RESEARCH REPORT

PORK SAFETY

Title: Evaluation of Isolation Method for Arcobacter in Pork - **NPB #98/159**

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ABSTRACT

Arcobacters are a group of emerging pathogenic organisms, that have caused outbreaks of foodborne illness in Europe. They are very similar to campylobacters, and it is thought that cases attributed to those organisms may have been caused by *Arcobacter* species instead. Arcobacters have been found in water, swine, cattle, poultry, and several food sources. These organisms have been found in ground pork, with incidences varying from 0 to 90% prevalence. The procedures used to isolate arcobacters are cumbersome and may not be accurate enough to reveal their true incidence in food products. Thus, the goal of this study was to compare three methods for the isolation of arcobacters (JM, Collins, and deBoer) in terms of sensitivity and specificity, both in buffer and in ground pork systems. In addition, we sought to determine the incidence of arcobacters in ground pork from various slaughter facilities across the U.S., testing the three methods to ascertain the true impact that these pathogens may have on the safety of such products. Finally, we conducted a small-scale study to determine whether meat fat content and/or age of the animal at slaughter would impact the incidence of arcobacters in ground pork.

Of the three methods evaluated, the Johnson-Murano (JM) method was superior to the others, able to detect *A. butzleri* and *A. cryaerophilus* in buffer down to a level of 10^1 cells/ml(g). This method was also the most successful in consistently detecting these organisms in ground pork in various trials. Evaluation of these methods in ground pork samples obtained from various plants revealed that 64/200 samples were contaminated with arcobacters as detected by the JM method, compared with 52/200 as detected by the Direct Collins method. Thus, given its accuracy and sensitivity, as well as its procedural ease, the JM method was superior to the others at detecting arcobacters from ground pork. The level of contamination was found to vary among plants, ranging from 0 to 68% incidence, with 32% overall for all four plants tested.

Ground pork low in fat had a higher contamination frequency (20%) when compared with high-fat pork (4%). Results also showed that meat from younger animals was more frequently contaminated than that from older animals, but the difference was not statistically significant.

INTRODUCTION

Food safety has gained tremendous importance in the last few years, mainly due to outbreaks of foodborne illness where "new" pathogens were the causative agents. These emerging threats to the safety of our food supply are not usually studied very much until an outbreak takes place. At such a time, scientists, regulatory agencies, and the industry scramble to learn as much as possible about the organism, but by then the damage has been done. Thus, it is now recognized that in order to truly be proactive, one must study potential threats to food safety before they become a problem.

Arcobacter has been implicated in outbreaks of foodborne illness in Europe, and has been found in a myriad of environments and food products, including pork. Arcobacters are very similar to campylobacters, the latter which are the leading cause of diarrhea in the world. Some scientists believe that such similarities may result in misidentification of the cause of an outbreak, with cases due to arcobacters being incorrectly attributed to campylobacters.

In order to investigate the prevalence of arcobacters in pork and other sources, it is imperative to use a method that is both sensitive (can detect very few cells) and specific (can correctly detect the organism in question). In our laboratory, we recently developed a procedure to isolate arcobacters in foods, termed the JM method. In this project, we evaluated the JM method, in comparison with two other procedures (Collins and deBoer) in terms of sensitivity and specificity in buffer, and in ground pork samples obtained from several commercial slaughter plants throughout the U.S. In addition, we performed a small-scale pilot study to determine whether fat content of the meat, and age of the animal at slaughter play a role in the prevalence of arcobacters in pork.

OBJECTIVES

The objectives of this research were two-fold: (1) to compare three methods (JM, Collins, and deBoer) in terms of their ability to detect arcobacters at high vs. low levels in buffer; and (2) to compare the three methods in terms of their ability to detect the presence of arcobacters in ground pork samples. A third objective was added after consultation with industry at the NPPC Post-Harvest Food Safety Committee meeting in 1999. It was suggested that we investigate the effect of fat content of the meat, and the age of the animal, if possible, on the presence of the pathogens.

