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Survival of *Clostridium perfringens* During Simulated Transport and Stability of Some Plasmid-borne Toxin Genes under Aerobic Conditions

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Johansson A, Engström BE, Frey J, Johansson K-E, Båverud V: Survival of *C. perfringens* during simulated transport and stability of some plasmid-borne toxin genes under aerobic conditions. Acta vet. scand. 2005, 46, 241-247. – *Clostridium perfringens* is a pathogen of great concern in veterinary medicine, because it causes enteric diseases and different types of toxaemias in domesticated animals. It is important that bacteria in tissue samples, which have been collected in the field, survive and for the classification of *C. perfringens* into the correct toxin group, it is crucial that plasmid-borne genes are not lost during transportation or in the diagnostic laboratory. The objectives of this study were to investigate the survival of *C. perfringens* in a simulated transport of field samples and to determine the stability of the plasmid-borne toxin genes *cpb1* and *etx* after storage at room temperature and at 4°C. Stability of the plasmid-borne genes *cpb1* and *etx* of *C. perfringens* CCUG 2035, and *cpb2* from *C. perfringens* CIP 106526, JF 2255 and 6 field isolates in aerobic atmosphere was also studied. Survival of *C. perfringens* was similar in all experiments. The *cpb1* and *etx* genes were detected in all isolates from samples stored either at room temperature or at 4°C for 24-44 h. Repeated aerobic treatment of *C. perfringens* CCUG 2035 and CIP 106526 did not result in the loss of the plasmid-borne genes *cpb1, cpb2* or *etx.* Plasmid-borne genes in *C. perfringens* were found to be more stable than generally reported. Therefore, *C. perfringens* toxinotyping by PCR can be performed reliably, as the risk of plasmid loss seems to be a minor problem.

Clostridium perfringens; survival; plasmid-borne genes; stability

Introduction

Clostridium perfringens is known to be a universal pathogen in humans, domestic animals and wildlife and is certainly the primary cause of clostridial enteric disease in domestic animals. *C. perfringens* also causes severe toxaemias in many domesticated animals (*Songer* 1996). Members of the species *C. perfringens* can be subtyped into five toxinotypes (A, B, C, D and E) according to the production of four major toxins: α, ß, ε and ι. Previously, laboratory animal tests and serological methods were used for toxinotyping of *C. perfringens* (*Petit et al.* 1999), but nowadays a multiplex PCR is used by which all the major toxin genes, alpha toxin gene (*plc*), beta toxin gene (*cpb1*), epsilon toxin gene (*etx*) and iota toxin gene (*iap*) can be detected (*Engström et al.* 2003). Of the four major toxin genes, only *plc* is located on the chromosome while the others are located on plasmids (*Petit et al.* 1999). An additional toxin

and virulence factor, designated ß2-toxin, has recently been found and associated with enteric diseases in domestic animals, especially piglets (*Garmory et al.* 2000, *Klaasen et al.* 1999, *Waters et al.* 2003) and horses (*Herholz et al.* 1999). The ß2-toxin gene (*cpb2*) is also located on a plasmid.

The stability of the plasmid-borne genes in *C. perfringens* is crucial for the correct typing of a *C. perfringens* isolate. It has been discussed previously whether the plasmid-borne genes *cpb1, etx* and *iap* may be lost when strains are stored for extended periods (*Yamagishi et al.* 1997). It is assumed that *C. perfringens* plasmid-borne genes are unstable, a matter that has to be taken into consideration in microbiological laboratory diagnosis of clostridial infections (*Buogo et al.* 1995). For typing of *C. perfringens* isolates, it is therefore recommended to include 5-10 individual colonies in the determination of the toxin type (*Buogo et al.* 1995). Another reason for including several colonies is that more than one toxinotype of *C. perfringens* may be present in a clinical sample. For diagnosis of infection by anaerobic pathogenic bacteria such as *C. perfringens,* it is necessary to protect the clinical samples from oxygen (*Citron et al.* 2000, *Hudspeth et al.* 1997, *Roelofsen et al.* 1999). A representative sampling in the field and safe transport to the laboratory is crucial for successful culturing and identification of the bacteria. For years, swabs have been used to sample and transport clinical material for bacteriological examination. Swab transport systems containing semisolid media have been developed for the transport of samples for subsequent anaerobic cultivation. These systems have been shown to protect both anaerobic and fastidious aerobic organisms (*Hindiyeh et al.* 2001). Preferably the infected material should be cultured immediately after sampling, although delay is common.

