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Comparison of *Arcobacter* Isolation Methods, and Diversity of *Arcobacter* spp. in Cheshire, United Kingdom▽

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The aims of this study were, firstly, to compare five published methods for the isolation of *Arcobacter* spp. from animal feces in order to determine the most sensitive and specific method. Second, we analyzed the resulting isolates by multilocus sequence typing (MLST) in order to investigate the diversity of the isolates recovered. Third, we investigated the ability to recover *Arcobacter* spp. from frozen fecal samples. Seventy-seven fecal samples from cattle, sheep, and badgers were subjected to five isolation methods, based on published methods for the isolation of *Arcobacter* and *Campylobacter* spp. Thirty-nine *Arcobacter butzleri* isolates were analyzed using a multilocus sequence typing scheme. The survival of *Arcobacter* spp. in frozen samples was investigated by freezing the fecal samples at −80°C for 7 days and then applying the same five isolation methods. The most sensitive and specific method used an *Arcobacter*-specific broth in conjunction with modified charcoal cefoperazone deoxycholate agar (mCCDA) with added antibiotics. Freezing of fecal samples led to a reduction in the recovery of *Arcobacter* spp. by approximately 50%. The 39 allelic profiles obtained by MLST could be divided into 11 sequence types (STs). We have identified the most sensitive and specific method for the isolation of *Arcobacter* spp. from animal feces and demonstrated that the freezing of fecal samples prior to isolation reduces arcobacter recovery. MLST analysis of the isolates revealed a high level of diversity.

Arcobacter spp. are Gram-negative bacteria that differ from the closely related *Campylobacter* spp. in that they are able to grow at temperatures as low as 15°C and under aerobic conditions. 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 (3, 4, 5, 7, 8, 9, 14, 17, 18, 20, 30, 32); *A. butzleri*, *A. skirrowii*, and *A. cryaerophilus* have been isolated from human fecal samples (15, 18, 24, 27, 29, 31, 33, 34, 35, 43).

It has been reported that *Arcobacter* spp., which were originally isolated from aborted bovine fetuses, can cause disease in cattle (7) although the true role of *Arcobacter* spp. as veterinary pathogens is yet to be definitively proven. *Arcobacter* spp. have also been isolated from healthy cattle in Belgium (37), Japan (20), Turkey (2, 23), the United States (8, 42), and New Zealand (25) and from beef and/or beef products from Thailand (39), Northern Ireland (32), Turkey (2, 28), Australia (30), Japan (21), Mexico (38), Czech Republic (40), the United States (8), and Netherlands (4). These strains show that the organism can be present in healthy animals and their products, illustrating the importance of cattle and beef as potential sources of *Arcobacter* infection of humans.

A variety of methods have been employed for the isolation of *Arcobacter* spp. from fecal samples, ranging from modified *Campylobacter* and *Leptospira* techniques to those involving *Arcobacter*-specific media. The first reported isolation of an *Arcobacter* used *Leptospira* Ellinghausen-McCullough-Johnson-Harris (EMJH) medium supplemented with 5-fluorouracil (7). Atabay and Corry (1) evaluated the use of *Arcobacter* broth (Oxoid, United Kingdom) with an added cefoperazone, amphotericin, and teicoplanin (CAT) supplement. Johnson and Murano (19) developed JM broth and plates, and more recently Houf et al. (13) developed an *Arcobacter*-specific isolation method involving the use of *Arcobacter* medium (Oxoid, United Kingdom) with a supplement consisting of five antibiotics (cefoperazone, trimethoprim, amphotericin, novobiocin, and 5-fluorouracil). This method has been used in a number of studies on the prevalence of *Arcobacter* spp. (12, 14, 15, 16, 17) and was modified for isolation of *Arcobacter* from animal feces by Van Driessche et al. (36). Other isolation methods used have included EMJH p-80 and brucella broth (28) and direct inoculation onto agar without antibiotics via filtration through a membrane (6).

Despite the range of isolation methods used previously, no single standard method for the isolation of *Arcobacter* spp. from fecal samples has been established. The lack of a standard method means that comparing data from different studies is difficult. It has been suggested that the lack of a standard *Arcobacter* isolation method may mean that many human cases go undetected and that such a method could lead to more accurate reporting of human infections (29) and thus improve efforts to control infection.