PROCEDURES

Objective #1

Arcobacter butzleri and *A. cryaerophilus* were grown overnight at 30^{o}C under microaerobic conditions (6% oxygen) in Brucella broth overnight. Each culture was then diluted in 0.1% peptone buffer to yield 4 cell concentrations: 10^4 cells/ml, 10^3 cells/ml, 10^2 cells/ml and 10 cells/ml. In addition, each cell suspension was inoculated into ground pork obtained from the Texas A&M University Rosenthal Meat Science & Technology Center, to yield the following concentrations: 10^4 cells/g, 10^3 cells/g, 10^2 cells/g, and 10 cells/g. Inoculation was carried out by separately adding 1.0ml of each cell suspension into 25g of pork, and homogenizing the mixture in a Stomacher blender for 2 min to distribute the inoculum. The inoculated buffer and pork samples were

evaluated for the presence of *Arcobacter* by the JM method, the Collins method, and the deBoer method.

For the JM method, a 0.1ml aliquot from each cell suspension was separately inoculated into a Johnson-Murano (JM) broth tube, and incubated for 48 hr at 30°C under aerobic conditions. For the pork samples, 0.1g from each was separately inoculated into JM broth and incubated as described above. After incubation, growth was removed from 2mm under the surface of each JM broth tube and streaked onto Johnson-Murano (JM) agar. The plates were incubated at 30°C for 48 hr under aerobic conditions. The identity of suspected colonies was confirmed by Polymerase Chain Reaction (PCR) assay, developed by Wesley et al.(1995).

For the Collins method, a 0.1ml aliquot from each cell suspension was inoculated into Ellinghausen-McCullough-Johnson-Harris Polysorbate 80 (EMJH-P80) broth, and incubated under microaerobic at 7°C for 9 d. For the pork samples, 0.1g from each was separately inoculated into EMJH-P80 broth and incubated as described above. After incubation, growth from each EMJH-P80 tube was streaked onto Campylobacter Vancomycin agar (CVA), and the plates incubated microaerobic for 48 hr at 42°C. Identity of suspected colonies was confirmed by PCR. For the deBoer method, growth from each cell suspension was inoculated into Arcobacter Selective broth (ASB) and incubated at 25°C for 48 hr under aerobic conditions. For the pork samples, 0.1g from each was separately inoculated into ASB and incubated as described above. After incubation, growth from each ASB tube was transferred to Arcobacter Selective agar (ASA), and incubated at 25°C for 48 hr under aerobic conditions. Confirmation of zones of motility was carried out by PCR.

Objective #2

Fifty (50) samples of ground pork were requested from four pork slaughter facilities across the U.S: Plant #1 (Pennsylvania), Plant #2 (Iowa), Plant #3 (Oklahoma), and Plant #4 (North Carolina). Samples were collected by plant personnel aseptically, and placed into sterile plastic collection tubes. These were packed with frozen ice packs in insulated boxes, and shipped overnight to Texas A&M University. Immediately upon arrival, samples were homogenized in 0.1% peptone buffer, and examined for the presence of *Arcobacter* species by the JM, Collins, and deBoer methods as described in Objective #1. Identity of suspect colonies was confirmed by PCR. In addition, PCR was also carried out directly from growth in EMJH-P80, which we termed the "direct Collins" method. This was carried out as per suggestions from Dr. Irene Wesley of the National Animal Disease Center in Ames, Iowa, who cited this protocol as one that she has used to successfully isolate *Arcobacter* from swine tissue samples (personal communication).

Objective #3 (new)

Fifty (50) samples each of low-fat (20-25%) and high-fat (>50%) ground pork were obtained from each of three slaughter plants: Plant #5 (Missouri), Plant #6 (Illinois), and Plant #7 (Iowa). These were collected and shipped to Texas A&M University as described for Objective #2. In addition, 50 samples each of ground pork obtained from young (5-6 months old) vs. old (2-3 year old) animals were obtained from plant #8 (Iowa). Samples were then processed for the presence of *Arcobacter* by following the JM method only. Please note that even though they are located in Iowa, Plants #7 and #8 are not the same, nor are they the same as Plant #2 used for Objective #2 above.

RESULTS

Isolation of *A. butzleri* and *A. cryaerophilus* from peptone buffer at various concentrations was carried out using the JM, Collins, and deBoer methods. Both the JM and the Collins methods successfully isolated both species of *Arcobacter* at every inoculum level (Table 1). In contrast, the deBoer method was unable to detect *A. cryaerophilus* at any level, even though it was able to detect *A. butzleri* at every inoculum concentration.

Table 1: Cell concentrations of two *Arcobacter* species detected in peptone buffer by three isolation methods.

