Client Report

Prepared for DEEResearch

December 2003

Development of Rapid PCR Kit for Detection of 'Blown Pack'-Causing Clostridia

J.A. Boerema & D.M. Broda

CR 966

C O N F I D E N T I A L

Client Report

This report has been prepared for DEEResarch and is CONFIDENTIAL to that organisation and AgResearch. AgResearch will not disclose its contents to third parties unless directed to do so by DEEResarch.

Every effort has been made to ensure this publication is accurate. However, because research and development can involve extrapolation and interpretation of uncertain data, AgResearch will not be responsible for any error or omission in this publication unless specifically agreed otherwise in writing. To the extent permissible by law, neither AgResearch nor any person involved in this publication accepts any liability for any loss or damage whatsoever that may directly or indirectly result from any advice, opinion, representation, statement or omission, whether negligent or otherwise, contained in this publication.

James Suttie
Manager – Food Science

Inquiries or requests to:
Jackie Boerema
Email: Jackie.boerema@agresearch.co.nz
Food Safety Group - Food Science
AgResearch -MIRINZ Centre
Private Bag 3123, Hamilton, New Zealand

Development of Rapid PCR Kit for Detection of ‘Blown Pack’-Causing Clostridia

Prepared for DEEResearch

December 2003

J.A. Boerema & D.M. Broda

Keywords: ‘blown pack’ spoilage; clostridia; rapid PCR detection

Summary

‘Blown pack’ spoilage of vacuum-packed refrigerated venison is caused by low-temperature-growing *Clostridium* spp. Classical microbiological methods for detection of these microorganisms are laborious and time consuming. In a previous Meat NZ/GIB/FRST funded study, DNA-based procedures have been established that allow specific detection of ‘blown pack’-causing clostridia in meat, water, faecal and soil samples. However, application of these procedures to meat plant environmental and/or dressed carcass samples has frequently required employment of a 3 week long cold enrichment step. Building on the previous work, the aim of the present study was:

- to develop a rapid (less than 12 h) and sensitive (less than 10 cells) procedure for detection of *C. estertheticum* spores on dressed deer carcasses, and
- to convert this procedure into a kit format.

The results of the present study indicated the feasibility of direct (without enrichment), rapid and sensitive molecular detection of *C. estertheticum* spores in a carcass swab/dilution fluid matrix. Using ‘nested’ PCR developed in this study, as few as 10 spores ml^{-1} were detected in enzyme-treated *C. estertheticum* spore preparations and approx. 1 spore ml^{-1} was detected in sonicated spore preparations. In comparison with conventional PCR, ‘nested’ PCR was shown to increase the limit of molecular detection of *C. estertheticum* spores by one logarithmic unit. The detection using ‘nested’ PCR can readily be accomplished in less than 12 h. A major effort was made in the present study to assure and optimize the release of genomic DNA from *C. esthertheticum* spores. The bead mill/detergent DNA extraction protocol proved to be the most effective for this purpose.

In its current format, following training in molecular techniques and providing the availability of testing facilities, the procedure we have developed can be used immediately for routine screening of deer carcasses for the presence of *C. estertheticum*. However, to be of value to venison producers in the selection of processing procedures prior to packaging/processing of the product and/or in estimating the expected shelf life of cervine cuts carrying *C. estertheticum* spores, the spore carcass carriage threshold for pack blowing needs to be determined. The study significantly advances progress towards the development of a kit format for rapid detection of *C. estertheticum*. Further work, including conversion of the PCR mix to a freeze-dried format, incorporation of an additional specific primer

into a ‘nested’ PCR procedure and adaptation of a user-friendly format for a bead mill/detergent DNA extraction, is required to progress the development of this kit to the market-ready stage.

Contents

Page

1. Introduction

Low-temperature-growing *Clostridium* spp. have been recognised as causative agents of 'blown pack' spoilage of vacuum-packed refrigerated meats. These microorganisms have maximum growth temperatures below 25°C and are obligately anaerobic. To-date, *Clostridium estertheticum* and *Clostridium gasigenes* have been named as two clostridial species responsible for New Zealand incidents of 'blown pack' spoilage. Classical microbiological methods for detection of these microorganisms include laborious and time consuming multi-step isolation and differentiation conducted at low temperatures. This isolation and differentiation are, when necessary followed by confirmation of the pack 'blowing' ability of isolated clostridia.

In a Meat NZ/GIB/FRST funded study, PCR primers have been designed that enable specific detection of clostridial species causing 'blown pack' spoilage. Similarly, procedures have been established that allow specific detection of 'blown pack'-causing clostridia in meat, water, faecal and soil samples. These methods offer reliable alternatives for specific detection of 'blown pack' causing clostridia without recourse to their isolation and are now routinely used in the MIRINZ Centre Food Safety laboratory for detection of these microorganisms in blown packs.

