RESEARCH REPORT
PUBLIC HEALTH/WORKER SAFETY

Title: Evaluation of methodologies for qualitative and quantitative detection of methicillin-resistant Staphylococcus aureus (MRSA) in retail pork **-NPB #08-219**

Investigator: J Scott Weese

Institution: University of Guelph

Date Submitted: Feb 10, 2010

Industry Summary:

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a critically important cause of disease in people. It has been a leading cause of infections of people in hospitals, and subsequently emerged as a common cause of infection in people in the general population. As MRSA has moved from a hospital-based problem and into the community, concerns about the role of animals in community-associated disease have emerged. A strong link between human MRSA infections and contact with pigs has been made in Europe, and concerns have arisen about the potential for food to act as a vehicle for MRSA transmission. Preliminary studies from Europe and North America identified MRSA in retail pork, yet the relevance of this finding has been unclear. There are theoretical concerns about the potential for people to become colonized (carriers) of MRSA following contact with contaminated food if good food handling practices are not used. Improper handling and storage of food could also lead to MRSA food poisoning, if MRSA contaminates food and is able to grow and produce enterotoxins. It is important to investigate MRSA contamination of retail pork, both for evaluation of human health risks and to provide important information for the pork industry that will be needed to address and/or allay public and regulatory concerns. One important aspect is development of methods of detect MRSA in meat and determine the level of contamination that is present. Preliminary studies have used different methods, none of which have been validated. Further, these studies have relied on methods that could likely detect very low levels of MRSA. Determination of the level of MRSA contamination in pork and detection thresholds for different methods may be very important aspects for understanding potential risks and for future studies evaluating food as a source of MRSA infection, sources of food contamination and measures to reduce MRSA contamination.

This study evaluated different methods to detect MRSA in meat, and to determine the level of contamination. Results indicated that enrichment culture methods that are commonly used are able to detect very low levels of MRSA in meat, as low at 10 bacteria per gram. Methods to determine the actual level of contamination are possible and practical, and while they cannot detect concentrations of MRSA quite as low as enrichment culture, they can be used to provide a better understanding of contamination.

Optimal methods for detection of MRSA and for determination of the level of contamination were chosen based on the first component of the study and used to test ground pork and pork chops. MRSA was isolated from 8/127 (6.3%) ground pork samples and 14/89 (14%) pork chops, for an overall rate of 9%. Seven of 22 positive samples were only positive using the enrichment culture method, mean the level of contamination was likely less than 20 bacteria per gram of meat. Of the samples where enough MRSA was present to determine a number, only 20 bacteria per gram were present in 9/15 (60%), while the remaining 6 samples had 30, 90, 100, 110, 340 and 3590 CFU/g.

MRSA strains that were isolated were typed and all were classified as the USA100 clone, a human-origin MRSA strain that is the most common strain found in human carriers and people with hospital-acquired infections in North America. This study confirmed previous reports of the presence of MRSA in retail pork, however it is clear that contamination is quite low-level. The relevance of contamination of this level of MRSA is currently unclear. While it should not be dismissed, care must be taken not to over-react to the presence of this organism is meat because of the low level of contamination that is present. Further, it was interesting that all MRSA isolates were a human-origin strain. This strain has been found in pigs in Canada, so it is not surprising that it would be present in some samples, however the most common strain, sequence type 398 (ST398, the strain one that has generated all of the concern regarding pigs) was not present in any sample. This raises questions about the source of MRSA contamination and whether humans or processing environments could be possible sources of contamination, or whether ST398 is less able to survive in the slaughterhouse and processing environments. The presence of USA100 in meat indicates that greater investigation of possible sources of contamination are needed, because if contamination occurs more commonly during meat processing, efforts may need to be focused on post-slaughter measures as opposed to on-farm measures.

Contact information: J Scott Weese, jsweese@uoguelph.ca

Scientific Abstract:

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a critically important human pathogen and has emerged as an important cause of infections of people in the community. MRSA can also be found in various animal species, including pigs. Studies have identified MRSA colonization in pigs in various countries internationally, including the United States and Canada. In Europe, numerous studies have reported an associated between contact with pigs and MRSA infections, specifically infections caused by sequence type 398 (ST398), an MRSA clone that appears to have originated in pigs. The widespread presence of MRSA in pigs has raised concern about the potential for contamination of meat, and meat as a vehicle for MRSA dissemination to people in the community. Recent studies have identified MRSA in retail meat in Europe and North America, yet the relevance of this is currently unclear. One aspect that needs investigation is the level of contamination of pork, since available studies have relied on enrichment culture methods that could potentially detect very low levels of contamination. Further, studies using proper sampling schemes are needed to avoid sampling bias. This study involved evaluation of various methods for qualitative and quantitative detection of MRSA in retail pork, through the use of experimentally inoculated samples. Optimal methods were then used to test evaluate retail pork collected through the systematic sampling methods of the Canadian Integrated Program for Antimicrobial Resistance Surveillance.