ISOLATION METHOD	CELL CONCENTRATION (cells/ml *A. butzleri*)	CELL CONCENTRATION (cells/ml *A. cryaerophilus*)
Johnson-Murano	10^4, 10^3, 10^2, 10^1	10^4, 10^3, 10^2, 10^1
Collins	10^4, 10^3, 10^2, 10^1	10^4, 10^3, 10^2, 10^1
deBoer	10^4, 10^3, 10^2, 10^1	none

When *A. butzleri* was inoculated into ground pork, only the JM method was able to successfully isolate it at every concentration and on each of the 4 trials (Table 2). The Collins method was also able to detect the organism at every concentration, but not consistently (only 2 out of 4 times at 10^4 cells/g and 10^1 cells/g). Similarly, the deBoer method was able to detect the organism at every level, but not in every trial (Table 2).

Table 2: Number of trials in which various cell concentrations of *A. butzleri* were detected by three isolation methods in inoculated ground pork.

ISOLATION METHOD	10^4 cells/g	10^3 cells/g	10^2 cells/g	10^1 cells/g
Johnson-Murano	4/4	4/4	4/4	4/4
Collins	2/4	2/4	3/4	2/4
deBoer	3/4	3/4	2/4	1/4

When *A. cryaerophilus* was inoculated into ground pork at various concentrations, the JM method was the most successful procedure, able to detect the organism at every level of inoculum (Table 3). This is in contrast to the other methods, which were unable to detect *A. cryaerophilus* at some concentrations. In addition, the JM method was more consistent, able to detect the organism in ground pork at least on 3 out of 4 trials. In contrast, the Collins method detected the organism in 2 out of 4 trials, but only at the highest inoculum concentration, unable to detect it at all at the lowest concentration (Table 3). The deBoer method was only able to detect *A. cryaerophilus* 1 out of 4 trials at best.

Table 3: Number of trials in which various cell concentrations of *A. cryaerophilus* were detected by three isolation methods in inoculated ground pork.

ISOLATION METHOD	10^4 cells/g	10^3 cells/g	10^2 cells/g	10^1 cells/g
Johnson-Murano	3/4	4/4	3/4	3/4
Collins	2/4	1/4	1/4	0/4
deBoer	0/4	1/4	1/4	1/4

It appears that the JM method and the Collins methods are equally sensitive at detecting both species of *Arcobacter* in the absence of other microorganisms, as was the case in sterile buffer. However, the JM method was superior to the other two at isolating both organisms in ground pork, probably due to its ability to select for *Arcobacter* species while not inhibiting them greatly. In that regard, *A. cryaerophilus* was not detected as often, even by the JM method, perhaps due to its sensitivity to selective agents in the medium, or to its inability to compete with other microflora as well as *A. butzleri*.

On isolation of arcobacters from ground pork obtained in slaughter facilities across the country, both the JM and the Direct Collins methods were able to detect a greater number of positive samples, compared with the deBoer and the Collins methods (Table 4). Interestingly, in some cases the Direct Collins method was able to detect positives where the JM method was not, and vice versa. Overall, however, the JM method detected arcobacters in 64 of 200 total samples, compared with 52 with the Direct Collins method. Given the higher number of positives, and the time savings involved in the JM method over the Direct Collins method (4 days vs. 9 days), the JM method should be used preferentially for isolation of arcobacters from ground pork.

Table 4: Number of positive samples for *Arcobacter* species of ground pork obtained from commercial slaughter plants in the U.S. by various isolation methods.

PLANT	JM	DeBOER	COLLINS	DIRECT COLLINS
1 (Pennsylvania)	20/50	2/50	3/50	21/50
2 (Iowa)	34/50	5/50	5/50	22/50
3 (Oklahoma)	10/50	0/50	0/50	8/50
4 (N. Carolina)	0/50	0/50	0/50	1/50
TOTAL	64/200	7/200	8/200	52/200

The deBoer and Collins methods were only able to detect 7/200 and 8/200 positives (Table 4). These results correlate with those presented in Table 3, showing that both of these methods were not as consistent as the JM method at detecting *Arcobacter* species from ground pork. We were unable to distinguish the identity of the *Arcobacter* isolates, so it is possible that *A. cryaerophilus* may have been present in the pork samples, posing a problem for the deBoer and Collins methods. At this time, it is

difficult to determine whether differences within plants are due geographical location or to other factors, such as cleanliness of the plant, etc. A more thorough study involving several plants from each region should be conducted.