In its current format, the molecular method can detect 10^4 clostridial cells in non-enriched meat or water samples, but as few as 10 cells if low temperature enrichment is performed. A previous Meat New Zealand funded study has determined that carcass carriage as few as 4 to 6 *C. estertheticum* spores per cm^2 can result in gross pack distension within 41 days of storage at 0°C. Consequently, to deliver the required level of assay sensitivity, enrichment must be employed for detection of 'blown pack' causing clostridia. At present, a rapid and sensitive detection method is being sought that would allow carcass screening for 'blown pack' causing clostridia prior to packaging/distribution of the product and that would permit early assessment of the expected shelf life of meat cuts carrying these microorganisms. This approach necessitates the development of a direct detection system that is capable of rapid (less than 12 h) and sensitive (less than 10 cells) detection of these microorganisms on dressed deer carcasses. In addition, the method must be able to detect spores, rather than vegetative cells, of 'blown pack'-causing clostridia.

The aim of the present study was to develop a rapid and sensitive procedure for carcass testing that would allow venison producers to routinely screen dressed deer carcasses for the presence of 'blown pack'-causing clostridia and, subsequently, to select appropriate processing procedure prior to packaging/processing of the product.

2. Objectives

- To develop a 'nested' PCR procedure for detection of spores and vegetative cells of 'blown pack'-causing clostridia on dressed deer carcasses and/or cervine cuts.
- To determine detection limits of 'primary' and 'nested' PCR procedures.
- To initiate the development of a kit format for the 'nested' PCR procedure.
- To test the reproducibility and stability of the kit.

3. Materials and Methods

3.1 Microorganisms

The reference strain of *C. estertheticum* DSM 8809^T was obtained from the Deutsche Sammlung von Mikrooganismen und Zellkulturen GmbH, Braunschweig, Germany. This strain was revived in sterile anaerobic milk and was sub-cultured onto Columbia Blood Agar (CBA, Oxoid) containing 5% v/v sheep's blood. The strain was grown anaerobically at 10°C for 3 weeks, after which time it was maintained by regular subculture on CBA. Before being used in spore preparation, the strain was checked for purity by plating on CBA agar.

3.2 Spore Preparation and Enumeration

Spore production and harvesting. Spores were produced in pre-reduced Peptone, Yeast, Glucose, Starch (PYGS) broth (Lund *et al.*, 1990). A volume (500ml) of PYGS broth in a culture bottle was inoculated with an exponentially growing culture of *C. estertheticum* and incubated anaerobically at 15°C for 12 weeks.

Spores were harvested when typical spore appearance, evidenced by the presence of refractive spheres, was observed with phase contrast microscopy. The broth culture was centrifuged, washed four times with sterile physiological saline and treated at 60°C for 10 min to eliminate any remaining vegetative cells. Spore suspensions were stored at -12°C prior to use.

Since extracellular DNA present in the cell preparations can bias the observed DNA extraction results, spores were further treated by either enzymatic digestion or sonication prior to DNA extraction step.

Enzymatic digestion. Extraneous cellular material was digested in thawed spore preparations by incubation with digestion buffer (30 mg ml^{-1} lysozyme, 2 mg ml^{-1} DNase I, 15 mM $MgCl_2$, in phosphate buffered saline), for 60 min at 37°C. Afterwards, 10 mg trypsin was added to the spore suspension and the incubation was continued for 90 min. To destroy residual DNase activity, the enzyme-treated suspension was heated at 90°C, centrifuged and washed four times in sterile physiological saline. Microscopic observation of the enzyme-treated spore suspension was then conducted to determine the presence of intact spores and complete removal of digested cellular material.

Sonication. Extraneous cellular material was eliminated from a spore preparation by sonication, followed by spore washes. Initially, the spore suspension was sonicated on ice, with an ultrasound probe at maximum output of 90 W with two 45 s bursts, followed by one 30 s burst. Cooling on ice was performed between each burst. Sonicated suspensions were subsequently centrifuged and washed four times in physiological saline. Microscopic observation of the sonicated spore suspension was conducted to confirm the presence of clean, intact spores, in the absence of vegetative cellular material.

Testing of spore preparations for the presence of extracellular DNA. Enzyme-treated and sonicated spore preparations were checked for the presence of extracellular DNA by PCR detection of *C. estertheticum* DNA in spore supernatants, as described in section 3.4.

Enumeration of spores. For enumeration of spores, serial dilutions of sonicated and enzyme-treated spore preparations were spread on freshly prepared CBA

plates and incubated in Gas Pak^{TM} pouches, at 10°C for 3 weeks, to determine an approximate number of colony forming units (cfu).

3.3 DNA Extraction and Purification

Bead mill/detergent protocol. A DNA extraction method based on a combination of physical disruption and enzymatic treatment was developed for efficient release of genomic DNA from *C. estertheticum* spores. Initially, 0.5 ml volumes of enzyme-treated or sonicated spore suspensions were homogenised in 0.5 ml SDS lysis solution (10 % SDS, 10 mM NaCl, 500 mM Tris pH 8.0) by two 40 s cycles at 4,200 rpm in a mini-bead beater cell disruptor (Bio-Spec products). Homogenised suspensions were centrifuged at 8,000 x *g* for 5 min to pellet the bead mix. Supernatants containing DNA were collected into 1.5 ml Eppendorf tubes, 40 µl of proteinase K and 200 µl of guanidine-HCl binding buffer were added, and the mixtures were incubated at 72°C for 10 min. Nucleic acids were precipitated with 100 µl of isopropanol and centrifuged on silica spin columns to bind DNA. Following centrifugation and ethanol washes, DNA was eluted from spin columns and stored at 4°C pending PCR amplification.

