B-5280
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SHORT COMMUNICATION Indian Journal of Anim

Pathogenic Potential Abilities of Seafood and Environmental Derived *Arcobacter butzleri* Strains on HeLa and Vero Cell Lines

Srinu Rathlavath[1,2], Parmanand Prabhakar[3], Sanath Kumar[1], Binaya Bhusan Nayak[1] 10.18805/IJAR.B-5280

ABSTRACT

The genus *Arcobacter* includes emerging pathogenic potential species routinely reported from various foods, animals and environmental samples. Among which *Arcobacter butzleri* is one of the major notable pathogen known to cause gastroenteritis and bacteraemia in humans. HeLa cell lines are used for the adhesion and invasion assay for 15 *A. butzleri* strains derived from seafood (fish, shellfish) and environmental (coastal water) samples. Quantification of *A. butzleri* cytotoxicity on Vero cell lines was done by using the WST-1 assay. Eleven of fifteen *A. butzleri* strains displayed the ability to adhere to and invade HeLa cells. Four strains (CW4, SF5, F1 and F2) exhibited only adhesion and showed no signs of invasion into HeLa cells. However, all the fifteen *A. butzleri* strains revealed a high percentage of cytotoxic effects on the Vero cells. It is apparent from this study that the environmental as well as seafood-derived strains can be as pathogenic as clinical strains.

Key words: *Arcobacter butzleri,* Cytotoxicity, Environment, Pathogenic potential, Sea food.

The genus *Arcobacter* consists of spiral-shaped, gram-negative, motile and fastidious bacteria resembling that of campylobacter species (Vandamme *et al.,* 1991). *Arcobacter butzleri, A. cryaerophilus* and *A. skirrowii* are the notable emerging pathogens to both humans and animals (Ferreira *et al.,* 2016; Sekhar *et al.,* 2018). In the year 2002, ICMSF declared *A. butzleri* as one of the potential biological hazard to human health from various foods and food sources (ICMSF, 2002). Infections arising from arcobacters are often associated with symptoms such as nausea, vomiting, diarrhoea, abdominal cramps and fever (Ramees *et al.,* 2017). *A. butzleri* is known to be recovered from the human patient stools suffering from bacteraemia, peritonitis and diarrhoea (Ferreira *et al.,* 2016; Arguello *et al.,* 2015; Vandenberg *et al.,* 2004). Ferreira *et al.* (2016) documented the relationship of *A. butzleri* with human gastroenteritis and recovery from many food, animal and environmental sources. Pathogenic characterization of any bacteria causing human gastroenteritis mainly depends on the capabilities of adhesion to and invasion related factors (Konekel *et al.,* 1999). Adhesion and invasion abilities of *A. butzleri* onto several extra-intestinal and intestinal cell lines along with potent cytotoxic effects were previously documented (Levican *et al.,* 2013; Ferreira *et al.,* 2016; Karadas *et al.,* 2016), such activities can lead to impairments in the epithelial barrier of intestine (Bücker *et al.,* 2009).

Very recently, high pathogenic potentials with significant adhesion and invasion abilities have been documented in arcobacters derived from food and water sources, where *A. butzleri* was found to be more virulent (Baztarrika *et al.,* 2024). Rathlavath *et al.* (2017a) described seafood and environmental derived *A. butzleri* strains displayed potent virulence genes for pathogenesis and resistance to several antimicrobial agents. Such strains, if found capable of adhesion and invasion, will effectively colonize and cause infections in humans. With this background, this study investigated the pathogenic potential abilities of *A. butzleri* strains for their adhesion, invasion in the HeLa cells and cytotoxicity on Vero cells.

[1]Quality Control Laboratory, Department of Post-Harvest Technology, ICAR-Central Institute of Fisheries Education, Mumbai-400 061, Maharashtra, India.
[2]School of Fisheries, Centurion University of Technology and Management, Paralakhemundi Campus, Gajapati-761 211, Odisha, India.
[3]College of Fisheries, Bihar Animal Science University, Kishanganj-855 107, Bihar, India.

Corresponding Author: Binaya Bhusan Nayak, Quality Control Laboratory, Department of Post-Harvest Technology, ICAR-Central Institute of Fisheries Education, Mumbai-400 061, Maharashtra, India. Email: nayakbb@cife.edu.in

How to cite this article: Rathlavath, S., Prabhakar, P., Kumar, S. and Nayak, B.B. (2024). Pathogenic Potential Abilities of Seafood and Environmental Derived *Arcobacter butzleri* Strains on HeLa and Vero Cell Lines. Indian Journal of Animal Research. DOI: 10.18805/IJAR.B-5280.

