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PCR detection of psychrotolerant clostridia associated with deep tissue spoilage of vacuum-packed chilled meats

J.A. Boerema, D.M. Broda and R.G. Bell
AgResearch, Hamilton, New Zealand

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Aims: To develop a practical molecular procedure that directly (without isolation) and specifically detects the presence of clostridia, which cause the deep tissue spoilage condition.

Methods and Results: A primer set was designed and a PCR amplification procedure developed to detect the presence of *Clostridium algidicarnis* and *Cl. putrefaciens* 16S rDNA gene fragments in meat. The procedure yielded amplicons of the expected size with homologous DNA templates, but failed to give PCR products with DNAs from 47 food clostridia and common meat spoilage micro-organisms. The minimum level of detection was 10^4 cfu g^{-1} for nonenriched meat samples. Based on the established specificity of these primers, as well as DNA sequencing of amplicons, the presence of *Cl. algidicarnis* and/or *Cl. putrefaciens* was confirmed in a swab sample taken from the cartilage of an ovine stifle joint, which on opening exhibited strong offensive odours.

Conclusions: The developed method can be used for rapid detection of clostridia causing deep tissue spoilage in commercial vacuum packs.

Significance and Impact of the Study: The paper reports practical procedures that can be used for rapid confirmation of the causative agents of deep tissue clostridial spoilage in commercial vacuum-packed chilled meats.

INTRODUCTION

Deep tissue or 'bone taint' spoilage is characterized by the presence of sour or putrid odours in deep musculature, bone marrow, hip or stifle joints, or shoulder the region in both fresh and cured meats (Haines 1941; Mundt and Kitchen 1951; Ingram 1952). Traditionally, this spoilage condition was associated with inadequate carcass cooling which, with a concomitantly high pH and tissue temperature and low muscle tissue-oxygen concentration, may allow germination and proliferation of mesophilic clostridia in deep tissues (Callow and Ingram 1955; Ingram and Dainty 1971). More recently, however, incidents of deep tissue spoilage of vacuum-packed chilled meats have been reported where temperature abuse has not occurred (Broda *et al*. 1996).

Two psychrotolerant clostridial species, *Clostridium putrefaciens* and *Cl. algidicarnis*, have been associated with deep tissue spoilage conditions. The former species has been implicated in instances of bone taint in cured hams (Ingram 1952; Ross 1965), while the latter has been associated with stifle joint taint in chilled vacuum-packed lamb (Broda *et al*. 1996) and with bone taint in temperature-abused beef (De Lacy *et al*. 1998). However, the recognition of psychrotolerant clostridia as the primary causative agents of deep tissue spoilage has been confounded, among other factors, by difficulties in their isolation from tainted tissues in numbers that indicate proliferation and active metabolism in deep tissues (Cosnett *et al*. 1956; Shank *et al*. 1962; Broda *et al*. 1996). However, it has been recognized that poor quantitative recovery or even complete inhibition of psychrophilic and psychrotolerant clostridia may occur on conventional microbiological media (Kalchayanand *et al*. 1993; Broda *et al*. 1996). Therefore, with the 'bone taint' spoilage condition, it is possible that the growth of clostridia in deep tissues might have occurred but high numbers of these micro-organisms would not be detected with conventional microbiological methods.

The aim of this study was to develop a molecular procedure that will detect and semiquantify *Cl. algidicarnis* and *Cl. putrefaciens* in meat without recourse to their isolation in pure culture.

Correspondence to: Dorota Broda, AgResearch Limited, Ruakura MIRINZ Centre, Private Bag 3123, Hamilton, New Zealand (e-mail: dorota.broda@agresearch.co.nz).
Present address: J.A. Boerema, HortResearch, Hamilton, New Zealand.

MATERIALS AND METHODS

Bacteria

The 50 bacterial strains used in this study are listed in Table 1. Reference strains of *Clostridium* spp. were either original New Zealand strains previously deposited in culture collections, or strains obtained from these collections or from Dr E.A. Szabo, CSIRO, Sydney, Australia. In addition, four local strains obtained from vacuum-packed chilled meats were used. The strains were maintained as recommended by respective culture collections or as described previously (Broda *et al*. 2000).

Before being subjected to DNA extraction, all strains were checked for purity by plating onto solid media.

PCR detection

DNA from pure bacterial cultures was extracted using a HighPure DNA preparation kit (Boehringer Mannheim GmbH), as described previously (Broda *et al*. 2000).