Roche High Pure Template Preparation kit protocol. The efficacy of the bead mill/detergent DNA extraction protocol was compared with the 'standard' method used by the MIRINZ Food Safety laboratory, that is, the protocol using the Roche High Pure Template preparation kit. DNA extraction was performed according to the kit manufacturer's recommended protocol for isolation of nucleic acids from bacteria, except that prior to extraction, spores were resuspended in 200 µl of 10 mg ml^{-1} lysozyme and incubated at 37°C for 60 min. Eluted DNA was stored at 4°C pending PCR amplification.

3.4 PCR Amplification of Specific DNA Fragments

'Primary' PCR. For amplification of *C. estertheticum*-specific gene fragments, genomic DNAs extracted from spores using the bead mill/detergent protocol or the Roche kit, were used as templates in PCR with the primer set 16SEF and 16SER (Broda et al., 2003). The PCR mix contained PCR buffer (10x) 5 µl, 0.2 mM of each deoxynucleoside triphosphate, 0.5 µM of each primer, 2.5 units of *Taq* polymerase and 5 µl of template DNA in a total volume of 50 µl. Amplifications were performed in a heated lid thermal cycler. After initial denaturation for 3 min

at 93°C, target DNA was amplified in 30 cycles. Each cycle consisted of denaturation for 1 min at 92°C, annealing for 1 min at 55°C and extension for 2 min at 72°C. Two controls, one containing only the reagent, and the other containing *C. estertheticum* DNA were included in each PCR run. PCR reaction tubes were held at 4°C pending further analysis.

For visualisation of DNA, an 18 µl aliquot of PCR product was electrophoresed on a 1.5 % (w/v) agarose gel at 90 V for 1 h. The DNA molecular weight marker VI (Roche Diagnostics) was used as a size marker. DNA was stained with ethidium bromide and visualised by u.v. transillumination.

'Nested' PCR. To improve the yield of PCR product and sensitivity of the PCR detection assay, a 'nested' PCR procedure was developed. For 'nested' PCR, 0.5 µl of product obtained in the 'primary' PCR amplification was used as template in a second round of PCR. PCR amplification was performed as described for the 'primary' PCR.

3.5 Limit of Detection of PCR Assay

Sensitivity of PCR detection in bead mill/detergent vs. Roche kit DNA extractions. For comparison of the sensitivity of detection in DNAs obtained using two DNA extraction methods, serial dilutions of enzyme-treated spores were prepared in sterile dilution fluid. Aliquots (0.5 ml) of serial dilutions were then used in DNA extractions using the bead mill/detergent and the Roche High Pure Template Preparation kit protocols. Extracted DNAs were used as templates in the 'primary' and 'nested' PCRs, as described in section 3.4. To determine numbers of viable *C. estertheticum* spores present in enzyme-treated spore preparation, serial dilutions were plated on CBA agar and enumerated, as described previously.

Sensitivity of PCR detection in enzyme-treated vs. sonicated C. estertheticum *spore preparations.* To determine the sensitivity of PCR detection, enzyme-treated and sonicated spore preparations were serially diluted in a carcass swab/dilution fluid matrix, subsequently determined to be free of target DNA. These serial dilutions were used in DNA extraction using the bead mill/detergent protocol, and the extracted DNAs were then used in 'primary' and 'nested' PCRs, as described in section 3.4. To determine numbers of viable *C. estertheticum*

spores present in enzyme-treated and sonicated spore preparations, serial dilutions were plated on CBA agar and enumerated, as described previously.

3.6 Detection of *C. estertheticum* in Deer Carcass Swabs

Sample collection. Dressed carcass swabs were collected from a venison meat plant that had in the past experienced incidents of 'blown pack' spoilage. Swab samples were either obtained from hot carcasses on the slaughter floor prior to their entry to the carcass chiller, or from cold carcasses that had been subjected to overnight chilling, immediately prior to their entry to the boning room. Single swab samples were obtained using pre-moistened gauze swabs to sample an area of approximately 300 cm^2, in the vicinity of the NMD hind leg site. In addition, composite samples, representing a total sampled surface area of approximately 900 cm^2, were obtained by swabbing an area of approximately 300 cm^2 in the vicinity of each of the three NMD sites (hind leg, sternum and inside foreleg) and combining the obtained three swabs into one sample. Single and composite swab samples were transported to the AgResearch MIRINZ Centre on ice and were stored at 2°C pending DNA extraction.

