Isolation and Identification of *Arcobacter* species Recovered from Rabbits in Zagazig, Egypt

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ABSTRACT

Although *Arcobacter* species is emerging foodborne pathogen, the role of rabbit as a source of Arcobacter infections is unknown. The present study was designed to study the isolation rate of *Arcobacter* species in rabbit and its drinking water.The molecular fingerprinting of 10 *Arcobacter*species isolates was carried out by ERIC-PCR technique. From 50 samples including 20 rabbit meat, 20 rabbit stool and 10 drinking waters, 36 (72%) *Arcobacter* species strains were isolated. Recovered isolates from the examined meat, stool and water samples were 15, 20 and 0 with the percentages of 75, 100 and 0, respectively. Concerning, *A. butzleri*, it was isolated from 2(10%) of meat samples and 3(15%) of stool samples. *A. skirrowii*was isolated from meat and stool with the frequency of 6(30%) and 8(40%), respectively. *A. cryaerophilus* was isolated from 7(35%) and 9(45%), respectively.

ERIC-PCR grouped the total examined 10 isolates of *Arcobacter* species based on the presence or absence of the major amplified bands (A-M) ranged from >1400 bp to 200 bp. The conservative common bands in all isolates were 300 and 400 bp bands. It was found that there are both intra-species and inter-species molecular diversity among the examined *Arcobacter* species clones. The circulating *Arcobacter* species clones in the tested rabbit farm have multiple genotypes (9/10). This may be attributed to the variant sources of infections.

Key words: Arcobacter, isolation, rabbit, ERIC-PCR, water, Aerotolerant.

INTRODUCTION

Arcobacters are considered potential emerging food and waterborne pathogens.*Arcobacter* species are members of family Campylobacteriaceae, and cause a variety of diseases in human and animal. They have the ability to grow aerobically at 30^{o}C which is a distinctive feature that differentiates *Arcobacter* species from *Campylobacter*species ***(Gonzales and Ferrus, 2011)***. The genus Arcobacter is relatively new, proposed by ***Vandamme and De Ley (1991)***, and encompasses a group of organisms known initially as aerotolerant campylobacters ***(Amare et al, 2011)***.The genus Arcobacter currently contains 10 species, of which seven may be considered emerging human food-

borne pathogens. *A. butzleri*, *A. skirrowii*, *A. cryaerophilus*, *A. cibarius*, *A. mytili*, *A. thereius*, and A. *trophiarum* have all been isolated from foodstuffs, including meat , shellfish and water, or from the feces of livestock ***(Collado et al, 2009-a).*** ***A.****butzleri*, *A. skirrowii* and *A.cryaerophilus* have been isolated from human ***(Houf and Stephan, 2007),*** poultry products ***(Houf et al, 2005)*** and feces of healthy farm animals ***(Van Driessche et al, 2003)***. Furthermore, the majority of isolated Arcobacters belong to one of the three species *A. butzleri*, *A.cryaerophilus* or*A. Skirrowii* ***(Miller et al, 2009)***. Raw meat is considered as a source of Arcobacter infection in human ***(Gonzales and Ferrus, 2011)***. Rabbit meat production is developing. In Egypt, rabbit meat represents 2.9% of the total meat consumption ***(Yamani, 1990)***. *Arcobacter* species was previously studied in different sources other than rabbits in Egypt by ***Mohamed et al (2004)*** and ***Soliman (2006)***. *Arcobacter* species were isolated from rabbit meat in a prevalence of 10% in Spain ***(Collado et al, 2009-b)***. *Arcobacter* species was recorded in the most fecally contaminated groundwater wells that provided potable water to the public ***(Fong et al, 2007).*** Different methods have been applied for distinguishing one strain of Arcobacter from another, for studying transmission routes or for tracing sources of outbreaks, including several PCR methods, one of them the enterobacterial repetitive intergenic consensus (ERIC-PCR) ***(Houf et al, 2002)***. The most used typing technique has been the ERIC-PCR, which has been successfully applied for investigating outbreaks ***(Vandamme et al, 1993)***. The genetic diversity of *Arcobacter* species was previously studied using ERIC-PCR profiling ***(Collado et al, 2010)***.

The objective of this research was to study the prevalence of *Arcobacter* species in rabbit meat, feces and their drinking water. Moreover, genotypic characterization of the most common isolated *Arcobacter* species was carried out.

MATERIAL AND METHODS

Sample collection

A total of 50 different samples were collected from apparently healthy rabbit farm of Faculty of Vet Medicine, Zagazig University, Zagazig in January 2012 including rabbit meat (n=20),feces (n=20) and drinking water (n=10). The samples were immediately transported to the laboratory in a cool box and processed within 2-4 h of sampling.

