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Detection and Identification of *Arcobacter* species in Poultry in Assiut Governorate, Upper Egypt

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ABSTRACT

This work aimed to detect, identify and study the epidemiology of *Arcobacter* species in avian species in Upper Egypt. A total 600 samples, including cloacal swabs and intestinal samples were collected from chickens, turkeys and ducks in Assiut Governorate in Upper Egypt. Using conventional phenotypic methods for isolation and identification, *Arcobacter* species could be isolated and identified with percentage 25.5% in chickens, 9.5% in turkeys and 14% in ducks. Sixteen randomly selected phenotypically identified *Arcobacter* species isolates were confirmed using one step multiplex PCR assay. In conclusion, *Arcobacter* species could be detected and identified from various avian species with variable incidence. Conventional phenotypic methods for detection and differentiation of *Arcobacter* species are often hampered by many limitations, while molecular methods, and PCR, in particular can provide a sensitive and rapid alternative method for detection and identification of *Arcobacter* species in different domestic poultry species.

Introduction

The genus *Arcobacter* is a motile, gram negative, slender curved, non-spore forming bacteria, it is a member of the family Campylobacteraceae that need microaerophilic or aerobic atmosphere to grow. *Arcobacter*s can also be grown at 15°C, which is a characteristic ability that differentiates *Arcobacter*s from Campylobacters (Vandamme *et al*., 1991; Lehner *et al*., 2005; Vandamme *et al*., 2005; Figueras *et al*., 2011). In recent years, *Arcobacter* species have required more importance because they have been classified as emergent enteropathogenic and potential zoonotic microorganisms (Ho *et al*., 2006).The *Arcobacter* genus includes 18 species that have been isolated from different sources, including domestic and wild animals, pets, primates, birds, foods of animal origin, water and vegetables (Collado *et al*., 2008; Shah *et al*., 2011; Levican *et al*., 2013). Most of *Arcobacter* isolates from different animals are one of three species, *A. butzleri, A. cryaerophilus* and *A. skirrowii* (Miller *et al*., 2009). Besides, *Arcobacter*s have been considered as waterborne and potential food pathogens (Gonzales *et al*., 2007; Shah *et al*., 2011; Lee and Choi, 2013). In 2002, the International Commission on Microbiological Specifications for Foods (ICMSF) reported *A. butzleri* as a serious hazard to human health (ICMSF, 2002). *Arcobacter* species are commonly isolated as a contaminant of broiler carcasses during processing in poultry slaughter houses (Van Driessche *et al*., 2003; Aydin *et al*., 2007). It was reported that contaminated poultry products are the most important source of transmission of *Arcobacter* species to human beings (Rivas *et al*., 2004). Despite of the frequent incidence of *Arcobacter*s in broiler carcasses and in the environment of poultry processing plants (Houf *et al*., 2002; Son *et al*., 2007), some researchers have reported low levels of intestinal colonization of *Arcobacter* species in poultry (Atabay *et al*., 2006) and believed that living birds were not the major source of *Arcobacter*s on chicken meat (Gude *et al*., 2005; Van Driessche and Houf, 2007). However, the existence, of *Arcobacter* species in the poultry slaughterhouses and water processing equipments can be the primary cause of contamination (Houf *et al*., 2002; Gude *et al*., 2005). Contamination of food with antibiotic resistant bacteria can be also considered as a significant hazed

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to human health, because antibiotic resistance genes can be moved to other pathogens, leading to weak response to the treatment of severe bacterial infections. In recent decades, there was increase in the incidence of antibacterial resistance among food born pathogenic bacteria (Van Driessche *et al*., 2005) and this may be due to the uncontrolled use of antibacterial drugs during rearing of food producing animals, besides that, unregulated use of antimicrobial drugs by human beings in developing countries (Van den Bogaard and Stobberingh, 2000).

Currently, there is limited information regarding the prevalence, isolation and identification of *Arcobacter* species in different poultry species. Therefore, the present study was conducted to determine the prevalence, isolation and phenotypic and molecular identification of *Arcobacter* spp. from different poultry species in Assiut Governorate, Upper Egypt.

