Research Note

Sensitivity of Three Methods Used in the Isolation of Arcobacter spp. in Raw Ground Pork

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ABSTRACT

Arcobacter, an aerotolerant Campylobacter-like organism, has been designated an emerging pathogen because of its newly recognized ability to cause diarrheal illness in both humans and animals and its presence in the human food supply. Because there is no standard isolation method for its detection, the true occurrence of this pathogen is largely unknown. In addition, the lack of a standardized isolation protocol limits the ability of investigators to compare field data. Arcobacter has been detected in whole muscle and ground pork at various levels by two different isolation methods (those of deBoer and Collins). In this study, these methods were tested along with the Johnson-Murano (JM) method, developed in our laboratory. The sensitivity of each method was tested for ground pork inoculated with Arcobacter butzleri and Arcobacter cryaerophilus 1A at levels of $10^2$, $10^3$, $10^4$, and $10^5$ CFU/g. Controls included tubes with uninoculated pork and broth tubes without pork. All samples that were morphologically similar to Arcobacter were analyzed by Gram staining and by catalase and oxidase reactions. Presumptive positive samples were confirmed by the polymerase chain reaction. The JM method was determined to be the most sensitive, detecting A. butzleri down to a level of $10^3$ CFU/g in 100% of the samples and detecting A. cryaerophilus 1A at a level of $10^4$ CFU/g in 75% of samples. In a pure buffer system, the Collins method was as effective as the JM method in isolating both organisms to levels of $10^4$ cells per g.

Arcobacter species exhibit marked similarities to Campylobacter with respect to phenotypic and genotypic characteristics such as morphology, nutrient requirements, and the ability to induce illness. Although closely related to Campylobacter, Arcobacter is distinctly different. Arcobacter organisms differ from those of Campylobacter in %G+C content, fatty acid methyl ester composition, electrophoretic protein profiles, and growth conditions. With regard to the latter, Arcobacter organisms have the ability to grow in aerobic conditions at low temperatures. Some strains of Arcobacter have been reported to grow at 5 to 40°C, and growth can be observed under aerobic, microaerophilic, and anaerobic conditions. Campylobacter fetus is the only Campylobacter species that has been found to grow at temperatures of <37°C; however, C. fetus is a strict microaerophile. Thus, the ability to withstand both low temperatures and atmospheric oxygen levels seems to be unique to Arcobacter and has been the basis of the development of current isolation methods.

The proper identification of Arcobacter is essential for an understanding of its role in causing human foodborne illness. Traditional plating methods and dark-field microscopy have led to misdiagnosis based on the aforementioned similarities to Campylobacter. It has been speculated that the absence of an effective standard isolation procedure for Arcobacter may be leading to significant underreporting of this organism in foods and clinical samples. Some scientists believe that because of the similarities between Arcobacter and Campylobacter, outbreaks attributed to Campylobacter may in fact be due to Arcobacter instead. Jacob et al. (10) isolated Arcobacter from a drinking water reservoir in eastern Germany in 1993. Their findings suggest that “campylobacter-like” bacteria from different water sources that were formerly typed as Campylobacter jejuni or Campylobacter jejuni-like should now be considered Arcobacter spp. in many cases. Arcobacteriosis has been found to produce symptoms similar to those of campylobacterial illness, including persistent diarrhea, abdominal pain, vomiting, fever, chills, and malaise. In a study of an outbreak of diarrhea in Thai children, both Campylobacter and Arcobacter were recovered from 15% of the children. C. jejuni comprised 67% of the isolates detected, and Arcobacter comprised 16%, with other campylobacters making up the remainder. This study allowed for the isolation of Arcobacter because of the lower incubation temperatures used in the procedure. However, in previous epidemiological studies, the standard Campylobacter isolation techniques used were not able to isolate Arcobacter because the use of higher temperatures (42°C) in these studies was not selective for Arcobacter. Therefore, current isolation methods have focused more on the differing temperature and oxygen requirements as a basis to select for Arcobacter.