There was some variability in the consistency and detection threshold of qualitative methods, with methods using larger samples of meat and selective agar being more consistent and with a lower detection threshold. Rinse methods were equivalent to more labor-intensive homogenization methods. Optimal methods are able to detect 10 CFU/g, or lower. Similarly, quantitative methods varied, with methods using larger samples and selective agar being superior.

MRSA was isolated from 8/127 (6.3%) ground pork samples and 14/89 (14%) pork chops, for an overall rate of 9%. Seven of 22 positive samples were only positive using the enrichment culture method, mean the level of contamination was likely less than 20 bacteria per gram of meat. Of the samples where enough MRSA was present to determine a number, only 20 bacteria per gram were present in 9/15 (60%), while the remaining 6 samples had 30, 90, 100, 110, 340 and 3590 CFU/g. All isolates were spa type t002, negative for Panton Valentine leukocidin genes and classified as USA100 by PFGE. Two different USA100 subtypes were present.

Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a critically important cause of disease in people. It has been a leading cause of infections of people in hospitals, resulting in large numbers of infections and deaths, and tremendous healthcare costs. In the 1990s, MRSA emerged as an important cause of infection in people in the general population, causing infections ranging from mild skin infections to fatal pneumonia and necrotizing fasciitis ('flesh-eating disease'). As MRSA has moved from a hospital-based problem and into the community, concerns about the role of animals in community-associated disease have emerged, and the high profile nature of the bacterium have lead to sensationalized responses to some studies and theoretical concerns. A strong link between human MRSA infections and contact with pigs has been made in Europe, and concerns have arisen about the potential for food to act as a vehicle for MRSA transmission. Theoretically, if good food handling practices were not observed, MRSA on pork could be transferred to someone's hands, then to their nose (the prime site where MRSA can reside) result in colonization or to a compromised site (i.e. cut, wound) and cause an infection. Improper handling and storage of food could also lead to MRSA food poisoning, if MRSA contaminates food and is able to grow and produce enterotoxins. Preliminary studies from Europe and North America have identified MRSA in retail pork, yet the relevance of this finding is unclear. It is important to investigate MRSA contamination of retail pork, both for evaluation of human health risks and to provide important information for the pork industry that will be needed to address and/or allay public and regulatory concerns. One important aspect is development of methods of detect MRSA in meat and determine the level of contamination that is present. Preliminary studies have used different methods, none of which have been validated. Further, these studies have relied on methods that could likely detect very low levels of MRSA. Determination of the level of MRSA contamination in pork and detection thresholds for different methods may be very important aspects for understanding potential risks and for future studies evaluating food as a source of MRSA infection, sources of food contamination and measures to reduce MRSA contamination.

Considering the potential public health concerns and the equal potential for overreaction, more information is needed to properly assess risks and determine optimal approaches. Only with more information will the pork industry be able to proactively deal with any risks, should they be present, and to properly communicate the true concerns to the public.

Objectives:

1) To compare methods for qualitative (yes/no) detection of methicillin-resistant *Staphylococcus aureus* in retail pork.
2) To determine the threshold for qualitative detection of MRSA in retail pork.
3) To compare methods for quantification (number of bacteria per gram) of MRSA in pork.
4) To determine the quantitative detection limit of MRSA in retail pork.
5) To determine the prevalence of MRSA in retail pork using optimal methods.
6) To quantify MRSA in retail pork using optimal methods.
7) To compare MRSA strains from pork with those from concurrent studies in pigs.

Materials & Methods

Preparation of test materials

1) Retail pork chops were purchased. Multiple cultures using enrichment (as described below) were performed to confirm that samples were unlikely to be contaminated naturally. (Sterilized meat samples were not used because of the need to evaluate test procedures in the presence of other bacteria that can be found on retail meat.)
2) Pure culture of an isolate of ST398 MRSA (pig origin) was grown in tryptone soya broth. After 24h of incubation, the broth was centrifuged and the pellet suspended in phosphate buffered saline (PBS, pH 7.4). Serial dilutions of the MRSA suspension were performed on blood agar to quantify the suspension.
3) Varying concentrations of MRSA were made to facilitate inoculation of pork with different MRSA concentrations but the same volume.

4) Pork chops were randomly assigned to different inoculum groups. The surface of samples were inoculated with MRSA ranging from 10-100000 CFU/g of meat.
5) Half of each inoculated pork chop was homogenized in a laboratory blender, mixed 1:1 (w:w) with PBS.

