Inactivation at various temperatures of bovine viral diarrhea virus in beef derived from persistently infected cattle

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ABSTRACT: Bovine viral diarrhea virus (BVDV) is a pestivirus that is enzootic in most cattle populations throughout the world. This virus is present throughout the body of persistently infected (PI) cattle. Previous research has not assessed the cooking temperature at which BVDV in meat from PI cattle can be inactivated. Therefore, muscle tissue from 6 PI cattle was harvested, refrigerated, frozen, and heated to various internal temperatures. The concentration of virus present was determined by virus isolation. Average cell culture infective doses (50% endpoint; CCID50) of BVDV per gram of frozen, uncooked meat from PI cattle were 105.85 CCID50/g of whole cuts and 106.02 CCID50/g of ground meat. The virus in whole and ground meat was consistently inactivated when cooked to temperatures greater than or equal to 75°C. A second objective of this research was to thoroughly reassess if Vero cells were permissive to BVDV infection in our laboratory to provide further indication of whether primates, including humans, might be susceptible to BVDV. Vero cells were not permissive to infection with any of 43 different strains of BVDV that readily replicated in Madin Darby bovine kidney cells. In conclusion, this bovine pathogen, which is not considered to be a human pathogen, can be inactivated by cooking ground or whole cuts of meat to 75°C or higher. Care should be taken to ensure that susceptible hosts such as pigs are not fed improperly cooked meat, meat by-products, or waste food originating from PI cattle.

Key words: beef, bovine viral diarrhea virus, inactivation, internal temperature, persistently infected, virus isolation

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INTRODUCTION

Bovine viral diarrhea virus (BVDV) is a pestivirus of the family Flaviviridae (Ridpath, 2005). This virus is highly mutable and endemic in the United States (Hamers et al., 2001). Persistent infection of cattle with BVDV occurs when a bovine fetus is infected with a noncytopathic strain of virus before approximately 125 d of gestation (Grooms, 2004). Persistently infected (PI) animals develop immunotolerance to the strain they have been infected with and exhibit presence of virus throughout the body (skin, semen, secretions, milk, blood; Thurmond, 2005; Marley et al., 2009; VanderLey et al., 2011).

Approximately 141,600 PI calves are born each year in the United States, based on a PI prevalence of 0.4% of the 35.4 million animal calf crop estimated by the USDA, National Agricultural Statistics Service (Yan et al., 2011). Persistently infected calves exhibit a 50% greater death rate in the first year of life than uninfected calves (Duffell and Harkness, 1985). Thus, approximately 70,800 PI carcasses are processed yearly for meat in the United States. Persistently infected animals can appear normal, so many pass USDA inspection. Bovine viral diarrhea virus has been shown to be inactivated in milk by heating to 85 to 92.2°C for 10 min (Marley et al., 2009). The temperature at which BVDV is inactivated in beef is unknown. The first objective of this study was to determine if cooking ground

1This research was supported by the USDA appropriations for continuing Animal Health and Disease Research Programs (Sec. 1433).
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Received May 3, 2011.
Accepted September 27, 2011.
and whole meat from PI animals to various internal temperatures would inactivate BVDV.

Bovine viral diarrhea virus is considered to be of little or no concern to human health. However, research regarding the susceptibility of primates to BVDV is conflicting. Some published studies describe the ability of BVDV to replicate in primate cell lines (Harasawa et al., 1993; Audet et al., 2000), whereas others do not (Toth and Hesse, 1983; Agnello et al., 1999; Studer et al., 2002; Maurer et al., 2004). The second objective of this study was to reassess if primate (Vero) cells were permissive to BVDV to provide further indication of whether primates (including humans) might be susceptible to BVDV after consumption of PI meat.

MATERIALS AND METHODS

Animals

Six crossbred beef cattle (Auburn University Institutional Animal Care and Use Committee Protocol Review #2009-1616) PI with BVDV were used for this study. These six animals consisted of 4 infected with a genotype 1a strain of BVDV (1 steer and 3 heifers), 1 with genotype 1b (bull) and 1 with genotype 2 (steer). Animals were 14 to 48 mo of age. Animals were previously demonstrated to be PI with BVDV by antigen capture ELISA of ear notch samples, reverse-transcription nested PCR of serum samples, and sequential virus isolation of serum and nasal samples (Rodning et al., 2010).