Atabay and Corry (1) showed the importance of using both a direct plating step and an enrichment step when ex-
amining poultry for C. jejuni and Arcobacter. These authors concluded that without the direct plating step, Campylobacter could not be isolated and that without enrichment, Arcobacter was undetectable. Thus, Atabay and Corry recommended that isolation procedures include enrichment because of the larger numbers of C. jejuni on poultry carcasses, which can mask lower numbers of Arcobacter, thus hindering the isolation of Arcobacter organisms.

Numerous isolation methods have been used to test swine tissue for Arcobacter since it was first detected by Ellis et al. (7). Some common isolation methods used today to detect Arcobacter in food and environmental systems include those described by Lammerding (14), Atabay and Corry (the Oxoid system) (2), Collins et al. (the Collins method) (5), deBoer et al. (the deBoer method) (6), and Johnson-Murano (the JM method) (11). The selective abilities of these isolation systems are based on aerotolerance and growth at lower temperatures (15 to 30°C), as these traits distinguish arcobacters from campylobacters and related taxa (18).

Isolation methods are chosen for efficiency of isolation and degree of selectivity for the organism of interest. In the present study, we selected the Collins and deBoer isolation methods because they have proved effective in isolating Arcobacter in pork systems. The third method we tested, the JM method, was previously shown in our laboratory to outperform these two methods in the detection of Arcobacter on poultry carcasses (12). The JM method isolated Arcobacter strains in 42 of 50 broiler chicken samples, while the deBoer and Collins methods detected the organism in only 15 and 24 of 50 samples, respectively. No research to date has tested the sensitivity of each of these methods in isolating different concentrations of Arcobacter in pork to determine whether one or more of the methods is more sensitive than others. The purpose of this research was to determine the most sensitive isolation tool for the detection of this pathogen in pork.

MATERIALS AND METHODS

Bacterial cultures. Arcobacter butzleri (49616) and Arcobacter cryaerophilus 1A (43158) were received from the American Type Culture Collection (Rockville, Md.). Upon receipt, the cultures were rehydrated and incubated under microaerophilic conditions with 6% O2 and 20% CO2 in GasPak jars (Becton Dickinson Inc., Gaithersburg, Md.) at 37°C in brain heart infusion (BHI) broth (Difco Laboratories, Detroit, Mich.) supplemented with 5% sheep’s blood (Cleveland Scientific, Cleveland, Ohio). Arcobacter was confirmed by polymerase chain reaction (PCR) prior to testing. For routine maintenance, bimonthly subculturing of the stock cultures was carried out throughout the study with brucella broth (Difco) and BHI agar supplemented with 10% defrinated sheep’s blood and held aerobically at 37°C.

Sample preparation in buffer solution. Cultures of A. butzleri and A. cryaerophilus 1A were grown overnight in brucella broth at 30°C. The cultures of Arcobacter were diluted into a 0.1% buffered peptone solution to visually approximate the 0.5 MacFarland standard to obtain 1.5 × 10^8 CFU/ml. This initial concentration was diluted in 0.1% peptone water to achieve cell concentrations of 10^8, 10^7, 10^6, and 10^5 CFU/ml. For each isolation method, 1 ml of each of the four concentrations of bacterial culture was inoculated into each of the enrichment broths (9 ml for protocols 1 and 3, 10 ml for protocol 2). One uninoculated vial of peptone water served as a control. There were 6 tubes per isolation method, including tubes containing each of the four Arcobacter levels being tested, a control tube with uninoculated pork, and a blank tube that contained only the enrichment broth to assure that the broth was not contaminated before the addition of the inoculum, for a total of 18 tubes.

