Original Article

BIOTYPING OF *Arcobacter butzleri* ISOLATED FROM POULTRY PRODUCTS: RESCUING A PHENOTYPIC METHOD

BIOTIPAGEM DE Arcobacter butzleri *DESDE PRODUTOS AVIARES: RESGATANDO UM MÉTODO FENOTÍPICO*

Heriberto FERNÁNDEZ[1]; Karen RINGLER[2]; María Laura ARIAS[3]

1. Professor, Doutor, Institute of Clinicall Microbiology, Universidad Austral de Chile, Valdivia, Chile. hfernand@uach.cl; 2. Medical Technologist, Clinical Laboratory and Blood Bank, Traumatology Institute, Santiago-Chile; 3.Professor, Mestre, Tropical Disease Research Center and Microbiology Faculty, Universidad de Costa Rica.

ABSTRACT: *Arcobacter butzleri* is an emergent zoonotic foodborne pathogen associated to enteritis and occasionally to bacteremia in human beings. Biotyping of this bacterium is important in order to establish the circulating strains and its dissemination routes. The purpose of this work was to determine the circulating *A. butzleri* biotypes in poultry products for human consumption in Southern Chile using the method proposed by Lior and Woodward, in order to explore the possibility of introducing this biotyping scheme as a routine laboratory tool. From the 60 strains studied the prevalent biotypes were 8A, 8B, 7A, 4A and 4B. The most frequently isolated biotype, independently of the sample of origin, was 8A with (44 strains, 73.3%). The less frequently isolated biotype was 4B (two strains 3.3%).
The biotyping method used results to be simple, easy to handle and yields stable results. Therefore, it might be rescued to be used as a phenotypic tool for epidemiological marking of *A. butzleri*.

KEYWORDS: *Arcobacter butzleri*. Biotyping. Epidemiology.

INTRODUCTION

The genus *Arcobacter* was originally proposed in 1991 (VANDAMME et al.,1991). Currently included 21 species (WHITEDUCK-LÉVEILLÉE et al., 2015). Four of them, including *Arcobacter butzleri, A. cryaerophilus, A. skirrowii* and *A. thereius* have been isolated from human infectious processes, especially diarrhea (3). From these, *A. butzleri* stands out as the most frequent isolate from clinical, animal, environmental and food samples, and also is considered as a zoonotic emergent pathogen transmitted by food (VANDENBERG et al, 20014, FERNÁNDEZ et al. 2010, COLLADO; FIGUERAS, 2011, HSU; LEE, 2015).

The epidemiology of *A. butzleri* seems to be complex due to its wide distribution in nature, it has been isolated from environmental water, mammal aborted fetus, domestic and wild birds and mammal fecal material and from foods of animal origin used for human consumption such as meat, poultry and lactic products (VANDENBERG et al, 20014, FERNÁNDEZ et al. 2010, COLLADO; FIGUERAS, 2011, HSU; LEE, 2015).

The identification of the epidemiological factors related to the transmission of *A. butzleri* as well as the characteristics of the strains associated to different ecological niches is necessary in order to clarify the propagation mechanisms, its distribution in nature or tracing sources of outbreaks. For these purposes, different genotyping molecular-based methods have been used, including whole genomic sequencing, multilocus sequence typing (MLST), single-enzyme amplified fragment length polymorphism (s-AFLP), pulsed-field gel electrophoresis (PFGE) profiling, enterobacterial repetitive intergenic consensus-PCR (ERIC-PCR) and randomly amplified polymorphic DNA-PCR (RAPD-PCR) (COLLADO; FIGUERAS, 2011, GONZÁLEZ et al., 2007, MERGA et al., 2013).

Unfortunately, these methods are not available in laboratories with reduced or low budget and here is where biotyping methods appear as the adequate tool to be used for epidemiological purposes. In 1993, Lior and Woodward (LIOR; WOODWARD, 1993) developed a biotyping scheme for *A. butzleri* and *A. butzleri*-like organisms using four biochemical tests: urease, rapid H_2S and DNAse production, and the utilization of sodium acetate.

Probably, because *A butzleri* is an emergent microorganism that still does not have a generalized research methodology, this biotyping method has not been widespread and in general, has been overshadowed by research and advanced laboratories that use molecular-based methods.

The main purpose of this work was to determine the circulating *A. butzleri* biotypes in poultry food for human consumption, to validate the Lior and and Woodward biotyping method and to establish the possibility of introducing this biotyping scheme as a routine lab tool.

Received: 20/04/16
Accepted: 05/10/16

MATERIAL AND METHODS

A total of 60 *A. butzleri* strains was used in this study. Nine of them were isolated from poultry gizzards, 18 from poultry livers and 33 form poultry meat. All strains were identified at species level using the multiplex PCR proposed by Houf et al. (HOUF et al., 2000).

