.5 µl; forward primer (10 pmol/µl) - 1 µl; reverse primer (10 pmol/µl) - 1 µl; *Taq* DNA polymerase (1 U/µl) - 1 µl and nuclease free water – 16.5 µl; under the following standardized cycling conditions: initial denaturation of 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 58°C for 30 sec and extension at 72°C for 30 sec. Final extension was done at 72°C for 10 min.

RESULTS AND DISCUSSION

In the phenotypic screening test, resistance to aztreonam was observed in 27 (65.8%) isolates, cefotaxime in 26 (63.4%), ceftazidime in 24 (58.5%) and ceftriaxone in 22 (53.6%) isolates (Table 1). A total of 33 out of 41 *Arcobacter* isolates were found to be resistant to one or more of the cephalosporin antibiotics tested giving an overall frequency of 80.4% (33/41) beta-lactam resistance and were designated as 'suspect ESBL producers' encompassing 12 (75.0%, 12/16) *A. butzleri* isolates, 11 (84.6%, 11/13) *A. cryaerophilus* and 10 (83.3%, 10/12) *A. skirrowii* isolates (Table 2). The high level of resistance to third generation cephalosporins and monobactams in *Arcobacter* isolates observed in the present study agrees with the findings of previous studies[6,7, 16-18].

ESBL production was confirmed in 15 isolates (out of 33 suspected) encompassing 6 (37.5%, 6/16) *A. butzleri*, 5 (38.4%, 5/13), *A. cryaerophilus* and 4 (33.3%, 4/12) *A. skirrowii* isolates (Table 2). All these 15 isolates were resistant to atleast one of the indicator cephalosporin in screening test, but were found susceptible to combination of indicator cephalosporin with clavulanic acid or sulbactam in the confirmatory test. As clavulanic acid or sulbactam are beta-lactamase inhibitors, we can conclude that in these 15 *Arcobacter* isolates the cephalosporin resistance mechanism could be mediated by beta-lactamase production[13,19]. In the remaining 18 *Arcobacter* isolates, beta-lactamase inhibitor synergy (i.e. 5 mm principle) was not detected, likely due to existence of other resistance mechanisms conferring resistance to beta-lactam antibiotics, like presence of porin proteins or efflux pumps, which are unaffected by the beta-lactamase inhibitors[20,21]. The present findings were in accordance with earlier studies on beta-lactama antimicrobial resistance in *Arcobacter* species, where ESBL production was confirmed in two *Arcobacter* isolates using combination discs[19].

Out of 41 *Arcobacter* isolates screened, one or more beta-lactamase genes

were detected in a total of 26 isolates (63.4%, 26/41), with bla_{TEM} being the predominant gene detected (51.2%, 21/41) followed by bla_{CTX-M} group 1 (36.5%, 15/41), bla_{AmpC} (29.2%, 12/41), bla_{OXA} (29.2%, 12/41), bla_{SHV} (14.6%, 6/41) and bla_{CTX-M} group 2 (14.6%, 6/41) (Table 3 and Fig. 1). Overall frequency of beta-lactamase genes in *Arcobacter* isolates was found to be 63.4%. To our knowledge, this was the first report of detection of beta-lactamase genes in *Arcobacter* species.

Among the *Arcobacter* isolates (15) that were confirmed as 'ESBL' resistant phenotype, multiple beta-lactamase genes co-existed in all the isolates with bla_{CTX-M} group 1 being the predominant beta-lactamase gene detected (15/15, 100%), followed by bla_{TEM} gene (12/15, 80%), bla_{OXA} (9/15, 60%), bla_{CTX-M} group 2 gene (6/15, 40%) and bla_{SHV} (5/15, 33.3%). The present findings corroborate with the global dominance of CTX-M type ESBLs among Gram negative bacteria [22]. Among the *Arcobacter* isolates (18) that exhibited 'non-ESBL' resistant phenotype (positive screening and negative confirmatory test), beta-lactamase genes were detected in 11 (61.1%) isolates, whereas no beta-lactamase genes were detected in a total of 7 (38.8%) isolates. Among these 11 isolates exhibiting 'non-ESBL' resistant phenotype, bla_{AmpC} gene was the predominant beta-lactamase gene detected (10/10, 90.9%), followed by bla_{TEM} gene (9/11, 81.8%), bla_{OXA} (3/11, 27.2%) and bla_{SHV} (1/11, 9.09%). Several explanations have been put forward by many workers for the possible expression of resistant phenotype in the absence of beta-lactamase genes. One explanation could be the presence of ESBL genes that were not detected with the primers used in the present study or the contribution of other resistance mechanisms, such as enhanced expression of efflux pumps[13, 20, 21].