For PCR detection of *Cl. algidicarnis* and *Cl. putrefaciens*, primers complementary to variable regions V1 and V6 of the 16S rRNA genes were designed. The primer sequences were: CAF (forward) 5′-AGT TAT TCC TTC GGG RA-3′ and CAR (reverse) 5′-ACG GAG GAT TGG TAT CC-3′. These primers bind at positions 63–79 and 818–802, respectively, of the *Cl. algidicarnis* and *Cl. putrefaciens* 16S rDNA sequences (GenBank accessions AF127023 and AF127024, respectively). The expected size of the PCR product obtained with these primers is 756 bp. The primers were custom-made by Life Technologies Ltd. (Gibco BRL Custom Primers, Penrose, Auckland, New Zealand).

Polymerase chain reaction amplification and detection of PCR products were conducted as described previously (Broda *et al*. 2000).

Sensitivity of PCR detection in meat

To test the sensitivity of PCR detection of deep tissue clostridia in meat, 10-g meat samples were inoculated with 1 ml of 0, −1, −2, −3 and −4 dilutions of actively growing *Cl. algidicarnis* NCFB 2931^T culture. A 10-g uninoculated mince sample was included as a negative control.

Eighty-nine ml of sterile dilution fluid was added to each inoculated mince sample and to the uninoculated control, before they were stomached for 2 min in a Colworth Stomacher. A 1-ml sample of this meat/dilution fluid homogenate was used for DNA extraction. DNA was extracted using a HighPure DNA preparation kit, except that the cells from the 1-ml volumes of meat homogenates were treated without adjusting the O.D. of the TE buffer suspensions. Polymerase chain reaction amplifications with CAF/CAR primers and amplicon analysis were performed, as described for the pure bacterial cultures.

Detection of deep tissue clostridia in naturally contaminated meat

A single commercial pack of vacuum-packed lamb leg exhibiting stifle joint taint was used. For molecular detection, the pack was opened aseptically and 1 ml of drip was removed for DNA extraction. In addition, the external

Table 1 Polymerase chain reaction products obtained during amplification of DNA with primers CAF and CAR

Micro-organism	Strain source and designation	Detection of PCR product
Aeromonas hydrophila	ATCC 7966	–
Alcaligenes faecalis	ATCC 19018	–
Alteromonas putrefaciens	NZRM B5944	–
Bacillus cereus	ATCC 10702	–
Brochothrix thermosphacta	ATCC 11509^T	–
Clostridium algidicarnis	NCFB 2931^T	+
Clostridium algidicarnis	Local SPL4*	+
Clostridium algidixylanolyticum	DSM 12273^T	–
Clostridium botulinum-like	Local PP1	–
Clostridium botulinum type A	ATCC 25763^T	–
Clostridium botulinum type B	CSIRO 17B	–
Clostridium botulinum type E	CSIRO Beluga	–
Clostridium botulinum type F	CSIRO 202F	–
Clostridium butyricum	ATCC 43755	–
Clostridium carnis	ATCC 25777^T	–
Clostridium chauvoei	ATCC 19399	–
Clostridium difficile	ATCC 9689^T	–
Clostridium estertheticum	DSM 8809^T	–
Clostridium estertheticum-like	Local K21	–
Clostridium fimetarium	DSM 9179^T	–
Clostridium frigidicarnis	DSM 12271^T	–
Clostridium gasigenes	DSM 12272^T	–
Clostridium gasigenes	Local R26	–
Clostridium perfringens	ATCC 13124^T	–
Clostridium putrefaciens	ATCC 25786^T	+
Clostridium septicum	ATCC 12464^T	–
Clostridium sporogenes	ATCC 19404	–
Clostridium subterminale	ATCC 25774^T	–
Clostridium tetanomorphum	ATCC 15920	–
Clostridium tyrobutyricum	NZDRI 20079	–
Clostridium vincentii	DSM 10228^T	–
Enterobacter aerogenes	ATCC 13048^T	–
Enterococcus faecalis	ATCC 19433^T	–
Escherichia coli	ATCC 25922	–
Hafnia alvei	ATCC 13337^T	–
Lactobacillus brevis	ATCC 14869^T	–
Lactobacillus fermentum	ATCC 9338	–
Lactobacillus plantarum	ATCC 8014	–
Lactobacillus rhamnosus	ATCC 7469^T	–

Table 1 (*Continued*)

