Detection of *C. botulinum* in PCR-positive pig faecal samples by traditional isolation and characterization methods.

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**Summary:** A recently developed semi-quantitatively PCR procedure, specific in the detection of part of the types B, E and F neurotoxin genes, have been used to establish the prevalence of *C. botulinum* in faeces from slaughter pigs in Sweden. To verify PCR-positive samples, tests were performed to isolate and to identify *C. botulinum* by traditional microbiological methods and by mouse bioassay. Of twenty isolates from selective and non-selective media, two isolates were found positive by the preliminary screening e.g. lipase reaction and gelatine liquefaction. None of the isolates were confirmed positive by RapID ANA II, PCR or by the mouse bioassay. This describes the difficulties in isolating *C. botulinum* from heavily contaminated samples such as faeces, because of the lack of satisfactory selective media for *C. botulinum* and the presence of toxic compounds other than botulinum toxin in the sample.

**Keywords:** Prevalence, confirmation, phenotypic, mouse bioassay

**Introduction:** Today, there is an increasing demand for convenient foods of high quality. This has resulted in the development of refrigerated processed foods of extended durability. Non-proteolytic strains of *C. botulinum* type B, E and F are considered as a hazard in these foods due to mild processing that may favour germination of spores and subsequently toxin formation. A recently performed survey in Sweden displayed a high prevalence (62%) of *C. botulinum* type B in faeces from pigs by using a combined selection and enrichment PCR procedure, specific for type B, E and F (Dahlenborg et al., unpublished). No samples were positive for *C. botulinum* types E and F. Of the positive faecal samples 71% had a spore load of less than 4 spores per g. The aim of this study was to verify the PCR-positive pig faecal samples by detecting *C. botulinum* by traditional methods based on phenotypic characteristics of the bacteria and by the mouse bioassay.

**Materials and methods:** Five faeces homogenate (3 g faeces in 27 ml TPGY medium) that were identified as positive for the type B neurotoxin gene when
analysed by a combined selection and enrichment PCR procedure (Dahlenborg et al., unpublished) were used in this study. Suspected *Clostridium* colonies were isolated and identified according to a conventional isolation procedure for *Clostridium botulinum* in clinical specimen (Moore et al., 1977). Two selective media, *Clostridium botulinum* isolation (CBI) agar (Dezfalian et al., 1981) and blood agar containing kanamycin, and one non-selective media, fastidious anaerobe agar (FAA) were used. All incubations were performed anaerobically at 37°C for 2-4 days. After Gram staining and morphology testing (rod shape and endospore formers) suspected isolates were confirmed phenotypically according to methods described in table 1, genetically by the specific PCR assays for types B, E and F and for toxin production by mouse bioassay.

**Table 1. Phenotypic confirmation of suspected *Clostridium* isolates.**

<table>
<thead>
<tr>
<th>Phenotypic characteristics</th>
<th>Identification method</th>
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<tbody>
<tr>
<td>Lipase activity</td>
<td>Egg Yolk Agar (EYA)</td>
</tr>
<tr>
<td>Lechitinase activity</td>
<td>Egg Yolk Agar (EYA)</td>
</tr>
<tr>
<td>Proteolysis of meat</td>
<td>Cooked Meat Medium (CMM)</td>
</tr>
<tr>
<td>Liquefication of gelatine</td>
<td>20% gelatine</td>
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<tr>
<td>Liquefication of milk</td>
<td>Skim Milk Medium</td>
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<tr>
<td>Milk curdling</td>
<td>Skim Milk Medium</td>
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<tr>
<td>Various biochemical tests</td>
<td>RapID ANA II test system</td>
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</tbody>
</table>

**Results:** Of twenty colonies picked from the selective and non-selective agar plates, only two isolates were identified to be Gram positive, rod shaped and endospore forming with positive lipase and negative lechitinase reaction when incubated on EYA. Both isolates were confirmed to liquefy gelatine and to have proteolytic activity of the cooked meat. One of the isolates liquefied milk and the other curdled the milk. None of the isolates were confirmed positive by the RapID ANA II test system, by the types B, E and F specific PCR assay or by the mouse bioassay.

**Discussion:** No traditional microbiological method based on biochemical and metabolic characteristics are specific for *C. botulinum*. Therefore identification of *C. botulinum* in clinical, food and environmental samples depends primary upon the demonstration of the neurotoxin. In a recently performed survey in Sweden of *C. botulinum* in slaughter pigs a PCR based technique was used instead. The presence of the type B neurotoxin gene was observed in 62% of the samples. In the present investigation a traditional isolation procedure and the mouse bioassay were used to verify the PCR-positive samples. Of the suspected isolates, 10% were identified as positive in the preliminary screening but no isolates were confirmed as *C. botulinum* after further biochemical tests, PCR analysis or by the mouse bioassay. There are no wholly satisfactory selective media for isolation of *C.
botulinum and several biochemical test systems have shown to be insufficient in identifying C. botulinum (Lindström et al., 1999). This is due to the presence of biochemically similar clostridia such as C. sporogenes and other putrefactive anaerobes that behave similarly to C. botulinum in the samples that are under investigation. Also the mouse bioassay has shown to be unreliable in heavily spoiled foods or on enrichment cultures from material that is initially heavily contaminated. This is because of toxic compounds other than C. botulinum toxin that may be present in the sample (Anonymous, 1996) or to degradation of the toxin by proteolytic enzymes (Lynt et al., 1982).

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References:


