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Research Note

Isolation and Identification of *Arcobacter* Species from Costa Rican Poultry Production and Retail Sources

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ABSTRACT

Arcobacter is a gram-negative rod recognized as a potential food- and waterborne pathogen; nevertheless, little is known about the effects of this pathogen on human and animal health. Although *Arcobacter* species are commonly found in nature, poultry is suspected to be the main vehicle for the transmission of this pathogen. The aims of this work were to determine the prevalence of *Arcobacter* spp. in broilers produced in Costa Rica for human consumption and to analyze the pathogenic capacity of the isolates through the detection of virulence genes. One hundred fifty-two samples of cecal content (87 farms), 104 samples of carcass rinse after chiller (six processing plants), and 96 carcass rinses from as many retail stores were analyzed. The suspicious isolates were identified using genus-specific PCR, and species-level identification was achieved with a multiplex PCR. Virulence genes were identified using the protocol described by L. Douidah, L. de Zutter, J. Baré, P. De Vos, P. Vandamme, O. Vandenberg, A.-M. Van den Abeele, and K. Houf (*J. Clin. Microbiol.* 50:735–741, 2012), which includes nine different virulence genes. The overall isolation frequency of *Arcobacter* was 6.5% ($n = 23$). Eight (34.8%) of the isolates came from cecal content, 2 (8.7%) were isolated from samples taken after chiller, and 13 (56.5%) were from retail stores. The species isolated included *A. thereius* (30.4%), *A. butzleri* (21.7%), *A. skirrowii* (4.3%), and *A. cibarius* (4.3%). The remaining samples were classified as *Arcobacter* sp. Gene *tlyA* was the most prevalent virulence gene, present in 9 of 23 samples analyzed; genes *hecA* and *pldA* were present in one only strain each. A strain of *A. butzleri* isolated from a retail store presented the highest number of virulence genes (five), and 11 samples did not present any of the genes analyzed. The results obtained suggest that the presence of virulent *Arcobacter* isolates in the poultry production chain from Costa Rica could be a risk for individuals who consume the contaminated product.

Key words: *Arcobacter*; Chicken production line; Virulence genes

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Arcobacter is a gram-negative rod that is recognized as a potential food- and waterborne pathogen *(23)*. Nevertheless, little is known about the effects of this pathogen on human and animal health. This bacteria was formerly known as aerotolerant *Campylobacter*-like bacteria *(19),* and the *Arcobacter* genus was proposed by Vandamme et al. *(25)* more than 20 years ago. *Arcobacter* species have been isolated worldwide. Reports of its presence in diverse products of animal origin include poultry *(3),* beef *(21),* pork *(26),* shellfish *(5),* and milk *(13)*. Similarly, *Arcobacter* isolation from drinking water polluted with fecal matter has been reported *(6, 15)*.

Arcobacter isolation from meat products has been reported in various countries, including New Zealand, the United States, Turkey, Australia, and Mexico *(1)*. Poultry is the meat most often associated with the spread of this pathogen. Isolation frequencies of *Arcobacter* from poultry vary from 23 to 100% *(1)*. Costa Rica has also reported the isolation of this bacteria from poultry meat *(2),* with isolation frequencies that vary from 17.3% in the viscera to 56% in chicken breast; the species *A. butzleri, A cryaerophilus,* and *A. skirrowi* are the most common isolates *(8, 10)*.

In Costa Rica, poultry production is a growing industry for both the domestic and the international market. The annual per capita consumption of poultry meat or poultry products in Costa Rica is 23.42 kg, which is higher than the consumption rates reported for other Central American and South American countries *(30)*. Higher consumption of poultry meat in Costa Rica may represent a risk for consumers if the product is contaminated with *Arcobacter* and other bacterial pathogens associated with this type of food product. The isolation and characterization of *Arcobacter* from poultry products could help to better estimate the risk for the population. Although, in 2002, the International Commission of Microbiological Specifications for Food recognized *Arcobacter* as a risk for human health, little is known about the virulence mechanisms and pathogenicity associated with this microorganism. Better descriptions of virulence genes

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such as *cadF, ciaB, cj1349, hecA, hecB, irgA, mviN, pldA, tlyA,* and *iroE* found in *A. butzleri* could be made after the most recent descriptions of the genome of this pathogen. Some of these virulence genes present strong homology with similar genes found in *Campylobacter jejuni,* whose functionality is already known *(11)*.