Fat content of the meat appeared to have an effect on detection of arcobacters. Ground pork with low-fat content had a higher incidence of these organisms (30/150) than pork containing >50% fat (6/150) (Table 5). It is difficult to ascertain the reason for this phenomenon. Pork containing a lower fat content may be handled more during trimming operations, and thus may harbor more arcobacters because of cross-contamination from workers and utensils. In addition, having a lower fat content may simply allow for more contaminants to be present simply because the bacteria probably reside on the muscle rather than on the fat tissue of the meat. Given the fact that samples were only available in three plants, it is difficult to attribute differences between plants to any specific factor. We speculate that Plant #7 may have received fewer animals contaminated with arcobacters. A more thorough study is needed to determine the cause for the differences observed.

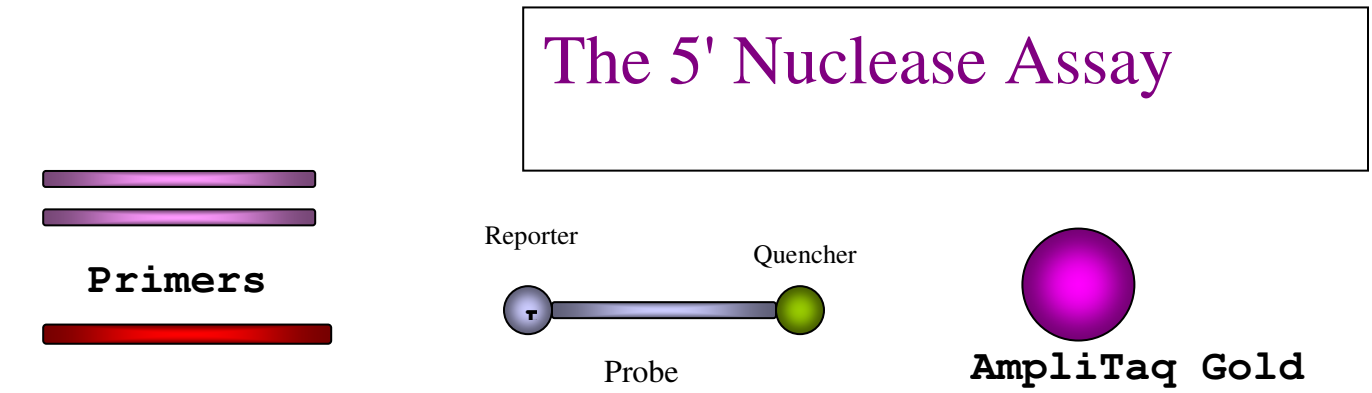
Table 5: Effect of meat fat content on prevalence of *Arcobacter* species in ground pork obtained from various commercial slaughter facilities.

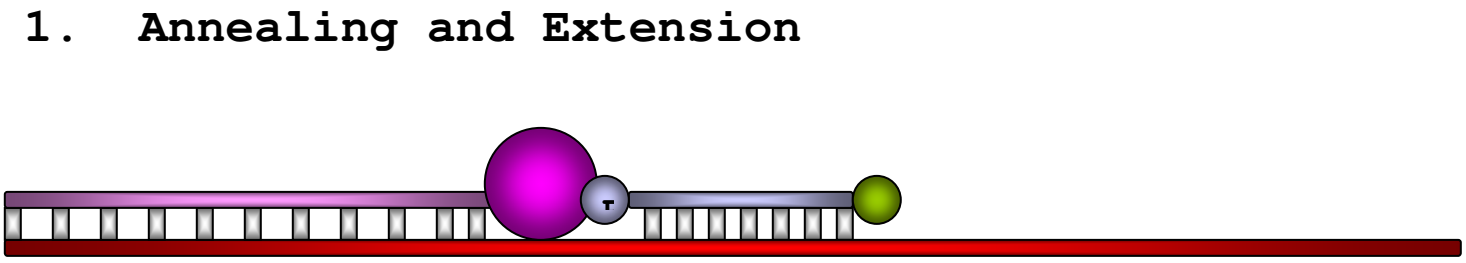
PLANT	LOW FAT (20-25%)	HIGH FAT (>50%)
5 (Missouri)	13/50	2/50
6 (Illinois)	15/50	3/50
7 (Iowa)	2/50	1/50
TOTAL	30/150	6/150