Table 1: Frequency of beta-lactam antimicrobial resistance detected in *Arcobacter* isolates

Species/ Source	Number tested	Cefotaxime No. (%)	Ceftriaxone No. (%)	Ceftazidime No. (%)	Aztreonam No. (%)
1. A. butzleri					
Poultry faeces	2	1 (50.0)	1 (50.0)	-	1 (50.0)
Pig faeces	2	1 (50.0)	1 (50.0)	1 (50.0)	1 (50.0)
Cattle faces	1	1 (100)	1 (100)	1 (100)	-
Chicken meat	2	2 (100)	1 (50.0)	2 (100)	2 (100)
Pork	1	-	-	1 (100)	1 (100)
Milk	1	1 (100)	1 (100)	-	1 (100)
Veterinary students	2	1 (50.0)	1 (50.0)	1 (50.0)	1 (50.0)
Farm workers	3	1 (33.3)	2 (66.6)	1 (33.3)	2 (66.6)
Diarrhoeic humans	2	2 (100)	1 (50.0)	1 (50.0)	2 (100)
TOTAL	**16**	**10 (62.5)**	**9 (56.2)**	**8 (50.0)**	**11 (68.7)**
2. A. cryaerophilus					
Poultry faeces	2	1 (50.0)	2 (100)	1 (50.0)	2 (100)
Pig faeces	3	1 (33.3)	-	1 (33.3)	2 (66.6)
Cattle faeces	1	-	-	-	-
Chicken meat	3	3 (100)	2 (66.6)	3 (100)	2 (66.6)
Pork	3	2 (66.6)	2 (66.6)	2 (66.6)	2 (66.6)
Milk	1	1 (100)	-	1 (100)	-
TOTAL	**13**	**8 (61.5)**	**6 (46.1)**	**8 (61.5)**	**8 (61.5)**
3. A. skirrowii					
Poultry faeces	5	3 (60.0)	3 (60.0)	2 (40.0)	4 (80.0)
Pig faeces	3	2 (66.6)	2 (66.6)	1 (33.3)	1 (33.3)
Sheep faeces	2	2 (100)	1 (50.0)	1 (50.0)	2 (100)
Mutton	2	1 (50.0)	1 (50.0)	2 (100)	1 (50.0)
TOTAL	**12**	**8 (66.6)**	**7 (58.3)**	**6 (50.0)**	**8 (66.6)**
GRAND TOTAL	**41**	**26 (63.4)**	**22 (53.6)**	**24 (58.5)**	**27 (65.8)**

Table 2: ESBL screening and confirmatory test results of *Arcobacter* isolates

Species	Source	Number of isolates	ESBL Screening test	ESBL Confirmation by combination disc method
***Arcobacter butzleri* (n=16)**	Poultry faeces	2	1 (50.0%)	-
	Pig faeces	2	1 (50.0%)	1 (50.0%)
	Cattle faces	1	1 (100%)	-
	Chicken meat	2	2 (100%)	2 (100%)
	Pork	1	1 (100%)	1 (100%)
	Milk	1	1 (100%)	1 (100%)
	Veterinary students	2	1 (100%)	-
	Farm workers	3	2 (66.6%)	-
	Diarrhoeic humans	2	2 (100%)	1 (50.0%)
	TOTAL	**16**	**12 (75.0%)**	**6 (37.5%)**
***Arcobacter cryaerophilus* (n=13)**	Poultry faeces	2	2 (100%)	-
	Pig faeces	3	2 (66.6%)	1 (33.3%)
	Cattle faeces	1	-	-
	Chicken meat	3	3 (100%)	2 (66.6%)
	Pork	3	3 (100%)	1 (33.3%)
	Milk	1	1 (100%)	1 (100%)
	TOTAL	**13**	**11 (84.6%)**	**5 (38.4%)**
***Arcobacter skirrowii* (n=12)**	Poultry faeces	5	4 (80.0%)	-
	Pig faeces	3	2 (66.6%)	1 (33.3%)
	Sheep faeces	2	2 (100%)	1 (50.0%)
	Mutton	2	2 (100%)	2 (100%)
	TOTAL	**12**	**10 (83.3%)**	**4 (33.3%)**
GRAND TOTAL		**41**	**33 (80.4%)**	**15 (36.5%)**