The strains were biotyped using the method proposed by Lior and Woodward (LIOR; WOODWARD, 1993), based on the following four biochemical tests: urease, rapid H_2S production, DNAse production and the sodium acetate test. This biotyping scheme recognizes 16 biotypes numbered 1A and 1B to 8A and 8B (Table 1).

Table 1. Biotypes of *Arcobacter butzleri* types according to the method of Lior and Woodward

TESTS	BIOTYPES															
	1 A	1 B	2 A	2 B	3 A	3 B	4 A	4 B	5 A	5 B	6 A	6 B	7 A	7 B	8 A	8 B
Urease	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-
H_2S	+	+	+	+	-	-	-	-	+	+	+	+	-	-	-	-
DNAse	+	+	-	-	+	+	-	-	+	+	-	-	+	+	-	-
Sodium acetate	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-

Urease test is based on the hydrolysis of urea by the enzyme urease, producing ammonium and CO_2, with alkalization of the culture media, which is revealed through a color change from yellow to fuchsia. Culture media has 2.9 g brain-heart infusion broth, 0.118 g anhydrous Na_2HPO_4, 0.023 g KH_2PO_4, 0.2 g L28 agar and 100 ml distilled water. pH is adjusted to 7.0 and ~~is~~ sterilized in autoclave. Then 10.4 ml of a 20% (p/v) urea aqueous solution sterilized by filtration is added. Once inoculated, it was incubated aerobically at 37°C for up to 72 h.

The rapid H_2S production test was done putting an abundant loop of a 24 h bacterial culture in the upper third of H_2S media, trying to keep the inoculum as a compact ball. It was incubated in a thermo-regulated bath at 37°C for 2 h. The reaction is considered as positive when a black coloration appears around the inoculum, indicating the production of H_2S. In this study, strains were incubated for 72h.

DNAse production was determined seeding a dense 24-48 h bacterial culture inoculum in the DNAse Lior medium and incubated aerobically at 37°C up to 72 h. The reaction is considered positive if around the inoculum appears a clear pink halo, revealing the DNA hydrolysis. The reaction is considered negative when there is no color change in the culture media. Media is composed of 0.3 g DNA, 1 ml $CaCl_2$, 10 g NaCl, 15 g L28 agar and 1000 ml Tris Buffer 0.05 M pH9. Ingredients are dissolved in hot Tris buffer heated to 90-95°C, cooled and 2.5 ml of toluidine blue 3% is added and distributed into petri dishes.

The sodium acetate test is used to determine the capacity of *A. butzleri* to use acetate as the only source of carbon, making the media alkaline. A tube of acetate agar is inoculated with the test bacteria and incubated at 37°C for up to 72 h. A positive reaction is produced when there is a change of the media color to blue (alkaline) and is considered negative if keeps its original green color. Media contains 5 g NaCl, 0.2 g $MgSO_4$ $7H_2O$, 1 g $(NH_4)H_2PO_4$, 1 g K_2HPO_4, 2 g sodium acetate, 0.08 g bromothymol blue, 20 g agar and 100 ml distilled water. The ingredients are mixed, dissolved by boiling; final pH is 6.8 +/- 0.2 at 25°C and distributed and autoclaved for 15 m at 121°C. Tubes are cooled in a slanted position until its solidification.

All strains were tested three times, under the same atmospheric, temperature and incubation periods, and the results recorded by two or three different observers.

RESULTS

None of the strains studied, independently of its origin or incubation periods (2, 24, 48 and 72 h) produced H_2S.

Only 5 (8.3%) strains were DNAse positive after 24 h incubation and no other strain were positive after 72 h incubation.

Three (5.0%) strains were urease positive after 24 h incubation. When incubation was extended to 48 h, the number of positive strains increased to 5 (8.3%) and no further positive strains appeared after 72 h incubation.

After 24 h incubation, 32 (53.3%) strains hydrolyzed the sodium acetate. However, after 48 and 72 h incubation, the number of acetate positive strains increased to 46 (76.7%) and 52 (86.7%) respectively.

Five different biotypes were isolated (8A, 8B 7A, 4A and 4B) being 8A biotype the most frequent in poultry meat, gizzard and liver, followed by 8B biotype. The biotype 4B was the less often found in those three types of food with only two isolates (3.3%). Tables 2 and 3 show the biotypes found and their frequency after 72 h incubation.

All strains showed identical results in each of the three repetitions performed.