The aims of the study were to investigate the survival of *C. perfringens* and the stability of the plasmid-borne genes *cpb1* and *etx* in a simulated *C. perfringens* containing tissue sample at 20°C and at 4°C. Furthermore the stability of plasmid-borne genes (*cpb1, cpb2* and *etx*) was assessed after exposure of *C. perfringens* strains to atmospheric oxygen.

Material and Methods

Bacterial strains and growth conditions

The type strain *C. perfringens* type B CCUG 2035 (*cpb1, etx*) was obtained from the Culture Collection University of Gothenburg, Sweden. This strain was chosen because it contains two plasmid-borne genes (*cpb1* and *etx*). Strain *C. perfringens* type A CIP 106526 (*cpb2*) was obtained from the Culture Collection of Institute Pasteur, Paris, France. The type A strain JF 2255 (*cpb2*) was isolated from a horse with typhlocolitis. The strains were cultured on Fastidious Anaerobe Agar (FAA) (LabM, Bury, Lancashire, England) with 10% defibrinated horse blood and incubated anaerobically over night at 37°C.

Survival of C. perfringens and stability of the plasmid-borne genes cpb1 and etx after simulated transport

One colony of *C. perfringens* CCUG 2035 was inoculated into 10 ml of Fastidious Anaerobe Broth (FAB) (LabM, Bury, Lancashire, England), which was further incubated over night under anaerobic conditions at 37°C. Tenfold serial dilutions of *C. perfringens* in saline were prepared. The number of CFU/ml from the dilutions was determined by plating 100 µl from each dilution on two FAA plates. In order to simulate a sample of infected tissue, 2 g of chicken liver and 2 ml of a tenfold serial dilution of *C. perfringens* were added to a stomacher bag. The bacterial count in the prepared suspensions was 0.5×10^1 - 0.5×10^5 CFU/ml. The mixture in the bag was homogenized by vi-

bration for 2 min. Two cotton-tipped swabs were dipped in each mixture for at least one minute. Each swab was then placed in a tube with Amies medium with charcoal (Copan International, Corona, Italy). Two experiments were performed to compare different transport conditions at different temperatures according to Table 1. After the simulated transport, each cotton-tipped swab was streaked onto two FAA plates, which were incubated under anaerobic conditions as described earlier. Growth of the bacteria was classified as shown in Table 1.

From experiment I, 20 colonies were collected from samples stored at 4°C for 24 h and 20 colonies from samples stored at room temperature (+20°C). DNA preparations were made according to the direct lysis method of *Herholz et al.* 1999. The DNA preparations were stored at –20°C and were all retested for the presence of the major toxin genes one year later. The bacteria were mixed with liver tissue. Organ tissue, invariably present in a swab sample, can act as a substrate for the bacteria and thereby influence the survival of the bacteria. In experiment I, we also investigated if the *C. perfringens* count was higher after the transport than before (Table 2).

Stability of the plasmid-borne genes cpb1, cpb2 and etx under aerobic conditions on agar plates

The strains, *C. perfringens* CCUG 2035 (*cpb1, etx*) and *C. perfringens* type A CIP 106526 (*cpb2*) were cultured on FAA plates and incubated in anaerobic atmosphere at 37°C. After overnight incubation, material (approximately half a colony) from three colonies was harvested for further PCR investigation. The remaining materials from the three colonies were left on the FAA plate under aerobic conditions at room temperature (20°C) for 8 h, and then plated out on three FAA plates. After over night incubation, material from a total of ten colonies from the three FAA plates was harvested and DNA preparations for further PCR investigation were made as described previously. The remaining material from the ten colonies was left on the FAA plates under aerobic conditions for 8 h, and then plated out on one FAA plate each. The same procedure as above, with one colony from ten plates, was repeated six times. Altogether 73 colonies were collected for each isolate. The last samples of bacteria had been exposed to aerobic atmosphere for 8 h, repeated seven times (56 h). As a control, isolates were grown and kept under constant anaerobic conditions and subcultured seven times. Three colonies per plate were harvested and DNA preparations for further PCR investigation were done as described above.

Stability of the plasmid-borne genes cpb1, cpb2 and etx under aerobic conditions in liquid medium

Strains CCUG 2035 (*cpb1* and *etx*), CIP 106526 (*cpb2*), JF 2255 (*cpb2*) and *6 cpb2* positive *C. perfringens* type A Swedish field isolates (2 from chicken, 2 from pig and 2 from horse) were subcultured twice on FAA plates and incubated in anaerobic atmosphere at 37°C. The six field isolates were included in this experiment, as reference strains are adapted to laboratory growth conditions and hence are expected to be more stable with respect to plasmid-borne genes. One colony was inoculated in 10 ml of Brain Heart Infusion (BHI) (Difco, Detroit, MI, USA), which was incubated anaerobically over night at 37°C. After incubation, the suspension was diluted 1:10 in BHI, and approximately 5 ml was transferred to a petri dish. The petri dishes containing the suspensions were kept aerobically at room temperature. As a control, one sample was collected at the start of the experiment and it was plated on FAA agar plates. After 8 hours of aerobic exposure, material was collected, diluted and plated on FAA agar. These plates were incubated in

Table 1. Survival of *Clostridium perfringens* CCUG 2035 on swabs in Amies medium after simulated transport.