Micro-organism	Strain source and designation	Detection of PCR product
Leuconostoc mesenteroides	NZRM 1564	–
Listeria monocytogenes	ATCC 35152	–
Proteus vulgaris	ATCC 13315	–
Pseudomonas aeruginosa	ATCC 25668	–
Pseudomonas alcaligenes	NZRM B296	–
Salmonella Menston	NCTC 7836	–
Serratia marcescens	ATCC 13880^T	–
Staphylococcus aureus	ATCC 9144	–
Streptococcus pyogenes	ATCC 12344^T	–
Weisella viridescens	ATCC 12706^T	–
Yersinia enterocolitica	ATCC 9610^T	–

* previously designated as DB4 (Broda *et al.* 1996).
^{T}, type strain; ATCC, the American Type Culture Collection, Manassas, VI, USA; CSIRO, Division of Food Science and Technology, Sydney, Australia; DSMZ, the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany; NCFB, the National Collection of Food Bacteria, Reading, UK; NCTC, National Collection of Type Cultures, London, UK; NZDRI, New Zealand Dairy Research Institute, Palmerston North, New Zealand; NZRM, New Zealand Reference Material, Communicable Disease Centre, Porirua, New Zealand.

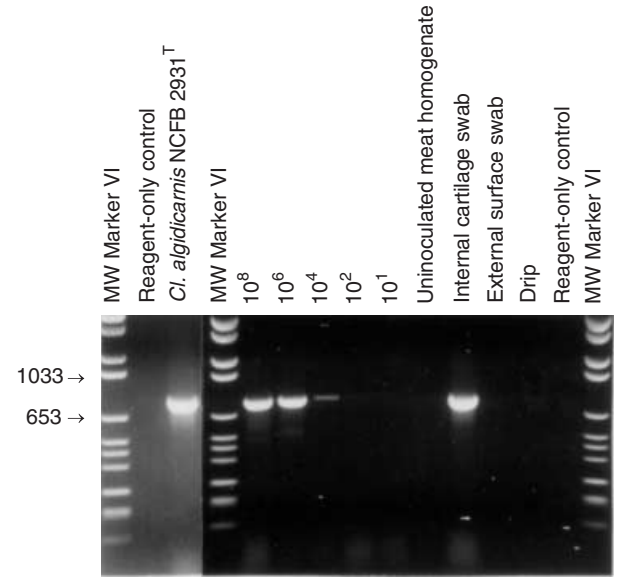

Fig. 1 Polymerase chain reaction detection of *Clostridium algidicarnis* and/or *Cl. putrefaciens* with CAF and CAR primers in artificially inoculated beef mince samples and in swab and drip samples from a commercially vacuum-packed chilled lamb leg showing stifle joint taint spoilage symptoms. The numbers above the lanes indicate the level of inoculation of meat homogenates, as confirmed by conventional enumeration

surface of the leg was swabbed using one dry and then one wet swab. The surface tissue covering the stifle joint was then seared with a hot iron. The joint was opened by an aseptic cut transverse to the long axis of the leg and an additional swab sample was taken from the internal cartilage of the stifle joint. The two sampling swabs were placed into universal bottles containing 10 ml of dilution fluid and shaken for 2 min. The resulting suspension was then used for DNA extraction.

One ml of drip or dilution fluid suspension was subjected to the DNA extraction using a HighPure DNA preparation kit except that the O.D. of the TE buffer suspensions was not adjusted. Polymerase chain reaction amplifications with CAF/CAR primers, and amplicon analysis were performed as described for the pure bacterial cultures.

To confirm the identity of the PCR product, the amplified DNA fragment was purified with the Wizard purification kit (Promega, Dade Diagnostics) according to the manufacturer's instructions. The purified DNA fragment was sequenced by the Waikato DNA Sequencing Facility, University of Waikato, Hamilton, New Zealand. Sequencing was conducted with forward primer CAF. The sequence was aligned against sequences of *Cl. algidicarnis* and *Cl. putrefaciens* (GenBank accessions AF127023 and AF127024, respectively) using Sequence Navigator (Perkin Elmer), and the alignment was corrected manually as required. Sequence similarity of the amplified DNA fragment with *Cl. algidicarnis* and *Cl. putrefaciens* was calculated using 523 nucleotide sites of the aligned sequences.

