Prevalence and Distribution of \textit{Arcobacter} spp. in Raw Milk and Retail Raw Beef

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ABSTRACT

A total of 106 beef samples which consisted of local (n = 59) and imported (n = 47) beef and 180 milk samples from cows (n = 86) and goats (n = 94) were collected from Selangor, Malaysia. Overall, 30.2\% (32 of 106) of beef samples were found positive for \textit{Arcobacter} species. Imported beef was significantly more contaminated (46.8\%) than local beef (16.9\%). \textit{Arcobacter butzleri} was the species isolated most frequently from imported (81.8\%) and local (60\%) beef, followed by \textit{Arcobacter cryaerophilus} in local (33.3\%) and imported (18.2\%) beef samples. Only one local beef sample (10\%) yielded \textit{Arcobacter skirrowii}. \textit{Arcobacter} species were detected from cow’s milk (5.8\%), with \textit{A. butzleri} as the dominant species (60\%), followed by \textit{A. cryaerophilus} (40\%), whereas none of the goat’s milk samples were found positive for \textit{Arcobacter}. This is the first report of the detection of \textit{Arcobacter} in milk and beef in Malaysia.

The genus \textit{Arcobacter} belongs to the family \textit{Campylobacteriaceae} (52), and its nomenclature was revised several times from its description in 1977 until 1991. Initially, it was called aerotolerant \textit{Campylobacter} because members could grow aerobically (38); in 1985 it was named \textit{Campylobacter aerophilus} (37), and finally, it was moved to a new genus named \textit{Arcobacter} (52).

The genus \textit{Arcobacter} is distinguished from \textit{Campylobacter} on the basis of its growth properties (a wide range of growth temperatures, from 15 to 42\(\)C), hydrogen requirement (members do not require hydrogen), and oxygen requirement (microaerobic and aerobic nature) (52).

\textit{Arcobacter} was first isolated from aborted bovine fetuses (8, 36), followed by its isolation from aborted porcine fetuses (9). Currently, the genus \textit{Arcobacter} consists of 13 species (15) that have been detected in various farm animals and foods of animal origin (14, 42, 49, 54).

\textit{Arcobacter} species are recognized as potential food- and waterborne pathogens (17, 18, 32, 35, 41). Water has a significant role in the transmission of \textit{Arcobacter} species both to animals and humans, and it has been estimated that 63\% of \textit{Arcobacter butzleri} infections in humans originate from the consumption of or contact with contaminated water (5, 29, 33, 44). \textit{Arcobacter} species have been associated with at least three waterborne outbreaks, in Slovenia (31) and in Idaho (43) and Ohio in the United States (16), although no clinical or environmental isolates have ever been available for comparison. Moreover, \textit{Arcobacter} organisms have recently been isolated from drinking water in Kayseri, Turkey (11).

Foods of animal origin, such as beef, pork, chicken meat, milk, turkey meat, duck meat, and rabbit meat, have been found to be contaminated with \textit{Arcobacter} (5, 45, 49). It is also reported that the \textit{Arcobacter} species occur in seafood and marine environments (5).

With the increase in its frequency of detection, \textit{Arcobacter} is considered an emerging foodborne pathogen (51). The consumption or handling of undercooked contaminated foods of animal origin and seafood are regarded as the major sources of \textit{Arcobacter} transmission to humans (49). Moreover, new findings have indicated that \textit{Arcobacter} transmission may occur through close contact with pets, such as dogs and cats (13, 26).

\textit{Arcobacter} species have been isolated from healthy humans (24, 46) and humans with diarrhea, enteritis, and bacteremia (10, 43), as well as from patients with pneumonia and a traffic accident victim (27, 58). \textit{A. butzleri}, \textit{Arcobacter cryaerophilus}, and \textit{Arcobacter skirrowii} are three species commonly reported as pathogenic in humans (12). Clinical signs of \textit{A. butzleri} infections are watery diarrhea with abdominal pain, nausea, and vomiting and sometimes fever (50). Compared with diarrhea from a \textit{Campylobacter jejuni} infection, diarrhea caused by \textit{A. butzleri} is more persistent and watery (4, 53, 56).

