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ORIGINAL ARTICLE

A Rapid Screening Identification of *Clostridium perfringens* Alpha and Epsilon Toxins Recovered from Mastitic Bovine Milk by SDS-PAGE Electrophoresis

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ABSTRACT

The objective of this work was to develop an electrophoretic assay (SDS-PAGE) for the detection of *Clostridium perfringens* toxin types that correlated to mouse lethality and dermonecrotic tests. Currently, the mouse lethality and guinea pig dermonecrotic tests are two of several tests used world-wide to evaluate serological responses in animals immunised with vaccines containing toxoids in addition to toxin typing. The mouse lethality test involves injecting mice with a toxin and then determining the number of mice that survive. The mouse lethality test requires large numbers of animals and causes severe distress to the animals. Organisations world-wide are working towards alternatives to animals in the development and control of biological products for human and veterinary use. Additionally, the mouse lethality and guinea pig dermonecrotic tests are labour-intensive, costly and lacks robustness and may be difficult to reproduce and interpret between different technicians. Exposure of seeded milk samples to the procedure resulted in a conclusion that SDS-PAGE is a highly reproducible, easy-to-use, economical and relatively fast method which can be applied for the detection of alpha and epsilon toxins in a crude medium and thereby to the early diagnosis of anaerobic mastitis caused by *C. perfringens* α and ε toxins.

Key words: *C. perfringens*, SDS-PAGE, mastitis, α, ε toxins

Introduction

Clostridium perfringens is an important pathogen in veterinary and medical fields. All types of *C. perfringens* may be normal inhabitants of the intestine of various animal species. Thus, the mere presence of this microorganism in intestinal contents from affected animals is not of diagnostic significance.

Diseases caused by this organism are in many cases life threatening or fatal (Sawires and Songer, 2006). *C. perfringens* type A food poisoning is one of the more common in the industrialised world (Brynestad and Granum, 2002). Cows suffering from mastitis discharge large numbers of pathogens into the milk, like *C. perfringens* (James, 2005).

Despite the clinical significance of clostridia, reliable, practical, and fast identification methods are few. Although simple tests can serve to identify most commonly isolated *Clostridium* species, the identification of other clostridia by conventional biochemical testing and gas-liquid chromatography is still laborious, expensive, and time-consuming. Furthermore, several commercial identification systems for anaerobic bacteria have failed to accurately identify *Clostridium* species (Sperner *et al.,* 1999). Due to these evident drawbacks of conventional methods, there is a growing trend toward toxin detection.

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For the epidemiologist and veterinarian every *C. perfringens* mastitis is a rare and unforgiving challenge, a public health emergency that requires rapid recognition and smooth cooperation between authorities to prevent additional cases. Just as veterinarians have to be prepared to quickly make the correct diagnosis of *C. perfringens* mastitis, the epidemiologist must prepare for such cases by surveying potential *C. perfringens* hazards in the community. Gram-positive bacilli, such as *C. perfringens*, are rare in ruminant mastitis although they have been reported on occasions (Contreras *et al.,* 2003). Over the past century microbiologists have searched for more rapid and efficient means of microbial identification (McCourt *et al.,* 2005; Rivera *et al.,* 2006). The identification and differentiation of microorganisms has principally relied on microbial morphology and growth variables. The problem has been that the standard assay is a serum neutralization test performed in guinea pigs or mice which has become increasingly undesirable, due to expense, complexity, disfavor on humanitarian grounds, and lack of sensitivity and specificity (Songer and Meer, 1996). The mouse and guinea pig bioassay, although sensitive enough, felt rather- illfitting in the era of molecular biology and growing concern over animal experimentation. Also, for the epidemiologist interested in the dissemination and biodiversity of pathogenic agents in the food chain, the lack of a meaningful, easier and quicker detection methods has effectively restricted most of the work with *C. perfringens* and its toxins.

Therefore, we developed a simple screening technique, with special emphasis on pathogenic clostridia of medical or veterinary interest, for the characterisation of α and ε toxins elaborated by a strain of *C. perfringens* achieved using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), to assess the ability whether SDS-PAGE is a suitable tool for the identification of two of the various *C. perfringens* toxins α and ε-toxin (a bioterrorism select agent) in a crude medium. It should be emphasized that this procedure describes the direct identification of the *C. perfringens* toxins in milk (food) and not from culture.