Harvesting of Meat

Animals were slaughtered within a biosecure area of the Auburn University Lambert-Powell Meats Laboratory under federal inspection. A serum sample (20 mL of whole blood centrifuged at 1,000 × g for 10 min at room temperature) was collected from each animal on the day of slaughter to determine the concentration of BVDV. On the day of slaughter (d 0), 5-g tissue samples of fresh beef were collected immediately after removal of the hide from the right side of each animal carcass from each of the 4 major primals (chuck, rib, loin, and round) for virus titration. Samples were collected in the same area on each carcass and trimmed of external fat cover. Carcasses were refrigerated and subsequently fabricated on d 2. Primals from the left side of each animal were fabricated into 2.54-cm-thick whole muscle steaks from each primal, utilizing the chuck arm, ribeye, striploin, and top round. Each meat sample was individually divided into 0.2-kg samples and vacuum sealed. Samples from each primal were randomly assigned to storage and cooking treatments.

Primals from the right side of each animal carcass were fabricated for ground meat samples from each of 3 ground products. Primals were ground into ground chuck containing meat from only the chuck, ground beef containing meat from the rib and the loin, and ground round containing meat from only the round. After grinding through a 0.3-cm plate, the ground meat was individually divided into 0.5-kg samples and vacuum sealed. Samples were randomly assigned to storage and cooking treatments.

Treatments

Treatments consisted of a) refrigeration for various durations, b) freezing, and c) cooking after freezing. One whole muscle sample from each primal was tested for virus titration and isolation on the day of fabrication (d 2), whereas other samples from each primal were refrigerated in vacuum-sealed bags (Ultravac UV21C, Kansas City, MO) individually (3 mm; 30 to 50 mL of O2/m2 for 24 h at 101,325 Pa and 23°C; Koch, Kansas City, MO) to be aged at 4 ± 2°C for a total of 7, 14, or 21 d postmortem. One sample from each primal was frozen at −20°C on the day of fabrication (d 2) for subsequent testing as a frozen but uncooked control. Six samples from each primal were also frozen at −20°C on the day of fabrication (d 2) for subsequent cooking at 55, 60, 65, 70, 75, or 85°C. One 2.2-kg package of ground meat sample from each of the 3 ground products was frozen at −20°C for subsequent testing as a frozen but uncooked control. Six 2.2-kg packages of ground meat samples from each of the 3 ground products were also frozen at −20°C for subsequent cooking at 55, 60, 65, 70, 75, or 85°C.

Cooking Treatment

Meat was allowed to thaw in the vacuum packages under refrigeration at 4 ± 2°C for 24 h before cooking. After thawing, ground meat samples were formed into 113.4-g patties pressed to 2.54 cm thick. All samples were cooked on a Hamilton Beach style grill model 25331 (Hamilton Beach Brands Inc., Southern Pines, NC) preheated for 15 min. A Type K insulated copper constantan beaded wire thermocouple (Omega Engineering Inc., Stamford, CT) was inserted into the geometric center of each steak, and temperature was monitored throughout the duration of the cooking process with an Omega HH309A Data Logger (Omega Engineering Inc.). The meat was removed from the grill at the specified endpoint cooking temperature for each sample. Samples were removed with tongs, placed on a clean piece of aluminum foil, and allowed to cool at room temperature for 5 min. They were then wrapped in the piece of aluminum foil and placed into zippered plastic bags to prevent meat juices from leaking from 1 sample to another. Between handling and cooking of each sample, all equipment including grill plates and thermostats were wiped with 70% ethanol to ensure virus inactivation.
Laboratory Sample Preparation