Isolation media

Arcobacter enrichment broth (AEB) was prepared using Arcobacter enrichment basal media (Oxoid, CM965, Hmpshire, UK) with cefoperazone-amphotericin-teicoplanin (CAT) selective supplement (Oxoid, SR174E) as described previously ***(Atabay et al, 2002)***. Blood agar was prepared by

adding 5% (v/v) defibrinated sheep blood in blood agar base.

Isolation procedures

Samples preparation

Rabbit carcass samples

Each carcass sample was rinsed with sterile distilled water by thorough shaking for approximately 1 min. One ml portion of well mixed samples was inoculated into 9 ml portion of AEB supplemented with CAT.

Fecal samples

Each sample (1 g) was homogenized in a sterile saline. One ml of each suspension was then inoculated into 9 ml of AEB containing CAT supplement.

Drinking water samples

Tap water samples (1 ml from each sample) were added to 9 ml of AEB containing the CAT supplement.

Method of isolation *(Atabay et al, 2003)*

Isolation of *Arcobacter* species by microfilter techniques using sterile, individually packaged filter-AC (Sartorius Goettingen, 82122-001-51) by picking up the membrane filter together with the yellow protective disc using sterile forceps, fixed onto the blood agar plates, then the protective disc was removed. 120 µl portions were taken from each previously enriched homogenate dropped onto this microfilter membrane (pore size 0.45 µ) and were incubated aerobically at 30^{o} C for one hour and was allowed to filter passively. The filter was removed and the plates were incubated aerobically at 30^{o} C until visible colonies were obtained (up to 7 days).

Identification of presumptive Arcobacter isolates

Suspected colonies were picked and purified by subcultring onto blood agar (BA) and identified according to ***Atabay et al (2003)***. Isolates were preserved for molecular characterization.

Extraction of genomic DNA

Arcobacter isolates were grown on BA at 30 ^{o}C for 48 -72 h under microaerophilic conditions. After incubation, one or two colonies of each strain grown on BA plate was suspended in 1 ml of sterile distilled water and centrifuged for 5 min at 13000 RPM and the supernatant was discarded. DNA extract were prepared by re-suspending the cell pellets in 1 ml of sterile distilled water and boiling the suspension for 10 min, centrifugation, the supernatant was used as DNA templates in PCR.

ERIC PCR technique

Ten isolated *Arcobacter* species were genotyped using enterobacterial repetitive intergenic consensus (ERIC) PCR technique with the protocol described by ***Houf et al (2002).*** ERIC-PCR was carried out in Department of genetics, Faculty of Agriculture, Zagazig University. The concentration of each DNA template was determined at A260 and adjusted to 25 ng µl-1.

Each 50 µl PCR mixture was composed of 5 µl of 10X PCR buffer (Invitrogen), 5 U of *Taq* DNA polymerase, and a mixture of

each dNTP at 0.2 mM. The primers ERIC 1R and ERIC 2 designed by ***Versalovic et al (1991)*** (Table 1) were each used at concentrations of 25 pmol. The PCR consisted of 40 cycles of 94°C for 1 min, 25°C for 1 min, and 72°C for 2 min prior to cycling, samples were heated at 94°C for 5 min. The PCR products were size separated by electrophoresis of 8 µl portions of the reaction mixtures in ethidium bromide-stained 2% agarose gels with 1X TBE buffer for 2.5 h at 100 V. The DNA profiles were visualized by UV transillumination and photographed. Patterns with at least one different band were considered as different genotypes.

Table 1. Primers used in ERIC-PCR fingerprinting for *Arcobacter* species strains

Primer	Sequence 5' to 3'	Gene	Reference
ERIC 1R	ATGTAAGCTCCTGGGGATTCAC	genome	[**Versalovic *et al*. (1991)**]
ERIC 2	AAGTAAGTGACTGGGGTGAGCG	genome	[**Versalovic *et al*. (1991)**]

RESULTS

The occurrence of *Arcobacter* species in the examined rabbit and drinking water samples are shown in table 2. From 50 samples including 20 rabbit meat, 20 rabbit stool and 10 drinking waters, a total number of 35 (72%) *Arcobacter* species strains were isolated. Recovered *Arcobacter* species isolates from the examined meat, stool and water samples were 15, 20 and 0 with the percentages of 75, 100 and 0, respectively. Concerning, *A. butzleri*, it was isolated from 2(10%) of meat samples and 3(15%) of stool samples. *A. skirrowii* was isolated from meat and stool with the frequency of 6(30%) and 8(40%), respectively. *A. cryaerophilus* was isolated from 7(35%) and 9(45%), respectively.