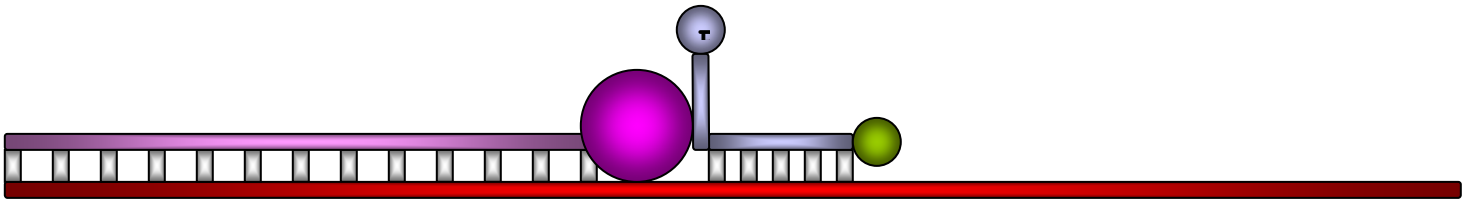
In examining pork samples obtained from old vs. young animals in Plant #8 (Iowa), 3/50 samples of meat from old animals were positive, compared with 11/50 of meat from young animals. It must be noted that only 50 samples of each type were collected for this study, thus there is no statistical difference between these. It is possible that significant differences may be revealed with a higher number of samples. For instance, if 200 samples had been collected of each type of meat, we may have found 12 positives in meat from old animals and 44 positives in meat from young animals, which would constitute a statistically significant difference.

In conclusion, the JM method was superior to the other isolation methods in that it was able to successfully isolate both *Arcobacter* species at levels down to 10^1 cells/ml in peptone buffer every time, and in inoculated ground pork at least 75% of the time. The JM method was as successful as the Direct Collins method at isolating arcobacters from field samples. Due to the ease and speed of the procedure, however, we conclude that the JM method should be the method of choice. Arcobacters were found more frequently in ground pork containing less than 25% fat than in meat containing 50% fat or more. We speculate that this may be due to the extra handling that is involved in trimming cuts to make lean ground pork, but other reasons may be at play. We recommend that a future study be conducted, where the practices involved in producing lean vs. high-fat ground pork be examined to determine whether the risk of contamination is dependent on the operation. In addition, the data suggests that meat obtained from young animals may be more frequently contaminated with this pathogen than pork obtained from older animals. Given the low number of samples available for this part of the study, it is difficult to make any conclusions. Thus, we recommend that a

future study be carried out with a higher number of samples in order to ascertain the true impact of age on contamination of ground pork with arcobacters.







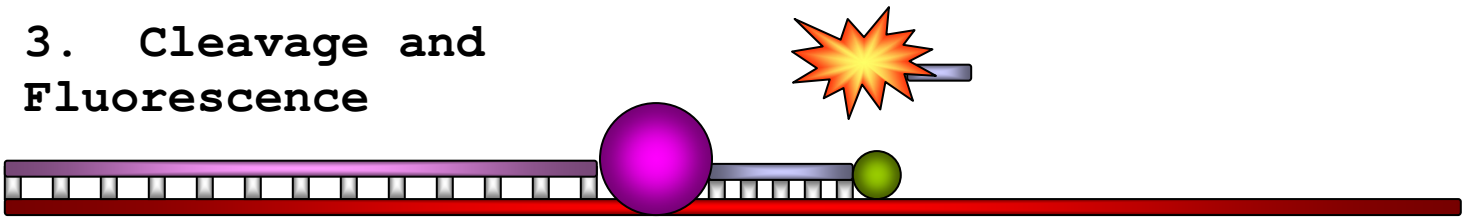


FIGURE 1.
The 5' nuclease assay utilizes the inherent 5' -> 3' nuclease activity of *Taq* DNA polymerase to cleave an internal fluorogenic probe. The probe is labeled with both a reporter and a quencher dye. (1) During PCR, the fluorogenic probe anneals to the target DNA between the two primers. (2) The probe is cleaved during amplification by the 5' nuclease activity of *Taq* DNA polymerase. (3) Cleavage releases the fluorescent reporter from the probe and the attached quencher. This increases the fluorescent emission of the reaction. An increase in fluorescence indicates amplification of target *ail* DNA of *Y. enterocolitica*.