Materials and Methods

Sampling and Bacteriological Examinations:

Udder secretions were collected and were distinguished, for all samples, for the inflammatory signs of mastitis to perform the Californian mastitis test (CMT). The result of the CMT divided the milk samples into two categories: Normal non-mastitic milk samples and mastitic milk samples. The collected milk samples were centrifuged at 3000 rpm for 20 min. The cream and the supernatant were discarded then the sediment of the mastitic milk samples were inoculated into cooked meat broth (CMB), and then heated at 80^0C for 10 minutes. After an overnight incubation anaerobically at 37^0C by using anaerobic jars containing 95% H_2 and 5% CO_2, a loopful was streaked onto sheep blood agar plates (BA) containing 150 µg /ml neomycin sulphate and incubated anaerobically at 37^0C for a further 24 hr (Murray *et al.,* 2003). Bacterial species were identified tentatively by their gross colony morphology. The identity of all *C. perfringens* type A cultures was confirmed by characteristic colonial morphology on Colombia blood agar, the presence of a zone of partial haemolysis and a zone of complete haemolysis and a positive Nagler reaction, by Gram staining, immobility and further confirmatory tests were used as necessary. None of the samples were from animals with arthritis, conjunctivitis or pneumonia; therefore, the mammary secretions were not checked for the presence of mycoplasms. The *C. perfringens* reference strains Types A & D used in this study as controls for the different toxins were obtained from the Animal health Research Institute, Dokki.

Biological Assays:

The isolated *C. perfringens* strains were then typed for their toxigenicity and typing by the mice neutralization test and dermonecrotic test in guinea pigs (Murray *et al.,* 2003).

Animal Model:

Adult guinea pigs weighing 350-450 g (used for the dermonecrotic reaction) in addition to immature BALB/c mice with an average weight of 15-20 g (used for the toxin neutralization test), were maintained in air-conditioned (22 ± 1°C) quarters under uniform husbandry conditions with a light cycle from 06:00 to 20:00. The animals were quarantined and acclimatized for 7 days prior to the initiation of the experimental treatment. During this acclimatization period, the animals were given pellet diet and tap water *ad libitum*. General procedures for animal care and housing were in accordance with the U.S. Department of Agriculture through the Animal Welfare Act (7USC 2131) 1985 and Animal Welfare Standards incorporated in 9 CFR Part 3, 1991.

Simplex PCR. Brain heart infusion agar (Becton-Dickinson) plates were inoculated with a putative type A isolate and then grown anaerobically overnight at 37°C. Three or four colonies were picked from each plate and used to prepare template DNA as described previously (Sritharan and Barker, 1991). These DNA preparations were then subjected to a simplex PCR assay (Riffon *et al.,* 2001) to detect the genes encoding *C. perfringens* lethal toxins i.e., the alpha toxin gene (*cpa*), the beta-toxin gene (*cpb*) and the CPE gene (*cpe*). Products from each simplex PCR were electrophoresed on 2% agarose gels; after electrophoresis, these gels were stained with ethidium bromide for visualization. Isolates carrying both *plc* and *etx* genes are genotypically type A and henceforth are referred to as genotype A isolates.

SDS-Polyacrylamide Gel Electrophoresis:

SDS-PAGE was used for detection and separation of *C. perfringens* major toxins in milk of cows and buffaloes suffering from *C. perfringens* mastitis The flow chart picturing the processing of the milk samples is indicated in Figure 1.