The aim of this work was to determine the prevalence of *Arcobacter* spp. in broilers for human consumption, using a simultaneous sampling design in three levels of the Costa Rican poultry production chain: farm, processing plant, and point of sale. Also, this study determined the presence and frequency of several virulence genes in the isolates obtained in order to define their potential risk as agents of foodborne disease *(18)*.

MATERIALS AND METHODS

Sampling. A cross-sectional study was conducted from March to July 2015 to determine the national prevalence of *Arcobacter* spp. in broilers for human consumption, using a simultaneous sampling design in three levels of the Costa Rican poultry production chain. The Win Episcope program *(24)* was used to determine the number of samples to analyze. One hundred fifty-two samples of cecal content (87 farms), 104 samples of carcass rinse after chiller (six processing plants), and 96 carcass rinses from as many retail stores were collected.

Processing plants were sampled from throughout the country, including 11 from Alajuela, 2 from Heredia, 2 from Puntarenas, 2 from San José, and 1 from Limón (Zumbado 2014 *(31)*); samples from points of sale included 48 from municipal markets and 48 from retail stores. Samples were transported at 4 to 6°C to the Food Microbiology Laboratory, University of Costa Rica, and were processed within 24 h.

Bacteriological isolation. Each sample was rinsed with 90 mL of 0.1% sterile peptone water; of this, 1-mL aliquots were transferred to tubes containing 10 mL of Houf selective broth (Oxoid, Ogdensburg, NY) *(14)*. Enrichment media were incubated aerobically at 30°C for 48 h. After this, *Arcobacter* isolation was performed using the membrane filtration technique. Briefly, 100 μL of enrichment media was filtered through sterile 0.45-μm-pore-size nitrocellulose membrane filters that were subsequently placed on the surface of nonselective blood agar plates. The plates were left at room temperature for 1 h, so that *Arcobacter* cells, owing to their mobility, could pass through the membrane filter. After removing the filter, plates were incubated aerobically at 30°C for up to 5 days. Suspicious colonies were selected, and gram-negative, oxidase-positive isolates were purified on blood agar before performing PCR identification.

Molecular identification of *Arcobacter*. DNA extraction was performed using the boiling lysis method. The PCR technique described by Harmon and Wesley *(12)* was used for identification at the genus level. Briefly, PCR amplification was performed in reaction mixture (50 μL) containing Tris-HCl buffer (pH 7.4), 0.2 mM deoxynucleoside triphosphates (dNTPs), 1.5 mM $MgCl_2$, 1 μM each primer, 1.5 U/μL *Taq* polymerase (Oxoid, Basingstoke, England), and 5 μL of template DNA. The *Arcobacter* genus–specific 16S rRNA fragment was amplified using the forward primer Arco I (5′-AGA GAT TAG CCT GTA TTG TAT C-3′) and the reverse primer Arco II (5′-TAG CAT CCC CGC TTC GAA TGA-3′) *(19)*. The thermocycling program was 94°C for 4 min; then 25 cycles of 94°C for 1 min, 56°C for 1 min, and 72°C for 1 min; and 72°C for 7 min. Analysis of PCR products was done by electrophoresis (60 V, 1.5 h) in 1.5% agarose gels (w/v) with Mass Ruler (100 to 1,000 bp; Fermentas, St. Leon-Rot, Germany) and Fast Red staining (DakoCytomation, Glostrup, Denmark). Positive *Arcobacter* identification was reported when a product of 1,223 bp was obtained.

