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Genotyping of various *Arcobacter* species isolated from domestic geese by randomly amplified polymorphic DNA (RAPD) analysis

Genotypisierung von aus Hausgänsen isolierten Arcobacter Spezies mittels Randomly Amplified Polymorphic DNA Analyse (RAPD)

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Summary

The present study was undertaken to genotype *Arcobacter* (*A.*) *butzleri*, *A. cryaerophilus* and *A. skirrowii* isolates from domestic geese from three different flocks in Turkey. Fifteen *Arcobacter* isolates were analysed to determine the RAPD profiles based on the amplified DNA fragment patterns using a universal primer for genotyping. 7 *A. cryaerophilus*, 2 *A. butzleri* and 6 *A. skirrowii* isolates produced 6, 2 and 3 distinct profiles, respectively. The isolates of the same patterns originated from the same flocks. The findings of the present study may support previous reports of the existence of a large degree of heterogeneity among *Arcobacter* isolates. Observation of such levels of genetic diversity may suggest that there are multiple contamination sources in the environment and/or the determined genotypes may have undergone genetic rearrangements. This first report of genotyping of various *Arcobacter* species isolated from healthy geese is expected to improve the understanding of the ecology and epidemiology of this emerging pathogen.

Keywords: *Arcobacter*, genotyping, RAPD-PCR, geese

Zusammenfassung

Die vorliegende Untersuchung wurde durchgeführt, um *Arcobacter* (*A.*) *butzleri*, *A. cryaerophilus* und *A. skirrowii* Isolate von Hausgänsen aus drei unterschiedlichen Herden in der Türkei zu genotypisieren. Fünfzehn *Arcobacter* Isolate wurden analysiert, um die auf den Fragmentmustern der amplifizierten DNA basierenden RAPD Profile zu ermitteln.7 *A. cryaerophilus*, 2 *A. butzleri* und 6 *A. skirrowii* Isolate ergaben jeweils 6, 2 bzw. 3 verschiedene Profile. Isolate mit identischen genotypischen Profilen stammten jeweils von der gleichen Herde. Die Ergebnisse der vorliegenden Untersuchung bestätigen Literaturangaben bezüglich des Bestehens von großen Unterschieden zwischen *Arcobacter* Isolaten. Die Beobachtung einer so großen genetischen Diversität kann damit erklärt werden, dass verschiedene Kontaminationsquellen in der Umgebung exisitieren und/oder genetische Mutationen stattgefunden haben. Dieser erste Bericht über die Genotypisierung verschiedener *Arcobacter* Spezies sollte zum besseren Verständnis der Ökologie und der Epidemiologie dieses „Emerging Pathogens" beitragen.

Schlüsselwörter: *Arcobacter*, Genotypisierung, RAPD-PCR, Gänse

Introduction

The genus *Arcobacter* (*A.*) was formed to include a number of microorganisms initially referred to as 'aerotolerant campylobacters' (Neill et al., 1979; Vandamme et al., 1991). These bacteria are distinguished from *Campylobacter* by their ability to grow at aerobic conditions and at lower temperatures (Vandamme et al., 1992). *Arcobacter* currently contains six validly described species: *A. butzleri*, *A. cryaerophilus* (with two subgroups), *A. skirrowii*, *A nitrofigilis*, *A. cibarius* and A. halophilus (Vandamme et al., 1992; Houf et al., 2005; Stuart et al., 2005).

A. butzleri, *A. cryaerophilus* and *A. skirrowii* have been associated with various animal diseases including abortion, diarrhoea and mastitis (Logan et al., 1982; Kiehlbauch et al., 1991; On et al., 2002). These three species have also been isolated in cases of human diseases such as enteritis and bacteremia with *A. butzleri* being the most frequently reported species (Mansfield and Forsythe, 2000; Vandenberg et al., 2004; Wybo et al., 2004; Snelling et al., 2006). *Arcobacters* were initially isolated from bovine foetuses (Ellis et al., 1977). They have also been isolated from different foods such as poultry carcasses, pork, beef and various water samples (Atabay et al., 1998a, 2002a, 2006; Rice et al., 1999; Kabeya et al., 2004; Gude et al., 2005), which suggests that *arcobacters* are transmitted via food and water. The studies conducted to determine the prevalence of *Arcobacter* spp. in foods of animal origin reported that arcobacters are more prevalent in poultry than in red meat (Corry and Atabay, 2001). Four species of *Arcobacter*, *A. butzleri*, *A. cryaerophilus*, *A. skirrowii* and *A. cibarius*, have so far been isolated in poultry and poultry products (Houf et al., 2005). *Arcobacter* spp. were also isolated in healthy livestock (Ongor et al., 2004; Van Driessche et al., 2005).