Prepared suspension (CFU/ml)	Experiment I[1,2]		Experiment II[1]	
	Room temperature, 24 h	4°C, 24 h	4°C, 24 h	4°C, 44 h
0.5×10^5	2	2	2	1
0.5×10^4	1.5	1.5	1	1
0.5×10^3	1	0.5	0.5	0.5
0.5×10^2	0	0	0	0.5
0.5×10^1	0	0	0	0

[1] On the basis of the number of colonies, bacterial growth was classified; no growth (0), sparse < 20 colonies (1), moderate 20 to 100 colonies (2) or profuse > 100 colonies (3).
[2] This experiment was duplicated with identical results.

anaerobic atmosphere at 37°C. Two DNA preparations were made from the control sample and 20 DNA preparations from the colonies originating from the 8 h aerobic exposure. Altogether, 198 DNA preparations were collected.

Multiplex and duplex PCR

The three toxin genes *plc, cpb1* and *etx* of *C. perfringens* 2035 were detected by a modified version of a multiplex PCR assay by *Engström et al.* 2003. One µl of template DNA, prepared by a direct lysis method of *Herholz et al.* 1999, was added to a 50 µl reaction mixture, with the following reagents: 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2.5 mM $MgCl_2$, 50 nM of each deoxynucleotide, 1 U of AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA, USA), 50 nM of each CPA (α-toxin) primer, 25 nM of each CPB (ß1-toxin) primer, 100 nM of each CPE (ε-toxin) primer and 25 nM of each CPI (ι-toxin) primer. In this study the CPI primers were included in order to get optimal concentrations in the PCR mastermix, although no *iap* gene was detected. The thermocycling (incubations for 1 min at 94°C, 55°C and 72°C, respectively, repeated 35 times) was preceded by incubation for 10 min at 94°C. The presence of the ß2-toxin gene was also determined. ß2 primers (250 nM) (*Herholz et al.* 1999) and enterotoxin primers (50 nM) (*Kadra et al.* 1999) were used in a duplex PCR. The conditions were as in the multiplex PCR, except for the annealing temperature, which was 59°C. The amplicons were analysed by electrophoresis on a 1.5% agarose gel according to standard procedures.

Results

The survival of *C. perfringens* CCUG 2035 stored under different conditions is shown in Table 1. Survival was similar under all the different conditions applied during the simulated transports. Chilling of the sample did not increase the survival rate of the bacteria. It was found that at least 0.5×10^3 CFU/ml of *C. perfringens* must be present in the sample in order to detect the bacterium under the conditions applied in this study (Table 1). The results presented in Table 2 show clearly that *C. perfringens* did not multiply on swabs stored in Amies transport medium. All toxin genes (*plc, cpb1* and *etx*) were detected in all the 40 DNA preparations from experiment I by PCR.

The plasmid-borne genes *cpb1* and *etx* of *C. perfringens* CCUG 2035 and *cpb2* from CIP 106526 were detected by PCR in all the DNA

Table 2. Number of *C. perfringens* CCUG 2035 on Amies swab before and after storage.

Prepared suspension (CFU/ml) of *C. perfringens*	No.* of *C. perfringens* on swab before transport	No.* of *C. perfringens* after transport at room temperature for 24 h	No.* of *C. perfringens* after transport at 4°C for 24 h
0.5×10^5	200	65	60
0.5×10^4	17	15	11
0.5×10^3	4	1	1
0.5×10^2	1	0	0
0.5×10^1	0	0	0

* The average number (n = 2) of *C. perfringens* colonies on two FAA plates.

preparations originating from colonies exposed repeatedly to aerobic atmosphere on agar plates. The *cpb2* gene of the isolates CIP 106526, JF 2255 and the six field isolates were also retained after 8 h of aerobic exposure in liquid medium, judging by PCR. As all the toxin genes were detected after exposure to oxygen, it was deemed unnecessary to examine the controls, which were not exposed to oxygen.

Discussion

For accurate microbiological diagnostics it is important that bacteria in a tissue sample taken in the field should survive and that plasmid-borne genes if present are not lost during transport to the laboratory. In this study we examined the effect of chilling on the survival of *C. perfringens* in a tissue sample stored in a tube with Amies medium up to 44 h.