Positive isolates were then analyzed to species level with a multiplex PCR. Amplification of the specific fragments was done with the forward primer Arco F (5′-GCT AGA GGA AGA GAA ATC AA-3′) and the reverse primers ButR (5′-TCC TGA TAC AAG ATA ATT GTA CG-3′), TherR (5′-GCA ACC TCT TTG GCT TAC GAA-3′), CibR (5′-CGA ACA GGA TTC TCA CCT GT-3′), and SkiR (5′-TCA GGA TAC CAT TAA AGT TAT TGA TG-3′) for *A. butzleri* (2,061 bp), *A. thereius* (1,590 bp), *A. cibarius* (1,125 bp), and *A. skirrowii* (198 bp), respectively *(11)*. Amplification of the 395-bp fragment from *A. cryaerophilus* was done with the primers CriF (5′-CAG AGG AAG AGA AAT CAA AT-3′) and CriR (5′-CCC ACT ATT CCA TCA GTG AG-3′) *(11)*. Template preparation and reaction mixture were as described above. The thermocycling program was 94°C for 4 min; then 30 cycles of 94°C for 45 s, 58°C for 45 s, and 72°C for 2 min; and finally, 72°C for 7 min. The size of the products was estimated by electrophoresis (120 V, 2 h) in 2% agarose gels (w/v) using the Mass Ruler (100 to 1,000 bp; Fermentas) and Fast Red staining (DakoCytomation).

Identification of virulence genes. Virulence genes were identified using the protocol described by Douidah et al. *(7)*, which includes nine different virulence genes. Primers used are described in Table 1.

PCR amplification was performed in reaction mixture (50 μL) containing Tris-HCl buffer (pH 7.4), 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 2 μM each primer, 1.5 U/μL *Taq* polymerase (Oxoid, Ogdensburg, NY) and 2 μL of template DNA.

The thermocycling program was 94°C for 3 min; then 32 cycles of 94°C for 145 s, 56°C for 45 s, and 72°C for 45 s; and finally, 72°C for 3 min. Analysis of PCR products was done by electrophoresis (60 V, 1.5 h) in 1.5% agarose gels (w/v) with Mass Ruler (100 to 1,000 bp; Fermentas) and GelRed (Biotium, Hayward, CA) staining.

RESULTS

A total of 23 strains of *Arcobacter* were isolated from the 352 samples analyzed (6.5% frequency). Eight (34.8%) of the isolates came from cecal content, two (8.7%) were isolated from samples taken after chiller, and 13 (56.5%) were from retail stores.

Multiplex PCR allowed classification of these isolates as *A. thereius* (30.4%), *A. butzleri* (21.7%), *A. skirrowii* (4.3%), and *A. cibarius* (4.3%). The remaining samples were classified as *Arcobacter* sp.

Results obtained for virulence genes are shown in Table 2. Note that gene *tlyA* was the most prevalent, present in 9 of the 23 samples analyzed, and that genes *hecA* and *pldA* were least prevalent, isolated from only one strain each. A strain of *A. butzleri* isolated from a retail store presented the highest number of virulence genes, with five of the nine genes analyzed at the same time. Eleven samples did not present any of the genes analyzed.

DISCUSSION

A 6.5% isolation frequency was obtained for *Arcobacter* species for the whole production chain. Isolates were

TABLE 1. *Sequence of nucleotides used as primers for the detection of virulence genes of* Arcobacter

Primer	Target gene	Sequence	Size (bp)
cadF-F	*cadF*	TTACTCCTACACCGTAGT	283
cadF-R		AAACTATGCTAACGCTGGTT	
ciaB-F	*ciaB*	TGGGCAGATGTGGATAGAGCTTGGA	284
ciaB-R		TAGTGCTGGTCGTCCCACATAAAG	
cj1349-F	*cj1349*	CCAGAAATCACTGGCTTTTGAG	659
cj1349-R		GGGCATAAGTTAGATGAGGTTCC	
mviN-F	*mviN*	TGCACTTGTTGCAAAACGGTG	294
mviN-R		TGCTGATGGAGCTTTTACGCAAGC	
pldA-F	*pldA*	TTGACGAGACAATAAGTGCAGC	293
pldA-R		CGTCTTTATCTTTGCTTTCAGGGA	
tlyA-F	*tlyA*	CAAAGTCGAAACAAAGCGACTG	230
tlyA-R		TCCACCAGTGCTACTTCCTATA	
irgA-F	*irgA*	TGCAGAGGATACTTGGAGCGTAACT	437
irgA-R		GTATAACCCCATTGATGAGGAGCA	
hecA-F	*hecA*	GTGGAAGTACAACGATAGCAGGCTC	537
hecA-R		GTCTGTTTTAGTTGCTCTGCACTC	
hecB-F	*hecB*	CTAAACTCTACAAATCGTGC	528
hecB-R		CTTTTGAGTGTTGACCTC	

obtained from the three different sources, with those from retail stores having the higher isolation rates.

