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Molecular investigation of Arcobacter species isolated from some poultry products in Menofia governorate.

EL-toukhy, E.I.[*]; Dalia, A. Salim[**] and Samar, M.M. Moustafa[***]

[*]Biotechnology Department, Animal Health Research Institute, ARC
[**]Food hygiene Department, Animal health research institute, Shebin El kom.
[***]Lecturer, Department of Zoonoses, Faculty of Vet. Med. Benha University.

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Abstract

A total of 120 fresh random samples of chicken liver, chicken gizzard and chicken fillet,40 of each were collected from different poultry slaughter shops in Menofia governorate in sterile bags , placed in ice box and sent to the laboratory under complete aseptic conditions for microbiological examination. A total of 29(24.1%) of samples was contaminated with *Arcobacter spp.* chicken liver samples were the most contaminated 12 (30%) followed by chicken fillet 9 (22.5%) then chicken gizzards 8 (20%). The isolates were confirmed by multiplex PCR using species specific primers. *A. butzleri, A. cryaerophilus* and *A. skirrowii* were present with different percentages in the examined samples, *A. butzleri* and *A. cryaerophilus* were detected in chicken liver 66.6% and 33.3% respectively and present in chicken fillet 33.3% and 66.7% respectively. *A. butzleri* and *A. skirrowii* were detected (50% in each) in chicken gizzard.

Keywords: *Molecular investigation, Arcobacter species, poultry products.*

Introduction

The current validated taxonomy places the *Arcobacter* genus within the *Campylobacteraceae* family (belonging to the class *Epsilonproteobacteria* of the phylum *Proteobacteria*) together with *Campylobacter* and *Sulfurospirillum* genera**. (Fanelli *et al*., 2019)** *.A rcobacter* spp. has been identified as an emerging food-borne zoonotic pathogen worldwide **(Ho *et al*., 2006)** and associated with enteritis and abortion in animals and bacteraemia, gastroenteritis and diarrhea in humans **(Jiang *et al.*, 2010; Figueras *et al.*, 2014** and **Van denAbeele, 2014).** Three species of Arcobacter namely *A. butzleri, A. cryaerophilus* and *A. skirrowii* are more commonly associated with clinical conditions **(Collado and Figueras 2011**and **Ramees *et al*., 2017).** In particular, *A. butzleri* has been classified as a serious hazard to human health by the International Commission on Microbiological Specifications for Foods **(ICMSF, 2002)**. *Arcobacter spp*. have been isolated from a variety of food products for human consumption (chicken, pork, beef meat, raw milk and dairy products, seafood, vegetables) **(Ramees *et al.*, 2017)**. Chicken meat particularly has been reported with highest prevalence for *Arcobacter* spp. *(A. butzleri* is the most prevalent) followed by pork and beef **(Shah *et al.*, 2011)** Like for Campylobacter, a high prevalence of *A. butzleri* and *A. cryaerophilus* is observed on chicken carcasses both from markets and abattoirs **(Kabeya *et al*., 2004)**. The bacteria were detected on almost all slaughter house carcasses tested during a large-scale study in Belgium and up to several thousand bacteria were present per gram of neck skin prior to evisceration **(Houf *et al*., 2002)**. Although a high prevalence of *Arcobacter* species on chicken meat is reported worldwide, the bacteria are rarely detected in the intestinal contents of these animals. It was therefore assumed that contamination of meat probably arises from other sources and occurs during processing (**Atabay and Corry, 1997)**. To de-

tect the source of carcass contamination *Arcobacter* species were recovered from 85% of swab samples taken before slaughter and after a period of a few days with no slaughter activity **(Houf *et al.*, 2002)**. Before the onset of slaughter, higher numbers of bacteria were detected on the last 2 days of a working week compared to the first day, which might indicate accumulation of contaminating bacteria on the processing line due to insufficient decontamination **(Houf *et al.*, 2003).** The bacteria were also recovered from the water outlet of defeathering machines, the neck-skin cutter and the washer. Molecular approaches have been used to analyse the relationship between isolates from environmental sources and carcasses and to track the route of transmission, but the data was not conclusive probably due to the high genetic heterogeneity of *Arcobacter* species. Thus despite many attempts, the original source of the high contamination level of chicken carcasses and the way of spreading the contamination still remain unknown **(Hoa *et al.*, 2006)**. *A. butzleri* could still be detected on the surface of the housing several weeks after the birds were removed suggesting a high capacity for survival in the environment. Therefore, contaminated farming facilities and insufficient hygiene probably add to the transmission of the bacteria through farms and among stocks.