Materials and methods

Samples tested

Samples for this study were collected from different poultry species (chickens, turkeys and ducks). Samples were collected from different breeds (native and foreign breeds). Different ages were subjected for study, ranging from day-old chicks to fifty-two-weeks old birds. Sampling process was done from birds suffering from enteritis as well as from apparently healthy birds. Samples were obtained from poultry farms at different localities in Assiut Governorate. A total of 600 samples were collected, including, 300 cloacal swabs (100 from chickens, 100 from turkeys and 100 from ducks), 300 intestinal samples (100 from chickens, 100 from turkeys and100 from ducks). Samples were collected into sterile tubes containing brain heart infusion (BHI) broth and were transferred immediately to the laboratory in an icebox, where they subjected to bacteriological examination with special reference for *Arcobacter* species.

Isolation of Arcobacter species

Samples were inoculated into BHI broth containing *Arcobacter* species growth supplement [Vancomycin (5.0 mg), Trimethoprim (2.5 mg) and Cefoprazone B (1250 IU)] 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 25ºC for 48 hours. The growth was examined for typical *Arcobacter* species colonies.

Phenotypic identification of Arcobacter species *isolates*

To confirm the presence of *Arcobacter* species on suspected isolates, gram stained films, motility test, biochemical reactions, including catalase production, oxidase production, urease production, hippurate hydrolysis, and nalidixic acid/cephalothin resistance/sensitivity tests and growth profile, including growth with 1% glycine, growth with 2% NaCl, growth at 25°C, growth at 37°C, and growth at anaerobic atmosphere were used.

Molecular identification of isolated Arcobacter species *by one step multiplex-PCR*

The protocol of the one step multiplex-PCR was done according to Kabeya *et al*. (2003). Phenotypically isolated and identified *Arcobacter* species isolates were cultured on BHI broth containing *Arcobacter* species growth supplement at 25°C for 48 hours. They were harvested from the broth and washed twice with phosphate buffered saline (PBS). The DNA for PCR amplification was prepared from each isolate by using a commercial DNA extraction kit (QIAamp DNA mini kit, Qiagen, USA). The concentrations of DNA materials were determined from absorbance at E260 nm and adjusted to 10 ng/Al. PCR amplifications were performed in 20 Al of the mixtures containing 20 ng of template DNA, 10 mM Tris (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 AM each of dATP, dCTP, dGTP, and dTTP (Promega, WI, USA), 0.5 AM each of (N.c.1A and ARCO-U), and 0.05 AM each of the primers (N.butz, N.c.1B and N.ski), along with 1.0 U of Taq DNA polymerase (Super Taq DNA polymerase, Promega, USA). The sequences of primers used in this study are shown in Table 1. Amplifications were performed in a thermal cycler (Techneuk. Co., UK) under the following conditions: initial denaturation at 94°C for 3 minutes then 30 cycles of 94°C for 45 seconds for denaturation, annealing at 62°C for one minute and extension for one minute at 72°C. The final extension took 10 minutes at 72°C. Each 10 Al of the PCR products was run on a 2% agarose gel (Molecular Biology Certified Agarose, BIORAD), and was visualized by staining with ethidium bromide on an UV transilluminator (Image Saver AE-6905C, ATTO).

Results

Incidence of Arcobacter species

As shown in Table 2 and Fig. 1, results of *Arcobacter* species incidence revealed that; concerning to incidence of *Arcobacter* species from cloacal swabs, the highest incidence was from the cloacal swabs of chickens (7%) followed by cloacal swabs of ducks (6%) then cloacal swabs of turkeys (4%). Concerning to incidence of *Arcobacter* species from intestinal samples, The highest incidence was from the intestinal samples of chickens (44%) followed by intestinal samples of ducks (22%) then intestinal samples of turkeys (15%). Generally, it was observed that there was higher incidence rate obtained from intestinal samples than from cloacal swabs in all species (chickens, turkeys and ducks). Also, it was observed that the incidence rate of isolation was higher in cloacal swabs obtained from birds showing clinical signs of enteritis than that of cloacal swabs obtained from apparently healthy birds.