Frozen meat was thawed at 4 ± 2°C for 24 h before laboratory sample preparation. Cooked meat was processed for laboratory sample preparation within 2 h after cooking. All meat samples were cut through the center into equal halves using a disposable sterile scalpel blade to facilitate collection of an uncontaminated 5-g laboratory sample from the direct center of the original intact meat sample or ground patty. Preliminary testing in our laboratory indicated very little variation in viral titers if laboratory samples from PI cattle were obtained from the very center or slightly off the center (but not on the extreme periphery) of ground or intact meat cooked to 55°C. This 5-g laboratory sample of meat was placed in a sterile 100-mL beaker and rinsed 1 time with 25 mL of PBS (Invitrogen, Carlsbad, CA) containing 1% penicillin, streptomycin, and amphotericin B (Sigma-Aldrich, St. Louis, MO). The meat was then minced into pieces 2 to 3 mm in each dimension using a sterile scalpel blade. The minced tissue was blended in a Waring MC1 mini blender (Waring Pro, Torrington, CT) container that had been previously cleaned, disinfected, rinsed, and chilled at 4°C. Between laboratory samples, these mini-blender containers were thoroughly washed and subsequently disinfected by filling with a sodium hypochlorite solution at a final concentration of 0.6% for at least 1 h of contact time before thorough rinsing. Ten milliliters of minimum essential medium (MEM, Invitrogen) containing 2% penicillin, streptomycin, and amphotericin B was added to the blended tissue. The mixture was blended on high speed for 60 s and the contents placed into a 50-mL centrifuge tube. Samples were centrifuged at 500 × g for 5 min at room temperature. Supernatant was collected and refrigerated less than 24 h before assaying by virus titration and amplification in Madin Darby bovine kidney (MDBK) cells. Minimum essential medium used in the sample preparation contained 10% (vol/vol) equine serum (HyClone, Logan, UT), sodium bicarbonate (0.75 mg/mL, Invitrogen), L-glutamine (0.29 mg/mL, Invitrogen), penicillin G (200 IU/mL), streptomycin (200 µg/mL), and amphotericin B (0.5 µg/mL).

Virus Titration

Virus titration was performed from serum and the supernatants of whole muscle and ground meat after each treatment and laboratory sample preparation. Virus titration procedures involved multiple 10-fold dilutions performed in triplicate on MDBK cells in a 96-well plate and employed the statistical method of Reed and Muench (Reed and Muench, 1938) to determine the quantity of BVDV present. Plates were incubated for 3 d at 38.5°C in a humidified atmosphere of 5% CO₂ and air and then underwent the immunoperoxidase monolayer assay. The immunoperoxidase monolayer assay was performed as a labeling technique to confirm the presence of BVDV (Afshar et al., 1991; Givens et al., 2003).

Amplification of BVDV in Monolayers of MDBK Cells

Supernatants of whole and ground meat samples were also passaged in monolayers of MDBK cells to attempt to isolate viable BVDV that might be present in very low concentrations. The procedure was performed as described previously (Walz et al., 2008) with some modifications. Culture plates (9.6 cm²) were seeded 24 h earlier with MDBK cells in MEM. Cells were inoculated with 768 µL of sample diluted in 192 µL of MEM. After a 1-h adsorption period, samples were removed from the plate and the cells washed with PBS to remove tissue debris. Minimum essential medium was added to the samples (3 mL). Minimum essential medium as described above was used except that 1% penicillin G, streptomycin, and amphotericin B were used. Plates were incubated for 4 d. After incubation, the samples underwent a single freeze-thaw cycle to release intracellular virus. Lysates from this procedure were assayed in triplicate by adding 10 µL of lysate sample and 90 µL of MEM to a well of a 96-well culture plate (0.36 cm²) followed by the addition of 50 µL of MEM containing MDBK cells. The plates were incubated for 3 d. After incubation, plates underwent the immunoperoxidase monolayer assay procedure for detection of BVDV as described above.

Amplification of BVDV in Monolayers of Vero Cells

To fulfill the second objective of this research, 43 strains of BVDV were amplified in monolayers of MDBK cells and Vero cells (African green monkey kidney). The strains of BVDV tested included 33 serum samples from PI animals with field strains of BVDV and 10 samples of laboratory strains of BVDV propagated for research purposes. The viruses tested included both genotype 1 and 2 BVDV, as well as cytopathic and noncytopathic strains. The monolayers of both the MDBK and Vero cells were grown in MEM containing fetal bovine serum (FBS; Sigma-Aldrich). The FBS had been previously confirmed negative for BVDV by virus isolation and also had a very low concentration of neutralizing antibodies to BVDV. Samples were tested in 96-well plates by performing 10-fold dilutions in triplicate and adding 50 µL of the respective cells to the wells. Plates were incubated at 38.5°C with 5% CO₂ for 3 d followed by the immunoperoxidase monolayer assay.