RESULTS

With CAF and CAR primers, PCR products of the expected size (approx. 756 bp) were amplified using DNA templates from reference strains *Cl. algidicarnis* NCFB 2931^T and *Cl. putrefaciens* ATCC 25786^T, and local strain *Cl. algidicarnis* SPL4. No PCR products were amplified with these primers for any other reference strains (Table 1). No amplification products were present in the reagent-only control.

With CAF and CAR primers, PCR products of 756 bp were evident in amplification with DNA templates prepared from nonenriched meat homogenates inoculated with *Cl. algidicarnis* NCFB 2931^T (Fig. 1). With these primers, the minimum level of detection was 10^4 cells g^{-1} beef mince (10^3 cells ml^{-1} meat homogenate) for nonenriched meat samples.

Amplification with DNA extracted from the stifle joint cartilage swab sample produced a single amplicon of the expected size (Fig. 1). Visual comparison of the yield of this amplicon with those obtained for meat homogenates indicated that the DNA template obtained from the cartilage swab suspension represented 10^7 or more target cells. No amplicons were produced with CAF and CAR primers when DNA extracted from drip or the external surface swab samples were used as PCR templates.

The sequence of the PCR product obtained with CAF and CAR primers showed 99·5% sequence similarity with the sequence of the 16S rDNA gene fragment of either *Cl. algidicarnis* (GenBank accession AF127023) or *Cl. putrefaciens* (GenBank accession AF127024).

DISCUSSION

In the past, two psychrotolerant clostridial species, *Cl. putrefaciens* and *Cl. algidicarnis*, have been associated with the occurrence of the deep tissue spoilage condition. The two species display considerable diversity of their morphological and biochemical characteristics, with the former being proteolytic but incapable of fermenting carbohydrates (McBryde 1911), while the latter, isolated and described more than 80 years after *Cl. putrefaciens*, is saccharolytic and cannot digest meat or milk (Lawson *et al*. 1994). Surprisingly, the ecological niche(s) in which these two micro-organisms have been encountered appear to be congruous and include internal animal tissues (Ingram 1952; Broda *et al*. 1996). Recently, it has been established that 16S rDNA gene sequences of *Cl. putrefaciens* and *Cl. algidicarnis* are highly homologous, displaying 99·5% nucleotide similarity (Broda *et al*. 2000). Regardless of the implications the DNA sequence data might have on the future taxonomic assignment of these two micro-organisms, high homogeneity of 16S rDNA gene sequences of *Cl. putrefaciens* and *Cl. algidicarnis* explains why primers designed from highly variable and species-specific V1 and V6 regions of the 16SrDNA gene are capable of detecting the micro-organisms described as two separate species.

Primers CAF and CAR permitted the specific molecular detection of *Cl. algidicarnis* and *Cl. putrefaciens*, as evidenced by their collective failure to produce any amplicons with DNA templates obtained from 47 nontarget micro-organisms. The lower limit of detection in beef mince was 10^4 cells per gram. As demonstrated by the amplification of the specific 16S rDNA gene fragment from the DNA template obtained from the stifle joint cartilage swab, the sensitivity level obtained in the present study appears to be sufficient for confirmation of the presence of a causative clostridial agent in commercial deep tissue spoilage incidents. This sensitivity level is, however, unlikely to allow direct detection of *Cl. algidicarnis* and *Cl. putrefaciens* in meat or environmental swab samples containing low numbers of target micro-organisms. Consequently, further development of rapid and sensitive PCR detection, for example incorporating low temperature enrichment, will be essential if molecular detection methods are to become an effective tool in the trace back of deep tissue spoilage incidents and the eventual control of this spoilage condition.

In the past, the numbers of clostridia found in deep tissues were frequently very low, suggesting that these bacteria may have been inadvertently introduced during sampling (Gill 1979). Contrary to these previous findings, the semiquantitative assessment of the PCR product yield obtained in the present study for the cartilage swab suggests that high numbers of clostridia were present within the stifle joint and, consequently, are unlikely to have been introduced during sampling. In addition, failure to obtain a PCR product with DNA extracted from drip and an external lamb leg swab indicates that these clostridia were present in the stifle joint at slaughter rather than introduced as a surface contaminant during dressing and packaging. Therefore, the results of the present study point to psychrotolerant clostridia as being the primary causative agents of, as well as intrinsic contaminants in, the deep tissue spoilage condition. Further work, however, is needed to establish the route of entry of clostridia into internal tissues and the sources of these micro-organisms in trace back from vacuum-packed products to the farm environment.

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