The frequency obtained is smaller than in previous studies reported in Costa Rica. Fallas et al. *(8)* reported a 56% frequency from chicken breast samples and Villalobos et al. *(27)* a 17.3% frequency from chicken viscera; both of these works were done in chicken obtained from retail stores. The isolation frequency obtained in this work contrasts with the 95% prevalence in Turkey determined by Atabay et al. *(3),* 23% in Japan reported by Kabeya et al. *(16),* and 0% in Mexico found by Villarruel-López et al. *(28)*.

In this study, the most frequently isolated species was *A. thereius,* followed by *A. butzlerii*. The finding of the first is unusual in poultry; *A. butzlerii* is reported to be the most frequently isolated species, followed by *A. cryaerophilus (13)*. These differences in isolation frequencies may be explained in terms of differences in sample size, isolation methodologies, and the hygienic conditions of retail stores and processing plants *(3)*.

Of the total number of samples analyzed, 13 (56.5%) were positive in retail stores, 2 (8.7%) in carcasses after chiller treatment in the plant, and 8 (34.8%) in cecal content. Cross-contamination during meat handling and the use of contaminated equipment are practices that may allow the spread of these bacteria at the retail level *(1, 21)*. Also, *Arcobacter* has the ability to adhere to different surfaces and form biofilms that protect it and stimulate further spread *(23)*.

The isolation of these bacteria from animal carcasses has been reported, most frequently in poultry, followed by pork and bovine meat. The low level of isolation from samples after chiller treatment shows the high resistance of these bacteria to usual decontamination processes, including the use of sodium hypochlorite and low temperatures *(4)*.

The low level of isolation of *Arcobacter* from cecal content shows that poultry meat is largely contaminated after sacrifice *(20)*. A possible explanation for this is that the bacteria goes through the digestive tract of poultry but does not colonize it, possibly because of the high corporal temperature of birds (41°C) *(29)*; nevertheless, its dissemination through feces or after sacrifice is evident *(9, 13)*.

Virulence genes are denominated as putative due to the amino acid structures and functions corresponding to other genomic structures that have already been characterized *(22)*. According to the data obtained, genes *mviN* and *tlyA* were the most frequent in the samples analyzed, followed by *ciaB, hecB,* and *cadF*; this correlates with other research done worldwide *(11)*. *mviN* gene traduces the mviN protein and is related with peptidoglycan synthesis in the host's cell, helping to create cellular lysis; *tlyA* generates a pore in the host's cellular membrane, facilitating lysis. Note that 75% of the *A. butzleri* isolates coming from retail stores had virulence genes, and of these, 100% were positive for *cadF, ciaB, mviN,* and *tlyA*. Similar frequencies have been reported by Karadas et al. *(17)*.

TABLE 2. *Frequency of different virulence genes from* Arcobacter *isolates collected from poultry in Costa Rica*[a]

Source	*cadF*	*ciaB*	*cj1349*	*irgA*	*hecA*	*hecB*	*mviN*	*pldA*	*tlyA*
After chiller	0/2	1/2	1/2	1/2	0/2	1/2	1/2	1/2	1/2
Cecal content	1/8	2/8	0/8	1/8	0/8	2/8	1/8	0/8	1/8
Retail stores	3/13	5/13	1/13	0/13	1/13	3/13	7/13	0/13	7/13

[a] Number of isolates containing virulence gene/number of isolates tested.

Growth conditions have been shown to be an important factor in the determination of virulence factors. Rivera *(22)* says that the microaerophilic incubation of Bolton and blood agar plates at 37°C is the optimum condition for determining virulence genes in poultry samples, a factor that may cause divergence in the frequencies reported worldwide.

The results obtained from this study demonstrate that *Arcobacter* isolates with virulence features are present at the three levels of the poultry production chain in Costa Rica. These findings could motivate future research to understand the risk for consumers and to devise control strategies for *Arcobacter* at both industrial and commercial levels.

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