Table 2. Frequency of *Arcobacter butzleri* biotypes isolated from poultry food products

BIOTYPE	FREQUENCY	PERCENTAGE (%)
8A	44	73.3
8B	6	10.0
7A	5	8.3
4A	3	5.0
4B	2	3.3
TOTAL	60	100

Table 3. *Arcobacter butzleri* biotypes isolated from three types of poultry food products

ORIGIN	BIOTYPE	FREQUENCY	PERCENTAGE (%)
Chicken meat (32 strains)	8A	28	87.5 %
	7A	2	6.3 %
	8B	1	3.1 %
	4A	1	3.1 %
Gizzards (9 strains)	8A	5	55.6 %
	8B	2	22.2 %
	7A	1	11.1 %
	4A	1	11.1 %
Livers (19 strains)	8A	11	57.9 %
	8B	3	15.8 %
	7A	2	10.5 %
	4B	2	10.5 %
	4A	1	5.3 %

DISCUSSION

A. butzleri is an emergent zoonotic foodborne pathogen that affects human beings and associated with enteritis and occasionally to bacteremia. The infection is acquired by the fecal oral route, through the consumption of water or contaminated food or through close contact with animals. It is important to perform epidemiological studies that allow us the establishment of their dissemination routes. Molecular typing methods are used in highly complex laboratories, but they are not accessible for lower resource laboratories. However, less sophisticated laboratories could access to phenotypical methods that would allow them the biotyping of these bacteria (HENRÍQUEZ, 2006).

In this study, the biotyping scheme proposed by Lior and Woodward (LIOR; WOODWARD, 1993) was used, being the only biotyping method available for *A. butzleri*. Of the 60 strains studied, all of them isolated from (poultry products (meat, liver and gizzard), were biotyped. We found that the prevalent circulating biotypes in Southern Chile were 8A, 8B, 7A, 4A and 4B. The biotype most frequently isolated, independent of the sample of origin, was 8A with a total of 44 strains (73.3%). The biotype less frequently isolated was the 4B with two (3.3%) strains.

In the original study done by Lior and Woodward (LIOR; WOODWARD, 1993) samples of avian origin were also analyzed, the biotypes most often isolated were 8A, 8B, 7A and 4A, that were also the ones found among our isolated *A. butzleri* strains. In our study biotypes 8A and 8B were the most common ones.

Although this method is the only phenotypic available method for *A. butzleri*, it has not been widely use neither well known among diagnostic laboratories, maybe because few labs are interested in *A. butzleri,* or because it is an emerging pathogen and for that reason less studied yet. This may explain the fact that there are no more studies using biotyping for *A. butzleri* that could allow for comparison of results. This paper represents the first study performed in Latin America in which *A. butzleri* was biotyped.

Because of differences in the results related to the incubation period, we suggest that in order to avoid false negative results, it is necessary to increase the incubation period up to 48 h in the case of urea hydrolysis and up to 72 h in the case of sodium acetate. Incubation period increase for the H_2S and DNAse test did not result in the appearance of new positive strains.

In the opinion of some authors (ON, 2003) biotyping methods are of limited use due to the instability and low reproducibility of the phenotypic characteristics. However, in this study all strains showed identical results in each of the three repetitions performed showing to be a reliable method.

Probably a limitation of this study is the absence of a molecular method for a comparative analysis, in order to determine the discriminatory power of biotyping. However, this biotyping scheme is a cheaper alternative method to be used as a screening tool for the selection of representative strains to be confirmed by clonal relatedness using a molecular method.

In our experience, this phenotypic biotyping method is simple, easy to handle and yield stable results. This is why we consider that having the precaution of making longer the incubation periods for urease and sodium acetate tests, this method might be rescued to be used as a phenotypic tool for epidemiological marking of *A. butzleri*.

ACKNOWLEDGEMENTS

To Professor, Ph.D. Guillermo Pérez-Pérez (New York University Langone Medical Center, U.S.A.) for their scientific advice and critical review. This work was supported by Projects DID S-2004-01 and Fondecyt N° 1030245.

RESUMO: *Arcobacter butzleri* é um patógeno emergente, zoonótico e de transmissão alimentar, associado a enterite e, ocasionalmente, a bacteremia em seres humanos. A biotipagem desta bactéria é importante para estabelecer os biótipos circulantes e suas rotas de disseminação. Os objetivos deste trabalho foram determinar os biótipos de *A. butzleri* circulantes em alimentos de origem aviar para consumo humano, no sul do Chile, explorando a possibilidade de introduzir o método de biotipagem proposto por Lior e Woodward como uma ferramenta de rotina no laboratório.
Entre as 60 cepas estudadas, os biótipos 8A, 8B, 7A e 4B foram os mais prevalentes. O biótipo mais frequentemente isolado, independentemente da amostra de origem, foi o biótipo 8A (44 cepas, 73,3%). O biótipo 4B apresentou a menor frequência de isolamento (duas cepas, 3,3%). O método de biotipagem utilizado resultou ser simples de executar, fácil de manusear e produz resultados estáveis. Portanto, pode ser resgatado para ser usado como uma ferramenta fenotípica para marcação epidemiológica de *A. butzleri*.

