

Presence of *Arcobacter* species in pet cats and dogs in the Czech Republic

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ABSTRACT: This study was conducted to evaluate the occurrence of the genus *Arcobacter* in cats and dogs in the Czech Republic. These animals may be carriers of the bacteria and potential sources of human infection. Oral smears were collected from animals using smear swabs and brushes. Based on previous studies, commercially available DNA kits were used for DNA isolation. Samples were analysed using polymerase chain reaction (PCR) and evaluated using gel electrophoresis. Overall, 178 oral smears were tested, of which 108 were from dogs and 70 were from cats. Out of all smears, five were positive, of which four samples were from dogs and one from a cat. In all five positive cases, PCR confirmed the presence of *Arcobacter butzleri*. In follow-up sampling, the presence of *Arcobacter butzleri* was demonstrated in two samples from a dog.

Keywords: *Arcobacter butzleri*; cat; dog; polymerase chain reaction; oral smears

Arcobacter species are Gram-negative, slender, spiral-shaped rods and, along with the genus *Campylobacter*, belong to the family Campylobacteraceae (Vandamme and De Ley 1991; Ursing et al. 1994). In recent years, a number of new species have been classified into this genus, which currently includes 21 species. The last species to be included was *Arcobacter lanthieri* (Whiteduck-Leveillee et al. 2015). These species have been isolated in particular from poultry (Atabay et al. 1998; Amare et al. 2011), meat (Van Driessche and Houf 2007; Pejchalova et al. 2008), faeces of humans and animals suffering from gastrointestinal tract diseases, and aborted cattle foetuses (*A. butzleri*, *A. cryaerophilus*, *A. skirrowii*, *A. cibarius*, *A. the-reius*, and *A. trophiarum*). Recently, however, these species have also very often been found in samples from wastewater (Gonzalez and Ferrus 2011; Hausdorf et al. 2013) and marine environments (*A. marinus*, *A. molluscorum*, *A. mytili*, *A. ellisii*, *A. bivalviorum*, and *A. venerupis*) (Fera et al. 2004;

Collado et al. 2009; Levican et al. 2012; Levican et al. 2013). Other representatives include *A. defluvii* from sewage water (Collado et al. 2010), *A. nitrofi-gilis* and *A. halophilus* from salt marshes (Levican et al. 2012), and the newly classified *A. cloacae*, *A. suis* (Levican et al. 2013), and *A. anaerophilus* (Jyothsna et al. 2013).

Cases of pets such as dogs and cats acting as carriers of arcobacter species have also been described (Lehner et al. 2005; Ho et al. 2006). A 2007 study by Petersen et al. (2007) tested for the presence of arcobacters in saliva samples from humans and domestic cats and dogs in Denmark. None of the humans suffered from dental problems but the pets did. *Campylobacter* spp. were found in both groups. *A. butzleri* was isolated from cat saliva samples and *A. butzleri* and *A. cryaerophilus* were confirmed in dog saliva samples. A year later, arcobacters were isolated from oral smears and stool samples from dogs and cats in Belgium (Houf et al. 2008). In dogs, both sample types gave positive results, although

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arcobacters were not detected simultaneously in both sample types in a single dog. The detected species were *A. cryaerophilus* and *A. butzleri*. Arcobacters were not found in any samples from cats. This is in contrast to a 2008 study from southern Italy in which tested clinical material was collected from cats (Fera et al. 2009). In that study, oral smears, peripheral blood samples, and fine needle lymph node aspirate samples were tested. Overall, 78.8% of samples tested positive for arcobacters. The presence of *A. butzleri* and *A. cryaerophilus* was confirmed in the samples.

Arcobacters are aerotolerant and are able to grow at lower cultivation temperatures than campylobacters (Atabay et al. 1998). Optimal conditions for arcobacters include the presence of oxygen, i.e. aerobic or microaerophilic conditions, and temperatures of 15–30 °C (Wesley et al. 2000). In general, growth and identification of arcobacters is difficult. Detection using culture methods is usually carried out after growth under aerobic conditions at 25–30 °C for 4–5 days. Given the demanding growth conditions of arcobacters, however, a standard procedure for isolating and growing arcobacters has not yet been successfully developed (Silha et al. 2015). The most reliable method for identifying arcobacters is polymerase chain reaction (PCR; Harmon and Wesley 1997; Houf et al. 2000). An enrichment step is frequently included prior to PCR to enable the captured micro-organisms to grow (Gonzalez and Ferrus 2011).