Information is lacking on the presence of \textit{Arcobacter} in Malaysia in animal origin food products, such as beef and the milk of cows and goats, which has led us to carry out this study to determine the occurrence of \textit{Arcobacter} in beef retailed in wet markets (fresh food markets) and fresh milk of cows and goats in Selangor State, Malaysia.
MATERIALS AND METHODS

Sample collection. A total of 106 beef samples, including local (n = 59) and imported (n = 47) beef, were collected from various stalls in wet markets in the Serdang, Puchong, Kajang, Sungai Besi, Putrajaya, and Bangi areas in Selangor State, Malaysia. The sampling was done during the months of April to August 2010. Each sample was collected with sterile gloves, individually placed in a sterile plastic bag, brought to the Veterinary Public Health laboratory in ice-packed containers, and processed within 3 to 4 h of collection.

Cow’s (n = 86) and goat’s (n = 94) raw milk samples were collected from 10 farms. From each farm, 15 to 20 healthy animals were randomly selected for sampling and a 20-ml milk sample per animal was collected in a sterile Bijoux bottle. The samples were transported to the Veterinary Public Health laboratory within 4 h of collection in containers packed with ice.

Enrichment of meat and milk samples. Ten grams of each beef sample was mixed with 90 ml of sterilized distilled water and homogenized in a stomacher (BagMixer 400 VW, Interscience, St. Nom, France) set at six strokes per second for 90 s. Subsequently, 1 ml of the homogenate was added to 9 ml of enrichment broth (Arcobacter broth, product code CM0965, Oxoid, Thermo Fisher Scientific, Basingstoke, UK) with an antibiotic supplement (cefoperazone, amphotericin, and teicoplanin supplement, product code SR0174, Oxoid, Thermo Fisher Scientific) added and incubated at 30°C under microaerobic conditions (BD CampyPak, BD, Franklin Lakes, NJ) for 48 h. One milliliter of milk was mixed into 9 ml of enrichment broth and incubated as described for beef samples.

Plating of enriched samples. Following enrichment of the samples, a membrane filtration technique as described by Atabay and Corry (1) was used for plating, with slight modifications as described by Shah et al. (48). Briefly, a 47-mm-diameter, 0.65-μm-pore-size cellulose acetate membrane filter (Sartorius Ltd., Epsom, UK) was laid on the surface of blood agar base no. 2 (product code CM0271, Oxoid, Thermo Fisher Scientific) incorporated with 5% defibrinated horse blood, and 4 drops (ca. 100 μl) of broth culture were dispensed onto the plates using a sterile Pasteur pipette. The plates were incubated aerobically at 37°C for 1 h before the filter was removed. After filter removal, the plates were incubated at 30°C under aerobic conditions for 48 h.

Presumptive identification of isolates. Three or four Arcobacter-like colonies were picked from each plate and subjected to Gram staining and oxidase, catalase, indoxyl acetate, and hippurate hydrolysis tests, and their motility was observed by microscopic examination of wet mounts. Isolates were then preserved in cryopreservation beads at −20°C until confirmation and species identification by multiplex PCR (mPCR).

Confirmation and species identification of isolates using mPCR. The mPCR assay was performed as described by Houf and Stephan (25) with some modifications in electrophoretic conditions. Briefly, DNA was extracted using a Wizard Genomic DNA purification kit (Promega, Madison, WI). The assay conditions were optimized using DNA extracts from known strains of A. butzleri (CCUG 17812), A. cryaerophilus (CCUG 17801), and A. skirrowii (CCUG 30483). PCR amplifications were performed in a 50-μl reaction mixture volume containing 25 μl of 2× master mix (Qiagen), 5 μl of 10× primer mix (primers ARCO, BUTZ, SKIR, CR1, and CR2), RNase-free water, and 2 μl of DNA. The reaction mixture was amplified in a thermal cycler (Bio-Rad, Hercules, CA) with the following cycling conditions: initial Taq, temperature 95°C for 15 min, followed by 32 cycles of denaturation at 94°C for 45 s, primer annealing at 61°C for 45 s, chain extension at 72°C for 30 s, and final extension at 72°C for 10 min. Amplified products were electrophoresed in 1% (wt/vol) agarose gel with 1× Tris-borate-EDTA buffer at 80 V for 60 min. Finally, the gel was stained with gel red (Biotium, Hayward, CA) and amplicons were visualized under a UV light in an Alphalmager gel documentation system (Bio-Rad).