Due to relative biochemial inertness and fastidious growth requirements, routine identification and differentiation of *Arcobacter* spp. are problematic (Atabay et al., 1998b). In addition, phenotypic similarities between *Campylobacter* and *Arcobacter* could lead to misidentification of *Arcobacter* as *Campylobacter* (Houf et al., 2000). Moreover, selective agent(s) used in *Arcobacter* isolation media can be detrimental to some species/strains of *Arcobacter* (Houf et al., 2001). Therefore, the true incidence and/or prevalence of *Arcobacter* species may be underestimated (Atabay et al., 2006).

In order to determine the epidemiology of *Arcobacter* spp. in detail, a variety of molecular genotyping techniques, which will help to elucidate epidemiological relationships among the various *Arcobacter* isolates, have previously been applied to arcobacters by a number of researchers (Houf et al., 2003; Morita et al., 2004). These methods include ribotyping (Kiehlbauch et al., 1991), amplification of the repetitive elements or random sequences using PCR (ERIC-PCR) (Atabay et al., 2002b; Houf et al., 2002; Van Driessche et al., 2005), RFLP analysis using PFGE (Hume et al., 2001), amplified fragment length polymorphism (AFLP) analysis of genomic DNA (On et al., 2004) and phylogenetic analysis using rpoB-rpoC (MoritaA et al., 2004).

This study was undertaken to genotype *A. butzleri*, *A. cryaerophilus* and *A. skirrowii* strains which were isolated from domestic geese in Turkey using RAPD-PCR. This analysis is expected to improve the understanding of the epidemiology of arcobacters in geese and in the surrounding environment.

Material and Methods

Arcobacter isolates

Fifteen *Arcobacter* isolates recovered from the cloacae of 90 domestic geese, which were from three different flocks on three different farms in Kars, Turkey (Location A, B and C), were used for genotyping in the current study. The origin of the samples and the method of sampling were previously described in detail (Atabay et al., 2007). Of these isolates, 7, 2 and 6 were *A. cryaerophilus*, *A. butzleri* and *A. skirrowii*, respectively. The procedures for isolation and identification of *Arcobacter* spp. were performed according to previously described methods and criteria (Atabay et al., 1998b, 2002a, 2006; HoufOUF et al., 2000).

Culturing of *Arcobacter* isolates

The organisms were sub-cultured on a non-selective blood agar (blood agar base No. II (Oxoid CM271, Basingstoke, UK) containing 7 % defibrinated sheep blood) by incubating the inoculated plates microaerobically at +30 °C for 2 days. After incubation, one loop of *Arcobacter* colonies was suspended in 200 µl physiological saline solution and used for genetic analyses.

Genotyping of *Arcobacter* isolates

RAPD-PCR was utilized for genotyping of *Arcobacter* isolates as previously described (MacGowan et al., 1993; Unver et al., 2006).

Extraction of genomic DNA

Arcobacter isolates suspended in 200 µl physiological saline solution were mixed with 2,5 µl Proteinase K (~600 U/ml, 20 mg/ml) (MBI Fermentas, Vilnius, Lithuania) and 400 µl lysis buffer (0.01 M NaCl, 1 mM EDTA, 0.01 M Tris-HCl pH 7.6, 0.05 % SDS), and the suspension was incubated at 65 °C for 30 min. An equal volume (600 µl) of chloroform and isoamyl alcohol (24:1 ratio) were added and mixed vigorously. The mixture was centrifuged at 13 000 rpm for 7 min. The aqueous layer was carefully removed to a clean tube and 0.1 volume of 3 M sodium acetate and 2.5 volume of absolute ethanol were added. The mixture was stored at –20°C for 2 h or longer. The precipitated DNA was pelleted after centrifugation of the mixture at 13 000 rpm for 7 min. After being washed with 70 % ethanol twice and air dried, the DNA pellet was resuspended with 60 µl dd H_2O. The DNA concentrations were spectrophotometrically calculated at A_{260}.