Statistical Analysis

Statistical analyses were performed using JMP software (JMP, SAS Inst. Inc., Cary, NC). The experimen-
tal unit of this research was the whole or ground cut of meat, which came from 1 of 6 PI animals, 1 of 4 subprimal cuts, and was maintained as whole or ground. A multivariate repeated measures ANOVA was used to evaluate the effect of animal, subprimal cut, and duration of refrigeration on the quantity of detected virus per gram of aged meat after refrigeration. A 1-way ANOVA was used to evaluate the effect of animal, subprimal, and maintaining as whole or grinding on the quantity of detected virus per gram of meat after freezing, thawing, or cooking, or combinations of all 3, to each endpoint temperature. The Wilson score interval test was used via JMP software to calculate the 95% confidence interval for inactivation of BVDV in meat from PI animals at various cooking temperatures. For the uncooked samples assayed as fresh or aged, 4 whole cuts from 6 animals resulted in 24 samples (n = 24) per treatment. For the frozen and cooked samples, 4 whole cuts plus 3 ground cuts from 6 animals produced a total of 42 samples (n = 42) per treatment.

**RESULTS**

It should be noted that all carcasses revealed no lesions that would have resulted in condemnation under current meat inspection guidelines. The concentration of BVDV in serum of PI cattle on the day of slaughter averaged $10^{5.35}$ cell culture infective doses (50% endpoint; CCID$_{50}$/mL with a range of $10^{4.3}$ to $10^{5.54}$ CCID$_{50}$/mL.

The concentration of BVDV in uncooked, fresh, and aged meat varied with the duration of refrigeration ($P = 0.05$; Table 1). After detecting $10^{6.37}$ CCID$_{50}$/g of meat on d 2, detectable BVDV decreased ($P = 0.05$) to $10^{5.98}$ by d 21 (Table 1). The concentration of BVDV in uncooked, aged meat did not vary ($P ≥ 0.15$) depending on the subprimal cut or the PI animal slaughtered.

The subprimal cut or PI animal slaughtered did not significantly ($P = 0.21$ and $P = 0.45$, respectively) affect the quantity of BVDV detected in frozen, uncooked meat. In contrast, grinding did affect the quantity of

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**Table 1. Inactivation of bovine viral diarrhea virus (BVDV) in whole and ground meat from 6 persistently infected cattle after refrigeration, freezing, and heating to various internal temperatures**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean viral concentration$^1$</th>
<th>Range$^1$</th>
<th>Sample testing free of virus$^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh d 0</td>
<td>5.83</td>
<td>4.4 to 6.4</td>
<td>0/24</td>
</tr>
<tr>
<td>Aged d 2</td>
<td>6.37</td>
<td>4.8 to 7.1</td>
<td>0/24</td>
</tr>
<tr>
<td>Aged d 7</td>
<td>6.20</td>
<td>4.7 to 7.1</td>
<td>0/24</td>
</tr>
<tr>
<td>Aged d 14</td>
<td>6.11</td>
<td>4.7 to 6.9</td>
<td>0/24</td>
</tr>
<tr>
<td>Aged d 21</td>
<td>5.98</td>
<td>4.9 to 6.7</td>
<td>0/24</td>
</tr>
<tr>
<td>Frozen on d 2 and thawed</td>
<td>5.91</td>
<td>4.9 to 6.2</td>
<td>0/42</td>
</tr>
<tr>
<td>Cooked to 55°C</td>
<td>3.90</td>
<td>VND$^3$ to 5.0</td>
<td>16/42</td>
</tr>
<tr>
<td>Cooked to 60°C</td>
<td>4.33</td>
<td>VND to 5.8</td>
<td>26/42</td>
</tr>
<tr>
<td>Cooked to 65°C</td>
<td>1.99</td>
<td>VND to 3.0</td>
<td>30/42</td>
</tr>
<tr>
<td>Cooked to 70°C</td>
<td>3.69</td>
<td>VND to 5.3</td>
<td>40/42</td>
</tr>
<tr>
<td>Cooked to 75°C</td>
<td>VND</td>
<td>—</td>
<td>42/42</td>
</tr>
<tr>
<td>Cooked to 85°C</td>
<td>VND</td>
<td>—</td>
<td>42/42</td>
</tr>
</tbody>
</table>

$^1$Log$_{10}$ cell culture infective doses/g of beef for samples testing BVDV positive.

