Occurrence of arcobacters in the Czech Republic and the influence of sample matrix on their detection using PCR

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Summary

Sixty nine meat and water samples were examined by multiplex-PCR (m-PCR) assay of the enrichment media. Twenty nine (42%) samples were contaminated with *Arcobacter* species. In 17 samples (24.6%), *A. butzleri* was confirmed by m-PCR. Direct detection of arcobacters from assay avoided cultivation on agar plates and reduced the time required for detection. Sensitivity of optimised m-PCR method was verified using four different enrichment broths artificially contaminated with pure cultures of arcobacters. Enrichment medium Formula 6 (composed of a special peptone No. 2, meat extract, yeast extract, sodium chloride, NaH_2PO_4, K_2HPO_4, defibrinated sheep blood, pyruvate, mercaptoacetic acid and cefoperazone) gave the best results of all tested recommended enrichment media. Influence of matrix samples was tested on genuine samples of chicken, pork and beef, in which suspension of definite density of arcobacters was added. It was shown, particularly, that a sample with higher fat content has required a longer incubation period in enrichment broth for successful detection by m-PCR assay.

Keywords

Arcobacter cryaerophilus; Arcobacter butzleri; multiplex-PCR; matrix of samples

Arcobacter species were previously known as aerotolerant *Campylobacter* species [1]. The genus *Arcobacter* was formed in 1991 as the results of a comprehensive taxonomic revision of *Campylobacter* and related bacteria involving DNA-DNA and rRNA-DNA hybridisation experiments [2].

Arcobacters were first isolated from aborted bovine and porcine foetuses [3-5]. In recent years, different studies reported *Arcobacter* occurrence in faeces, food of animal origin, and in water suggesting a ubiquitous distribution of these organisms in the environment [6-11]. At present, genus *Arcobacter* comprises six species, *Arcobacter butzleri, A. cryaerophilus, A. skirrowii. A. nitrofigilis, A. cibarius* isolated by HOUF et al. [12] and *A. halophilus,* characterised by STUART et al. [13], out of which *A. butzleri*, *A. cryaerophilus* and *A. skirrowii*, were considered pathogenic for both human and animals. These species were isolated from cases of human diarrhoea and septicaemia [4, 14, 15]. Poultry, contaminated water and infected persons could act as an environmental reservoir of arcobacters [10, 11, 16, 17]. Recently, *A. cryaerophilus* was isolated from naturally infected rainbow trout [18].

A number of enrichment and plating formulations have been described for the selective isolation and identification of *Arcobacter* sp. from food. Isolation and identification included an enrichment step in a selective broth followed by cultivation on selective agar. Then, physiological and morphological properties and the results of biochemical tests were assessed [19-24]. However, no generally recommended method has been accepted so far. Since the genus *Arcobacter* has low biochemical activity, molecular techniques such as PCR-based systems have been successfully applied to develop improved detection methods for arcobacters in the environment [25-27]. In this study a multiplex PCR assay, described by HARMON and WESLEY, for the rapid and simultaneous detection and identification of arcobacters [28].

Little is known about the prevalence of arcobacters in Czech Republic and their role in food-

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stuff is not fully understood. Our study aimed at investigating the prevalence of arcobacters in meat, develop a shortcut method for isolation and identification of arcobacters, and determine influence of matrix samples on sensitivity of m-PCR assay.

MATERIALS AND METHODS

Bacterial strains

Three species of arcobacters were used throughout this study. *Arcobacter cryaerophilus* CCM 3934 and CCM 3933 (Czech Collection of Microorganisms, Masaryk University, Brno, Czech Republic) and *Arcobacter butzleri* CCUG 30484 obtained from Culture Collection University of Göteborg, Sweden. Type cultures of arcobacters were incubated aerobically onto Casein-peptone Soymeal-peptone agar (CASO, Merck, Darmstadt, Germany) at 30 °C for 2–3 days. Stock cultures were maintained on the same agar in refrigerator at 5–8 °C. Cultures were transferred monthly at least.

Enrichment media

The following enrichment media were used: AM/CAT consisting of *Arcobacter* broth (Oxoid, Basingstoke, United Kingdom) with CAT supplement (cefoperazone, amphotericin, teicoplanin, SR 174E, Oxoid), EMJH P-80 and JM broth according to JOHNSON and MURANO [22], and Formula 6 (F6) which was developed by JOHNSON and MURANO [20].