Preparation of spore concentrates. To remove spores from gauze swabs, single or composite swab samples were moistened with 50 ml of sterile dilution fluid and stomached in a Seward Stomacher for 2 min. The resulting suspensions were centrifuged at 8500 rpm for 10 min and resuspended in 2 ml of supernatant.

PCR detection of *C. estertheticum* in deer carcass swabs was conducted either directly (without enrichment) or following cold enrichment.

Direct detection without prior enrichment. For direct detection, an aliquot (1.5 ml) of a spore concentrate was further concentrated to 0.5 ml by centrifugation. DNA was extracted using the bead mill/detergent protocol, and 'primary' and 'nested' PCR detection was performed as described in section 3.4.

Detection following cold enrichment. For cold enrichment, an aliquot (0.5 ml) of a spore concentrate was added to PYGS broth and incubated anaerobically at 10°C to promote germination and growth of *C. estertheticum* spores. Following 13 day and 3 week incubation, 1 ml sub-samples were removed from cold enrichments and used for DNA extraction. With 13 day enrichments, DNA was extracted using

the bead mill/detergent protocol. With 3 week enrichments, it was thought that spores present would have been given sufficient incubation for germination and growth, and, consequently, DNA was extracted using the Roche High Pure Template Preparation kit protocol. 'Primary' and 'nested' PCR detection of *C. estertheticum* in DNAs from enriched and non-enriched samples was conducted as described in section 3.4.

3.7 Assessment of the Stability of PCR Mix During Frozen Storage

To assess the stability and reproducibility of the PCR-ready mix, the mix was prepared as described in section 3.4, distributed into 90 µl reaction volumes and placed in frozen storage at -20°C. The stored mix was used for PCR detection of *C. estertheticum* at 2 weekly intervals over a 10 week period of frozen storage. *C. estertheticum* DNA was isolated using the Roche High Pure DNA Template Preparation kit, as described in section 3.4. Following the extraction, this DNA was distributed into 0.1 ml volumes and frozen at -20°C. A new tube of the same DNA preparation was used for the PCR mix stability assessment at each testing interval. A set of 5 reaction tubes containing one negative (reagent only) control and four tubes to which 10 µl of *C. estertheticum* DNA was added, was assayed after 0, 2, 4, 6, 8 and 10 weeks storage. At each testing interval, PCR was performed and PCR products analysed as described in section 3.4.

4. Results

Microscopic observation of the spore preparation determined that intact spores were present in the enzyme-treated spore suspension, but that digested cellular material was not removed completely. However, this material appeared to be completely removed following sonication treatment. With the PCR assay, free DNA was not detected in physiological saline washes from enzyme-treated or sonicated spore preparations at levels that could influence the limit of PCR detection of *C. estertheticum*.

4.1 Sensitivity of PCR Detection in Bead Mill/detergent vs. The Roche Kit DNA Extractions

The results of PCR amplification with DNAs obtained using the bead mill/detergent and the Roche kit protocols are shown in Figures 1 and 2.

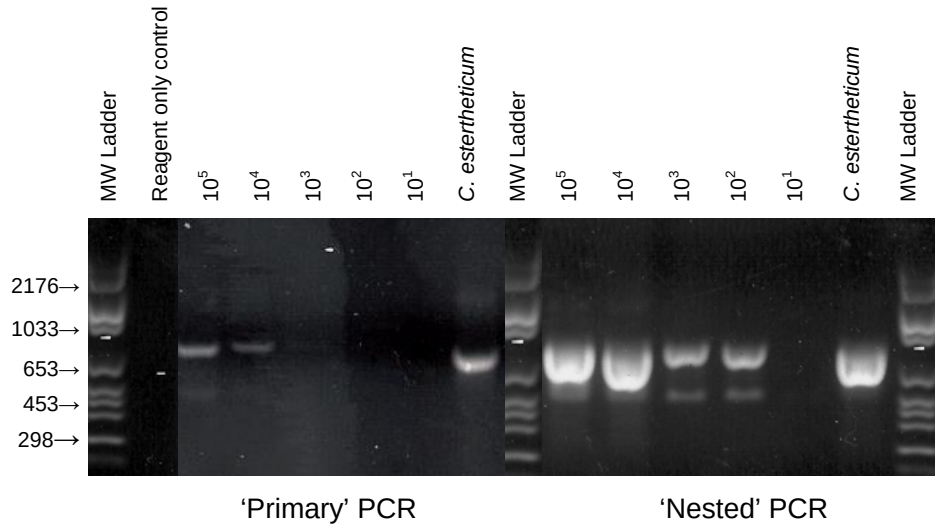

Figure 1. Sensitivity of PCR detection of *C. estertheticum* in a spore preparation, with DNA extracted from spores using the Roche High Pure PCR template preparation kit. Enzyme-treated spores were used and serial dilutions were prepared in sterile dilution fluid.

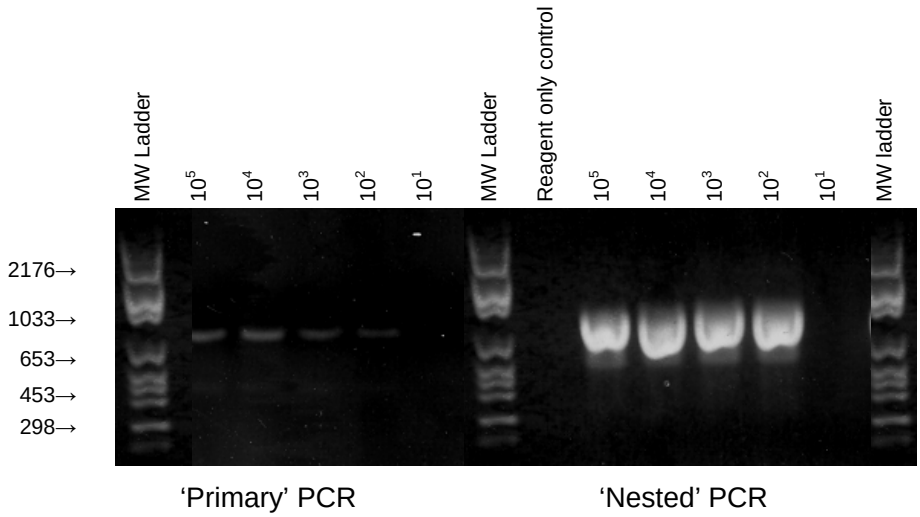

Figure 2. Sensitivity of PCR detection of *C. estertheticum* in a spore preparation, with DNA extracted from spores using the bead mill/detergent protocol. Enzyme-treated spores were used and serial dilutions were prepared in sterile dilution fluid.

Using DNA extracted with the Roche kit protocol, 10^3 ml^{-1} *C. estertheticum* spores were detected following the 'primary' PCR (Fig 1). The limit of molecular detection increased to 10^2 ml^{-1} *C. estertheticum* spores following 'nested' PCR amplification.

With DNA extracted using the bead mill/detergent protocol, 10^2 ml^{-1} *C. estertheticum* spores were detected following either 'primary' or 'nested' PCR (Fig 2). However, a substantially higher yield of PCR product was obtained at the lowest level of detection (10^2) using the bead mill/detergent DNA extraction than was obtained with the Roche kit.

An attempt was made to improve the limit of detection of the assay by introducing an overnight enrichment of *C. estertheticum* in PYGS broth prior to DNA extraction using the bead mill/detergent protocol. However, an overnight enrichment did not result in an improved sensitivity of the assay (results not shown).

No amplification products were detected in the reagent only controls included in 'primary' and 'nested' PCR assays. Positive control that included DNA from *C. estertheticum* consistently yielded PCR amplification products of the expected size.

4.2 Sensitivity of PCR Detection in Enzyme-treated vs. Sonicated *C. estertheticum* Spore Preparations

PCR detection of *C. estertheticum* in DNA templates obtained from enzyme-treated spore preparations that were serially diluted in a carcass swab/dilution fluid matrix, yielded bands of expected size when as few as 10^1 ml^{-1} spores were used (Fig 3). This level of detection was obtained for both 'primary' and 'nested' PCR, however, substantially higher DNA yield was obtained following 'nested' PCR.

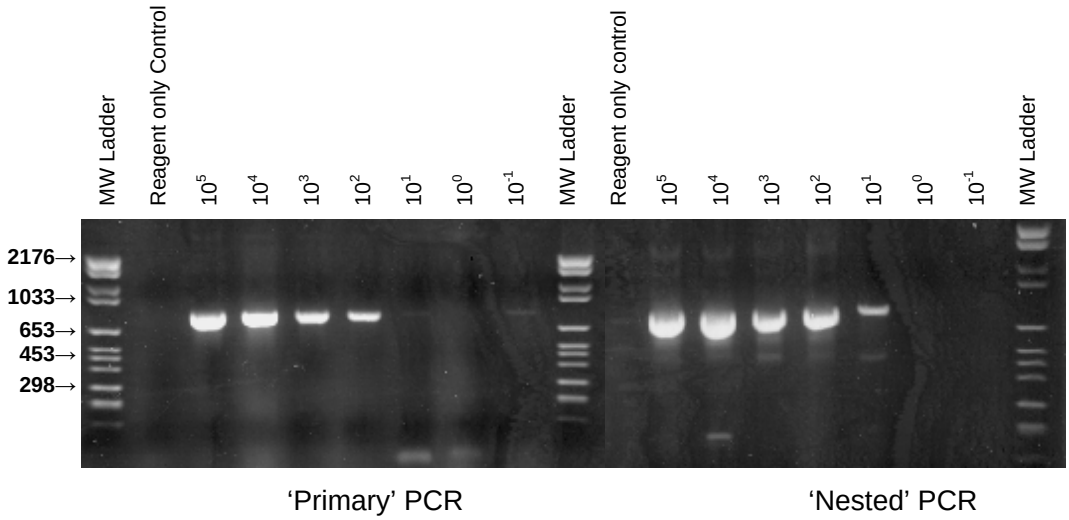

Figure 3. Sensitivity of PCR detection of *C. estertheticum* in a spore preparation that underwent enzyme treatment. DNA was extracted from spores using the bead mill/detergent protocol and serial dilutions were prepared in a carcass swab/dilution fluid matrix.