$^2$As determined by amplification of bovine diarrhea virus in monolayers of Madin Darby bovine kidney cells.

$^3$Mean viral concentration is only for samples testing BVDV positive. VND = virus not detected.

**Table 2. Detection of bovine viral diarrhea virus in frozen, uncooked whole and ground meat from 6 persistently infected cattle**

<table>
<thead>
<tr>
<th>Item</th>
<th>Mean viral concentration$^1$ in whole subprimal cuts (n = 4 per animal)</th>
<th>Mean viral concentration$^1$ in ground meat products (n = 3 per animal)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Persistently infected animal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>5.99</td>
<td>6.03</td>
</tr>
<tr>
<td>2</td>
<td>5.66</td>
<td>6.11</td>
</tr>
<tr>
<td>3</td>
<td>5.69</td>
<td>5.92</td>
</tr>
<tr>
<td>4</td>
<td>5.86</td>
<td>5.86</td>
</tr>
<tr>
<td>5</td>
<td>6.03</td>
<td>6.03</td>
</tr>
<tr>
<td>6</td>
<td>5.75</td>
<td>6.11</td>
</tr>
<tr>
<td>Cumulative$^2$</td>
<td>5.85</td>
<td>6.02</td>
</tr>
</tbody>
</table>

$^1$Log$_{10}$ cell culture infective doses/g of beef.

$^2P = 0.01$. 
BVDV detected in frozen uncooked meat \((P = 0.01;\) Table 2). For this frozen, uncooked meat, the mean CCID_{50}/g of a whole meat cut was \(10^{1.85}\) compared with \(10^{6.02}\) CCID_{50}/g of ground meat \((SE = 10^{1.92} \text{ and } 10^{3.98}, \text{ respectively})\).

The subprimal cut, PI animal slaughtered, or preparation as a ground product did not significantly \((P \geq 0.30, P \geq 0.33, \text{ and } P \geq 0.15, \text{ respectively})\) affect the quantity of BVDV detected after cooking to any temperature. The virus in meat from PI animals was consistently inactivated when cooked to temperatures greater than or equal to 75°C \((\text{Table 1})\). Sufficient tests were performed to permit the following 95% confidence statement: BVDV can be considered inactivated in at least 91.6% of all cuts of meat from PI cattle after cooking to a temperature of 75°C.

The virus was detected in only 2 of 42 cuts of meat cooked to 70°C. The cuts yielding viable BVDV after cooking to 70°C were a whole round and whole rib from 2 separate animals. Thus, the virus in ground meat was consistently inactivated when cooked to temperatures greater than or equal to 70°C. Sufficient tests were performed to permit the following 95% confidence statement: BVDV can be considered inactivated in at least 84.2% of all cuts of meat from PI cattle after cooking to a temperature of 70°C.

The virus was detected in 12 of 42 samples cooked to 65°C. The cuts yielding viable BVDV after cooking to 65°C were from whole and ground cuts from 5 separate animals. Virus was detected in 5 of 18 samples of ground meat cooked to 65°C. Sufficient tests were performed to permit the following 95% confidence statement: BVDV can be considered inactivated in at least 56.4% of all cuts of meat from PI cattle after cooking to a temperature of 65°C.

Vero cells were not permissive to infection with BVDV. All of the 43 strains of BVDV were detected in monolayers of MDBK cells, whereas none of the 43 strains of BVDV were detected in monolayers of Vero cells.