Submitted: 11-12-2023 **Accepted:** 03-07-2024 **Online:** 01-08-2024

A. butzleri strains and growth environment

The experiment was conducted in 2017-18 at Quality Control Laboratory, Central Institute of Fisheries Education, Mumbai, Maharashtra. A total of Fifteen *A. butzleri* strains, previously isolated, identified and characterized from seafood (fish [F] and shellfish [SF]) and environmental (coastal water [CW]) samples was used for this study (Rathlavath *et al.,* 2017b). *A. butzleri*, clinical strain (ATCC 49616) was used as a positive control for adhesion and

invasion analysis. Initially, each bacterium was streaked onto Arcobacter Blood Agar (ABA) and allowed for incubation; a single colony of each isolate was transferred to Brain Heart Infusion (BHI) broth (Hi-Media), allowed for incubation (microaerobic conditions) for 48 h at 30°C. After the incubation, the cultures were then subjected to centrifugation (3000 rpm) at 4°C for 5 min. The pellets obtained were resuspended in DMEM (Dulbecco Minimum Essential Medium) supplemented with 10% FBS without antibiotics maintained at 37°C. The bacterial cultures were adjusted to an OD of (600 nm) 0.05 (ca. 10^8 CFU /ml).

Cell lines

HeLa and Vero cell lines obtained from the National Center for Cell Sciences (NCCS), were upheld in DMEM supplemented with 10% foetal bovine serum (FBS) with antibiotics (penicillin [10,000 U] and streptomycin [10, 000 µg/ml]). The cell lines cultures were incubated at 37°C in 5% CO_2 humidified atmosphere until a confluent monolayer (upto 80%) for adhesion, invasion and cytotoxic assays.

***A. butzleri* adhesion and invasion assay**

A. butzleri adhesion and invasion ability assay study was performed as per the protocol suggested by Karadas *et al.* (2016) and Levican *et al.* (2013) with slight modifications. Briefly, for the adherence assay, 24-well tissue culture plates were seeded with HeLa cells (4×10^4 cells/ml) in DMEM with antibiotics and incubated till confluent monolayer is formed. Growth medium was pipetted out from the wells and were washed three times with 1 X PBS (Phosphate Buffered Saline, pH 7.2) to remove the traces of dead cells and growth medium. Bacteria harvested in DMEM without antibiotic was adjusted to 0.5 ($\approx 10^8$ CFU/ml). Suspension of each bacterium (10^8 CFU/ml) was added to each well in 24 well cell culture plates and incubated at 37°C for 2 h for checking adhesion. Bacterial cells which did not adhere were removed by pipetting the media and rinsing with 1 X PBS, followed by lysing the confluent monolayer with a detergent (1% Triton X-100) for at least 10 min.

To determine invasion ability, 24-well plates were inoculated with a bacterial suspension (10^8 CFU/ml) of each *A. butzleri* to allow adhesion and invasion by incubating for 3 h. The non-adherent bacteria were removed by pipetting the media followed by washing the wells thrice with 1 X PBS. HeLa cells were then treated with 0.5 ml of DMEM containing gentamicin (125 µg/ml) to kill adhered bacteria. Cells were further washed three times with 1 X PBS and lysed as mentioned before to remove the invasive bacteria from HeLa cells.

The lysates collected (after adhesion and invasion assay) were then subjected to serial dilution with 1 X PBS for plating (Arcobacter Blood Agar) and kept for incubation. All the experiments were carried out in triplicates; *A. butzleri* clinical strain (ATCC 49616) was used as a positive control and DMEM without bacteria as a negative control. Results of bacteria adhered or invaded to HeLa cells were expressed as the mean± standard deviation.

Vero cell cytotoxicity (WST-1 assay)

Cytotoxicity of the cells is established based on the salt reduction of water-soluble tetrazolium (WST) and forming a coloured product by the action of mitochondrial reductases. *A butzleri* cytotoxicity assay was performed as per the protocol suggested by Karadas *et al.* (2016) with slight modifications.

