Abattoir sources of psychrophilic clostridia causing blown pack spoilage of vacuum-packed chilled meats determined by culture-based and molecular detection procedures

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ABSTRACT

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Aims: To identify the abattoir source(s) of psychrophilic clostridia causing 'blown pack' spoilage of vacuum-packed chilled meats.

Methods and Results: Molecular procedures were used to detect the presence of specific 16S rRNA gene fragments of blown pack-causing clostridia in samples collected from a commercial abattoir and its environs. Blown pack-causing clostridia were consistently detected in hide, soil and faecal samples, as well as in samples collected at slaughter plant locations associated with handling of animals and animal carcasses prior to pelt removal.

Conclusions: The data indicate that pelts *per se* or soil particles/faecal material attached thereto are the most probable primary reservoir of blown pack clostridia in the abattoir.

Significance and Impact of the Study: The paper provides information critical for controlling blown pack spoilage in commercial meat-processing plants.

Keywords: 'blown pack', clostridia, meat spoilage.

INTRODUCTION

Premature spoilage of vacuum-packed chilled meats as a result of gas production and gross pack distension is principally attributed to the growth of psychrotolerant *Clostridium* spp. (Dainty *et al.* 1989; Kalchayanand *et al.* 1989; Broda *et al.* 1996). Currently, it is believed that the frequency and severity of commercial incidents of blown pack spoilage could be minimized, among others, by removing the abattoir source(s) of psychrophilic clostridia and/or by limiting transfer of these clostridia from their abattoir source(s) onto carcasses. Consequently, development of effective measures for control of blown pack spoilage depends, among other factors, on successful detection of blown pack-causing clostridia in the meat plant environment.

Culturing of microorganisms is a prerequisite for conventional detection of blown pack-causing clostridia. This process is both laborious and time consuming. Recently developed molecular methods offer a rapid and reliable alternative for specific detection of blown pack-causing clostridia without recourse to isolation (Broda *et al.* 2003). However, the use of molecular detection procedures in a DNA-based screening for source(s) of these microorganisms in a commercial abattoir has not yet been reported.

This study reports on a DNA-based investigation to determine the abattoir source(s) of clostridia causing blown pack spoilage of vacuum-packed chilled meats. The efficacy of 16S rDNA-based specific detection of blown pack-causing clostridia was compared with their culture-based detection.

MATERIALS AND METHODS

Bacteria

The reference strain of *Clostridium estertheticum* DSM 8809[T] (T = type strain) was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen

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GmbH, Braunschweig, Germany. The reference strain of *Clostridium gasigenes* DB1A[T] (DSM 12272[T]) was the original New Zealand strain obtained from an incident of blown pack spoilage of chilled vacuum-packed lamb (Broda *et al.* 2000). *Clostridium estertheticum*-like strains K21 and K24 were isolated using methods described previously (Broda *et al.* 1996). All strains were maintained as described previously (Broda *et al.* 2000).

Sample collection and handling

Sampling was conducted in a commercial abattoir that had experienced incidents of blown pack spoilage of vacuum-packed product. A total of 39 samples were collected from the abattoir and its environs. These samples included soil from outside the abattoir, faeces and hide samples from slaughter animals, and swab samples from work and structural surfaces, as well as processing water, taken from various points along the processing chain in the abattoir (Table 1).

Samples were packed on ice and transported to our laboratory in chilled insulated containers. On arrival, all samples were placed into anaerobic storage at 2°C pending microbiological examination.

Sample preparation

Enrichments of all samples were prepared inside an anaerobic chamber (Forma Scientific, Marietta, OH, USA) operating at 15°C. With soil samples, a loopful of soil was added to 10 ml of pre-reduced single-strength Peptone Yeast Extract Glucose Starch (PYGS, Lund *et al.* 1990) broth and, after mixing, the suspension was incubated anaerobically at 4°C for 3 weeks. With faecal samples, a single sterile swab stick was pushed into the faeces, retracted and then placed into a sterile universal bottle. An aliquot (15 ml) of pre-reduced single-strength PYGS broth was then added to each universal bottle and the contents were swirled gently to release microorganisms attached to the swab. Each faecal sample was then incubated anaerobically at 4°C for 3 weeks. With hide samples, a volume (75 ml) of pre-reduced single-strength PYGS was added to a stomacher bag containing approx. 2 cm[2] of hide. The contents were mixed and incubated anaerobically at 4°C for 3 weeks. With water samples, a volume (10 ml) of water was added to 10 ml of pre-reduced double-strength PYGS in a sterile universal bottle. Contents of each bottle were mixed and incubated anaerobically at 4°C for 3 weeks. With swab samples, an appropriate volume (15 or 50 ml) of pre-reduced single-strength PYGS was added to each universal bottle or stomacher bag containing swab sticks or gauze swabs, respectively. Each sample was then mixed and incubated anaerobically at 4°C for 3 weeks.

16S rDNA-based detection of blown pack-causing clostridia

Molecular detection of blown pack-causing clostridia was conducted using methods described previously (Broda *et al.* in press) using primers 16SEF and 16SER for PCR detection of 16S rDNA gene fragments of *Cl. estertheticum* and strains K21/K24, and primers 16SDBSF and 16SDBSR for detection of 16S rDNA gene fragments of *Cl. gasigenes*.

To confirm the identity of PCR products detected in enrichments, five of the 16S rDNA gene fragments that were amplified with *Cl. estertheticum*-specific primers and six that were amplified with *Cl. gasigenes*-specific primers were purified and sequenced with forward primers 16SEF and 16SDBSF, respectively. The DNA sequencing and subsequent sequence analyses were performed using methods described previously (Broda *et al.* 2003).

Culture-based detection of blown pack-causing clostridia

Loopfuls of the enrichments were directly streaked onto the surface of freshly prepared Columbia Blood Agar (CBA) with 5% v/v sterile sheep blood. The inoculated plates were incubated anaerobically at 10°C for 3 weeks. After incubation, isolates whose colony morphology resembled those of *Cl. estertheticum*, *Cl. gasigenes* or strains K21/K24 were streaked onto CBA agar and incubated anaerobically at 10°C for 7–10 days to confirm purity. Isolates that were unable to grow in air at 10°C were selected for identification.

Abattoir strains of presumptive blown pack-causing clostridia were identified at species level using restriction fragment length polymorphism analysis (RFLP) of 16S rDNA gene, as described previously (Broda *et al.* 2000). Abattoir and reference strains of *Cl. estertheticum*, *Cl. gasigenes* or strains K21/K24 were grouped on the basis of their banding pattern similarity as assessed by comparison of resolved fragments larger than 154 bp. Isolates whose PCR-amplified 16S rDNA genes digested with *Alu*I yielded indistinguishable banding patterns were considered the members of the same PCR-RFLP type, and, therefore, of the same clostridial species (Broda *et al.* 2000).

The genomic DNA of abattoir strains of blown pack-causing clostridia were also used as PCR templates in amplification of specific 16S rDNA gene fragments. PCR was performed, as with enrichments and DNA from *Cl. estertheticum* and *Cl. gasigenes* were included as positive controls.

Table 1 Detection of blown pack-causing clostridia in 39 enriched abattoir and environmental samples by molecular and/or conventional detection procedures