PCR primer and PCR conditions

A universal RAPD typing primer (Primer 6: 5'-AACAGCACTCTGTTCAGGC-3' (Integrated DNA Tech., Coralville, USA)) was used in the present study as previously described (MacGowan et al., 1993; Unver et al., 2006). The amplification was carried out in a 50 µl reaction mixture including PCR buffer, 1.5 mM $MgCl_2$, 25 pmol primer 6, 0.2 mM each of the dNTP mixture, 2 U Taq DNA polymerase (MBI Fermentas, Vilnius, Lithuania) and 450 ng DNA with 5 min of denaturation at 94 °C followed by 4 cycles each consisting of 1 min denatur-

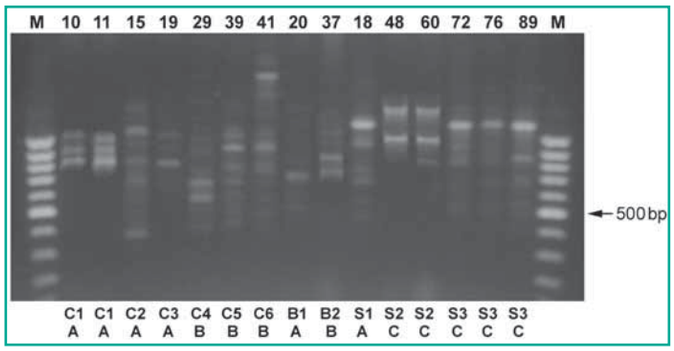

FIGURE 1: *DNA fingerprinting of various Arcobacter species by RAPD analyses using a universal genotyping primer (primer 6). The amplified products were resolved on 1.5 % agarose gels containing EtBr. Isolate ID numbers are shown on the top of photograph. RAPD profiles and locations (A, B, and C) of the geese are indicated on the bottom of the photograph. Lane M, molecular size markers [Gene Ruler "100 bp" MBI Fermentas, Vilnius, Lithuania]).*

ation at 94 °C, 2 min annealing at 26 °C, and 2 min extension at 72 °C. Another 35 cycles each consisting of 1 min denaturation at 94 °C, 2 min annealing at 36 °C and 1.5 min extension at 72 °C followed. The final extension was allowed to continue for 7 min. The template DNA was used at different amounts with independent experiments and 450 ng of DNA was used to get the highest number of discriminating fragments compared with other DNA amounts. Another PCR mixture was processed using the same procedures but without adding DNA template and PCR was carried out with the same reaction conditions as a negative control (no template control). Thermal cycles were performed in MJ Mini Cycler (BioRad, Hercules, USA). The PCR products were electrophoresed (1 h at 100 V) in 1.5 % agarose gels and visualized with ethidium bromide under a UV transilluminator (UVP, Upland, USA). The gels were photographed and RAPD profiles were determined based on the varied sizes of amplified DNA fragments. The PCR assay was performed twice with independent experiments to confirm the reproducibility of the technique.

TABLE 1: *RAPD patterns of Arcobacter strains isolated from domestic geese raised in three locations (flocks) in Turkey*

Arcobacter **species (no. of isolates examined)**	**RAPD patterns obtained**	**No. isolates with respective RAPD pattern**	**Location (flock)**
A. cryaerophilus (7)	C1	2	A
	C2	1	A
	C3	1	A
	C4	1	B
	C5	1	B
	C6	1	B
A. butzleri (2)	B1	1	A
	B2	1	B
A. skirrowii (6)	S1	1	A
	S2	2	C
	S3	3	C