The main aims of this study were to compare five methods

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for the isolation of *Arcobacter* spp. from animal fecal samples and to investigate the diversity of a selection of the *Arcobacter* isolates obtained using multilocus sequence typing (MLST). Additionally, the effect of freezing on *Arcobacter* in fecal material was tested in order to determine the reliability of isolating *Arcobacter* from archived frozen samples.

MATERIALS AND METHODS

Comparison of *Arcobacter* isolation methods. A total of 77 fecal samples were collected from cattle ($n = 47$), sheep ($n = 18$), and badgers ($n = 12$) on six farms in Cheshire and Lancashire and from a wildlife park in Gloucester, United Kingdom. Four cattle farms (comprising three dairy and one beef) and two sheep farms were sampled, along with the wildlife park (which contained farmland), which also had a large population of badgers. All of the locations were sampled once, with 6 to 12 fecal samples collected from each. Cattle samples were collected from unweaned calves, weaned calves, nonlactating adults, and lactating adults on each farm. Samples were collected using sterile plastic containers and were processed within 3 h of collection on all occasions, except for the badger samples, which were processed immediately after being received via post.

One gram of fecal material was transferred into 9 ml of enrichment broth, mixed by shaking, and incubated either aerobically or microaerobically, depending on the broth, for 24 h. The following broths were used. H broth was an *Arcobacter*-specific broth (Oxoid, Basingstoke, United Kingdom) with the addition of 5-fluorouracil (100 mg ml^{-1}), amphotericin B (10 mg ml^{-1}), cefoperazone (16 mg ml^{-1}), novobiocin (32 mg ml^{-1}), and trimethoprim (64 mg ml^{-1}) (Sigma-Aldrich, Dorset, United Kingdom), as described by Houf et al. (12). AC broth was an *Arcobacter*-specific broth comprising *Arcobacter* broth (Oxoid, United Kingdom) with the addition of cefoperazone (8 mg $liter^{-1}$), amphotericin B (10 mg $liter^{-1}$), and teicoplanin (4 mg $liter^{-1}$) (CAT supplement; Oxoid, United Kingdom), as described by Atabay and Corry (1). C broth was a *Campylobacter*-specific enrichment broth (Lab M, Bury, United Kingdom) containing 5% (vol/vol) defibrinated horse blood and cefoperazone (20 mg $liter^{-1}$), vancomycin (20 mg $liter^{-1}$), trimethoprim (20 mg $liter^{-1}$), and cycloheximide (50 mg $liter^{-1}$) (CVTC supplement; Lab M, Bury, United Kingdom) as described by Kemp et al. (23). H and AC broths were incubated aerobically at 30°C while C broth was incubated microaerobically at 37°C.

After incubation, 20 μl of enrichment broth was streaked onto solid medium and incubated again. The following solid media were used: H medium, which was the solid equivalent of H broth and contained the same five-antibiotic supplement (12); CC medium, which comprised modified charcoal agar (modified charcoal cefoperazone deoxycholate agar [mCCDA]; Lab M, Bury, United Kingdom) with added CAT supplement (Lab M, Bury, United Kingdom) (23); and C medium, which was a *Campylobacter*-specific isolation medium (Lab M, Bury, United Kingdom) containing mCCDA (Lab M, Bury, United Kingdom) with an added cefoperazone (32 mg $liter^{-1}$) and amphotericin B (10 mg $liter^{-1}$) supplement (Lab M, Bury, United Kingdom) as described by Kemp et al. (23). The five isolation methods used were named as follows: HH (Houf broth and Houf plates), HCC (Houf broth with mCCDA-CAT plates), ACH (*Arcobacter* broth-CAT broth with Houf plates), ACCC (*Arcobacter* broth-CAT broth with mCCDA-CAT plates) and CC (*Campylobacter*-specific broth and *Campylobacter*-specific plates). Enriched samples were plated onto solid media in duplicate, and each sample was subjected to each of the five isolation methods. Plates were incubated aerobically at 30°C (methods H and CC) or microaerobically at 37°C (method C) for 48 h. Even though the H method originally used an incubation temperature of 28°C, 30°C was used here after previous work found *Arcobacter* spp. to grow well at 30°C with little or no growth of contaminants (unpublished data). Up to 10 colonies per sample, per method, were then selected based on morphology (Gram-negative, small gray-white, round colonies) and streaked onto Columbia agar containing 5% (vol/vol) defibrinated horse blood and incubated as before, for 48 h. Table 1 shows the combinations of media used.

The sensitivity of each method was calculated as the ability of a method to detect *Arcobacter*-positive animals. The maximum possible number of *Arcobacter*-positive animals was taken as the total number of animals testing positive during this study using all five of the methods. The number of *Arcobacter*-positive animals using a particular method was then calculated as a percentage of the maximum possible number. Specificity was calculated as the number of *Arcobacter* isolates obtained using each method as a percentage of the total number of isolates retrieved overall (which included *Campylobacter* spp. and other bacterial isolates). The difference in specificity of each isolation method was tested for significance using Fisher's exact test using the GraphPad QuickCalcs free online calculator (http://www.graphpad.com/quickcalcs/contingency1.cfm).

TABLE 1. The five combinations of media and conditions used

Method	Broth type	Solid medium (plate)	Incubation conditions
HH	H	H	30°C, aerobic
HCC	H	CC	30°C, aerobic
ACH	AC	H	30°C, aerobic
ACCC	AC	CC	30°C, aerobic
CC	C	C	36°C, microaerobic

For each isolate, a cell lysate was prepared by creating a cell suspension in 150 μl of distilled water and heating the suspension at 100°C for 15 min before centrifuging at 13,000 rpm for 10 min; the supernatant was used as a template for PCRs.

Identification of isolates by PCR. An *Arcobacter* genus-specific PCR assay (9) was applied to all isolates. Any isolates positive using this PCR assay were then further identified to species level using the *Arcobacter* multiplex PCR assay of Houf et al. (11). All isolates negative in the genus-specific PCR assay were discarded. *Campylobacter* isolates were identified using the multiplex PCR of Wang et al. (41). All PCR assays were carried out using ReddyMix PCR Master Mix (Abgene, Loughborough, United Kingdom), which contains 1.5 μM $MgCl_2$, 1 U of ThermoPrime *Taq* DNA polymerase, and a 20 mM concentration of the deoxynucleoside triphosphates (dNTPs).

MLST analysis. In order to assess diversity, MLST profiles were obtained for 39 randomly selected isolates. The isolates were subjected to the *Arcobacter*-specific MLST scheme as described by Miller et al. (26). Sequence data were aligned using the in-built ClustalW alignment tool, and dendrograms were constructed for each locus using the neighbor-joining bootstrap test of phylogeny.

Investigation into the effect of freezing. All fecal samples were frozen at −80°C for 7 days and then defrosted and subjected to the five isolation methods described. Archived samples were available and stored at −80°C, so this investigation aimed to show whether these could be used for future work. The resulting isolates were tested using the *Arcobacter* genus-specific PCR assay (9).

RESULTS

Comparison of isolation methods. In total, 1,260 isolates were recovered from 77 animal fecal samples using five isolation methods. Of these isolates, 483 (38.3%) were identified as *Arcobacter* and further assigned to species using PCR assays. Of the remainder, 231 were identified as *Campylobacter* and assigned to species using PCR; the rest ($n = 546$) did not belong to the *Campylobacteraceae*, and the majority of these did not comply with typical *Arcobacter* morphology when recultured. A small number of isolates ($n = 24$) gave a positive result in the genus-specific PCR assay but were then negative when the species-specific PCR was attempted. These isolates were subjected to *groEL* gene sequencing (22) and identified using BLAST analysis as some *A. butzleri* isolates as well as some fecal flora, including *Pseudomonas* sp., *Acinetobacter* sp., *Psychrobacter* sp., and *Escherichia coli.*

The five isolation methods (HH, HCC, ACH, ACCC, and CC) (Table 1) were compared for sensitivity and specificity using all of the samples from which *Arcobacter* spp. were isolated. Table 2 shows the sensitivity and specificity of each method. HCC had the greatest sensitivity (70.7%) and specificity (63.9%) of the five methods tested. The sensitivities of the other four methods were lower than the sensitivity of the HCC method (Table 2). The difference in the specificity of each method was tested for significance using Fisher's exact test. HCC was significantly more specific than all four other methods ($P = 0.0139$ compared with ACCC, ACH, and CC; $P = 0.0249$ compared with HH).