Table 3: Frequency of beta-lactamase genes detected in *Arcobacter* isolates

Species	Source	No. of strains examined	No. of strains with beta-lactamase genes detected					
			bla_{TEM}	bla_{SHV}	bla_{OXA}	$bla_{CTX\text{-}M}$ group 1	$bla_{CTX\text{-}M}$ group 2	bla_{AmpC}
1. A. butzleri								
Poultry faeces		2	-	-	-	-	-	-
Pig faeces		2	1	-	1	1	1	-
Cattle faeces		1	-	-	-	-	-	-
Chicken meat		2	2	1	2	2	-	-
Pork		1	1	-	1	1	1	-
Milk		1	1	-	-	1	-	-
Veterinary students		2	1	-	1	-	-	1
Farm workers		3	-	-	-	-	-	2
Diarrhoeic humans		2	1	1	-	1	1	1
TOTAL		**16**	**7 (43.7%)**	**2 (12.5%)**	**5 (31.2%)**	**6 (37.5%)**	**3 (18.7%)**	**4 (25%)**
2. A. cryaerophilus								
Poultry faeces		2	-	-	-	-	-	-
Pig faeces		3	2	1	1	1	-	1
Cattle faeces		1	-	-	-	-	-	-
Chicken meat		3	3	1	1	2	1	1
Pork		3	3	-	2	1	1	2
Milk		1	-	-	-	1	-	-
TOTAL		**13**	**8 (61.5%)**	**2 (15.3%)**	**4 (30.7%)**	**5 (38.4%)**	**2 (15.3%)**	**4 (30.7%)**
3. A. skirrowii								
Poultry faeces		5	2	1	-	-	-	2
Pig faeces		3	1	-	1	1	1	1
Sheep faeces		2	2	1	1	1	-	1
Mutton		2	1	-	1	2	-	-
TOTAL		**12**	**6 (50%)**	**2 (16.6%)**	**3 (25%)**	**4 (33.3%)**	**1 (8.33%)**	**4 (33.3%)**
GRAND TOTAL		**41**	**21 (51.2%)**	**6 (14.6%)**	**12 (29.2%)**	**15 (36.5%)**	**6 (14.6%)**	**12 (29.2%)**

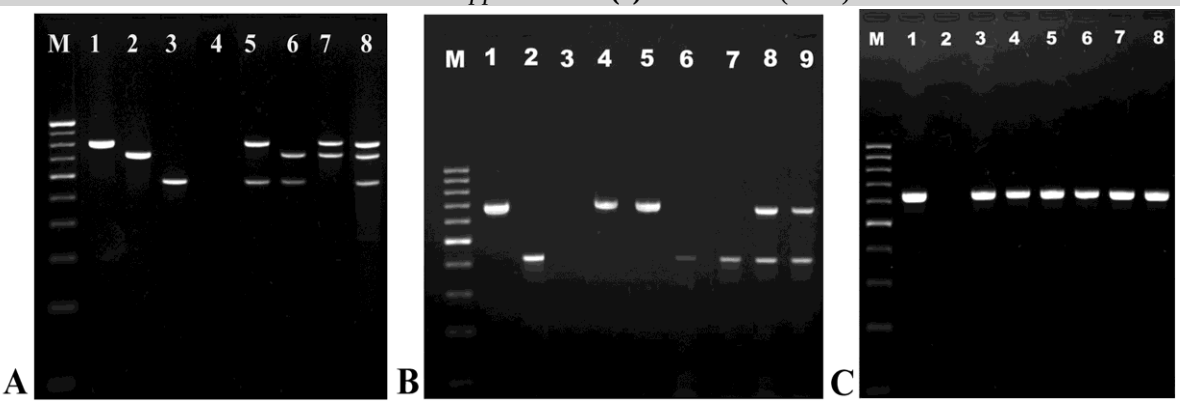

Fig. 1: (A). Gel photograph of multiplex PCR I targeting *bla*$_{TEM}$ (800 bp) *bla*$_{SHV}$ (713 bp) and *bla*$_{OXA}$ (564 bp) genes in *Arcobacter* species. (B). Gel photograph of multiplex PCR II targeting *bla*$_{CTX-M}$ group 1 (688 bp) and group 2 (404 bp) genes in *Arcobacter* species. (C). Gel photograph of uniplex PCR targeting *bla*$_{AmpC}$ (631 bp) gene in *Arcobacter* species.

CONCLUSION

Under the emerging era of "antibiotic resistance" and "one world one health", food borne pathogen prevalence and resistance monitoring are an essential basis for risk assessment that secures animal and public health equally. Beta-lactam resistance profiles of *Arcobacter* species of animal and human origin detected in the present study may pose threat to food safety, animal and human health in this region.

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