MATERIAL AND METHODS

Bacterial strains and culture conditions. *A. butzleri* CCUG 30484 (Culture Collection, University of Goteborg, Sweden), *A. skirrowii* LMG 6621 (Belgian Co-ordinated Collection of Micro-organisms, Ghent University, Belgium), *A. cryaerophilus* CCM

7050, and *Campylobacter coli* CCM 7227 (Czech Collection of Microorganisms, Masaryk University, Czech Republic) were used to prepare control microbial suspensions.

To prepare bacterial suspensions, cultures of *Arcobacter* strains were cultured for 48 h at 30 °C under aerobic conditions on tryptone soya agar growth medium and cultures of *Campylobacter* strains were cultured for 48 h at 42 °C in an microaerophilic environment on Campyloselect agar growth medium (BioMerieux, Marcy-l’Etoile, France).

Sample collection and processing. From September 2013 to May 2014, a total of 178 samples were collected, of which 70 were from cats and 108 from dogs. Samples were collected throughout the Czech Republic directly from pet owners, and in the Pardubice Region also in the veterinary clinic of MVDr. Marketa Haslova. Oral smears were collected using Amies medium transport cotton swabs with charcoal (COPAN, Brescia, Italy) as well as two Cytobrush Plus collectors (CooperSurgical, Trumbull, CT, USA). According to their owners, all tested animals were healthy at collection with no apparent disease symptoms. All collected samples were marked with a sample number and processed within 24 h of collection. For all samples, each animal’s age and sex were known as well as whether it was in contact with another tested animal species (cat, dog) in its home. The lifestyles of the tested animals (indoor/outdoor cats; backyard dogs) were also recorded (Table 1). In the case of positive results, another sample was collected two weeks after the first collection.

Culture examination. A total of 178 samples collected using cotton swabs were used for culture examination. The swabs were wiped over the surface of a Tryptone soya agar (TSA) medium (HiMedia, Mumbai, India; 15 g/l enzymatic casein

Table 1. Details of samples included in the study

	Number of			Age range of animals	Number of households ^a	Indoor ^b	Outdoor ^c
	samples	females	males				
Dogs	108	52	56	1 month–14 years	89	60	48
Cats	70	36	34	1 month–17 years	61	37	33

^anumber of households from which collections were taken

^banimals living only indoors

^canimals roaming freely

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hydrolysate, 5 g/l soy peptone, 5 g/l NaCl, 15 g/l agar). Culturing took place at 30 °C for 48 h in an aerobic environment. After culturing, suspected colonies (small, beige-to-white) were Gram-stained and tested for catalase and oxidase. Rough bacterial lysates were prepared from confirmed Gram-negative rods that tested positive for oxidase and were examined using PCR.

DNA isolation. Bacterial DNA was isolated from collected samples using Cytobrushes by three methods: (1) using a QIAamp DNA Mini Kit (Quiagen, Venlo, Netherlands), (2) through cell lysis using dry heat directly from the brush (rough bacterial lysate), and (3) through cell lysis from the culture medium (Arcobacter Broth CM0965, Oxoid, Basingstoke, UK).

(1) A total of 178 samples from smear brushes were placed into micro test tubes with 580 µl of phosphate-buffered saline (PBS, pH 7.4). Following vortex stirring, 20 µl of proteinase K (20 mg/ml) and 200 µl lysis buffer AL were added. The test tube contents were homogenised, and the test tubes were placed into a Stuart SBH130 DC block heater (BioCote, Coventry, UK) and incubated for 10 min at 55 °C. Then, 400 µl of 96% ethanol were added and the mixture was homogenized by vortexing. The mixture was then loaded onto the QIAamp spin column provided in the kit and centrifuged at 6000 g for 1 min. The QIAamp spin column was placed into a 2 ml collection micro test tube, and the tube containing the mixture was discarded. The column material was washed with 500 µl of each of the two wash buffers (Buffer AW1 and Buffer

AW2) provided in the kit. Finally, the DNA was eluted with 150 µl of a third buffer (Buffer AE) provided in the kit.