Positive PCR signals in sonicated spore preparations were obtained during 'primary' PCR when 10^1 ml^{-1} *C. estertheticum* spores were used. The sensitivity of this detection improved following 'nested' PCR, with main PCR amplification product of the expected size obtained when 10^0 ml^{-1} spores were used (Fig 4).

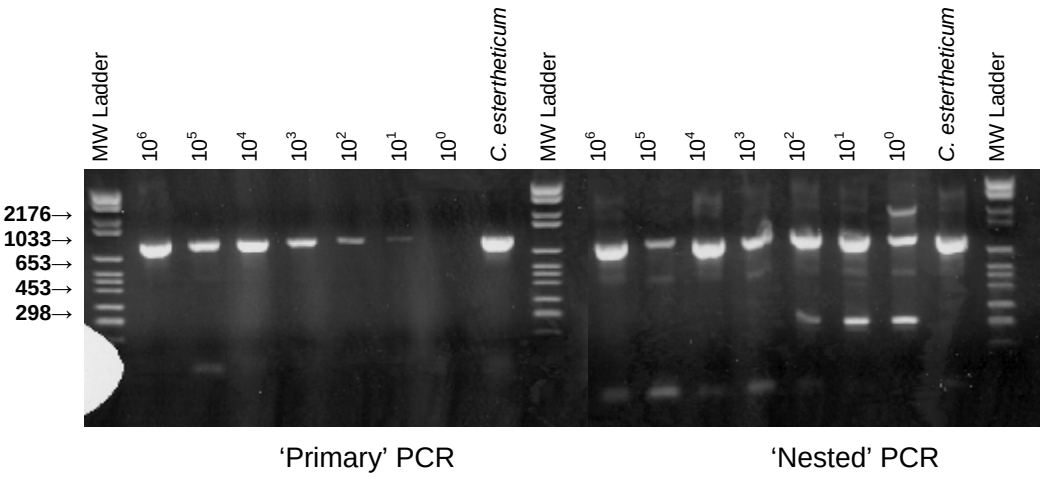

Figure 4. Sensitivity of PCR detection of *C. estertheticum* in a spore preparation that underwent sonication treatment. DNA was extracted from spores using the bead mill/detergent protocol and serial dilutions were prepared in a carcass swab/dilution fluid matrix.

No amplification products were detected in reagent only controls included in 'primary' and 'nested' PCR assays. Positive control that included DNA from *C. estertheticum* consistently yielded PCR amplification products of the expected size.

4.3 Detection of *C. estertheticum* in Deer Carcass Swabs

A total of 22 single or composite swab samples from hot or cold dressed deer carcasses were examined by direct detection, and following a 13 day and 3 week enrichment at 10°C. The results of the detection are shown in Table 1.

No PCR amplicons of the expected size (approx. 790 bp) were produced with the carcass swabs of hot or cold deer carcasses following direct PCR detection. With a single sample (SFC 8), direct PCR amplification yielded a PCR product of the size that was similar, but not identical to one typically obtained for *C. estertheticum*. Subsequently, with DNA sequencing, this PCR product has been confirmed as being non-specific.

PCR products of the expected size were obtained with samples SF6 and CC4 following a cold enrichment of 13 days. Three samples, SF14, SF6 and CC4, yielded positive PCR products when cold enrichment was extended to 3 weeks and DNA extraction was performed using the Roche kit.

No amplification products were detected in reagent only controls, and a positive *C. estertheticum* control consistently yielded PCR amplification products of the expected size.

Table 1. Detection of *C. estertheticum* in deer carcass swabs.

Sample designation	**Sample type**	**PCR signal following**		
		Direct detection[#]	13 day enrichment[#§]	3 week enrichment[@§]
SF13	Hot carcass single swab	-ve	-ve	-ve
SF14	Hot carcass single swab	-ve	-ve	**+ve**
SF19	Hot carcass single swab	-ve	-ve	-ve
SFC8	Hot carcass composite	-ve*	-ve	-ve
SFC10	Hot carcass composite	-ve	-ve	-ve
SFC15	Hot carcass composite	-ve	-ve	-ve
SFC1	Hot carcass composite	-ve	-ve	-ve
SFC3	Hot carcass composite	-ve	-ve	-ve
SFC5	Hot carcass composite	-ve	-ve	-ve
SFC6	Hot carcass composite	-ve	**+ve**	**+ve**
SFC11	Hot carcass composite	-ve	-ve	-ve
SFC12	Hot carcass composite	-ve	-ve	-ve
C1	Cold carcass single swab	-ve	-ve	-ve
C2	Cold carcass single swab	-ve	-ve	-ve
C4	Cold carcass single swab	-ve	-ve	-ve
C10	Cold carcass single swab	-ve	-ve	-ve
C11	Cold carcass single swab	-ve	-ve	-ve
C13	Cold carcass single swab	-ve	-ve	-ve
CC1	Cold carcass composite	-ve	-ve	-ve
CC2	Cold carcass composite	-ve	-ve	-ve
CC3	Cold carcass composite	-ve	-ve	-ve
CC4	Cold carcass composite	-ve	**+ve**	**+ve**