The reproducibility of ERIC-PCR for the tested 10 clones of *Arcobacter* species are shown in table 3 and figure 1. ERIC-PCR discriminate the examined 10 isolates of *Arcobacter* species based on the presence or absence of the major amplified bands (A-M) which ranged from >1400 bp to 200 bp. The molecular weights of the conservative bands in all isolates were 300 and 400 bp bands. It was found that there are both intra-species and interspecies molecular diversity of examined strains.

Table (2): *Occurrence of Arcobacter species in the examined rabbit farm samples.*

Source (No)	Meat (20)		fecal (20)		Water (10)		Total	
Species	No	%	No	%	No	%	No	%
A. butzleri	2	10	3	15	0	0	5	10
A. skirrowii	6	30	8	40	0	0	14	28
A. cryaerophilus	7	35	9	45	0	0	16	32
Total	15	75	20	100	0	0	35	70

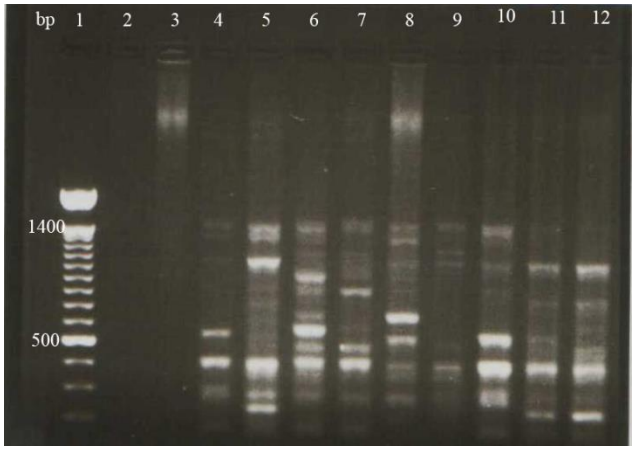

Fig. 1: *ERIC- PCR fingerprints of the 10 Arcobacter species isolates. The lane numbers correspond to the isolate numbers shown in table 3. Lane 1 contains 100 bp marker. Lane 2 contains master mix without template DNAas a control.*

Table 3: *ERIC-PCR product reproducibility of Arcobacter species isolated from apparently healthy rabbits.*

Amplicon bp	Band code	No of isolate/No of lane in Fig. 1									
		1/3	2/4	3/5	4/6	5/7	6/8	7/9	8/10	9/11	10/12
>1400	A	+					+				
1400	B			+	+	+	+	+	+		
1300	C			+			+				
1100	D			+							
1000	E				+		+	+	+	+	+
900	F				+						
800	G				+	+				+	+
700	H				+		+				
600	I		+		+						
500	J				+	+					
400	K	+	+	+	+	+	+	+	+	+	+
300	L	+	+	+	+	+	+	+	+	+	+
200	M			+						+	+
Genotype		1	2	3	4	5	6	7	8	9	9
Species		*A. butzleri*				*A. skirrowii*			*A. cryaerophilus*		

DISCUSSION

Due to the lack of available literature concerning the role of rabbit as a source for arcobacterioses, the present study was carried out to isolate and identify *Arcobacter* species from rabbit meat, stool and their drinking water. The isolation rate (75%) of *Arcobacter* species in rabbit meat in the present study is higher than that recorded in Spain ***(Collado et al, 2009-b)*** which was (1/10)10%. The variation in distribution frequency may be due to hygienic status of the herd and slaughtering sanitary procedures. Difference in isolation rate of Arcobacter from examined rabbit samples may be attributed to several factors such as hygienic conditions during the processing and sensitivity of the isolation method used ***(Gude et al, 2005)***.

The genus Arcobacter has gained increased attention as an emergent waterborne and foodborne enteropathogen. *A. butzleri*, *A. cryaerophilus* and *A. skirrowii* have been associated with gasterointestinal disease and bacteremia in humans and with abortion anddiarrhea in animals ***(Ho et al, 2006).***

A. *butzleri* is the most commonly isolated species and has been classified as a serious hazard to human health by ***ICMSF (2002).***Contamination of rabbit carcasses with Arcobacters poses a risk for both human and animal's infection.The presence of Arcobacter in the feces of healthy livestock at slaughter constitutes a significant risk of carcass and meat contamination ***(De Smet et al, 2010).*** Detection of several different Arcobacter strains may suggest multiple source of infection.In this study no Arcobacter was detected in drinking water. The obtained result agree with that of ***Collado et al (2010)***, ***Diergaardt et al (2004) and Aydin et al (2007)*** who could not find any Arcobacter in drinking water samples.

No Arcobacter were detected in drinking water samples examined may be due to proper disinfection practices as Arcobacter are sensitive to chlorine ***(Moreno et al, 2004).*** Nevertheless, water has a significant role in the transmission of *Arcobacter* species both to human and animals and it has been estimated that 63% of *A. butzleri* infection in humans is from the consumption of or contact with contaminated water ***(Mansfield and Forsythe, 2000)***.