Table 1. Nucleotide sequences of the primers used in this study (Kabeya *et al*., 2003)

Primer	Specific species	Position	Nucleotide sequences (5′–3′)
N. butz	*A. butzleri*	1174– 1199	AGCGTTCTATTCAGCGTAGAAGATGT
N.c.1A	*A. cryaerophilus* 1A	1135– 1162	ACCGAAGCTTTAGATTCGAATTTATTCG
N.c.1B	*A. cryaerophilus* 1B	1713–1736	GGACTTGCTCCAAAAAGCTGAAG
N. ski	*A. skirrowii*	1424–1443	CGAGGTCACGGATGGAAGTG
ARCO-U	Arcobacter	1865–1846	TTCGCTTGCGCTGACATCAT

Table 2. Incidence of *Arcobacter* spp. from chickens, turkey and ducks in Assiut Governorate, Upper Egypt.

Bird species	age	breed	Clinical signs*	Cloacal swabs (100)			Intestinal samples (100)			Total isolates	%
				No	isolates	%	No	isolate	%		
Chickens (100)	Young	Native	H	.15	0	0	.15	8	53.33	51	25.5
			D	5	1	20	5	2	40		
		Foreign	H	20	0	0	20	10	50		
			D	10	2	20	10	4	40		
	Semi-mature	Native	H	5	1	20	5	2	40		
			D	5	0	0	5	2	40		
		Foreign	H	5	0	0	5	2	40		
			D	10	1	10	10	4	40		
	Adult	Native	H	5	0	0	5	2	40		
			D	5	1	20	5	2	40		
		Foreign	H	5	0	0	5	2	40		
			D	10	1	10	10	4	40		
Turkeys (100)	Young	Native	H	30	1	3.33	30	6	20	19	9.5
			D	20	1	5	20	4	20		
	Semi-mature	Native	H	15	0	0	15	2	13.33		
			D	10	1	10	10	1	10		
	Adult	Native	H	15	0	0	15	1	6.67		
			D	10	1	10	10	1	10		
Ducks (100)	Young	Native	H	30	2	6.67	30	8	26.67	28	14
			D	20	2	10	20	4	20		
	Semi-mature	Native	H	15	0	0	15	3	20		
			D	10	1	10	10	2	20		
	Adult	Native	H	15	0	0	15	3	20		
			D	10	1	10	10	2	20		