HCC isolated the largest proportion of *Arcobacter* (37.6%).

TABLE 2. Sensitivity and specificity of each isolation method tested[a]

Method	No. of *Arcobacter*-positive samples	No. of *Arcobacter* sp. isolates	No. of *Campylobacter* sp. and non-*Campylobacteraceae* isolates	Sensitivity (%)	Specificity (%)
HH	17	92	62	41.5	59.7
ACH	18	63	201	43.9	23.9
ACCC	18	58	290	43.9	16.6
HCC	**29**	**175**	**99**	**70.7**	**63.9**
CC	18	95	125	43.9	43.2

[a] The method with the greatest sensitivity and specificity is shown in boldface.

Of these, almost equal proportions of *A. skirrowii* (29%) and *A. butzleri* (26%) were obtained, along with 6% *A. cryaerophilus* (Fig. 1). HCC was more selective for *A. skirrowii* than the HH method, which gave a much higher proportion of *A. butzleri* (41%) than *A. skirrowii* (15%). Of the isolates obtained using the CC method, the largest proportion of identifiable isolates (28%) were *Campylobacter* spp. (Fig. 1), as would be expected from a *Campylobacter*-specific method. This method isolated the largest proportion of *A. cryaerophilus* (14%). ACH, ACCC, and CC all appeared to isolate higher proportions of *A. butzleri* than *A. skirrowii*. Of these three, ACCC seemed to be more selective for *A. skirrowii* than *A. butzleri*, with 11% of the isolates being *A. skirrowii* and only 4% identified as *A. butzleri* (Fig. 1).

Overall, the most frequently isolated *Arcobacter* sp. was *A. skirrowii*, constituting 17.8% of the total isolates obtained (47% of all *Arcobacter* isolates). *A. butzleri* made up 15.5% of all isolates (41% of all *Arcobacter* isolates), and *A. cryaerophilus* comprised 4.5% (12% of all *Arcobacter* isolates). *Campylobacter* spp. accounted for 12.2% of all isolates.

Typing of the *Arcobacter* isolates by MLST. Thirty-nine allelic profiles were obtained by MLST, all of which belonged to the species *A. butzleri* and within which 11 sequence types (STs) were present (Table 3). Each ST was exclusive to one farm, meaning that no ST was found on multiple farms. Allelic density (number of alleles/number of strains) was as follows: *aspA*, 20.5%; *atpA*, 15.0%; *glnA*, 17.9%; *gltA*, 23.1%; *glyA*, 28.2%; *pgm*, 25.6%; and *tkt*, 20.5%. For the 11 *A. butzleri* sequence types identified, the allele sequences were concatenated in the order *aspA*, *atpA*, *glnA*, *gltA*, *glyA*, *pgm*, and *tkt* and aligned using ClustalW; a neighbor-joining tree was constructed from the aligned sequences (Fig. 2). ST-1 (*A. butzleri*), ST-206 (*A. cryaerophilus*), and ST-243 (*A. skirrowii*) from the MLST database were included in the tree for reference.

Use of frozen samples. A total of 750 isolates were recovered from the same fecal samples after they had been frozen at −80°c for 1 week. Of these, 149 (19%) were identified as belonging to the *Arcobacter* genus using a PCR assay (9). These isolates were not assigned to species. *Arcobacter* recovery from frozen samples was approximately 49% compared with the recovery from fresh samples.