Results and Discussion

Fifteen *Arcobacter* isolates from 90 cloacal samples were analysed to determine the RAPD profiles based on the distinct amplified DNA fragment patterns and they produced a total of 11 different RAPD profiles. The RAPD analysis was able to discriminate all the *Arcobacter* isolates examined. Distinct RAPD patterns obtained are shown in Figure 1. *A. cryaerophilus*, *A. butzleri* and *A. skirrowii* isolates produced 6 (C1-C6), 2 (B1, B2) and 3 (S1-S3) different profiles, respectively. Table 1 summarizes the RAPD patterns of the *Arcobacter* isolates and the locations where the organisms were isolated. The isolates producing the same patterns originated from the same flocks. The numbers of the distinct RAPD patterns were distributed as follows: 3 patterns (S3), 2 patterns (C1), 2 patterns (S2) and the rest of the distinct patterns produced were from a different isolate. No amplification was observed in the "no template control" (data not shown). RAPD results were found to be reproducible when analyzed in independent experiments.

High genetic diversity of *Arcobacter* strains isolated from numerous sources has been previously shown in independent studies from different regions. Manke et al. (1998) reported 86 different DNA patterns in 121 *A. butzleri* isolates from 223 turkey carcasses. Atabay et al. (2002b) reported that 11 subtypes were found in 35 *A. butzleri* isolates recovered from 35 chicken carcasses in Turkey. In another study, 91 *A. butzleri* and 40 *A. cryaerophilus* genotypes were detected in 182 *A. butzleri* and 46 *A. cryaerophilus* isolates from broiler carcasses in Belgium (Houf et al., 2002). Houf et al. (2003) also reported that 159 *A. butzleri* and 139 *A. cryaerophilus* subtypes were determined within 1079 *Arcobacter* isolates recovered from a poultry abattoir environment. Thirty five A. skirrowi, 121 *A. cryaerophilus* and 322 *A. butzleri* isolates from the faeces of healthy pigs in Belgium generated 30, 70 and 123 distinct DNA patterns (Van Driessche et al., 2004). Genetic diversity was also observed in *Arcobacter* strains isolated from cattle (Van Driessche et al., 2005). The RAPD-PCR genotyping technique, as in the present study, was utilised in all the studies mentioned above in order to subtype *Arcobacter* isolates from various sources. The current study revealed 10 different RAPD profiles in 15 *Arcobacter* isolates obtained from three locations in the Northeastern part of Turkey. A high degree of genetic diversity among the *Arcobacter* isolates detected in the current study is in line with the results of previous studies. These findings may suggest that there exist multiple contamination sources in the environment and/or the determined genotypes may have undergone genetic rearrangements. The mechanism(s) how arcobacters generate this genetic heterogeneity should be further studied.

In the present study, similar genotypes were detected only among *Arcobacter* isolates recovered from the same flock. This may show that there is a limited genetic diversity, the existence of a relatively small number of parent genotypes and/or shared contamination sources within the flocks or locations examined. However, larger epidemiological studies involving more locations and flocks with more isolates are required in order to confirm this hypothesis.

Since arcobacters show relative inertness to biochemical tests and they require fastidious growth conditions (Atabay et al., 1998b; On, 2001), a variety of molecular genotyping techniques such as ribotyping, ERIC-PCR,

PFGE, AFLP and sequencing of certain genes have been practically used to discriminate the isolates among and/or within species (Atabay et al., 2002b; Houf et al., 2002; Van Driessche et al., 2004, 2005). In the current study, the RAPD-PCR technique utilizing a universal typing primer was successfully used for genotyping the isolates suggesting that the primer used in this study has a considerable discriminatory power to differentiate genotypes of arcobacters. The limited reproducibility of RAPD-PCR is of general concern for using this method. However, this technique utilizing a universal primer was found to be highly repeatable in this study. Therefore, RAPD-PCR can be practically applied in most laboratories since it requires no special and/or complex equipment and takes less time and is less labourous as compared with some other genotyping methods such as PFGE and AFLP.

In conclusion, the detection of genetically diverse *Arcobacter* species in the faeces of geese in the current study may indicate the presence of multiple sources for contamination in the environment and/or the determined genotypes may have undergone genetic rearrangements. This is the first report of genotyping of various *Arcobacter* species isolated from the faeces of healthy geese. Detailed molecular epidemiological studies involving high numbers of isolates from different sources are needed in order to understand the epidemiology of arcobacters and to clarify their role(s) as foodborne human pathogens.

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