Sample preparation in raw ground pork system. Frozen ground pork was obtained from the Rosenthal Meat Science and Technology Center (Texas A&M University, College Station, Tex.). Cultures of A. butzleri and A. cryaerophilus 1A were grown overnight in brucella broth at 30°C. The ground pork was stored frozen and was thawed at 4°C for 24 h prior to use. The cultures of Arcobacter were then diluted to 0.1% buffered peptone (Oxoid, Basingstoke, Hampshire, UK) to visually approximate the 0.5 MacFarland standard to obtain 1.5 × 10^8 CFU/ml. This initial concentration was further diluted in 0.1% peptone to obtain cell concentrations of 10^8, 10^7, 10^6, and 10^5 CFU/g upon inoculation into pork. Ground pork was divided into samples of 25 ± 0.1 g with a TM400 Lab Balance (Ohas Inc., Florham Park, N.J.) and placed into filtered Whirl Pack bags (Nasco Plastics Corp., Modesto, Calif.). Each sample was inoculated by dispensing the four concentrations of bacterial culture into four separate 25-g batches of product, with one bag serving as the uninoculated control. The pork samples were then stomached for 2 min with a stomacher blender (Blender Model 400, Steward Lab., London, UK), diluted in 225 ml of 0.1% peptone water (resulting in final cell concentrations of 10^8, 10^7, 10^6, and 10^5 CFU/ml), and stomached for an additional 2 min to thoroughly disperse bacteria. Samples (1 ml) of each dilution were then added to the appropriate enrichment broth (9 ml for protocols 1 and 3, 10 ml for protocol 2) for each isolation method. There were 6 tubes for each of the three isolation methods (tubes containing each of the four levels of Arcobacter being tested, a control tube with uninoculated pork, and a blank tube containing only the enrichment broth to assure that the broth was not contaminated before the addition of the meat solution), for a total of 18 tubes. This procedure was repeated four times on four different days and was conducted separately for two species, A. butzleri and A. cryaerophilus 1A.

Isolation methods. Protocol 1, the Collins method, used Ellinghausen-McCullough-Johnson-Harris polysorbate 80 (EMJH-P80) as an enrichment medium, and the selective plating step involved plating onto cefalothin-vancymycin-amphotericin B (CVA) agar (Sigma, St. Louis, Mo.) (5). The enrichment broth was incubated for 9 days under aerobic conditions, after which a loopful of culture was removed from approximately 1 cm below the surface of the tube, streaked onto CVA agar, and incubated for 48 h at 30°C under microaerophilic conditions with Gas Pack jars (BBL, Sparks, Md.) and Campy Pack gas envelopes (BBL). Blood agar yielded round white, off-white, or grayish colonies that were convex with smooth edges. Isolates that were morphologically similar to Arcobacter were subcultured onto BHI agar with 10% sheep’s blood (Cleveland Scientific, Inc., Bath, Ohio).

Protocol 2, the deBoer method, used Arcobacter selective broth, containing 28 g of brucella broth powder per liter and supplemented with 32 μg of cepfodoxime (Sigma) per ml, 75 μg of piperacillin (Sigma) per ml, 20 μg of trimethoprim (Sigma) per ml, 100 μg of cyclohexamide (Sigma) per ml, and 50 ml of horse blood (Cleveland Scientific) per liter, as an enrichment broth. The broth was incubated aerobically for 48 h at 24°C. Following the enrichment step, Mueller-Hinton medium (Difco) supplemented with 2.5 g of agar and antimicrobial agents, including 32 μg of...
TABLE 1. Numbers of trials (out of four) in which A. butzleri was isolated from pork with three isolation methods

<table>
<thead>
<tr>
<th>Isolation method</th>
<th>$10^2$ CFU/g</th>
<th>$10^3$ CFU/g</th>
<th>$10^4$ CFU/g</th>
<th>$10^5$ CFU/g</th>
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<tbody>
<tr>
<td>Collins</td>
<td>2/4</td>
<td>2/4</td>
<td>3/4</td>
<td>2/4</td>
</tr>
<tr>
<td>DeBoer</td>
<td>3/4</td>
<td>3/4</td>
<td>2/4</td>
<td>1/4</td>
</tr>
<tr>
<td>JM</td>
<td>4/4</td>
<td>4/4</td>
<td>4/4</td>
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cefoperazone per ml, 75 μg of pipericillin per ml, 20 μg of trimethoprim per ml, and 100 μg of cyclohexamide per ml, was inoculated by dispensing 40 μl of the growth of the enrichment broth onto the center of the plating medium and incubating the plates for 72 h at 24°C under aerobic conditions. The plates were then examined for motility zones described by deBoer et al. (6) as gray and continuous, with growth from such zones being re-streaked onto BHI agar supplemented with 10% sheep’s blood. Growth on blood agar requiring additional incubation aerobically for 48 h at 30°C was necessary in order to carry out biochemical tests and Gram staining in a consistent manner (16).