DISCUSSION

The five methods tested isolated different proportions of *Arcobacter* spp. These differences are most likely due to various sensitivities to the antibiotic supplements used in the media. *A. skirrowii* was the most frequently isolated species overall, followed by *A. butzleri* then *A. cryaerophilus*; HCC appeared to be more representative of these overall results than the next most specific method, HH. The fact that *A. skirrowii* was the most frequently isolated species of *Arcobacter* in this study is surprising as this species is reported as being the most susceptible to some antimicrobial agents used in selective media (13). *A. skirrowii* may have been present in large enough numbers to make it culturable despite its sensitivity, or possibly the isolates recovered in this study were less sensitive to agents in the media.

A. butzleri, *A. skirrowii*, and *A. cryaerophilus* were all isolated from cattle in this study, whereas *A. butzleri* was the only *Arcobacter* species isolated from sheep. *Arcobacter* spp. were not isolated from badgers. This is the first study to our knowledge to isolate all three species from the feces of cattle in the United Kingdom. A small number of isolates could not be

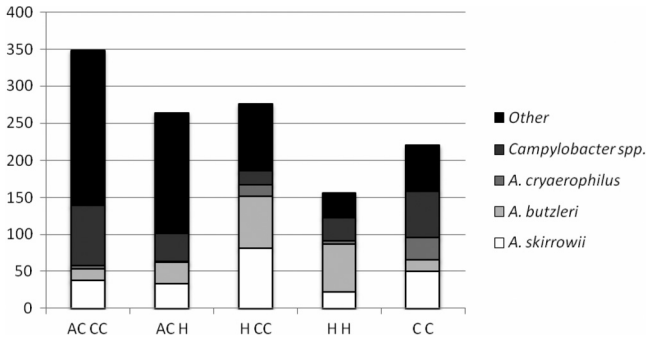

FIG. 1. The proportion of species isolated using each of the five methods. Methods are as described in Table 1.

TABLE 3. Distribution of the 11 *A. butzleri* sequence types present

ST	No. of times present	Location
292	11	Mixed dairy/sheep farm
295	2	Mixed dairy/sheep farm
297	1	Dairy farm 1
299	2	Dairy farm 1
300	2	Beef farm
305	1	Mixed dairy/sheep farm
307	3	Mixed dairy/sheep farm
308	11	Dairy farm 2
334	1	Dairy farm 2
347	2	Beef farm
355	3	Dairy farm 2

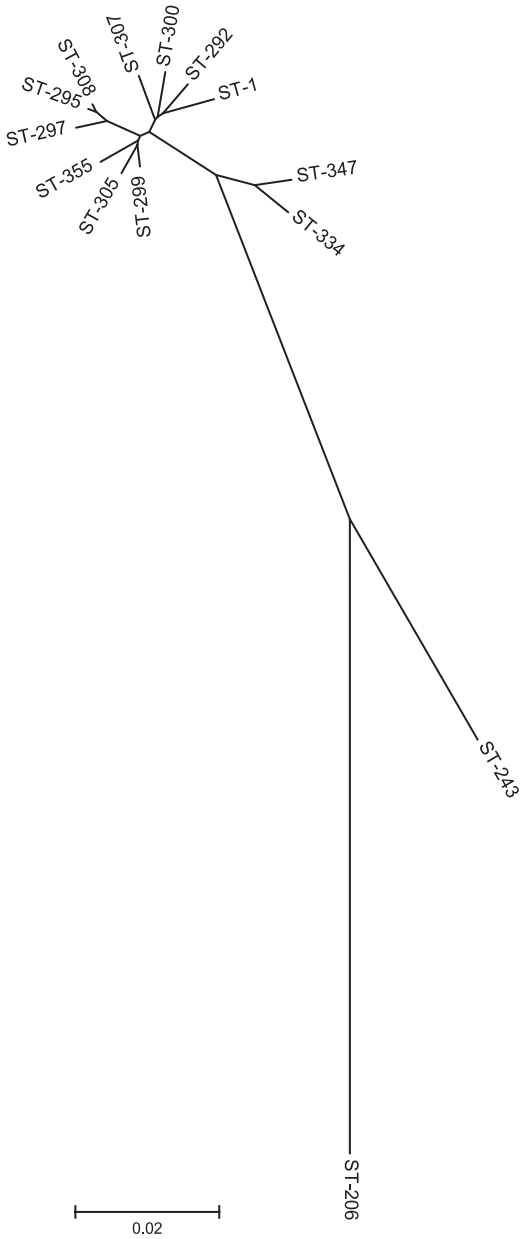

FIG. 2. Neighbor-joining tree constructed using alleles concatenated in the order *aspA*, *atpA*, *glnA*, *gltA*, *glyA*, *pgm*, and *tkt* from the 11 sequence types identified. ST-1 (*A. butzleri*), ST-206 (*A. cryaerophilus*), and ST-243 (*A. skirrowii*) were included from the MLST database for reference.