Data analysis. To compare the detection rate of Arcobacter species from local and imported beef samples and cow’s and goat’s milk, the Pearson χ² test was used (http://www.graphpad.com/quickcalcs/chisquared1.cfm). The results were considered statistically significant at a P value of ≤0.05.

RESULTS

Small, smooth, translucent, and watery colonies were presumptively identified as Arcobacter. All organisms were gram negative, motile (cork-screw type), positive for oxidase, catalase, and indoxyl acetate hydrolysis, and negative for hippurate hydrolysis. For confirmation of isolates and species identification, all suspected isolates were examined using the mPCR assay. Amplicons of the expected sizes of 257, 401, and 641 bp were generated for A. cryaerophilus, A. butzleri, and A. skirrowii, respectively.

Overall, the isolation rate of Arcobacter species from beef was 30.2% (32 of 106), as shown in Table 1. Imported beef was more contaminated (46.8%) than local (16.9%) products (P = 0.001). The frequency of detection of Arcobacter species from beef samples is shown in Table 1. A. butzleri was the species most frequently isolated from imported (81.8%; 18 of 22) and local (60%; 6 of 10) beef, followed by A. cryaerophilus, which was detected at rates of 33.3% (3 of 10) (local) and 18.2% (4 of 22) (imported) from beef samples. Only 1 of 10 local beef samples (10%) was found positive for A. skirrowii.

A total of 5.8% of the cow’s milk samples were found positive for Arcobacter species, whereas none were detected from any of the goat’s milk samples, which was a statistically significant difference (P = 0.0106) between cow’s and goat’s milk for the isolation of Arcobacter organisms (Table 2). Among the isolated species, A. butzleri was the most frequently (60%) isolated, followed by A. cryaerophilus (40%), whereas A. skirrowii was not detected from any of the milk samples.

DISCUSSION

To the authors’ knowledge, this is the first survey for the detection of Arcobacter spp. from retail beef and fresh milk in Malaysia. In the present study, a total of 30.2% of beef samples were found positive for Arcobacter, with A. butzleri as the most frequently isolated species, followed by A. cryaerophilus and then A. skirrowii (Table 1). In Turkey, 37% of the minced beef samples were found contaminated with Arcobacter spp., with A. butzleri the most frequently isolated species (33.3%), followed by A. cryaerophilus (3.7%) (3). Scullion et al. (47) detected Arcobacter species in 34% of beef samples (37 of 108), including A. butzleri (59.4%), A. cryaerophilus (45.9%), and A. skirrowii (5.4%).
A Japanese study accounted *A. butzleri* as the most frequent species found in meat, whereas *A. cryaerophilus* was the second-most commonly isolated species, followed by the rarely isolated species *A. skirrowii* (28). *A. skirrowii* is often not detected or detected at a low rate, which may be due to its low occurrence in meat, its slow-growing nature, or its being more difficult to isolate (22). Analysis of 88 and 45 beef samples in Australia (45) and Japan (28), respectively, yielded only *A. butzleri*. In Mexico, 38% (17 of 45) of beef samples were positive for *A. butzleri* and 9% (4 of 45) for *A. skirrowii* (56). However, the comparison of rates of occurrence in one study with the rates in other studies is not feasible because of the lack of a standardized isolation protocol (47). The types of meat examined, temperature, environmental differences, meat processing methods, breed of cattle, and age of cattle all contribute to regional differences.