One liter of EMJH P-80 contained 40 ml phosphate buffer (16.6 $g.l^{-1}$ Na_2HPO_4; 2.17 $g.l^{-1}$ KH_2PO_4), 50 ml salt solution (38.5 $g.l^{-1}$ NaCl; 5.35 $g.l^{-1}$ NH_4Cl; 3.81 $g.l^{-1}$ $MgCl_2$) 0.2 g of L-cystine (Sigma-Aldrich, Prague, Czech Republic), 1.2 ml of Tween 80 (Sigma), 200 mg 5-fluorouracil (Sigma), and following solutions were added; 1 ml of $CuSO_4$ (0.03%), 10 ml of $ZnSO_4$ (0.2%), 20 ml of $FeSO_4$ (0.25%), 2.0 ml cobalamine (0.2%, Sigma), 0.1 ml 0.2% thiamine hydrochloride (Sigma), and 200 ml albumine (5%, Sigma).

JM broth was composed of a special peptone No. 2 (Oxoid), 3% activated charcoal, 0.25% bile salts No. 3 (Oxoid), 0.05% pyruvate sodium salt (Fisher Scientific, Hampton, USA), 0.05% mercaptoacetic acid (Sigma), 32 $mg.l^{-1}$ cefoperazone (Sigma), 200 $mg.l^{-1}$ 5-fluorouracil (Sigma).

Formula 6 broth was composed of a special peptone No. 2 (Oxoid) as the basal component and meat extract (Difco Laboratories, Augsburg, Germany) 6.0 $g.l^{-1}$, yeast extract (Difco) 6.0 $g.l^{-1}$, sodium chloride 4.0 $g.l^{-1}$, NaH_2PO_4 2.0 $g.l^{-1}$, K_2HPO_4 1.6 $g.l^{-1}$, defibrinated sheep blood 5.0%, pyruvate 0.05% (Fisher), mercaptoacetic acid 0.05% (Sigma) and cefoperazone (Sigma) 32 $\mu g.l^{-1}$.

Plating media

Non-selective agar plates were used: CASO agar (Merck), Brain-heart infusion (BHI, Oxoid, United Kingdom), Blood agar (Blood agar base No. 2 with 5% sheep blood, HiMedia, Mumbai, India). Selective agars were MacConkey agar (Imuna, Šarišské Michaľany, Slovakia) and modified CIN/CAT agar (mCIN/CAT) [19]. The composition of mCIN/CAT was followed: CIN agar base (*Yersinia* selective agar base, Oxoid CM 653) 58.0 $mg.l^{-1}$, yeasts extract 3.0 $mg.l^{-1}$, Bacto agar (Difco) 2.5 $mg.l^{-1}$, sodium chloride 1.5 $mg.l^{-1}$, disodium succinate hexahydrate 2.0 $mg.l^{-1}$, disodium L-glutamate monohydrate 2.0 $mg.l^{-1}$, $MgCl_2$ $6H_2O$ 1.0 $mg.l^{-1}$ and 5-fluorouracil 0.2 $mg.l^{-1}$ [6]. Supplemented CAT SR 174E (Oxoid), JM agar according to JOHNSON and MURANO [21] had the same composition as the corresponding broth, besides an addition of 16 $g.l^{-1}$ agar.

Sampling procedure

Randomly collected samples of chicken, pork and beef meat were purchased on retail market, some samples were also obtained from slaughterhouses. Sixty three chicken, pork and beef samples were examined for the presence of *Arcobacter*. The collection of samples also comprised three samples of the cooled rests containing chicken blood and three samples of surface water obtained from Pardubice regions. Chicken skin, meat, bones and pork/beef meat surface were wiped off with sterile cotton swaband swab samples transferred to culture tubes containing 10 ml aliquot of AM/CAT enrichment broth. The meat samples and swab samples were stored in refrigerator and used for subsequent incubation within 24 hours. Water samples were immediately processed after being collected.