A great interest for *Arcobacter* in veterinary and human public health enhanced since the first report of the isolation of *Arcobacter* from food of animal origin (**de Boer *et al.*, 1996)**. Since then, studies worldwide have reported the occurrence of *Arcobacter* on food and have highlighted the possible transmission to the human population, so the aim of this work is the isolation and molecular identification of *Arcobacter* spp. from some poultry products in Menofia governorate.

Because of high sensitivity, specificity, and rapid results, PCR assays often are used instead of conventional microbiological culture methods to detect specific types of microorganisms in foods, water, and environmental samples **(Hill, 1996).**
:
A number of PCR assays have been developed for the detection and identification of *Arcobacter* species **(Gonza´lez *et al.* 2000)** Multiplex PCR (mPCR) systems targeting the 16S rRNA genes have been described for the simultaneous detection and identification of different *Arcobacter* species (**Harmon and Wesley (1997).** PCR assays to detect all members of the genus *Arcobacter* and that are specific for each *Arcobacter* species have been reported. Based on the knowledge of the *Arcobacter* nucleic acid composition of the 16S rRNA, a PCR product of 401-bp was generated for *A. butzleri*, 257-bp for *A. cryaerophilus* and 641-bp for *A. skirrowii*. Those three species were also identified by the PCR assay developed by **(Kabeya *et al.*, 2003).**

Materials and Methods

Sample collection: A total of 120 fresh random samples each of 40 chicken liver, chicken gizzard and chicken fillet were collected from different poultry slaughter shops in Menofia governorate in sterile bags, placed in ice box and sent to the laboratory under complete aseptic conditions for microbiological examination.

Preparation of samples (ISO 6887-1:2017 (E)): Poultry products were prepared for microbiological analysis by homogenizing 25 gm of the examined products for 2 minutes without diluents using stomacher blender (*Blender, Steward Laboratories, London, England)*, diluted in 225 ml of 0.1% peptone water as described by and homogenized for an additional 2 minutes. .

Isolation of *Arcobacter* species (Johnson and Murano, 1999): One ml of each homogenized fluid samples was aseptically inoculated into sterile test tubes containing 9 ml into BHI broth containing *Arcobacter* species growth supplement **[**CAT (cefoperazone, amphotericin, and teicoplanin) selective supplement (catalog no. SR0174E oxoid), then incubated at 25°C for 48–72 hours. Sub-culturing was carried out on BHI agar plates enriched with 5-10 % sheep blood and containing *Arcobacter* species growth supplement and incubation at 25°C for 48 hours. The growth was examined for typical *Arcobacter* species colonies Arcobacter -like colonies (round, 2–4 mm grey to whitish) were picked for phenotyping according the

standard biochemical tests recommended by **(Kayman, 2012)**.

Phenotypic identification of *Arcobacter* species:
To confirm the presence of *Arcobacter* species on suspected isolates, gram stained films, motility test, biochemical reactions, including catalase production, oxidase production, urease production, nitrate reduction and growth profile, including growth with 1% glycine, growth with 2%NaCl, grow that 25°C, growth at 37°C, and growth at anaerobic atmosphere were used.

Identification of isolated strains by polymerase chain reaction (PCR):
Extraction of genomic DNA: Genomic DNA Extraction using QIA amp kit**: (Shah *et al.*, 2009).**

Primer sequences used for PCR system according to Houf *et al.*, (2000):
Specific 16S rDNA fragments for *A. butzleri*, *A. skirrowii* as well as for *A. cryaerophilus* were applied for demonstration and characterization of such strains

Fragment	Primer	Oligonucleotide sequence (5′ → 3′)	Product size (bp)
A. butzleri 16S rDNA	BUTZ (F)	**5′** CCT GGA CTT GAC ATA GTA AGA ATGA **′3**	**401**
	ARCO (R)	**5′** CGTATTCAACCGTAGCATAGC **′3**	
A. skirrowii 16S rDNA	SKIR (F)	**5′** GGCGATTTACTGGAACACA **′3**	**641**
	ARCO (R)	**5′** CGTATTCACCGTAGCATAGC **′3**	
A. cryaerophilus 16S rDNA	CRY1 (F)	**5′** TGCTGGAGCGGATAGAAGTA **′3**	**257**
	CRY2 (R)	**5′** AACAACCTACGTCCTTCGAC **′3**	

Amplification reaction of *Arcobacter* species (Wesley *et al.*, 1995):
PCR reactions were performed in a reaction mixture (50 µl volume) containing 2 µl of lysed bacteria, 5 µl of Gibco BRL 10U PCR buffer, 1.5 µl of Taq DNA polymerase (Gibco), 0.2 mmol l31 of each deoxyribonucleotide triphosphate, 1.3 mmol l31 MgCl2, 5 Wl of loading buffer (4 mM cresol red, 60% sucrose) and 50 pmol of the primers ARCO, BUTZ, CRY1, CRY2, and 25 pmol of primer SKIR.