*, PCR amplification with this sample yielded a PCR product of the size that was similar, but not identical to one typically obtained for *C. estertheticum*. Subsequently, with DNA sequencing, this PCR product has been confirmed as being non-specific. [#], DNA extracted using bead mill/detergent protocol. [@], DNA extracted using the Roche High Pure PCR template preparation kit. [§], results obtained following 'primary' PCR.

4.4 Assessment of the Stability of PCR Mix During Frozen Storage

Results of the assessment of the stability and reproducibility of PCR-ready reagent mix following its frozen storage for 4 and 10 weeks are shown in Fig 2. Positive PCR signals were detected in all tested replicates of the mix regardless of the storage period. However, with the mix stored for 10 weeks, lower yield of PCR product was detected than with the PCR mix that did not underwent frozen storage.

No PCR amplicons were present in reagent only controls.

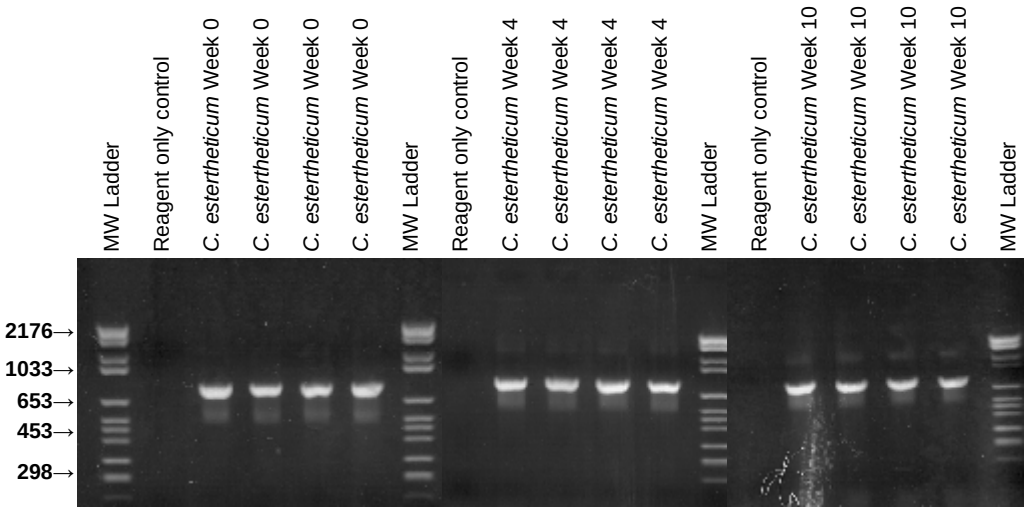

Figure 5. *C. estertheticum* amplicons obtained following frozen storage of the PCR mix.

5. Discussion

For a molecular detection procedure to be employed for on-line screening of dressed deer carcasses for the presence of 'blown pack'-causing clostridia, there are three main requirements to be satisfied:

1. With cold boning, time between the carcass exit from the slaughter floor and its entry to the boning room may be as short as 12 h. Therefore, the detection procedure must be rapid.

2. As few as 4 *C. estertheticum* spores per cm^2 of carcass surface can result in gross pack distension within 41 days of storage at 0°C. Therefore, the detection procedure must be sensitive.

3. Conditions that *C. estertheticum* require for growth include, among others, low temperature and low redox potential. These microorganisms are, therefore, likely to be present on carcass surface in a form of spores. The procedure must be able to detect spores.

The procedure developed in the present study fulfils all these requirements.

To date, the molecular detection of 'blown pack'-causing clostridia has incorporated a cold enrichment step that, typically, included anaerobic incubation for 3 weeks at 10°C. The cold enrichment has been incorporated in the detection of these microorganisms for a number of reasons. First, during the enrichment period clostridial spores present in a sample are given sufficient time and appropriate growth conditions to encourage germination and outgrowth and, consequently, ease DNA release from clostridial cells. Second, cold enrichment gives psychrophilic clostridia a competitive advantage over, and suppresses the growth of, mesophilic flora present in the sample. Third, the use of enrichment enables effective dilution of sample matrix substances that can potentially interfere with the PCR reaction e.g. humic acids. Although DNA extraction from enrichments, followed by PCR detection of 'blown pack' causing clostridia, can easily be achieved within 12 h, incorporation of the cold enrichment step extends the analysis to 3 weeks from sampling. The procedure developed in the present study does not require cold enrichment and, therefore, permits rapid analysis.