PCR assay

A polymerase chain reaction assay was performed according to HARMON and WESLEY [28] using specific primers based on 16S rRNA and 23S rRNA gene sequence data. A single suspect colony was suspended in 200 μl of a sterile TE buffer (pH 8.0, 100 $mmol.l^{-1}$ Tris, 10 $mmol.l^{-1}$ EDTA) in a microcetrifuge tube, incubated for 15 min at 110 °C in dry bath and centrifuged for 1 min at 16750 *g* (EBA 12, Hettich, Tuttlingen, Germany). An aliquot of the supernatant (2 μl) was used as a DNA template for PCR. Amplification was performed in a 50 μl volume containing the crude bacterial

lysate, 25 pmol of each of the PCR primers Arco I, Arco II, ARCO2, BUTZ (Generi, Biotech, Hradec Králové, Czech Republic), 1 μl dNTP Mix-PLUS (Roche, Mannheim, Germany) (200 μmol. l^{-1} of dATP, dCTP, dGTP, and 600 μmol.l^{-1} dUTP), 1.25 U of Taq™ DNA polymerase (TAKARA Biomedicals, Kyoto, Japan), 10 mmol.l^{-1} Tris-HCl, 50 mmol.l^{-1} KCl, and 3.5 mmol.l^{-1} $MgCl_2$ (pH 8.3). Then, 1 U uracil DNA glycosylase (Roche) was added to the reaction mixture. The mixture was overlaid with sterile mineral oil (MJ Research, San Francisco, CA, USA) and amplified in a thermal cycler (PTC-150, MJ Research). First, it was subjected to an initial incubation (20 °C for 10 min), then to a denaturation step (94 °C for 4 min), which was followed by 35 amplification cycles. Each amplification cycle consisted of three 1 min steps: denaturation at 94 °C, primer annealing at 56 °C, primer extension at 72 °C. The final amplification cycle was followed by a synthesis at 72 °C for 7 min.

PCR reaction products (8 μl) were detected in 1.5% agarose gel by electrophoresis for 1 h at 120 V (Serva, Heidelberg, Germany). DNA marker ΦX 174 DNA/*Bsu*RI (*Hae* III) Marker 9 (MBI, Fermentas, Vilnius, Lithuania) was included for comparison. The presence of an *Arcobacter* sp. was shown by the presence of a PCR product of 1223 bp, whereas for *A. butzleri* two products of 1223 and 686 bp were detected.

Shortcut method of isolation and identification

Serial 10-fold dilutions in sterile physiological solution were prepared using the 0.5 McFarland turbidimetric standard and from these dilutions, 1 ml aliquots were added to different enrichment broths (CAT, JM, F6, EMJH P–80) to obtain final concentration 10^7 to 10^1 CFU.ml^{-1}. Actual colony counts were enumerated on CASO agar plates after 2–3 days of incubation at 30 °C. One millilitre aliquot of each enriched suspension was centrifuged (16750 *g*, 15 min) after 24 and 48 hours of incubation at 30 °C. A portion of supernatant (800 μl) was removed, and then 400 μl of TE buffer was added. After lysis in dry bath (110 °C, 15 min) and subsequent centrifugation (16750 *g*, 5 min), an aliquot of 5 μl served as a DNA template for PCR.

Matrix influence assay

Chicken, pork and beef genuine samples were used to determine the possible influence of matrix. A 25 g sample was placed in a plastic bag containing 225 ml of enrichment broth (AM/CAT) and pummelled in a Stomacher homogeniser (IUL Instruments, Barcelona, Spain).

Serial 10-fold dilutions of 2–3 days culture of *Arcobacter butzleri* CCUG 30484 (aerobically incubated onto CASO agar at 30 °C) were prepared to obtain 10^3 CFU.ml^{-1} for artificial contamination of genuine samples. 1 ml aliquot of these serial dilutions was added to plastic bag with homogenized sample and after 24 and 48 hours of aerobic incubation at 30 °C detection of arcobacters by m-PCR assay was applied. Simultaneously, enriched suspension (100 μl) was streaked onto surface JM and mCIN/CAT selective agar plate for verification of amount of arcobacters.

Real sample analysis

A 25 g portion aseptically taken meat sample was homogenized with 225 ml of AM/CAT enrichment broth. A swab sample was transferred to 10 ml of AM/CAT enrichment broth. The enrichment procedure took 2–3 days at 30 °C. To obtain single colonies, enriched suspension aliquots were surface streaked onto JM selective agar plates and BHI agar plates. After aerobic incubation (30 °C, 2–3 days), suspected colonies were streaked onto JM and mCIN/CAT selective agar plates, and on CASO agar plate. The presence of *Arcobacter* both in agar plates (JM, mCIN/CAT and CASO) and in enrichment broth (F6) after appropriate incubation was detected by m-PCR assay.