The concentration of each DNA template was determined spectrophotometrically at 260 nm and adjusted to 10 ng/µl. Purity was determined as ratio of the absorbance at 260 nm divided by the reading at 280 nm. The determined values were greater or equal to 1.8.

(2) A total of 108 dog samples collected by smear brushes were placed into 1.5 ml micro test tubes with 580 µl PBS and incubated at 110 °C for 15 min in a block heater.

(3) A total of 70 cat samples were processed using the following procedure. Five ml of Arcobacter Broth (18 g/l peptone, 5 g/l NaCl, 1 g/l yeast extract) with CAT supplement (SR174E, Oxoid; 4 mg/l cefoperazone, mg/l amphotericin B5, 2 mg/l teicoplanin) were pipetted into sterile 10 ml plastic test tubes with smear brushes. Test tubes were incubated at 30 °C for 48 h under aerobic conditions. For the culture examination, 0.1 ml of incubated broth from each sample were inoculated onto a Petri dish with non-selective TSA medium using an L-shaped spreader. For DNA isolation, 1.5 ml of inoculated broth were centrifuged at 16 000 g for 3 min. The supernatant was removed with a micropipette. When the QIAamp DNA Mini Kit was used to isolate DNA, 580 µl PBS buffer were added to the cells in the micro test tubes. The procedure then continued as above. When the rough bacterial lysate was used, 200 µl PBS buffer was added to the cells in the micro test tubes after removing the

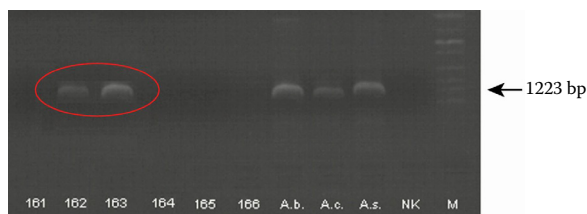


Figure 1. Results from isolated strains using genus-specific PCR for *Arcobacter* spp. (DNA isolated directly from smear brushes, lysis using dry heat)

M = DNA marker (200–1500 bp), **AC** = positive control *A. cry-aerophilus* CCM 7050, **AB** = positive control *A. butzleri* CCUG 30484, **AS** = positive control *A. skirrowii* LMG 6621, **NK** = negative control *Campylobacter jejuni* CCM 7227

Lanes 161–166 are isolates from samples: **161** = negative sample, **162** = *Arcobacter* spp., **163** = *Arcobacter* spp., **164** = negative sample, **165** = negative sample, **166** = negative sample

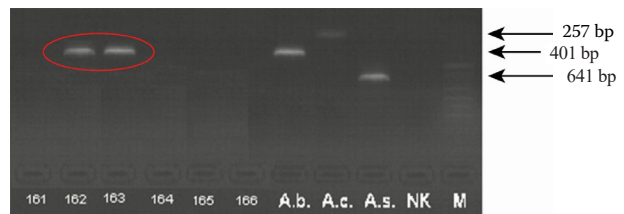


Figure 2. Results from isolated strains using species-specific multiplex PCR for *Arcobacter* spp. (DNA isolated directly from smear brushes, lysis using dry heat)

M = DNA marker (155–970 bp), **AC** = positive control *A. cry-aerophilus* CCM 7050, **AB** = positive control *A. butzleri* CCUG 30484, **AS** = positive control *A. skirrowii* LMG 6621, **NK** = negative control *Campylobacter jejuni* CCM 7227

Lanes 161–166 are isolates from samples: **161** = negative sample, **162** = *Arcobacter* spp., **163** = *Arcobacter* spp., **164** = negative sample, **165** = negative sample, **166** = negative sample

supernatant. Micro test tubes were placed into the block heater for 15 min at 110 °C. Following vortex stirring, 2 µl lysate were used for PCR.