The young bacterial suspensions initially grown in BHI broth was subjected to centrifugation (3000 rpm) at 4°C for 30 min. The supernatant collected from each bacterium was filter sterilized using 0.22 µm-membrane filter (Millipore). 96-well flat bottom plates were seeded with 100 µl of Vero cells and grown till they formed a confluent monolayer. Growth media was pipetted out and the cells were washed thrice with 1 X PBS. Vero cells were then added with 100 µl of filter sterilized supernatant. After an incubation of 48 h, WST-1 solution (one tenth volume) was added and incubated for 1 h in a humidified atmosphere of 5% CO_2 kept at 37°C in the dark room for substrate conversion. The colourful product formazan dye was measured at 450 nm using an EPSON LX-300 ELISA reader with a reference at 600 nm. Positive controls (1% Triton X-100; *A. butzleri* ATCC 49616) and negative control (respective medium) were involved in this assay. The absorbance values obtained from the blank has been deducted from sample absorbance. The value of cytotoxicity of each bacterium was expressed in percentage.

Statistical analysis

The analysis was carried out by one-way ANOVA with the Duncan test using R Studio software followed by a post-hoc test. Results were compared and considered statistically significant at $P<0.05$ level.

Adherence and invasion of *A. butzleri* to HeLa cell line

Fifteen *A. butzleri* strains derived from fish (5) and shellfish (5) and coastal water (5) samples, that tested positive for the putative virulence genes encoded for adhesion and invasion abilities by PCR analysis (Rathlavath *et al.,* 2017a) were selected for the investigation. *A. butzleri* though considered as emerging food-borne and water-borne pathogen, when compared to other enteric pathogens, data about the pathogenic potentials of this species are very scarce. All 15 *A. butzleri* strains showed their ability to attach to HeLa cells; however, their attachment varied from 4.86±0.08 to 6.45±0.02 $\log_{10}$ CFU /ml (Table 1), while positive control (*A. butzleri*; ATCC 49616) had 6.13±0.01 $\log_{10}$ CFU /ml. *A. butzleri* that exhibited the highest levels of adhesion even more than the pathogenic positive control were CW2, SF3, SF4, SF2, SF1 and CW3 (6.45±0.02 to 6.15±0.03 $\log_{10}$ CFU /ml). Shellfish derived strains, SF1-SF4 (6.28±0.02, 6.29±0.02, 6.42±0.03, 6.32±0.02 $\log_{10}$CFU/ml)

Table 1: Adhesion, invasion and cytotoxic capabilities of *A. butzleri* stains on Cell lines.

Strain	HeLa Cells		Vero cells
	Adhesion - log_{10} CFU/ml (Mean ± SD)	Invasion - log_{10} CFU/ml (Mean ± SD)	Cytotoxicity (%)
ATCC 49616	6.13±0.01	4.08±0.03	98
F1	5.65±0.06	NI	95*
F2	6.03±0.05	NI	92
F3	4.86±0.08	2.37±0.11	93
F4	4.92±0.05	2.50±0.03	94
F5	5.89±0.07	2.24±0.09	93
SF1	6.28±0.02	2.46±0.07	93
SF2	6.29±0.02	2.37±0.11	93
SF3	6.42±0.03	2.23±0.14	93
SF4	6.32±0.02	2.32±0.21	94
SF5	5.72±0.11	NI	95*
CW1	5.96±0.03	2.44±0.07	93
CW2	6.45±0.02	2.46±0.03	94
CW3	6.15±0.03	2.27±0.07	89
CW4	6.13±0.04	NI	95*
CW5	5.88±0.04	2.39±0.10	94
1 % Triton	-	-	100

F- Fish; SF- Shellfish; CW- Coastal water; NI- No Invasion detected; * Highest cytotoxicity but no invasion detected, the values for adhesion and Invasion on HeLa cell line were proportionately calculated to the inoculum of 10^8 CFU/ml for each strain. Cytotoxicity of each *A. butzleri* on Vero cells was measured by using WST-1 assay, recorded absorbance values are converted into percentage.

showed higher adhesive index compared to fish and coastal water derived strains (Table 1). The maximum adhesion index values was noticed in the CW2 (6.45±0.02 log_{10}CFU /ml) followed by SF3 (6.42±0.03 log_{10}CFU /ml). Lowest adhesions were recorded in the fish isolates F3 (4.86±0.08 log10CFU /ml). Such differences in adhesive capacities to HeLa cells are reported from *A. butzleri* strains derived from river water, sea water and zooplankton (Gugliandolo *et al.,* 2008; Carbone *et al.,* 2003). Until now, no studies have reported the adhesion and invasion abilities of fish and shell fish derived *A. butzleri* on the HeLa cells. Similar studies showed medium to highest adhesion (Hep-2 cell line) and low adhesion (INT407 cell line) of *A. butzleri* strains derived from seawater and river water (Fernández *et al.,* 2010; Musmanno *et al.,* 1997). *A. butzleri* derived from shellfish (mussels and clams) have presented their ability to adhere to Hep-2 cell line (Ferna´ndez *et al.,* 2010) and Caco-2 cell line (Levican *et al.,* 2013).