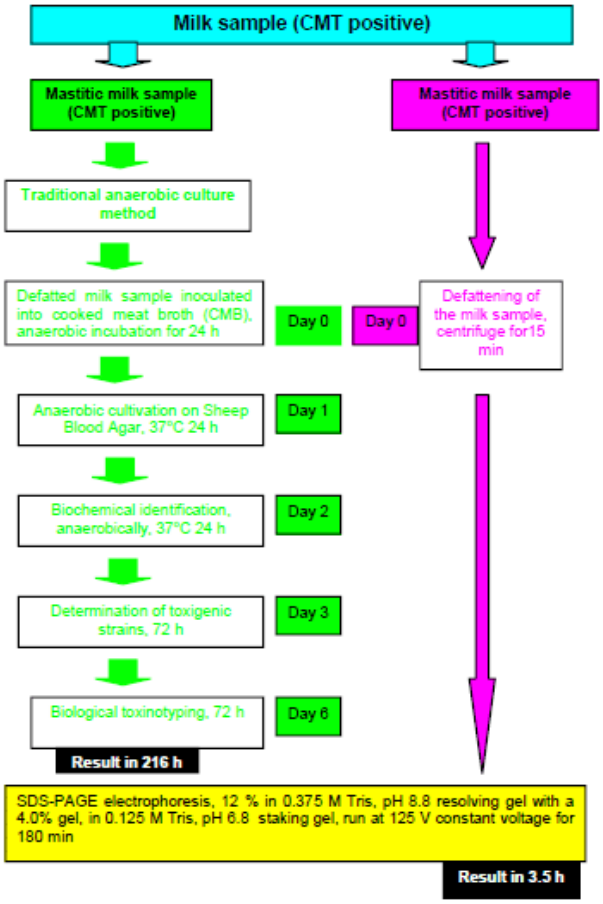

Fig. 1:

Preparation of Sample:.

Mastitic milk samples, that were either positive or negative for *C. perfringens*, were centrifuged at 6000xg for 15 minutes. The fat layer was removed and the supernatant was separated and lyophilized. The lyophilized samples were diluted in 100 µl 90 mM Tris-borate buffer (TB) pH 8.3 and ten µl of each sample was added to 10 µl of the sample buffer and boiled for 4 min immediately before running the SDS-PAGE.

The CMT negative milk sample was divided into two samples to be seeded with the standard strains of *C. perfringens* Types A and D each. The two samples were then treated in the same procedure. The milk samples that were injected into the gel and subjected to SDS-PAGE analyses were thus comprised of the following:

- Lane 1 was injected with a low range protein molecular weight marker (14.4–94kDa).
- Lane 2 was injected with the Toxin production medium without toxin and is considered as a negative control.
- Lane 3 was injected with a bacteriologically free milk sample (CMT negative) contaminated with standard *C. perfringens* type A (control positive alpha and epsilon toxins).
- Lane 4 was injected with a milk sample (CMT positive) proven to be contaminated with *C. perfringens* type D after toxinotyping.
- Lane 5 was filled with a field mastitic milk sample obtained from the naturally infected animals and was bacteriologically positive to *C. perfringens* (tested sample)
- Lane 6 was injected with a mastitic milk sample milk (CMT positive) but negative for *C. perfringens* (negative control).

Running of the SDS-Polyacrylamide Gel Electrophoresis:

SDS-PAGE consisted of a 12 % in 0.375 M Tris, pH 8.8 resolving gel with a 4.0% gel, in 0.125 M Tris, pH 6.8 staking gel and a total of 10 µl of each sample was applied to each lane compared with low range protein molecular weight marker (14.4–94 kDa) Pharmacia Biotech. Electrophoresis was run at 125 V constant voltage for 180 min and the run stopped after the bromophenol blue dye reached the end of the plate. After electrophoresis the gel was stained with Coomassie Brilliant Blue R 250 according to standard procedures.

Coomassie Gel Stain:

The gel was stained with Coomassie brilliant blue at 37°C for 2-3 hours in a shaker incubator. After staining, the gel was washed twice in distilled water to remove excess of the stain then the gel was covered with 200-250 ml of destaining solution (45:45:10% Methanol: Acetic acid: Water). The loosely covered gel/stain container was rocked on a slow rocker overnight. Three to four changes of destaining solution was usually sufficient to visualize bands with a clear background.

Photographing of the Gel:

Gels were visualised under UV illumination using a gel image analysis system (UVP Products, England) and all images archived as digital graphic files.

Results and Discussion

We isolated *C. perfringens* after conducting the traditional and classical identification and toxinotyping tests. The biological assays indicated the presence of Types A and D in the milk samples.

The result of the polyacrylamide gel electrophoresis performed in this investigation on milk samples in the mastitic and non-mastitic milk samples is seen in Figure 2. This was scanned and analyzed revealing the following:

In Lane 2, there were no evidenced bands as the Lane was filled with normal non-infected (CMT negative) milk sample and the electrophoresis conditions did not allow for any milk proteins to be seen.