As shown in Table 1, no differences were detected in the survival of *C. perfringens* CCUG 2035 under the conditions applied in the two experiments, which indicates that temperature during transport is not a critical factor for the survival of *C. perfringens* in clinical samples. Prolonged storage for 44 h at 4°C did not affect the survival of the bacteria. These observations confirm results where it was found that there was no significant effect on the survival rate when comparing 24 and 48 h storage in Amies medium, at room temperature (*Perry* 1997). However, *Österblad et al.* 2003 demonstrated that temperature has an impact on the survival of *C. perfringens* when incubated for a long time. In that study, 10% of the initial amount of the bacteria could be detected after 1-4 days at 20°C while at 4°C *C. perfringens* survived up to two weeks. In our study short storage periods were used, which is in accordance with practice in most European countries. The detection level of *C. perfringens* in this experiment was 500 CFU/ml in the suspension, which can be considered adequate for detection of *C. perfringens* in diseased animals. The ability of *C. perfringens* to form spores that can survive for very long times must also been taken into consideration when survival of *C. perfringens* is discussed. The presence of spores in a clinical sample can significantly influence survival times.

A common opinion is that *C. perfringens* plasmid-borne genes are apparently unstable, although no data to prove this hypothesis are available. In this study all toxin genes, *cpa, cpb1* and *etx* were detected in all the 40 DNA preparations originating from *C. perfringens* kept either at room temperature or at 4°C for 24 h and all were classified as *C. perfringens* type B. Furthermore all the *C. perfringens* plasmid-borne genes *cpb1, cpb2* and *etx* were retained when isolates were repeatedly exposed to aero-

bic environment on agar plates and after 8 h oxygen exposure in BHI. The stability of *C. perfringens* plasmid-borne genes has been discussed previously (*Yamagishi et al.* 1997). In that study it was stated that the plasmid-borne genes *cpb1, etx* and *iap* might be lost during long-term storage of isolates. It was found that 14 of 23 original type B and C strains lost their plasmid genes after storage for several years at –70°C. Initial studies with the primary culture of strain JF 2255 showed an apparent loss of the *cpb2* containing plasmid. However, in a cloned single colony of JF 2255 the *cpb2* gene showed full stability after exposure for 8 h to ambient atmosphere. The apparent loss of *cpb2* in the previous experiment can be explained under the assumption that the original isolate contained both *cpb2* positive and *cpb2* negative colonies that have different growth rates.

Our results indicate that the plasmid-borne genes *cpb1, cpb2* and *etx* are stable under normal laboratory conditions and under conditions normally used during transportation. Therefore, *C. perfringens* toxinotyping by PCR can be performed reliably, as the risk of plasmid loss and problems with misleading results seem to be a minor problem.

Conclusion

In this study the different temperatures used for storage did not affect the survival of *C. perfringens*. The three plasmid-borne genes *cpb1, cpb2* and *etx* were detected in all the DNA preparations that originated from colonies exposed to aerobic conditions. It is very important for classification of *C. perfringens* into the correct toxin group that the plasmid-borne genes are not lost during transport or lost in the clinical laboratory. Our results demonstrate that the plasmid encoded genes *cpb1, cpb2* and *etx* are stable under normal laboratory conditions and under conditions normally used during transportation.

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Sammanfattning

Överlevnad av C. perfringens och stabilitet av vissa plasmidburna toxingener.

Målet med denna studie var att undersöka hur *C. perfringens* överlever efter transport i ett simulerat vävnadsprov under 24-44 timmar vid +4°C och +20°C. Vidare undersöktes om de plasmidburna toxingenerna *cpb1, cpb2* och *etx* var stabila efter exponering i aerob miljö. Vi observerade att de olika temperaturerna, som har använts vid transport, inte påverkade överlevnaden av *C. perfringens.* Alla plasmidburna gener visade sig vara stabila efter upprepad aerob exponering på agarplatta samt vid aerob exponering i BHI buljong. Det är mycket viktigt att ett eventuella bakterier, som finns i ett prov, vilket skickas in till ett diagnostiskt laboratorium, överlever transporten. Dessutom är det av stor vikt att generna är stabila, så att isolaten kan analyseras och klassificeras med molekylärbiologiska metoder. Våra resultat visar att *C. perfringens* överlever väl, om proverna transporteras under kortare tid (upp till 44 timmar) och inom ett temperaturintervall av +4°C - +20°C. Vidare indikerar resultaten att de plasmidburna generna kan anses vara stabila under normala laboratorieförhållanden.

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