Because of the biochemical inertness of Arcobacters, the applications of these tests are often not adequate to differentiate *Arcobacter* spp. properly at the species level ***(On et al, 1996)***. Therefore, DNA- based methods have been established for rapid and correct identification and/ or differentiation of *Arcobacter* spp. at the species level ***(Houf et al, 2000)***.

The variant genotypes of the examined 10 clones of *Arcobacter* species were shown in table 3 and figure 1.***Van Driessche et al (2004)***

found that individual pig could excrete up to 7 *A. butzleri*, 10*A. cryaerophilus* and *6 A. skirrowii* genotypes. Similar results were recorded by ***Van Driessche et al (2005)*** who recorded shedding of 6 *A. cryaerophilus* and 2 *A. skirrowii* genotypes in cows by using ERIC-PCR. Moreover, ***Houf et al (2008)*** characterized *A. cryaerophilus* and *A. butzleri* clones by ERIC-PCR and reproduced banding patterns genotypes ranged from 100-2072 bp. The extreme genetic diversity of *Arcobacter* species on the carcasses of the same flock can be explained by cross-contamination within one flock and from flocks of different farms ***Ho et al, 2008)***. The genetic diversity 90% in the present results is near to that found by ***Collado et al (2010)*** who recorded genetic diversity of *Arcobacter* species ranged from 11 to 58.6% for isolates of *A. butzleri* and from 43.2% to 100% for the isolates of *A. cryaerophilus*.It was found that ERIC-PCR fingerprinting profiling of *Arcobacter* species is reproducible and discriminative. It could help in tracing the sources of infections. The circulating Arcobacter strains are diverse as the detected genotypes are 9 clones per 10 strains.Further studies are needed to trace the sources of infection to different farms at the national level and horizontal hygiene strategy.

In conclusion, rabbit meat and stool may be a potential source of arcobacterioses in both human and animal niches. Further molecular epidemiological studies are needed to trace the different sources of Arcobacter infection at national level. ERIC-PCR is an efficient method to detect the molecular diversity of *Arcobacter* species.

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الملخص العربي

عزل وتصنيف انواع الأركوباكتر المعزولة من الأرانب فى الزقازيق – مصر

ايمان ابراهيم عطية سويلم

المستشفى البيطرى التعليمى – كلية الطب البيطرى – جامعة الزقازيق

على الرغم من ان نوع الاركوباكتر من المسببات المرضية المارقة، إلا أن دور الأرانب كمصدرللعدوى بالاركوباكتر غير معروف. وقد صممت الدراسة الحالية لدراسة معدل تواجد نوع الاركوباكتر فى الارانب ومياه الشرب لها. وكذلك ايضا عمل البصمة الجزيئية لعدد ١٠ معزولات لأنواع الاركوباكتر بواسطة تقنية تفاعل البلمرة المتسلسل ERIC . وقد جمعت عدد ٥٠ عينة اشتملت على عدد ٢٠ لحوم ارانب وعدد ٢٠ عينة بعر ارانب وعدد ١٠ عينات مياه شرب. وقد تم عزل عدد ٣٥(٧٠٪) معزولة من نوع الاركوباكتر. وكانت المعزولات من اللحوم والبعر والمياه بعدد ١٥ ، ٢٠ ، صفر بنسبة مئوية ٧٥ ، ١٠٠ ، صفر على التوالى.

وبخصوص الاركوباكتر بوتزلارى فقد عزلت من عدد ٢(١٠٪) من عينات اللحوم و ٣(١٥٪) من عينات البعر. أما الاركوباكتر سكيروى فقد عزلت من عينات اللحوم والبعر من عدد ٦(٣٠٪) و ٩ (٤٥٪) على التوالى بينما الاركوباكتر الكرايروفيليس فتم عزله من عدد ٧(٣٥٪) و ٩ (٤٥٪) على التوالى. وقد تم التمييز بين العشر معزولات الممثلة لنوع الاركوباكترمعتمدين على وجود أو غياب الحزم المضخمة الكبيرة (ا-م) والتى تتراوح اوزانها الجزيئية بين أقل من ١٤٠٠ زوج قواعد الى ٢٠٠ زوج قواعد.

وقد كانت اكثر الحزم شيوعا وثباتا متراوحة بين ٣٠٠-٤٠٠ زوج قواعد. وقد وجد تنوع جينى داخل النوع وكذلك ايضا بين الأنواع التى تم فحصها من نسخ انواع الاركوباكتر. وقد وجد أن العترات السارية لأنواع الاركوباكتر فى مزرعة الأرانب التى تم الدراسة لها تمتلك العديد من الأنماط الجينية (١٠/٩). وقد يعزى ذلك التنوع الى تعدد مصادر العدوى.