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

Maria Dahlenborg\(^1,2\), Elisabeth Borch\(^2\) and Peter Rådström\(^1\)

1: Applied Microbiology, Center for Chemistry and Chemical Engineering, Lund Institute of Technology, Lund University, P.O. Box 124, SE-221 00 Lund, Sweden, Phone: +46-46-222 06 49, Fax: +46-46-222 42 03, E-mail: maria.dahlenborg@tmb.lth.se
2: Swedish Meats R&D, Kävlinge, Sweden

**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from pigs by using a combined selection and enrichment PCR procedure, specific for type B, E and F (Dahlenborg et al., unpublished).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 (Dezfuiian 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>
<thead>
<tr>
<th>Phenotypic characteristics</th>
<th>Identification method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipase activity</td>
<td>Egg Yolk Agar (EYA)</td>
</tr>
<tr>
<td>Lechitinsase 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>
</tr>
<tr>
<td>Liquefication of milk</td>
<td>Skim Milk Medium</td>
</tr>
<tr>
<td>Milk curdling</td>
<td>Skim Milk Medium</td>
</tr>
<tr>
<td>Various biochemical tests</td>
<td>RapID ANA II test system</td>
</tr>
</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 lechitinsase 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).

Acknowledgments: This work was financially supported by the Swedish
Foundation for Strategic Research through a national, industry-orientated
programme for research and PhD education, LiFT-Future Technologies for Food
Production.

References:
Chapman & Hall, pp. 69.


Dahlenborg, M., Borch, E., and Rådström, P. Unpublished. Prevalence of Clostridium botulinum
types B, E and F in fecal samples from slaughter pigs using a combined selection and
enrichment PCR procedure.

foods, New York: Marcel Dekker.

Clostridium botulinum with API 20A, Rapid ID 32A and RapID ANA II. FEMS Immunol.

and nonproteolytic strains of Clostridium botulinum types A, B, E and F: a review. J. Food
Prot. 45(5): 466-474.

Polytechnic Institute, State University, Blacksburg, Wa, U.S.A.