Phase 1

1) Each concentration of inoculated meat was tested as follows:
    a. Direct impression of a 1" x 1" sample of pork chop onto blood agar.
    b. Direct impression of a 1" x 1" sample of pork chop onto Chromogenic MRSA agar
    c. Inoculation of 100 ug of homogenized pork onto blood agar
    d. Inoculation of 100 ug of homogenized pork onto Chromogenic agar.
    e. Inoculation of a 1" x 1" sample of pork chop into 5ml of selective enrichment broth (10 g tryptone T/L, 75 g sodium chloride/L, 10g mannitol/L, and 2.5 g of yeast extract/L) followed by incubation at 35^0C for 24 h and subculture onto Chromogenic agar with 24-48h incubation.
    f. Inoculation of a 1" x 1" sample of pork chop into 5ml of selective enrichment broth (10 g tryptone T/L, 75 g sodium chloride/L, 10g mannitol/L, and 2.5 g of yeast extract/L) followed by incubation at 35^0C for 24 h and subculture onto blood agar with 24-48h incubation.
    g. Inoculation of 5 g (equivalent to a 1" x 1" piece) of homogenized pork into 45ml of enrichment broth followed by incubation at 35^0C for 24 h and subculture onto Chromogenic agar with 24-48h incubation.
    h. Inoculation of 5 g (equivalent to a 1" x 1" piece) of homogenized pork into 45ml of enrichment broth followed by incubation at 35^0C for 24 h and subculture onto blood agar with 24-48h incubation.
    i. For all studies, MRSA was identified by colony morphology, Gram stain appearance, *S. aureus* latex agglutination test and PBP2a latex agglutination test (for determination of methicillin resistance).
    j. The detection threshold will be evaluated for each method. This will be determined by both the lowest concentration at which at least one replicate was positive, and the lowest concentration at which all 3 replicates were positive.
2) Un-inoculated samples were run in parallel as negative controls.
3) All testing was performed in triplicate.
4) Subjective comparison of the methods were performed, with detection threshold, repeatability, ease and cost being considered to determine the optimal method.

Phase 2: Evaluation of quantitative methods

1) Each concentration of inoculated meat was tested as follows:
    a. Serial dilution of 1g of homogenized meat into 9ml of PBS, followed by inoculation of 100 ul onto blood agar.
    b. Serial dilution of 1g of homogenized meat into 9ml of PBS, followed by inoculation of 100 ul onto Chromogenic MRSA agar.
    c. Rinsing of ½ of a pork chop in 50 ml PBS, followed by serial dilution in PBS and inoculation onto blood agar.
    d. Rinsing of ½ of a pork chop in 50 ml PBS, followed by serial dilution in PBS and inoculation onto Chromogenic agar.
    e. All results were adjusted to account for a value of contamination in CFU/gram of meat sample tested.
    f. Subjective comparison of the methods was performed, with detection threshold, repeatability, ease and cost being considered to determine the optimal method.

Phase 3: Determination of the prevalence of MRSA in retail pork

1) 230 retail pork samples were collected through routine CIPARS sampling in the active CIPARS sampling sites (British Columbia, Saskatchewan, Ontario, Quebec and the Maritimes (comprising Nova Scotia, New Brunswick and Prince Edward Island as a single sampling region). CIPARS sampling sites are randomly selected within each province/region based on population weighting; the number of

samples per province is also population weighted. No more than 1 sample was collected per retail store during a given visit, and each store was only visited once.
2) Samples were shipped on ice to the Investigators' laboratory.
3) Based on results from above, Method 'e' from Phase 1 and Method 'd' from Phase 2 were chosen.
4) The prevalence of retail meat contamination was evaluated.
5) The quantity of MRSA present in each sample was determined.
6) Spa typing, PFGE and PVL PCR were performed to characterize isolates.

Results:

1) To compare methods for qualitative (yes/no) detection of methicillin-resistant *Staphylococcus aureus* in retail pork.
    a. Methods that used enrichment broth followed by inoculation onto Chromogenic agar were considered superior. All analysis was subjective, however the growth was more consistent and differentiation of MRSA from other organisms was much easier with this type of approach, thereby increasing the accuracy of testing and decreasing the time required. Methods that involved immersion of the pork chop were similar to those that involved large volumes of homogenized meat, and since homogenization is more labour intensive, immersion of meat into enrichment broth is the preferred method. Methods using smaller pieces of meat or small amounts of homogenized meat did not perform as well at the lower concentrations.
2) To determine the threshold for qualitative detection of MRSA in retail pork.
    a. The detection threshold for enrichment culture was 10 organisms/gram or lower. The optimal methods were able to detect MRSA at this level on all replicates.
3) To compare methods for quantification (number of bacteria per gram) of MRSA in pork.
    a. Methods based on use of the piece of pork chop were superior to those involving smaller amounts of homogenized meat, particularly at lower concentrations. Methods using Chromogenic agar were superior to those using blood agar, as discussed above.
4) To determine the quantitative detection limit of MRSA in retail pork.
    a. The detection threshold was more variable and higher for direct culture, with the lower limit being 20 bacteria per gram for the optimal methods.
5) To determine the prevalence of MRSA in retail pork using optimal methods.
    a. MRSA was isolated from 8/127 (6.3%) ground pork samples and 14/89 (14%) pork chops, for an overall rate of 9%.
6) To quantify MRSA in retail pork using optimal methods.
    a. Seven of 22 positive samples were only positive using the enrichment culture method, mean the level of contamination was likely less than 20 bacteria per gram of meat. Of the samples where enough MRSA was present to determine a number, only 20 bacteria per gram were present in 9/15 (60%), while the remaining 6 samples had 30, 90, 100, 110, 340 and 3590 CFU/g.
7) To compare MRSA strains from pork with those from concurrent studies in pigs.
    a. All MRSA isolates were spa t002 and classified as USA100 on PFGE. There were two different PFGE subtypes (subtypes of USA100). All isolates were PVL negative. This is in contrast with concurrent studies of pigs, where USA100 can be found, but where ST398 predominates. Results are similar to parallel studies of beef, where USA100 MRSA was found in 5.6% of samples.

Discussion

This study demonstrated that enrichment culture methods are able to detect very low concentrations of MRSA and to do so consistently. This is useful for surveillance programs but the low detection threshold must be considered. The ability to quantify MRSA using standard methods is important, and this study has shown that it can be done consistently even with reasonably low MRSA concentrations. The methods that were shown to be optimal here are relatively user-friendly and are amenable to application in most microbiology laboratories, thereby facilitating surveillance activities.

The prevalence of MRSA contamination of retail meat in this study is somewhat similar to other reports from The Netherlands (various meats, 11.9%), the US (pork 5.6%, beef 3.3%) and Canada (pork, 5.8%). Care should be taken in comparing prevalence data between different studies because of a lack of standardized culture methodologies. The use of different sampling techniques must also be considered, and this study is the first to report use of a systematic sampling method to reduce the impact of sampling bias.

A novel and important aspect of this study was quantitation of contamination. Considering most samples were either only positive on enrichment culture or had <100 CFU/g, it is apparent that MRSA contamination is typically low-level. This must be taken into consideration when evaluating potential risks and while low levels may be less concerning, they should not be dismissed.

The predominance of USA100/CMRSA-2 in pork was somewhat surprising. Laboratory error or contamination are unlikely to account for the strain distribution present here since this clone was uncommon in other MRSA studies performed concurrently in the same laboratory, and positive samples were identified sporadically throughout the study period. The additional discriminatory power of PFGE also indicates that contamination should not be a cause because different PFGE types were identified. USA100/CMRSA-2 has been found in pigs in Canada, as well as pork in Canada and the US. Further, spa types consistent with USA100/CMRSA-2 were reported in the large Dutch retail meat study. However, while USA100/CMRSA-2 can be found in pigs, ST398 is the most common strain found in pigs in Canada and the US yet it was not identified in any sample. If MRSA in meat were a direct refection of MRSA in food animals, frequent isolation of ST398 strains would be expected. These factors raise questions about the origin of MRSA in retail meat. The apparent discordance between prevalence and strain types in food animals and food suggests that other sources of contamination may be important, including food processing personnel and the food processing environment. USA100/CMRSA-2 is a common human clone and the most common strain found in colonized individuals in the US. Further, t242 was the 2nd most common CMRSA-2 spa type in a large study of MRSA from humans in Canada. Variation in PFGE pattern can be present within spa types, and 28 distinct patterns were present amongst t242 isolates in that study. Longitudinal 'farm-to-fork' study of the origin of MRSA contamination of food products is indicated.

The relevance of MRSA contamination of retail meat is unknown. While MRSA 'food poisoning', staphylococcal enterotoxin-associated diarrhea, has been reported with MRSA, there should be no clinical difference in disease caused by MRSA versus susceptible *S. aureus*, although it is plausible that MRSA food poisoning could result in subsequent colonization of the affected individual. Touching one's nose after handling contaminated meat could plausibly result in colonization and contact of contaminated meat with skin lesions could potentially result in infection. The true likelihood of these is unknown but deserve further study. Regardless, proper handling of raw meat, including prevention of cross contamination, adequate cleaning and disinfection and good personal hygiene practices (especially hand hygiene) would likely reduce or eliminate any risks. Therefore, while further study of the sources and implications of MRSA contamination of retail meat are indicated, continuing education of the public about safe meat handling practices may be as important.