Polymerase chain reaction. To classify bacteria to the genus *Arcobacter*, we used PCR with the primers ARCO I and ARCO II, which after amplification resulted in a product of 1223 bp in size (Harmon and Wesley 1997) (Figure 1). In the case of positive findings, species-specific multiplex PCR was subsequently carried out using the primers ARCO, SKIR, BUTZ, CRY1, and CRY2 (Houf et al. 2000). In multiplex PCR, a PCR product of 401 bp in size is characteristic of *A. butzleri*, 257 bp of *A. cryaerophilus*, and 641 bp of *A. skirrowii* (Figure 2).

The PCR mixture contained 10 × PCR buffer (100 mmol/l Tris-HCl, 500 mmol/l KCl), 25 mmol/l MgCl₂, 2.5 mmol/l dNTP mix, and *Taq*TM DNA polymerase (TaKaRa BIO, Kusatsu, Japan) as well as specific primers (Generi Biotech, Hradec Kralove, Czech Republic). Final mixture volume was 25 µl. The mixture for genus-specific PCR contained 3 mmol/l MgCl₂, 5 mmol/l Tris-HCl (pH 8.3), 25 mmol/l KCl, 0.2 mmol/l dNTP, 1.75 U *Taq*TM DNA polymerase, 25 pmol ARCO I primer, 25 pmol ARCO II primer, and 2 µl DNA solution. The mixture for the multiplex PCR contained 3 mmol/l MgCl₂; 10 mmol/l Tris-HCl (pH 8.3); 50 mmol/l KCl; 0.2 mmol/l dNTP; 0.75 U *Taq*TM DNA polymerase; 25 pmol SKIR primer; 50 pmol of the primers ARCO, BUTZ, CRY I and CRY II; and 2 µl DNA solution. The program for the genus-specific PCR was as follows: initial denaturation (94 °C, 4 min); 35 cycles of denaturation (94 °C, 1 min), primer annealing (56 °C, 1 min), and chain extension (72 °C, 1 min); and final DNA extension (72 °C, 7 min). The program for the multiplex PCR was as follows: initial denaturation (94 °C, 2 min); 32 cycles of denaturation (94 °C, 45 s), primer annealing (65 °C,

45 s), and chain extension (72 °C, 30 s); and final DNA extension (72 °C, 4 min).

Evaluation of PCR products. PCR products were separated by gel electrophoresis (50 min, 100 V) on 1.5% agarose gels with ethidium bromide (10 mg/ml, Top-Bio, Prague, Czech Republic). A mixture of PCR product and loading buffer was introduced into the gel and then also the 155-970 DNA marker (for genus-specific PCR) or 200-1500 DNA marker (Top-Bio, Czech Republic). Visualisation was conducted using a BIO-PRINT UV transilluminator with a CCD camera and BioCapt software (Vilber Lourmat, Marne-la-Vallee, France).

Negative (DNA lysate collection culture of *C. coli* CCM 7227) and positive (DNA lysate collection cultures of *A. butzleri* CCUG 30484, *A. cryaerophilus* CCM 7050, and *A. skirrowii* LMG 6621) controls were always used.

RESULTS

Of the total 178 examined animals, five tested samples were positive for arcobacters. One case involved a cat and four cases dogs (Table 2).

After the culture examination for which 178 oral smears were collected using Amies medium transport swabs with charcoal, bacteria from the *Arcobacter* genus were found in only one sample from a dog. After finding suspect colonies, Gram staining and testing for catalase and oxidase were conducted. Gram-negative spiral-shaped rods were found, and so genus-specific PCR was carried out from rough bacterial lysate. This confirmed bacteria of the *Arcobacter* genus in one sample from the dog (a 2-year-old Maltese Pinscher) and all positive samples from cats. Subsequent multiplex PCR confirmed the presence of *A. butzleri*.