Water samples analysis

10 ml aliquot of water sample was added to 90 ml of enrichment broth (AM/CAT, F6, JM, EMJH P–80) and after incubation at 30 °C for 4 days, enrichment broths were seeded onto JM agar plates and BHI agar plates by loop streaking. Suspected colonies were picked for identification by m-PCR assay.

RESULTS AND DISCUSSION

All the tested bacterial strains gave 1223 bp PCR specific product. In the case of *Arcobacter butzleri*, two PCR products were detected; genus specific 1223 bp and species specific 686 bp.

Four artificially contaminated enrichment broths (AM/CAT, JM, F6 and EMJH P–80) were used to shorten the time consuming detection of arcobacters from approximately 10 days (including conventional cultivation methods, morphology assessment, physiological and biochemical tests) to 2–3 days. This shortcut method involved PCR detection directly from enrichment broth without subsequent cultivation on selective agar plates. Detection of pure cultures of *A. cryaerophilus* CCM 3933 and *A. butzleri* CCUG 30484

Tab. 1. Detection of pure cultures of arcobacters directly from enrichment broths using m-PCR.

Arcobacter sp.	Concentration of *Arcobacter* [CFU.ml^{-1}]	Product of PCR							
		AM/CAT		JM		F6		EMJH P–80	
		after 24 h	after 48 h	after 24 h	after 48 h	after 24 h	after 48 h	after 24 h	after 48 h
Arcobacter cryaerophilus CCM 3933	10^4	–	+	–	+	+	+	–	+
	10^3	–	+	–	+	–	+	–	+
	10^2	–	+	–	+	–	+	–	+
	10^1	–	+	–	+	–	+	–	+
Arcobacter butzleri CCUG 30484	10^4	–	+	+	+	+	+	+	+
	10^3	–	+	–	+	+	+	+	+
	10^2	–	+	–	+	+	+	+	+
	10^1	–	+	–	+	+	+	+	+

+ - positive PCR product, – - no PCR product.

by m-PCR assay was successful after 24 hours of incubation in Formula 6 enrichment broth at initial levels of 10^4 and 10^1 CFU.ml^{-1}, respectively. Although after 24 hours of incubation in F6, JM and EMJH P–80 enrichment broth, *A. butzleri* CCUG 30484 was detected, 48 hours incubation period was necessary for successful detection of *A. cryaerophilus* CCM 3933. All of the tested enrichment media allowed detection both of pure cultures after 48 hours of incubation. The results are summarized in Table 1. The assay of naturally contaminated samples required 48 hours of enrichment. That is why we applied enrichment medium AM/CAT according to Antolín et al. [29], where accompanying microflora is suppressed in the best manner.

The influence of sample matrices was examined after artificial contamination of genuine samples of chicken, pork and beef by a suspension of *Arcobacter butzleri* CCUG 30484 of definite density. Results obtained in this study are listed in Table 2. In general, the ratio of fat-muscle depends on animal species, their age, and gender and the type of diet. Some animal products are high in fat, for example streaky pork, fatback, and goose and duck from poultry. Chicken meat and beef meat are lower in fat.

Tab. 2. Influence of sample matrix using enrichment broth AM/CAT.

Number of sample	Type of sample	PCR product	
		24 h	48 h
1	Chicken wings	+	+
2	Chicken necks	+	+
3	Pork legs–boneless	–	+
4	Beef meat	–	+
5	Rump beef steak-sliced	–	+
6	Beef flank and meat from beef ribs	–	+

+ - positive PCR product, – - no PCR product.