Protocol 3, the JM method, consisted of selective enrichment in JM broth followed by plating onto a selective agar known as JM agar. The broth was prepared with 1.0% special peptone no. 2 (Oxoid) as a base, to which 0.05% pyruvate, 0.05% thioglycolate, 3% activated charcoal, 0.2% bile salts no. 3 (Difco), 32 mg of cefoperazone per ml, 200 mg of 5-fluorouracil per ml, and 0.2% agar were added. The sample was inoculated into JM broth and incubated aerobically at 30°C for 48 h. The lids of the broth tubes were loosely screwed onto the tube bases to promote an aerobic atmosphere. After enrichment, a loopful of broth was extracted from approximately 1 cm below the surface and plated onto JM agar. The plating medium was composed of 1.0% special peptone no. 2, 0.05% pyruvate, 0.05% thioglycolate, 5% sheep’s blood, and 32 mg of cefoperazone per ml. Plates were incubated aerobically at 30°C for 48 h. Arcobacter growth on plates appeared as gray translucent or clear colonies. Presumptive Arcobacter growth was transferred to BHI agar supplemented with 10% sheep’s blood (Cleveland Scientific) for confirmatory tests.

Confirmation techniques. Morphologically presumptive colonies were tested for the presence of catalase and oxidase and were Gram stained for preliminary confirmation (8). Colonies obtained from each isolation protocol were confirmed to be Arcobacter colonies by a PCR assay developed by Harmon and Wesley (9). PCR primers containing genus-specific sequences were used for the identification of Arcobacter. Primers ARCOI (5’-AGAGTTAGGCTGTATTGTATC-3’) and ARCOII (5’-TAGCA-TCCCGCTTCAAAGA-3’) target a 1,223-bp region of the Arcobacter 16S rRNA gene (9).

RESULTS AND DISCUSSION

The three isolation methods tested were selected because they had successfully isolated Arcobacter in pork or because of their exemplary performance in poultry systems. In this study, they were evaluated not only for their sensitivity in detecting Arcobacter at low levels in pork systems, but also for their ease and practicality of use.

Since A. butzleri and A. cryaerophilus have been isolated from humans with gastroenteritis, we selected these pathogens for the evaluation of the three methods (13). There are two hybridization groups of A. cryaerophilus that cannot be phenotypically distinguished but vary somewhat in their abilities to survive in harsh growth conditions. Although A. cryaerophilus 1B is the hybridization group associated with cases of human enteritis, A. cryaerophilus 1A was chosen for testing because it has been found to be more sensitive to NaCl, high temperatures, and other growth parameters (13, 18). The aim of this study was to find the isolation method that was most sensitive in detecting Arcobacter at varying levels, so the more delicate hybridization group was selected to assess sensitivity.

A preliminary trial consisting of a buffer-only system inoculated with A. butzleri and A. cryaerophilus 1A at levels of $10^4$, $10^3$, and $10^2$ CFU/ml was used to determine how the isolation methods operated in the absence of pork meat. The Collins method and the JM method were found to perform equally well at isolating both species of Arcobacter at a level of $10^4$ CFU/ml. The DeBoer method was as sensitive for the isolation of A. butzleri at $10^4$ CFU/ml but not able to isolate A. cryaerophilus at any level. deBoer et al. (6) described the capability of this isolation method with respect to A. butzleri and Arcobacter skirrowii but included no information regarding the isolation performance of their method for A. cryaerophilus, suggesting that preliminary trials may have proven unsuccessful in isolating this species or that this method was not tested with A. cryaerophilus.

The Collins, deBoer, and JM methods were tested on four different days for their ability to detect Arcobacter in ground pork at different levels. The JM method was the most sensitive, detecting A. butzleri at $10^4$ CFU/g 100% of the time and detecting A. cryaerophilus 1A at $10^4$ CFU/g 75% of the time (Tables 1 and 2). Overall, more A. butzleri than A. cryaerophilus was detected on any given isolation medium.