Table 2. Detection of *Arcobacter butzleri* in oral smears collected in the Czech Republic

Animal	Sex	Age of animal	Applied methods
Dog	F	3 years	cytobrush; cell lysis using dry heat; m-PCR
Dog	F	3 years	cytobrush; cell lysis using dry heat; m-PCR
Dog	M	2 years	cotton swab; culture examination; cell lysis using dry heat; m-PCR
Dog	F	8 years	cytobrush; QIAamp DNA kit; m-PCR
Cat	M	5 years	cytobrush; cultivation; cell lysis using dry heat; m-PCR

F = female, M = male, m-PCR = multiplex PCR

^aspecies classified based on PCR

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Bacterial DNA for PCR was isolated from 178 oral smears collected with Cytobrush Plus® collectors. After processing using the QIAamp DNA Mini Kit arcobacters were found in one case. Bacterial DNA was also isolated from 108 dog samples through cell lysis using dry heat. In genus-specific PCR, arcobacters were found in two samples treated in this manner. *A. butzleri* was confirmed using multiplex PCR. The two samples from dogs were positive also in the subsequent collection after two weeks. These were samples from 3-year-old female dogs living together in a single household. One peculiarity in the results is that the two dogs lived together with another 3-year-old female dog for which no positive results were recorded. The method of cell lysis using dry heat from the culture medium was used for 70 feline samples. *A. butzleri* was confirmed in one case, a sample collected from a 5-year-old outdoor cat.

DISCUSSION

The samples collected in the course of this study, especially from cats, contained high concentrations of various accompanying microflora, considerably dominated by *Pseudomonas* spp., which had been encountered also in the Belgian study in 2008 (Houf et al. 2008). These high numbers of accompanying microflora may have been caused by both the low selectivity of the culture medium and the low density of arcobacters in the sample. The low number of positive samples after culture examination (1) could have been caused, however, by the high concentration of foreign DNA in the lysate from the culture medium that entirely suppresses the low concentration of arcobacters. Another potential cause of the negative response in samples treated in this manner could be the very low concentration of arcobacter DNA in the samples, which corresponds to the negative presence in the culture examination. It is also possible that arcobacters were washed out of the wells and therefore not recorded in the subsequent PCR.

DNA was isolated directly from samples collected with the Cytobrushes using the QIAamp DNA Mini Kit. Arcobacters were found in one sample (0.6%) treated in this manner. Both positive samples originated from dogs. A positive response was visible in the genus-specific PCR, manifesting as a product in the 1223 bp region, which is character-

istic of the genus *Arcobacter*. Using multiplex PCR, *A. butzleri* was found in sample as demonstrated by the presence of a band in the 401 bp region in gel electrophoresis. This DNA isolation method had also been used in the Italian study (Fera et al. 2009) where arcobacters were confirmed in 67% of samples tested. Using a QIAamp DNA Mini Kit to isolate arcobacter DNA directly from oral smears does not seem to be an ideal method.

Overall, the results of our study are in agreement with the study carried out in Belgium (Houf et al. 2008). The authors of this work employed the method of cell lysis using dry heat from the culture medium, which appears to be the most effective, and where there were positive results in 2.6% of cases. In our study, one out of 70 (1.4%) oral smears from cats was positive and two out of 108 (1.9%) samples from dogs were positive. Another advantage of this procedure is its low price.

Entirely different results were obtained in the study by Fera et al. (2009) from southern Italy. That study found that *Arcobacter* bacteria commonly occur in the oral cavity of cats. The study included 85 cats, of which 17 were healthy and 68 had clinical symptoms of oral disease or lymphadenomegaly. *Arcobacter*-specific DNA was detected in 67 of the 85 examined cats (78.8%), with 66 samples harbouring *A. butzleri* and 29 samples *A. cryaerophilus*. The high number of positives could be related not only to the clinical state of the animals but also to the large numbers of free-roaming cats and dogs in certain Italian regions (Slater et al. 2008).

The owners of the infected dogs had not observed any disease symptoms. This corresponds with the Belgian study (Houf et al. 2008), where one case of *A. butzleri* was identified in a sample from a 1-year-old female Labrador Retriever in two collections one week apart. Arcobacters were not found in a third sampling from this dog. The owners stated that they had observed no disease symptoms in the animal. Analysis performed by veterinarians showed that dental disorders in dogs and cats are relatively frequent in the Czech Republic (Kyllar and Witter 2005), which is in contrast with the observations of the owners (no disease symptoms noticed).

The presence of arcobacters in oral smears from cats and dogs in the Czech Republic suggests that these animals kept as pets can be potential sources of infection for *Arcobacter* bacteria in humans, as physical contact occurs frequently between pets and their owners.

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