Comparison of final PCR products, fat content in samples was marked (Table 2). *A. butzleri* was detectable after 24 hours of incubation in artificially contaminated chicken meat homogenates while none was detected in beef/pork meat homogenates. The visualised PCR products (bands) of arcobacters obtained from beef meat homogenate were more luminous than those of pork. The more luminous bands were also obtained from lean meat homogenates compared with fatty ones. Not only sample matrices but the accompanying flora could interfere with m-PCR assay. Thus a selective combination of enrichment broth (AM/CAT) and mCIN/CAT agar plates was used according to Antolín et al. [29]. Authors developed a protocol for the selective isolation of *Arcobacter* species from poultry meat with maximal suppression of naturally occurring flora. Whereas accompanying flora grows on BHI agar plates without constraints, JM agar plates resulted in a supplementary inhibition of accompanying flora, although some Gram positive cocci, yeasts and Gram negative rods, other than *Arcobacter* species remained. Beef meat homogenates were highly contaminated with accompanying flora. Plates with mCIN/CAT support mainly the growth of *Arcobacter*.

The results of analysis of real sample are shown in Table 3. Forty two percent of examined samples were contaminated with *Arcobacter* species. Multiplex PCR assay has distinguished *A. but-*

Tab. 3. Occurrence of arcobacters in genuine samples.

Type of samples and source	N	*A. butzleri*	*A. cryaerophilus*	*Arcobacter* spp.
rests of cooling with chicken blood[a]	3	0	1	1
swabs from chicken meat[a] (drumstick, with skin)	32	15	1	16
chicken meat in retail market	3	0	0	0
swabs from chicken meat[a] (breasts, without skin)	15	1	5	6
pork meat in retail market	2	0	0	0
beef meat[b]	1	0	0	0
swabs from beef meat[b]	7	0	5	5
beef meat in retail market	2	0	0	0
minced mixed pork/beef , retail	1	0	0	0
samples of water[c]	3	1	0	1
Total	69	17	12	29

N - number of samples, a - chicken meat-processing plant, b - pork and beef meat-processing plant, c - water locality Pardubice-river, ponds.

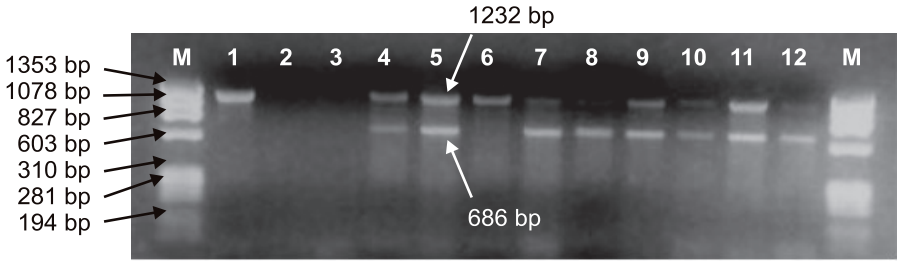

Fig. 1. Analysis of genuine samples (cooled chicken breasts–swabs).
1 - *A. cryaerophilus* CCM 3934, 2 - negative control, 3 - negative control, 4 - *A. butzleri*, 5 - *A. butzleri*, 6 - *A. cryaerophilus*, 7 - *A. butzleri*, 8 - *A. butzleri*, 9 - *A. butzleri*, 10 - *A.butzleri*, 11 - *A. butzleri*, 12 - *A. butzleri*, M - DNA marker ΦX 174 DNA/*Bsu*RI.

zleri (24.6%) from the rest of *Arcobacter* species (17.4%), which were confirmed as *A. cryaerophilus* using spectrum biochemical tests according to On et al. [30]. API Campy Set (BioMérieux, Marcy l'Etoile, France) could be used only as a complementary estimative test, since API Campy strips are not reliable for identification of *Arcobacter* species [19]. An example of visible PCR products of chicken meat sample is shown in Fig. 1. *A. butzleri* was frequently isolated from chicken skin and water samples. Chicken meat without skin and beef samples was mainly contaminated with *A. cryaerophilus*. Houf et al. [31] found neck skin from broiler associated with both *A. butlzeri* and *A. cryaerophilus* whereas *A. butzleri* alone was isolated from chicken breast without the skin. This contradiction probably occurred due to different isolation protocol. One of three water samples was found to be positive for *Arcobacter butzleri* although some authors reported that *Arcobacter* species was often isolated from different kind of water [8, 9, 32]. Our positive water sample was taken from a pond populated with aquatic birds. Conclusive evidence must be based on more samples of water from a wider range of localities. Further research will be necessary to investigate biochemical and physiological profile as well as antibiotic susceptibility of the first *Arcobacter* strains isolated from food and environment in Czech Republic.

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