Sample code	Sample description	Detection of *Cl. estertheticum* or strains K21/K24		Detection of *Cl. gasigenes*	
		DNA-based	Culture-based	DNA-based	Culture-based
	Stockyard pens				
SP1	Footwalk above the stockyard pens	+	+	+	+
SP2	Walls in the stockyard pens	−	−	+*	−
	Slaughter floor				
SF1	Underneath the gut stand, slaughter floor, after clean down	−	−	−	−
SF2	Underneath the legging stand, slaughter floor, after clean down	+vw	−	−	−
SF3	Air intake, slaughter floor, after clean down	−	−	−	−
SF4	Walls in the sticking area, slaughter floor	−	−	+	+
SF5	Floor at legging stand, slaughter floor	−	−	+*	−
SF6	Gut trolley, slaughter floor	+w*	−	−	−
SF7	Apron wash in the pelting area, slaughter floor	+vw	−	+*	−
SF8	Sides of carcasses, slaughter floor	−	−	−	−
SF9	Water from hand wash, slaughter floor	−	−	−	−
SF10	Water from sterilizer/grading	−	−	−	−
SF11	Water, slaughter floor	−	−	−	−
SF12	Knife swabbed after entering neck cavity, slaughter floor	−	−	−	−
SF13	Rail above gut stand, slaughter floor	−	−	+	+
SF14	Air intake outside building, above slaughter floor	−	−	−	−
	Chillers				
CH1	Floor, No. 1 chiller	−	−	−	−
CH2	Drip tray condenser, No. 1 chiller	−	−	−	−
CH3	Drip tray condenser, No. 2 chiller	−	−	−	−
	Boning room				
BR1	Vacuum-packaging machine, boning room, after clean down	−	−	−	−
BR2	Carcass before boning in shoulder area, boning room	−	−	−	−
BR3	Boning table in shoulder area, boning room	−	−	−	−
BR4	Floor in carcass breakdown area, boning room	−	−	−	−
BR5	Conveyer belt to vacuum packaging machine, boning room	−	−	−	−
BR6	Roll rails in dipping bath after vacuum packaging, boning room	−	−	−	−
BR7	Chop board by skinning machine, boning room	−	−	−	−
BR8	Silverskin from skinning machine, boning room	−	−	−	−
BR9	Skinning machine, boning room	−	−	−	−
BR10	Sawbench, boning room	−	−	−	−
BR11	Fan guard and surrounds by vacuum packaging machine, boning room	−	−	−	−
BR12	Chop board in legging area, boning room	−	−	−	−
BR13	Hind leg of boned carcass, boning room	−	−	+	+
	Hide				
H1	Hide 1	+*	−	+*	−
H2	Hide 2	+	+	+*	−
H3	Hide 3	+*	−	+*	−
	Faeces				
F1	Faeces 1	+*	−	+	+
F2	Faeces 2	+w	+	−	−
	Soil				
S1	Soil 1	−	−	+w†	+
S2	Soil 2	+*	−	+	+

+, positive (+w, weak positive PCR signal; vw+, very weak positive PCR signal; +w†, weak positive PCR signal was obtained on subsequent testing).
−, negative.
*identity of the PCR product was confirmed with DNA sequencing.

RESULTS

16S rDNA-based detection of blown pack-causing clostridia

With *Cl. estertheticum* and strain K21/K24-specific primers, PCR products of the expected size (approx. 775 nucleotides) were amplified using DNA templates from 10 of 39 enriched samples (Fig. 1). PCR product of similar size was also amplified from DNA of reference strain *Cl. estertheticum*. The sequenced PCR products from five positive samples (Table 1) showed between 99·6 and 100% sequence similarity with the sequence of 16S rDNA gene fragment of strains K21/K24 and between 99·1 and 99·6% sequence similarity with the sequence of 16S rDNA gene fragment of *Cl. estertheticum*.

With *Cl. gasigenes*-specific primers, PCR products of the expected size (approx. 920 nucleotides) were amplified using DNA templates from 13 of 39 enriched samples (Fig. 2). PCR product of similar size was also amplified from DNA of

reference strain *Cl. gasigenes*. The sequenced PCR products from six positive samples (Table 1) showed between 99·7 and 100% sequence similarity with the sequence of 16S rDNA gene fragment of *Cl. gasigenes* strains DB1A or R26.

Culture-based detection of blown pack-causing clostridia

Of the initial 107 isolates, 39 isolates unable to grow in air at 10°C were selected for identification. With RFLP of the 16S rDNA gene, five isolates obtained from three different abattoir samples were identified as strain K21 or K24, and 20 isolates obtained from seven different abattoir samples were identified as *Cl. gasigenes* (Table 1). Restriction fragment length polymorphism analysis patterns of the remaining 14 isolates showed little similarity to *Cl. estertheticum* or *Cl. gasigenes*.