The amplification was conducted with an initial DNA denaturing step at 94°C for 2 min, followed by 32 amplification cycles of denaturation (94°C for 45sec), primer annealing (61°C, 45 sec) and primer extension (72°C, 30 sec). A final step at 72°C for 5 min was included tonsure full extension of the product.
Detection of PCR products: amplified products were detected in 1.5% agarose gel electrophoresis pre-stained with ethidium bromide, at 80 V for 1 hour. The gel was photographed by using gel documentation system.

Results

Table (1). Prevalence of *Arcobacter species* in the examined poultry product samples

Types of poultry products	No. of examined samples	suspected samples by culture method		positive samples by bio-chemical test	
		No.	%	No.	%
Chicken liver	40	18	**45**	12	**30**
Chicken gizzard	40	10	**25**	8	**20**
Chicken fillet	40	15	**37.5**	9	**22.5**
Total	120	43	**35.8**	29	**24.1**

Table (2). Identification of Arcobacter species by using PCR:

Arcobacter spp / Poultry products	*A. butzleri*		*A. cryaerophilus*		*A. skirrowii*	
	No.	%	No.	%	No.	%
Chicken liver	8/12	66.6	4/12	33.3	0	0
Chicken gizzard	4/8	50	0	0	4/8	50
Chicken fillet	3/9	33.3	6/9	66.7	0	0

PCR Results

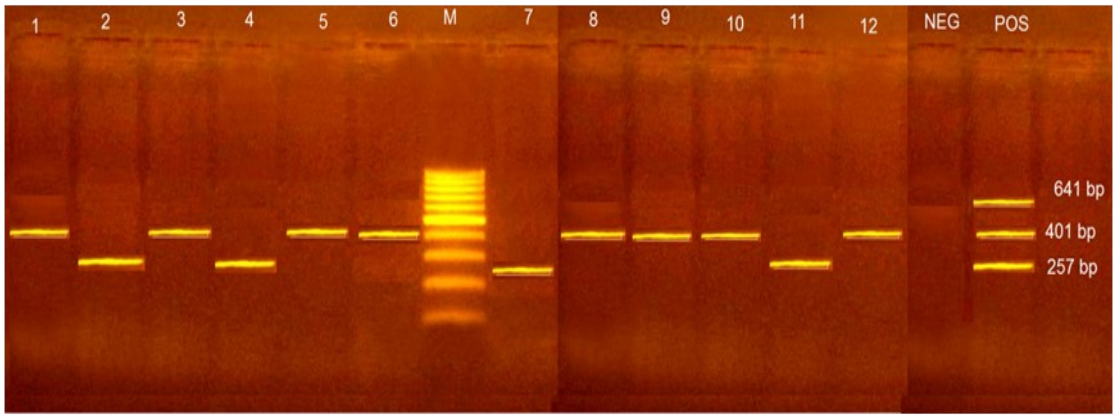

Fig. (1): Agarose gel electrophoresis 1.5% showing PCR products *of* the *Arcobacter spp.* in chicken liver samples.
Lane M: 100 bp molecular weight marker DNA Ladder.
Lane neg.: negative control.
Lane pos.: positive control.
Lanes 2, 4, 7, 11 are positive samples for ***A. Cryaerophilus*** giving a band at 257 bp. Lanes 1, 3, 5, 6, 8, 9, 10, 12 are positive samples for ***A. Butzleri*** giving band at 401 bp .