*H =apparently healthy birds, D= Diseased birds with enteritis

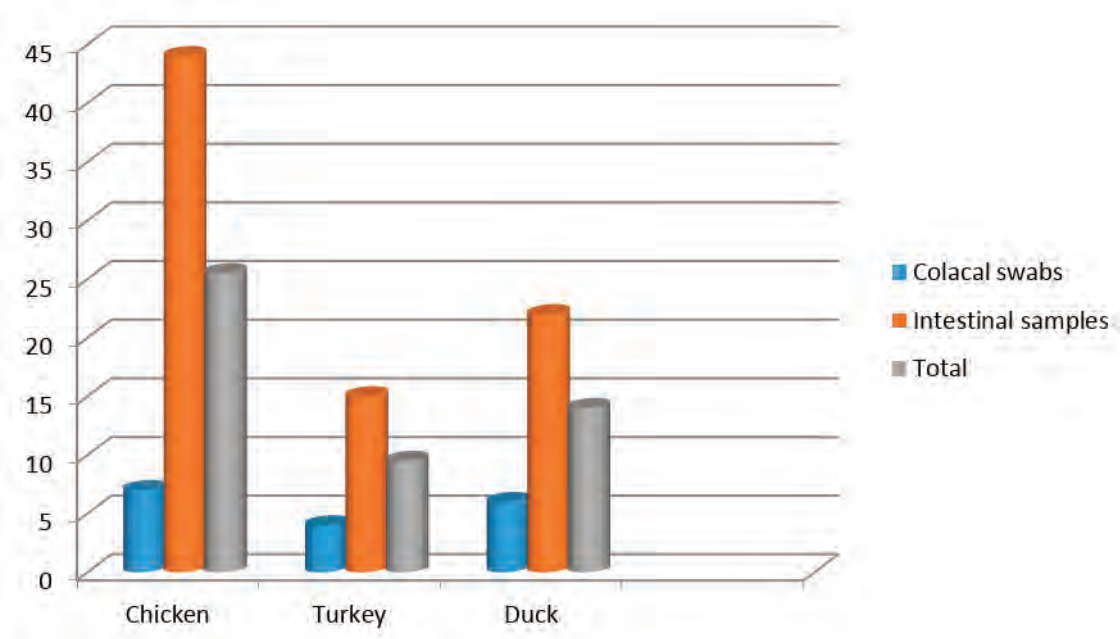

Fig. 1. Incidence of *Arcobacter* spp. in chickens, turkeys and ducks in Assiut Governorate, Upper Egypt.

Phenotypic identification of Arcobacter species

Arcobacter suspected colonies were observed among 98 of 600 samples (16.33%) obtained from chickens, turkeys and ducks included the cloacal swabs (17 out of 300), intestinal samples (81 out of 300). *Arcobacter* suspected growth on brain heart infusion broth supplemented with *Arcobacter* growth supplement appeared in the form of turbidity (turbid broth). *Arcobacter* suspected colonies on brain heart infusion blood agar shown as pinpoint to 1 mm in diameter, grayish white, non-pigmented, smooth, translucent and alpha-hemolytic at 48-72 hours post-inoculation. *Arcobacter* suspected colonies were observed and subjected to Gram staining and revealed that cells were Gram negative slightly curved short rods. *Arcobacter* suspected isolates were biochemically identified for confirmation on the basis of their reaction in the following tests: catalase test, oxidase test, urease test, hippurate hydrolysis test, resistance to naladixic acid and resistance to cephalothin. *Arcobacter* suspected strains were identified on the basis of their growth profile in the following conditions: growth with 1% glycine, growth with 2% NaCl, growth at 37°C and growth at anaerobic atmosphere. Results of biochemical reactions and growth profile were illustrated in Table 3.

Table 3. Biochemical reactions and growth profile of suspected *Arcobacter* spp. isolates.

Test/characteristic	Result	%
Catalase production	92/98	92.6
Oxidase production	98/98	100
Urease production	0/98	0
Hippurate hydrolysis	88/98	0
Resistance to naladixic acid	7/98	7
Resistance to cephalothin	86/98	100
Growth with 1% glycin	92/98	0
Growth with NaCl	95/98	0
Growth at 25°C	98/98	0
Growth at 37°C	98/98	100
Growth at 42°C	98/98	100
Growth at aerobic atmosphere	98/98	0
Growth at anaerobic atmosphere	0/98	100

Genotypic identification of Arcobacter species *isolates using one step multiplex-PCR*

In this study only 10 randomly selected *Arcobacter* species isolates which were previously identified on the basis of phenotypic reactions, were subjected to one step multiplex polymerase chain reaction. To differentiate among *Arcobacter* species, one step PCR was performed using a set of primers (N.butz, N.c.1A, N.c.1B, N.ski, and ARCO-U). The sequences of 23S rRNA genes predict that these primers should amplify cDNA of 692 b.p. (*A. butzleri*), 728 b.p. (*A. cryaerophilus* 1A), 152 b.p. (*A. cryaerophilus* 1B) and 448 b.p. (*A. skirrowii*), respectively. After amplification, the PCR products were subjected to electrophoresis, stained with ethidium bromide.

Discussion

However, *Arcobacter*s are currently not classified as pathogens of significant public health concern (Snelling *et al*., 2006), latest research reported that their importance in human infections may be underestimated, due to inadequate information about detection and identification methods (Vandenberg *et al*., 2004; Snelling *et al*., 2006; Figueras *et al*., 2008).