With *Cl. estertheticum* and strains K21/K24-specific primers, PCR product of expected size was amplified with
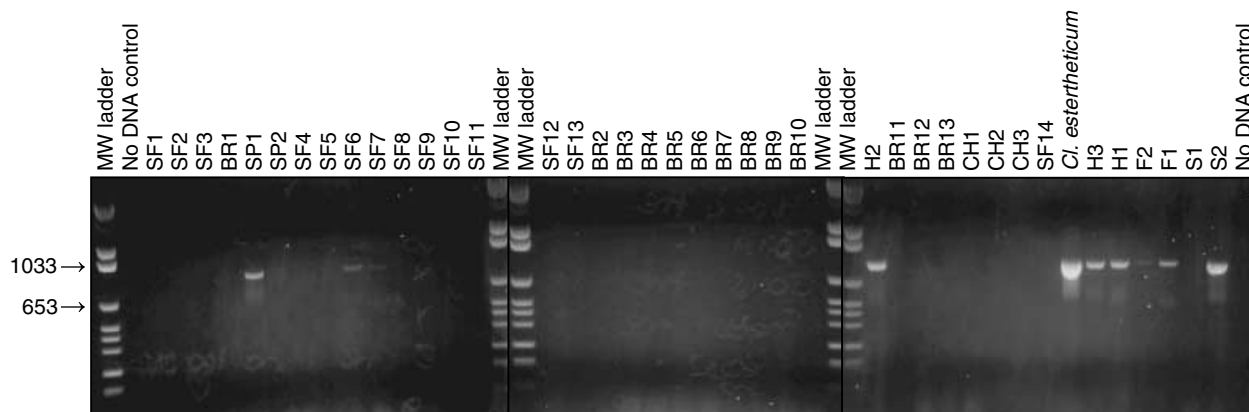

Fig. 1 PCR detection of 16S rDNA gene fragments in enriched abattoir and environmental samples using *Cl. estertheticum*- and strain K21/K24-specific primers. For sample codes above gel lanes see Table 1
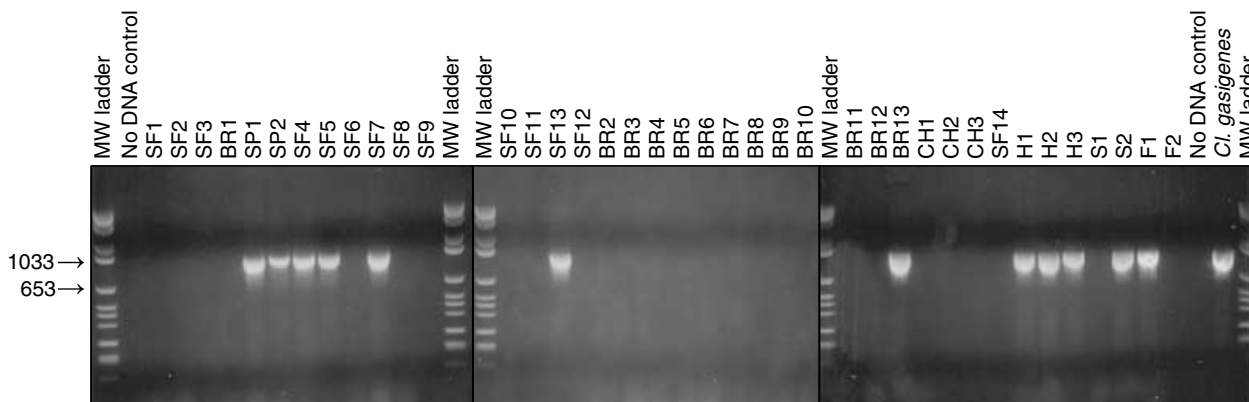

Fig. 2 Detection of 16S rDNA gene fragments in enriched abattoir and environmental samples using *Cl. gasigenes*-specific primers. Sample S1 was positive on subsequent testing

DNA of each isolate that was identified with RFLP analysis of 16S rDNA gene as blown pack-causing clostridial strain K21 or K24. Similarly, when *Cl. gasigenes*-specific primers were used, PCR product of expected size was amplified with DNA of each isolate that was identified with RFLP analysis of 16S rDNA gene as blown pack-causing clostridial strain of *Cl. gasigenes*.