PALAVRAS CHAVE: *Arcobacter butzleri*. Biotipagem. Epidemiologia.

REFERENCES

COLLADO, L.; FIGUERAS, M. J. Taxonomy, epidemiology, and clinical relevance of the genus *Arcobacter*. **Clin Microbiol Rev,** v. 24, p. 174-192. 2011. https:/doi.org/10.1128/CMR.00034-10

FERNÁNDEZ, H.; FLORES, S.; INZUNZA, F. *Arcobacter butzleri* strains isolated from different sources display adhesive capacity to epithelial cells in vitro. **Acta Scientiae Veterinariae,** v. 38, p. 283-287. 2010.

GONZÁLEZ, A.; FERRÚS, M.A.; GONZÁLEZ, R.; HERNÁNDEZ, J. Molecular fingerprinting of *Campylobacter* and *Arcobacter* isolated from chicken and water. **Int Microbiol,** v. 10, p. 85-90. 2007.

HENRÍQUEZ, R. Extracción de DNA plasmidial para la determinación de perfiles plasmidiales en cepas de *Arcobacter butzleri* aisladas de carcasas e hígado de pollo en la ciudad de Valdivia. 2006. 31 p. Disertación (Licenciatura en Tecnología Médica). Escuela de Tecnología Médica. Facultad de Medicina. Universidad Austral de Chile. 2006.

HOUF, K.; TUTENEL, A.; DE ZUTTER, L.; VAN HOOF, J.; VANDAMME, P. Development of a multiplex PCR assay for the simultaneous detection and identification of *Arcobacter butzleri*, *Arcobacter cryaerophilus* and *Arcobacter skirrowii*. **FEMS Microbiol Lett,** v. 193, p. 89-94. 2000. https:/doi.org/10.1111/j.1574-6968.2000.tb09407.x

HSU, T. D.; LEE, J. Global distribution and prevalence of *Arcobacter* in food and water. **Zoonoses Public Health,** v. 62, p. 579–589. 2015. https:/doi.org/10.1111/zph.12215

LIOR, H.; WOODWARD, D. L. *Arcobacter butzleri*: A biotyping scheme. **Acta Gastro-enterol Belg,** Suppl. 6, p. 28. 1993.

MERGA, J. Y.; WILLIAMS, N. J.; MILLER, W. G.; LEATHERBARROW, A. J. H.; BENNETT, M.; HALL, N.; ASHELFORD, K. E.; WINSTANLEY, C. Exploring the diversity of *Arcobacter butzleri* from cattle in the UK using MLST and whole genome sequencing. **PLoS ONE,** v. 8: e55240. doi:10.1371/journal.pone.0055240). 2013.

ON, S. L. W. Identification methods for campylobacters, helicobacters, and related organisms. **Clin Microbial Rev,** v. 9, p. 405-422. 1996.

VANDAMME, P.; FALSEN, E.; ROSSAU, R.; HOSTE, B.; SEGERS, P.; TYTGAT, R.; DE LEY, J. Revision of *Campylobacter*, *Helicobacter*, and *Wolinella* taxonomy: emendation of generic descriptions and proposal of *Arcobacter* gen. nov. **Int J Syst Bacteriol,** v. 41, p. 88-103. 1991. https:/doi.org/10.1099/00207713-41-1-88

VANDENBERG, O.; DEDISTE, A.; HOUF, K.; IBEKWEM, S.; SOUAYAH, H.; CADRANEL, S.; DOUAT, N.; ZISSIS, G.; BUTZLER, J.-P. VANDAMME, P. *Arcobacter* species in humans. **Emerg Infect Dis,** v. 10, p. 1863-1867. 2004. https:/doi.org/10.3201/eid1010.040241

WHITEDUCK-LÉVEILLÉE, K.; WHITEDUCK-LÉVEILLÉE, J.; CLOUTIER, M.; TAMBONG, J. T.; XU, R.; TOPP, E.; ARTS M. T.; CHAO, J.; ADAM, Z.; LÉVESQUE, C. A.; LAPEN, D. R.; VILLEMUR, R.; TALBOT, G.; KHAN, I. U. H. *Arcobacter lanthieri* sp. nov., isolated from pig and dairy cattle manure. **Int J Syst Evol Microbiol,** v. 65, p. 2709-2716. 2015. https:/doi.org/10.1099/ijs.0.000318