assigned to species using the multiplex PCR assay (11) after testing positive in the *Arcobacter* genus-specific PCR assay (9). It was initially assumed that these isolates were likely to be either *A. cibarius* (14), *A. thereius* (17), or a novel *Arcobacter* sp. However, after *groEL* gene sequencing and BLAST analysis of the resulting sequences, it was determined that the isolates included further *A. butzleri* and *A. skirrowii* isolates, as well as *Pseudomonas* sp., *Acinetobacter* sp., *E. coli*, and a *Saccharophagus* sp., indicating some unreliability of both the *Arcobacter* genus-specific and species-specific PCR assays.

Previous studies outside the United Kingdom reported low prevalence of *Arcobacter* spp. in sheep (0% [2]); 15% from lamb meat [29]; 16.1% from sheep feces [36]). The prevalence of *Arcobacter* spp. in the sheep samples in this study was 40% ($n = 10$), which is higher than found in a previous study in the United Kingdom, which isolated *Arcobacter* using the *Campylobacter*-specific method, CC (10). Season, climate, geographical location, and sampling and isolation methods may all contribute to the low prevalence of *Arcobacter* recovered from sheep.

MLST analysis revealed a high level of diversity among the isolates at all loci (data not shown). The *glyA2* locus was not used as it was found to be of limited use by Miller et al. (26). The allelic density of *A. butzleri* isolates in this study is lower than that observed by Miller et al. (26), possibly as a result of the small sample size. The greatest allelic density was observed at the *glyA* locus (28.2%), followed by the *pgm* locus (25.6%), in agreement with the study of Miller et al. (26). The *glyA* and *pgm* loci show the greatest variation in both *Arcobacter* MLST studies to date, with the lowest allelic density at the *atpA* locus (15.0%) in this study. Further MLST studies on the diversity of *Arcobacter* spp. in cattle will elucidate whether allelic density is consistently lower in *Arcobacter* isolates from cattle.

Figure 2 shows that ST-334 and ST-347 form an outlying group from the main group of *A. butzleri* STs found in this study. ST-334 was identified on one occasion from the feces of a dairy cow on a farm in Lancashire. Other STs found on the same farm were STs 308 and 355, and one of three samples from this farm was coinfected with both ST-308 and ST-355. None of the STs from this farm was detected elsewhere. ST-347 was recovered from the feces of a beef bull on a farm in Cheshire; ST-300 was also found on the same farm but not in the same animal. Neither ST-347 nor ST-300 was found elsewhere. Overall, none of the STs identified in this study was found on more than one farm (Table 3). As might be expected, the *A. butzleri* isolates from this study form a cluster along with ST-1 (*A. butzleri*) from the MLST database while ST-206 (*A. cryaerophilus*) and ST-243 (*A. skirrowii*) form separate, distinct branches.

Freezing of the fecal samples resulted in a 50% reduction in recovery of *Arcobacter* spp. It is therefore recommended that for optimal isolation of *Arcobacter* spp. from fecal samples, the samples used must be fresh and not frozen. It is possible that one or more species of *Arcobacter* may be more or less susceptible to freezing; therefore, an investigation into the effect of freezing where species is taken into account would be of value.

In conclusion, this study has determined a sensitive and specific method for the isolation of *Arcobacter* spp. from animal feces, which is recommended for use as a standard *Arcobacter* isolation method, and determined that frozen fecal samples are not recommended for use in the isolation of *Arcobacter*. MLST showed that a large amount of diversity exists among *Arcobacter* isolates from cattle.

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