Arcobacter spp. have repeatedly been isolated from samples of intestines and fecal matter of various domestic animals, but they apparently have the ability to induce infection in only some of them (Ho *et al*., 2006). It is well known that *Arcobacter*s are shedding in fecal matter of poultry including chickens, ducks, turkeys and domesticated geese, however it was reported that there was no diseases association in those animals, and so it has been believed that different poultry species could play as a natural reservoir of *Arcobacter* spp. (Atabay *et al*., 2008; Ho *et al*., 2008; Lipman *et al*., 2008).

Reviewing of the literature revealed that, little is known about how processing procedures may affect the prevalence of *Arcobacter*s. At present, the origin of contamination of *Arcobacter* spp. and the nature of their pathogenesis are still unknown. This is due in part to the lack of standardized isolation methods for *Arcobacter*s. However, a number of studies on the development and comparison of media and enrichment broths for the recovery of *Arcobacter*s from foods of animal origin such as poultry.

Regarding to isolation of *Arcobacter* species in cloacal swabs, in our work, isolation was 7% (7 out of 100 coloacal swabs) in chickens, 4% (4 out of 100 cloacal swabs) in turkey and 6% (6 out of 100 cloacal swabs) in ducks. Wesley and Baetz (1999) reported that the incidence rate of *Arcobacter* species in cloacal swabs of chickens and turkeys were 15% and 65% respectively. Ho *et al*. (2008) found that the isolation rate of *Arcobacter* species in cloacal swabs of chicken hens was 53% and chicken broilers was 27%.

Regarding to incidence rate of *Arcobacter* species in intestinal samples, our results point out an isolation rate of 44% (n=44) of *Arcobacter* species in intestinal samples of 100 chickens of different ages and breeds, 15% (n=15) of *Arcobacter* species in intestinal samples of 100 turkeys and 22% (n=22) of *Arcobacter* species in intestinal samples of 100 ducks. Bhandari (2006) found an incidence rate of 72% upon sampling the intestinal contents of 150 healthy broiler chickens and isolated *Arcobacter* species from 9 out of 18 intestine of laying hens affected by enteritis, while Ho *et al*. (2006) isolated *Campylobacter*-like organisms, Successfully identified as *Arcobacter* species , from 12 out of 50 intestinal samples (24%) and from 9 out of 15 fresh carcasses (60%) collected from two different farms, on the other hand, Son *et al*. (2007) found a high prevalence of *Arcobacter* species in intestinal samples of poultry: 96.8% of pre-scaled carcasses and 61.3% of pre-chilled carcasses.

In our study, it was observed that there was higher incidence rate obtained from samples (cloacal swabs and intestinal samples) collected from birds suffer from enteritis than that collected from apparently healthy birds (chickens, turkeys and ducks).

Our results of isolation of *Arcobacter* species may disagree with the findings reported by Wesley and Baetz (1999); Bhandari (2006); Ho *et al*. (2006); Son *et al*. (2007) and Ho *et al*. (2008). It was suggested that the reason that we found a lower incidence rate of *Arcobacter* species in cloacal swabs and intestinal samples than that found by the previous studies could be attributed to a variety of factors specially differences on isolation methods used for detection of this micro-organism.

Referring to incubational requirements of *Arcobacter* species, in our experiment, it was found that the organism require temperature ranges from 25°C to 30°C in aerobic atmosphere and requires incubation period 2-3 days. These findings are in agreement with these reported by (Wesley and Baetz 1999; Son *et al*., 2007; Lipman *et al*. 2008) and disagree with Ho *et al*. 2008, who used a micro-aerophilic atmosphere for incubation.

In the current study we could isolate *Arcobacter* species organism on brain heart infusion media supplemented with supplement that contains Trimethprim (2.5 mg), Cefoprazone (1250 IU) and Vancomycine (5.0mg). This result goes hand with hand with Wesley and Baetz (1999); Son *et al*. (2007); Ho *et al*. (2008) and Lipman *et al*. (2008).