**DISCUSSION**

Bovine viral diarrhea virus is a pestivirus of the family Flaviviridae which includes classical swine fever virus \((\text{CSFV})\) and border disease virus \((\text{Ridpath, 2005})\). Because CSFV is inactivated in pork at 65°C for 30 min or 71°C for 1 min \((\text{Terpstra and Krol, 1976; Stewart et al., 1979; Edwards, 2000})\), we hypothesized that similar results would be found with BVDV. Not all species are permissive hosts for BVDV or related pestiviruses \((\text{Bolin et al., 1994})\). Susceptibility to BVDV or closely related pestiviruses has been demonstrated for 7 of the 10 families in the order Artiodactyla \((\text{Passler and Walz, 2010})\). This includes antilocapridae \((\text{e.g., pronghorn})\), bovidae \((\text{e.g., cattle, sheep, goats})\), camelidae \((\text{e.g., camels, llamas})\), cervidae \((\text{e.g., deer, moose})\), giraffidae \((\text{e.g., giraffe})\), suidae \((\text{e.g., pigs})\), and tragulidae \((\text{e.g., mouse deer, Carberry et al., 1976})\).

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Bovine viral diarrhea virus is present at increased concentrations in muscle tissue of PI cattle that may be slaughtered for meat. As previously described \((\text{Taylor et al., 1997})\), some PI cattle will be considered acceptable for slaughter based on ante- and postmortem examinations. The detected concentration of this BVDV is greater in muscle tissue than in serum on the day of slaughter. Interestingly, harvesting of the beef carcass and aging for 2 d allowed detection of a significantly greater concentration of BVDV in meat than detected in meat sampled immediately after slaughter. This apparent change in viral concentration could be due to the disruption of cell membranes and subsequent release of noncytopathic intracellular virus after the first 2 d of aging. Thus, meat produced from PI cattle contains increased concentrations of BVDV, which is of concern if improperly cooked meat, meat by-products, or waste food are fed to susceptible hosts such as pigs.

The concentration of BVDV in meat declined with aging from 2 to 21 d after slaughter. Unfortunately, \(10^{3.98}\) CCID_{50} of BVDV per gram of meat remained after aging for 21 d at 4°C. Thus, aging or freezing of meat from PI animals does little to diminish the potential for transmission of BVDV via improperly cooked meat, meat by-products, or waste food.

More viable virus was detectable in ground, frozen, uncooked meat than in whole, frozen, uncooked meat. Yet, both ground and whole, frozen, uncooked meat from PI cattle contained more than \(10^{5.8}\) CCID_{50} of BVDV per gram of beef. Thus, processing as a ground or whole product does little to diminish the potential for transmission of BVDV via improperly cooked meat, meat by-products, or waste food.

The virus was not detectable when whole product was cooked to greater than or equal to 75°C. Bovine viral diarrhea virus is much more susceptible to inactivation via the heat of cooking conditions than was previously described for exposure of BVDV to dry heat from an instrument designed for disinfectant purposes \((\text{Sauerbrei and Wutzler, 2009})\). In that research, a time interval of \(2\ h\) at 95°C was considered necessary for a significant reduction in viral titer \((\text{Sauerbrei and Wutzler, 2009})\). The results of this current study are consistent with the results from research regarding the inactivation of CSFV in cooked hams from acutely infected pigs \((\text{Stewart et al., 1979})\). In that research, 9 of 13 samples of ham tested positive for CSFV after cook-
ing to 70°C for 1 min, whereas 0 of 15 samples tested positive after cooking to 71°C (Stewart et al., 1979).

The inability of 43 diverse BVDV strains to grow in Vero cells adds support to the conclusion that humans are not permissive hosts for various BVDV strains. These results are consistent with those of prior research involving human or primate cells (Toth and Hesse, 1983; Agnello et al., 1999; Maurer et al., 2004). Paradoxically, even a strain of BVDV reported to be isolated from peripheral white blood cells of a 30-yr-old woman did not grow on Vero or HeLa cells (Giangaspero et al., 1993). The body of available evidence continues to indicate that humans are not susceptible to BVDV (Walz et al., 2010).

In conclusion, infectious BVDV is detectable in increased concentrations in meat from PI animals and readily survives aging for 21 d at 4°C, freezing, thawing, and cooking to temperatures of less than or equal to 70°C. This bovine pathogen, which does not appear to be a human pathogen, can be inactivated by cooking ground or whole cuts of meat to 75°C or higher. Care should be taken to ensure that susceptible hosts such as pigs are not fed improperly cooked meat, meat by-products, or waste food originating from PI cattle.

**LITERATURE CITED**