To date, the molecular method for detection of *C. estertheticum* could detect 10^4 ml^{-1} or less clostridial cells in non-enriched meat or water samples. In contrast, the method developed in the present study can detect as few as 10 spores ml^{-1} in enzyme-treated spore preparations and 1 spore ml^{-1} in sonicated spore preparations. It appears that the sensitivity of the *C. estertheticum* can be enhanced using the 'nested' PCR procedure. In this study, 'nested' PCR consistently resulted in higher yield of PCR amplification products when compared with that obtained in the 'primary' PCR. In addition, 'nested' PCR was shown, in most instances, to increase the limit of molecular detection of *C. estertheticum* spores by one logarithmic unit.

Psychrophilic clostridia are likely to be present on carcasses as spores, rather than as vegetative cells. The release of nucleic acids from vegetative cells is relatively straightforward, and simple treatments, such as boiling, may liberate sufficient template DNA. With bacterial endospores, however, destruction of the spore is necessary to release DNA and this process is not one to be accomplished readily. A major effort was made in the present study to ensure and optimize the release of genomic DNA from *C. esthertheticum* spores. The bead mill/detergent DNA extraction protocol proved to be the most effective for this purpose.

In the present study, no PCR amplicons of the expected size were produced with the carcass swabs of hot or cold deer carcasses following direct (without enrichment) PCR detection. However, PCR products of the expected size were obtained with two and three out of 22 swab samples following a cold enrichment for 13 days and 3 weeks, respectively. It is thought that due to the high sensitivity of the method used in this study, as well as the large carcass surface area sampled, the failure to detect *C. estertheticum* by direct detection might have been due to the extremely low level of deer carcass contamination with spores of this microorganism. Arguably, this failure may indicate that a further improvement in the developed assay's limit of detection may be required. However, it is not known what the minimum numbers of *C. estertheticum* spores are that, when carried on the dressed carcasses, subsequently result in blowing of vacuum-packed venison, or, in other words, what the minimal threshold for blowing is. The possibility exists that, due to its high sensitivity and low limit of detection, the method developed in the present study detects *C. estertheticum* spores in numbers that are already below the threshold for blowing. It is recommended that future studies determine the threshold for pack blowing and/or include a large number of naturally contaminated samples (preferably during commercial incidents of 'blown pack' spoilage) to accurately assess the efficacy of the developed method.

The aim of the present study was to develop a rapid and sensitive procedure for dressed carcass screening that would allow the venison producers (1) to routinely screen dressed deer carcasses for the presence of 'blown pack'-causing clostridia, (2) to select appropriate processing procedures prior to packaging/processing of the product, and (3) to assess the expected shelf life of cervine cuts carrying *C. estertheticum* spores. It is believed that, following basic

molecular training and providing the availability of testing facilities, and once the threshold for pack blowing is established, venison processors should be able to accomplish the aims just listed using procedures developed in this study. However, it was also the intention of the present study to initiate the development of a kit format for rapid detection of *C. estertheticum*. It is thought that this study significantly advances progress towards the development of such a kit. Evaluation of the frozen PCR-ready mix revealed no marked deterioration in performance in the molecular detection of *C. estertheticum* during the 10 week storage. To assure longevity of this reagent, the PCR mix may now be converted into a freeze-dried format. To ensure high specificity of 'nested' PCR, another specific primer may be incorporated into a 'nested' PCR procedure. In addition, a user-friendly format for a bead mill/detergent DNA extraction is required. When supported by detailed stability, reproducibility and sensitivity data, the PCR and DNA extraction ingredients of the detection system described in the present study may form basis for the development of a market-ready kit for rapid and sensitive detection of *C. estertheticum*.

6. Conclusions and Recommendations

- A rapid and sensitive procedure was developed that enables detection of less than 10 spores ml^{-1} of pack blowing microorganism *C. estertheticum* in less than 12 h in dressed deer carcass swabs.

- Pending molecular techniques training and availability of testing facilities, the developed procedure can be used for routine screening of deer carcasses for the presence of *C. estertheticum*.

- While the developed procedure can be used immediately, its value in selecting processing procedures prior to packaging/processing of the product and/or in estimating the expected shelf life of cervine cuts carrying *C. estertheticum* spores may be limited until the spore carcass carriage threshold for pack blowing is determined.

- The study significantly advanced progress towards the development of a kit format for rapid detection of *C. estertheticum*.

- Further work, including conversion of the PCR mix to a freeze-dried format, incorporation of an additional specific primer into a 'nested' PCR procedure and adaptation of a user-friendly format for a bead mill/detergent DNA extraction, is required to progress the development of this kit to the market-ready stage.

7. References

Broda D.M., Boerema J.A and Bell R.G., (2003) PCR detection of psychrophilic *Clostridium* spp. causing 'blown pack' spoilage of vacuum-packed chilled meats. *Journal of Applied Microbiology* **94**, 515-522.

Lund B.M, Graham A.F, George S.M and Brown D. (1990) The combined effect of incubation temperature, pH and sorbic acid on the probability of growth of non-proteolytic type B *Clostridium botulinum. Journal of Applied Bacteriology* **69,** 481-492.