Ho *et al*. (2008) and Lipman *et al*. (2008) could isolate *Arcobacter* species using modified filter technique of Steele and McDermott (1984), In brief, 300 µl of each diluted sample was spread on a 47 mm, 0.65 µm pore size sterile filter (DAWP04700, Millipore Corporation, Bedford, MA 01730, USA) previously placed on the agar surface. In our study we used a completely different method depending on a selective supplement for inhibition of microorganisms other than *Arcobacter* species.

In the present work, cellular and colonial morphology of *Arcobacter* species were studied. The micro-organism was Gram negative, slightly curved short rods and motile. The isolated strains gave pinpoint to 1 mm in diameter, grayish white, non-pigmented, smooth, translucent and alpha-hemolytic colonies. This conclusion had been recorded by Wesley and Baetz (1999); Bhandari (2006); Son *et al*. (2007); Ho *et al*. (2008) and Lipman *et al*. (2008).

As far as biochemical tests and growth profile of *Arcobacter* species are concerned, our results are in accordance with the data of Wesley and Baetz (1999); Bhandari (2006); Son *et*

al. (2007); Ho *et al*. (2008) and Lipman *et al*. (2008). Results of biochemical reactions and growth profile were illustrated in Table 3.

Stock cultures of *Arcobacter* species can be maintained under micro-aerobic conditions by transfer onto common blood agar bases every 4–7 days. Cultures may be stored for many years by freezing at -80°C, or in liquid nitrogen. Cryoprotective agents such as 10% glycerol or dimethyl sulfoxide (DMSO) should be added to cultures before freezing and heavy cell concentrations should be used, these findings agree with those found by Vandamme *et al*. (1991).

Arcobacter species infection can produce an illness similar to that caused by *Campylobacter* and other food borne pathogens (Vandenberg *et al*., 2004). In our study, some biochemical reactions and growth profile were used to distinguish between *Arcobacter* species and *Campylobacter* species (*C. jejuni, C. coli* and *C. lari*), they were H_2S production on TSI [*Arcobacter* species (-ve), *C. jejuni* and *C. lari* (-ve) and C. coli (variable)], growth on MacConkey [*Arcobacter* species (-ve), *Campylobacter* species (+ve)], growth with 2% NaCL [*Arcobacter* species (+ve), *Campylobacter* species (-ve)] and resistance to naladixic acid and cephalothine [*Arcobacter* species (resistant, sensitive), *C. jejuni, C. coli* (sensitive, resistant) and *C. lari* (resistant, resistant).

It is worth mentioning that, the use of conventional bacteriological tests for identification and differentiation of *Arcobacter* species are often hampered by many limitations. In addition, these bacteria possess few distinguishing biochemical characteristics. Due to aforementioned limitations, molecular methods, and PCR in particular, have marked an important step forward in bacterial diagnostics. These presumptive *Arcobacter*s were confirmed by a species-specific multiplex PCR (m-PCR) either as *A. butzleri*, *A. cryaerophilus* or *A. skirrowii*. DNA sequencing was done for selected isolates from both species to further confirm the PCR results (Bhandari, 2006). We used a definitive, reliable and easy molecular method for identification of *Arcobacter* species, which is based upon PCR. We used this method due to its broad ability to detect and identify members of ε-group of proteobacteria (genera *Campylobacter*, *Helicobacter* and *Arcobacter*) which also colonize bird's intestinal tract.

In this study only 10 randomly selected isolates of *Arcobacter* species which were previously identified on the basis of phenotypic reactions, were subjected to one step multiplex polymerase chain reaction. Results revealed that 4 out of 10 isolates were positive for *A. cryaerophilus* 1B, 2 isolates were positive for *A. skirrowii*, one isolate were positive for *A. cryaerophilus* 1A and one isolate were positive for *A. butzleri*. Two isolates were negative. These results agree with results reported by Kabeya *et al*., 2003 who developed a species specific PCR assay for the identification of the *Arcobacter* species and found that the simple one-step PCR assay was found to be a powerful tool for the survey of *Arcobacter* infection than conventional methods of identification.

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