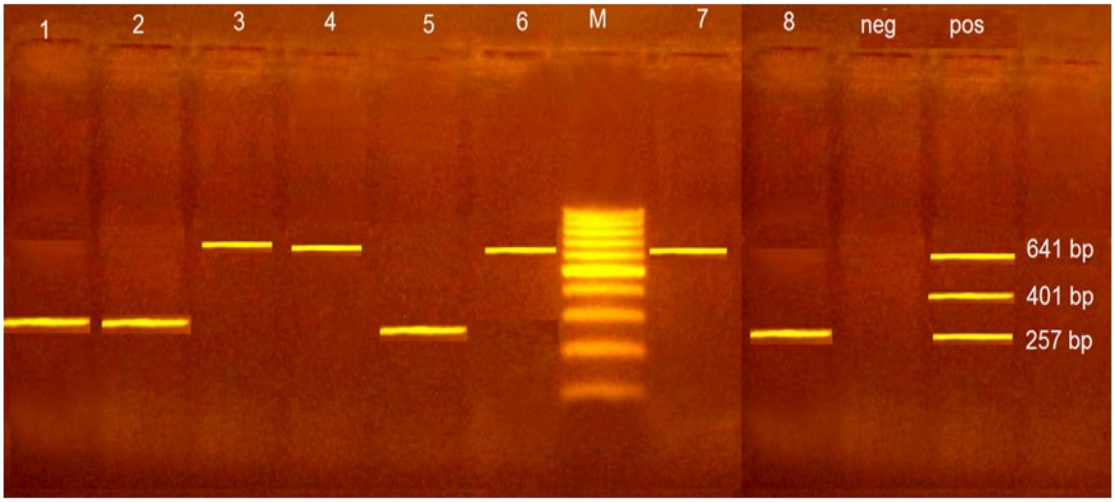

Fig. (2): Agarose gel electrophoresis 1.5% showing PCR products *of* the *Arcobacter spp.* in chicken gizzard samples.
Lane M: 100 bp molecular weight marker DNA Ladder.
Lane neg.: negative control.
Lane pos.: positive control.
Lanes 1, 2, 5, 8 are positive samples for ***A. Cryaerophilus*** giving a band at 257 bp. Lanes 3, 4, 6, 7 are positive samples for ***A. Skirrowii*** giving band at 641bp .

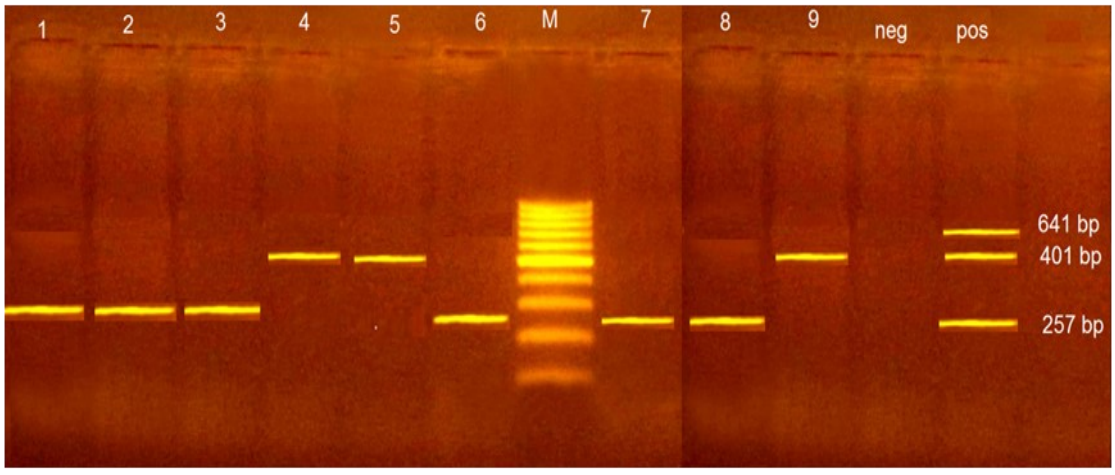

Fig. (3): Agarose gel electrophoresis 1.5% showing PCR products *of* the *Arcobacter spp.* In chicken fillet samples.
Lane M: 100 bp molecular weight marker DNA Ladder.
Lane neg.: negative control.
Lane pos.: positive control.
Lanes 1, 2, 3, 6, 7, 8 are positive samples for ***A. Cryaerophilus*** giving a band at 257 bp. Lanes 4, 5, 9 are positive samples for ***A. Butzleri*** giving band at 401bp.

Discussion

Arcobacter, like thermo-tolerant campylobacter's, have been reported more frequently from poultry products than from red meat. Recent studies have indicated that also *Arcobacter* are common on broiler carcasses. *Arcobacter* species has newly emerged meat-borne pathogen which mainly transmitted to consumers throw handling and consumption of foods of animal origin especially fresh chicken meat and their products.**(Abdelrahman *et al*., 2012).** *Arcobacter* spp. are considered 'emerging' pathogens based on the characteristics they share with Campylobacters, potentially extending from morphological similarities to infectious capabilities and transmission routes, **(Wesley, 1996).**