The Collins method was difficult to use because of the overgrowth of plates with white, mucoid colonies in most cases. Thus, the medium used was not found to be selective for Arcobacter. In fact, even when it did not appear that there were any Arcobacter cells present on the plates, Arcobacter was detected with Gram staining and PCR confirmation (data not shown). This finding demonstrates that although Arcobacter was in fact present on the plates, it was being concealed by the overgrowth of competing organisms. The only selective agent used in the broth was 200 μg of 5-fluorouracil per ml, which might not inhibit enough of the competing flora in the enrichment step.

The DeBoer method was cumbersome to use because of the semisolid consistency of the medium in the petri plates. There were often zones present that were cloudy and white rather than clear, gray, and continuous, and these
zones were confirmed not to contain Arcobacter (data not shown). However, anytime there was a clear, gray, and continuous zone of motility (as described by deBoer et al. (6)) present, Arcobacter was confirmed by PCR. This implies that there are also competing organisms that are not inhibited by the antimicrobial agents in the deBoer formulation, possibly allowing Arcobacter to go undetected in some cases. A. cryaerophilus grew with this method in the inoculated pork studies, whereas it did not grow in the aforementioned preliminary trial with pure buffer. The JM method resulted in little contamination and was able to isolate Arcobacter down to the lowest level in most cases.

These findings parallel those described by Johnson and Murano (12) for a comparison study of these three methods for isolating Arcobacter from poultry carcasses. These investigators reported that the isolation plates for both the Collins and the deBoer methods contained more contaminants than did those for the JM method. In fact, 28% of 50 samples tested with the Collins method were completely overgrown. Wesley (19) has eliminated the use of the selective agar plates and reports using the EMJH-P80 broth only because of contamination problems associated with the Collins method. Moreover, Johnson et al. (12) found that 16% of 50 samples isolated by using the deBoer method were contaminated enough to preclude detection.

The limitations of these isolation methods could prevent the isolation of Arcobacter from pork meat, since the enrichment broth of either the Collins method or the deBoer method may not allow the growth of Arcobacter to a detectable level in the presence of other contaminants. It is possible that the antimicrobial agents at the concentrations used, which should select for Arcobacter, could be inhibiting its growth. Also, competing microflora could be covering colonies of Arcobacter, making detection impossible, as the procedure for PCR confirmation involves picking colonies that are morphologically representative of Arcobacter. The latter of these two explanations is supported by the results presented here.

Overall, A. butzleri was detected more often than A. cryaerophilus 1A when these pathogens were inoculated at the same levels in pork samples. In addition, the growth characteristics of A. cryaerophilus were much different from those observed for A. butzleri for every isolation method tested. A. butzleri produced larger and more numerous colonies than did A. cryaerophilus. These findings are similar to those of Atabay et al. (3), who observed abundant growth and larger colony sizes for A. butzleri than for A. cryaerophilus, even after a 3-day incubation period.

One reason for the difference in detection between the two species could be that A. cryaerophilus has been found to grow less on blood agar than A. butzleri does. Kiehlbauch et al. (13) reports that since A. cryaerophilus strains (hybridization groups 1A and 1B) grew poorly on blood agar, they would most likely be missed in specimens containing contaminating flora. This idea is further supported by the results obtained for the one trial in which buffer was used without pork, for which both the JM and the Collins methods detected A. butzleri and A. cryaerophilus at every level.

In conclusion, although there are limits to the sensitivity of each isolation method, the JM method was proven to be the most sensitive in detecting the two strains of Arcobacter that induce illness in humans, and this method should therefore be considered in future clinical studies. In addition, it should be noted that the JM method, besides being the most sensitive in detecting Arcobacter in ground pork, also produced results in about half the time taken by the deBoer method and in one-third the time taken by the Collins method. Future research should be conducted to determine the contaminants inherent in pork that are inhibiting the effectiveness of these methods, and the results of such research should be used in formulation modification. Research efforts should also focus specifically on improving the JM method, since it was determined to be the most sensitive, the easiest to perform, and the most timely in producing results. The specificity and selectivity of the JM method should be evaluated to determine which contaminants effectively compete with Arcobacter. In addition, both hybridization groups of A. cryaerophilus and multiple strains of each species of Arcobacter obtained from various studies should be used for further testing for a more complete assessment of the capabilities of the JM method.

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REFERENCES


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