DISCUSSION

In this study, DNA-based methods successfully detected the presence of specific 16S rDNA gene fragments of known species of blown pack-causing clostridia in enriched samples from the abattoir and its environs. The identity of PCR products amplified from these samples was positively confirmed by DNA sequencing. Consequently, the recently developed molecular procedures for clostridial detection (Broda *et al*. 2003) appear to be suitable for specific detection of blown pack-causing clostridia in matrices that are likely to occur in the meat plant environment.

DNA-based detection of blown pack-causing clostridia was performed in this study with abattoir samples that were enriched in PYGS broth at low temperature. In the abattoir and its environs, clostridia are likely to be present in low numbers and may occur in various physiological forms, including spores. While the addition of an enrichment step extends the time required for detection, introduction of this step allows increased sensitivity of the DNA-based procedure. In addition, the use of enrichment promotes germination and outgrowth of clostridial spores and, consequently, improves the yield of extracted DNA. PCR has demonstrated a wide potential for specific recognition of bacteria without their prior enrichment (Scheu *et al*. 1998). However, PCR detects the presence of target DNA in the sample, rather than the presence of viable and/or culturable microorganisms. From the spoilage control perspective, the significance of the presence in the abattoir of the DNA of non-viable psychrotolerant clostridia is yet to be assessed. However, the detection of the presence of specific DNA in enriched samples requires growth and demonstrates the viability of blown pack-causing clostridia, and their potential to cause spoilage.

Of 10 abattoir samples that were positive for *Cl. estertheticum* or strains K21/K24 with DNA-based detection, only three samples tested positive for these clostridia when culture-based detection was conducted. Similarly, strains of *Cl. gasigenes* were isolated from only six of 13 samples that tested positive with DNA-based procedures. It is likely that the low cultural detection rate of blown pack-causing clostridia demonstrated in this study reflects the commonly experienced difficulties with the conventional isolation of psychrophilic and psychrotolerant clostridia (Dainty *et al*. 1989; Kalchayanand *et al*. 1989; Broda *et al*. 1996). Surpris-ingly, *Cl. gasigenes* strain was isolated from a single soil sample in the absence of a positive PCR amplification from this sample with *Cl. gasigenes*-specific primers. However, on subsequent retesting a weak band of PCR product of the expected size was amplified using DNA template from this soil sample. It is possible that the failure to amplify *Cl. gasigenes*-specific 16S rDNA fragment during the initial test was due to interference of PCR-inhibiting soil components, such as humic acids (Ogram *et al*. 1987; Smalla *et al*. 1993).

The specific 16S rDNA gene fragments of both species of blown pack-causing clostridia were produced with DNA templates from each of the three hide samples tested in this study. With the DNA-based method, blown pack-causing clostridia were also consistently traced back to preslaughter holding of animals and slaughter floor operations associated with handling of animal carcasses prior to pelt removal, e.g. sticking or dressing. These results point to animal hides as the probable abattoir source of carcass contamination with blown pack-causing clostridia. It is likely that during dressing, this contamination occurs through the introduction onto carcasses of spores that originate from the exogenous environment of the slaughter animal, such as soil, feed or plant surfaces (Bell 1997). If this hypothesis is confirmed by the results of a trace-back study that includes samples from farm environment of slaughter animals, the practical control of blown pack spoilage will be more likely achieved by limiting the transfer of clostridial spores from hides onto carcasses during dressing operations than by eliminating primary source(s) of clostridia from the farm environment. The spread of blown pack-causing clostridia in the abattoir environment, such as that effected via air movement may be minimized by physical containment of, and more stringent cleaning regimens within, those slaughter floor operations that involve handling of carcasses prior to pelt removal.

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