Arcobacters are biochemically inert and have fastidious growth requirements, which make their speciation problematic using standard phenotypic procedures, (**On *et al.,* 1995). Vytrasova*et al*. (2003)** stated that biochemical tests alone are not adequate to confirm *Arcobacter*spp., unless they are followed by PCR assay. The reasons were explained by **Milesi, (2010)** who mentioned that differentiating of *Arcobacter* spp. by using phenotypic tests might give false results because of the shortage of clear-cut differentiating tests, a phenomenon which has also been observed in the closely related genus *Campylobacter*. So, table (1) discussed the prevalence of Arcobacer species in poultry products in which the suspected samples by culture method were 45,25 and 37.5% for chicken liver, chicken gizzard and chicken fillet, respectively. Followed by biochemical test in which the positive samples were 30, 20and 22.5% for chicken liver, chicken gizzard and chicken fillet, respectively. In contrast, **Atabay *et al.* (2003)** mentioned that the occurance of *Arcobacter spp.* on fresh chicken carcasses was at a much higher contamination level (95%). Differences in published recovery rates of *Arcobacter* in poultry may be attributed to multiple factors, such as geographic and seasonal variation **(Collins, *et al*. 1996)** and **(Gonza´lez *et al*. 2000)**. variation in hygienic conditions during production and processing, or differences in the sensitivity and specificity of the isolation methods used **(Atabay *et al.* (2003)**. The differences among isolation methods seems to be the most probable cause .

The isolates were confirmed by multiplex PCR using species specific primers. *A. butzleri, A. cryaerophilus* and *A. skirrowii* were present in the examined samples. *A. butzleri* and *A. cryaerophilus* were detected in chicken liver 66.6% and 33.3% respectively (Fig 1) and present in chicken fillet 33.3% and 66.7% respectively (Fig 2). *A. butzleri* and *A. skirrowii* were detected in chicken gizzard 50% in each **(Fig 3)**.

Abdelrahman *et al.* (2012) showed that *Arcobacter* species were isolated from 72.7% (32/44), 66.7% (32/48) and 100% (40/40) of the examined chicken carcass, chicken liver and chicken burger samples respectively. While**, Ammar and AL-Habaty (2015)** examined 75 fresh samples of beef, minced beef, and fish (*Oreochromis niloticus*) ,47 % of samples were contaminated with *Arcobacter spp*. Beef was the most contaminated 13(52%) followed by minced beef 12(48%) then fish 10 (40%). By genotyping using PCR, only a total 11 (15%) of samples harbor *Arcobacter* spp. Five (20%) of beef, 2 (8%) of minced beef and 4 (16%) of fish were contaminated with *Arcobacter spp*.

Since only some *Arcobacter* strains grow at 41°C, it is feasible that the high body temperature of birds inhibited or suppressed *Arcobacter* growth and colonization. The origin of the almost ubiquitous presence of *Arcobacter* on poultry carcasses is still under discussion as the transmission routes of these bacteria are still not established. In contrast to the related *Campylobacter*, for which the contamination at broiler house level is well documented and easily detected by conventional

microbiological methods, *Arcobacter* seem however to display a different behavior. Several authors have suggested that *Arcobacter* are probably not normal inhabitants of the poultry intestine **(Eifert *et al.,* 2003**, **Houf *et al*., 2003**, **Gude *et al*., 2005**, and **Van Driessche and Houf, 2007)**. *Arcobacter* spp. are found on equipments along the processing line and in

processing water, water in the scalding tank, water outlets of the defeathering machine and washers **(Gude *et al.*, 2005)**. The bacteria were also isolated from the transportation crates used by flocks.

In this work, the isolated Arcobacer species from positive samples by PCR were *A. butzleri*, *A. cryaerophilus* and *A. skirrowii*. *A. butzleri* and *A. cryaerphilus* could be detected from chicken liver with (66.6 and 33.3%) and chicken fillet with (33.3 and 66.7%) while *A. butzleri* and *A. skirrowii* could be detected with 50% for each from chicken gizzard **(table 2)**. Patients with *A. butzleri* infection have reported suffering from diarrhoea associated with abdominal pain, with the occurrence of a variety of symptoms including nausea, vomiting and fever **(Vandenberg *et al*. 2004).** *A. skirrowii* has been isolated from a human stool sample from a 73-year-oldman with a prosthetic aortic heart valve, who was admitted to the hospital after 2 months of persistent diarrhea **(Wybo *et al.* 2004).**

Conclusion

This study revealed that the chicken liver, gizzard and fillet from the retail markets are critical sources of *Arcobacter spp.* that may have a role in the contamination of the environment and human food chain. *A. butzleri, A. cryaerophilus* and *A. skirrowii* could be isolated with different percentages**.** PCR is an efficient method to detect the molecular diversity of *Arcobacter* species. Further efforts are required to examine cases with diarrheal illness to elucidate the role of *A. butzleri* in veterinary public health. Such epidemiologic data is important for preventive roles and control of diarrheal diseases.

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