Imported (frozen) beef was more contaminated (46.80%) than local beef (16.9%), with the difference being statistically significant (*P* = 0.001). The disparity in rates of occurrence may be due to various factors, such as geographic and seasonal variation and hygienic conditions during production and processing (22). Survival studies of *Arcobacter* at different temperatures under different environmental conditions have shown that it has the potential to persist in a wide range of temperatures. In water, *A. butzleri* can remain viable for 196 days at 4 and 7°C and for ≥15 min at 60°C (55). *Arcobacter* organisms can survive in post-chilled beef after 24 h of forced-air cooling (7). In another in vitro study, *A. butzleri’s* survival period in chicken meat juice medium was recorded as 35 days at 5°C (30). Storage of cells from the stationary phase at 4 to 8°C caused a gradual decrease (4 log) over 21 days, whereas freezing caused a 2-log decrease in viability after only 24 h of storage, and thereafter, the viability remained constant (21). The data on survival temperatures provide enough clues that *Arcobacter* spp. can combat chilling temperatures. In a study carried out to test the effect of freezing (−8°C) on *Arcobacter* cells in fecal material, it was reported that 149 (19%) *Arcobacter* isolates were recovered from frozen samples after a 1-week period (34). The presence of *Arcobacter* in beef could possibly be encouraged by other factors, which could include water and the slaughtering equipment (22). The handling of beef and the market environment probably play vital roles as sources of contamination on meat (7). Apart from other sources discussed, there is the possibility of transmission of *Arcobacter* spp. by flies. Studies have shown that flies recovered from poultry houses harbor *Campylobacter, Salmonella,* and other pathogens (19, 23).

It is assumed that gastroenteric microorganisms found on raw meat are commonly of fecal origin (20). *Arcobacter* organisms are frequently present in healthy cattle, from which they may act as an unnoticed contamination risk during slaughter (7). This phenomenon has been proved true in pigs, with a prevalence of 43.9% prior to slaughter that resulted in contamination of 96.4% of carcasses and 21% of pork at retail (54). It is commonly assumed that enteric pathogens found on raw meat are mainly derived from fecal
In this study, *Arcobacter* species were found in 5.8% of milk samples of 86 clinically healthy dairy cattle. Species identification by mPCR showed that *A. butzleri* was the most common species (60%), followed by *A. cryaerophilus* (40%). None of the milk samples were positive for *A. skirrowii*. The results are in agreement with the study carried out in Brazil (42), but the isolation rate was low compared with those of Northern Ireland, 45%, where only *A. butzleri* was detected (47), and Turkey, 6% (11), where *A. butzleri* was the most frequently isolated species. The prevalence of *Arcobacter* species in milk depends on farm management (39, 47, 57). Water and animals harboring *Arcobacter* in their intestines may cause contamination of milk (11). Healthy animals carrying *Arcobacter* in their digestive tracts without showing any clinical signs shed the organisms in feces, which contaminate the teats of animals and, subsequently, the raw milk (3, 39).

In the present study, none of the goat’s milk samples were found positive for *Arcobacter*. In Belgium, *Arcobacter* organisms were isolated from goat feces, but goat’s milk samples were negative for *Arcobacter* species (6). It is assumed that the presence of the lactoperoxidase protein in goat’s milk retards the growth and multiplication of *Arcobacter* spp. Lactoperoxidase has antibacterial effects against *Vibrio cholera*, *Salmoneella Typhi*, *Klebsiella pneumonia*, *Shigella dysenteriae*, and *Staphylococcus aureus* (40). A similar study comparing goat and bovine lactoperoxidase showed that only goat’s milk is antibacterial (2).

In conclusion, *Arcobacter* spp. were detected from retail beef and fresh dairy cow’s milk in Malaysia, but none of the goat’s milk samples were found positive. *A. butzleri* was the most frequent species, followed by *A. cryaerophilus* and *A. skirrowii*. The presence of *Arcobacter* species in beef suggests that there is a need to further study the market environment and the processing line at slaughter houses and scrutinize control measures to curtail *Arcobacter* contamination in processing and retail environments.

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**REFERENCES**