Eleven out of 15 *A. butzleri* strains showed their ability to invade into HeLa cells; however, their invasion varied from 2.23±0.14 to 2.50±0.03 log_{10}CFU /ml (Table 1), while positive control (*A. butzleri*; ATCC 49616) had 4.08±0.03 log_{10}CFU/ml. The maximum invasion was observed in F4 (2.50±0.03 log_{10}CFU /ml) followed by CW2 and SF1 (2.46±0.03, 2.46±0.07 log_{10}CFU /ml). The lowest invasion was found in SF3 (2.23±0.14 log_{10}CFU / ml). ATCC 49616 strain, *A. butzleri* (Positive control) showed the highest invasion (4.08±0.03 log_{10}CFU /ml) to HeLa cells (Table 1). Lower invasion values were observed in six strains (CW2, SF3, SF4, SF2, SF1 and CW3) which showed more adhesion than clinical strain. Three of five fish (F3-F5) derived strains, four of five isolates from shellfish derived strains (SF1-SF4) and three of five coastal water derived strains (CW1, CW3, CW5) adhered and invaded HeLa cell line. Musmanno *et al.* (1997) reported 18 non-invasive (on HeLa and INT407 cell line) *A. butzleri* isolates derived from river water, whereas Baztarrika *et al.* (2024) reported all the tested arcobacter strains showed invasion on Caco-2 cell lines. In this study as well few strains (F1, F2, SF5 and CW4) though carried the virulence genes for invasions were not able to invade the HeLa cells (Table 1), such variations in adhesion to and invasion capabilities of *A. butzleri* could depend on the source of isolate and the cell lines used (Ferreira *et al.,* 2016). Levican *et al.* (2013) tested *A. butzleri* abilities of adhesion to and invasion into Caco-2, where *A. butzleri* derived from mussel did not show any invasion though the invasive gene (*ciaB)* was present. It is also evident from this study that adhesion and invasion abilities occur in HeLa cell lines by *A. butzleri* derived from seafood and environmental strains.

Cytotoxicity of *A. butzleri* to vero cells

Toxin production is the main principal mechanism by which most of the bacterial pathogens cause disease in humans and animals. Cytotoxic effects of toxin on vero cells morphologically (cell rounding and nuclear pyknosis) can be distinguished after 48 h of incubation (Hazarika *et al.,* 2024; Hussien *et al.,* 2023). Such morphological deviations by *A. butzleri* toxins were earlier reported in HeLa, Vero and INT-407 cell lines (Gugliandolo *et al.,* 2008; Carbone *et al.,* 2003; Johnson and Murano, 2002; Musmanno *et al.,* 1997).

Quantification of cytotoxic effects of *A. butzleri*, WST-1 assay (colorimetric assay) showed fifteen *A. butzleri* strains irrespective of their sources, adhesion and invasion abilities revealed high percentage (89-95%) of cytotoxicity on the Vero cells (Table 1). Similar results were obtained by Karadas *et al.* (2016) while examining the toxigenic potential of *A. butzleri* on IPEC-J2 and HT-29/B6 cell lines. F1, SF5 and CW4 showed the highest (95%) toxicity among tested isolates; interestingly, these three strains did not show any signs of invasion on HeLa cells. Studies have shown *A. butzleri* derived from coastal water and river water are cytotoxic to CHO, HeLa and INT407 and Vero cells (Carbone *et al.,* 2003; Musmanno *et al.,* 1997; Johnson and Murano, 2002). Even in this study coastal water derived *A. butzleri* showed cytotoxic effects on Vero cells apart from fish and shellfish derived *A. butzleri* strains. Presence of such toxins has the ability to cause damage to intestinal epithelial cells, initiate intestinal secretion and causes inflammation or gastroenteritis (Guerrant *et al.,* 1999).

CONCLUSION

We observed different adhesion, invasion and cytotoxicity profiles among different strains of *A. butzleri* from coastal water, shellfish and fish samples. Further, investigations on isolates with significant numbers from different sources can be studied on different cell lines to elucidate the nature of individual mechanisms related to adhesion, invasion, toxins involved and virulence related factors.

ACKNOWLEDGEMENT

Authors thank the Director and the Vice-Chancellor of the Central Institute of Fisheries Education (CIFE), Mumbai, for help in procuring cell lines and support to carry out this investigation.

Conflict of interest

The authors declare that they have no conflict of interest.

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