Detection of C. Perfringens Toxins in Artificially Contaminated Samples:

Lane 3 revealed a band at the 43 kDa region for alpha toxin and a band at the 30 kDa region for the mature epsilon toxin, respectively.

Detection of C. perfringens Toxins in Naturally Contaminated Samples with C. perfringens:

In Lane 5, a band at the 43 kDa region was recorded identical and at the same plane of the standard alpha toxin recorded in Lane 3.

Detection of C. pefringens Toxins in Blind Samples:

In Lane 4, the 30 kDa bands was clearly seen (the region of the mature epsilon toxin). In Lane 6, there were no bands in the 43 and 30 kDa region in the tested milk sample.

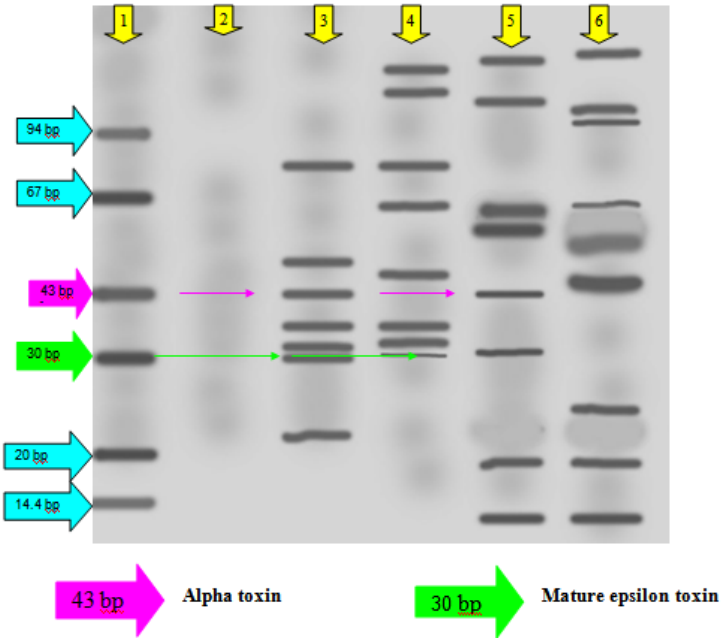

Fig. 2: SDS- PAGE of milk samples naturally (mastitic) and artificially infected with C. perfringens.

Discussion:

It is noteworthy to point out that, although the application of SDS-PAGE in the investigation of toxins is often the first step in purification of the protein molecule, where this technique is a tool in more complex research in the quantification of the toxin and to investigate its production patterns; yet, in the present investigation the SDS-PAGE was used as a rapid crude diagnostic procedure to indicate the electrophoretic band identical to the required toxin under investigation.

The α- toxin is produced by all five types of *C. perfringens* (A, B, C, D and E) and exhibits lecithinase activity in addition to its lethal and necrotic activities (Murray *et al.,* 2003). Typing of *C. perfringens* has traditionally been made by neutralization of lethality in mice or neutralization of dermonecrotic effect in guinea pigs (Murray *et al.,* 2003). However, these methods are both expensive, time-consuming and require large amounts of toxin, specific antiserum and laboratory animals.

C. perfringens toxin A has been identified by seroneutralization in laboratory animals, using specific antisera. Toxinotyping technique requires continuous supply of laboratory animals and use of diagnostic sera, which are increasingly difficult to find and extremely expensive. Moreover, toxinotyping results are obtained in 24 or even 48 h observation (Kadra *et al.,* 1999).

The 43 kDa band revealed in Lanes 3 and 5 is identical to the α-toxin (Hatheway, 1990). Yamakawa and Ohsaka (1977) estimated the molecular weight of Phospholipase C to be 43,000 by SDS-polyacrylamide gel electrophoresis. The recorded seven bands that range from 80-23 kDa represent the other minor toxins that could be present in the infected milk samples (Hatheway, 1990).

Different techniques are routinely used to detect epsilon toxin in body fluids. Stark and Duncan (1972) obtained molecular weights of 34,000 while an approximate molecular weight of 30,000 is indicated in the Factsheets on Chemical and Biological Warfare (http://www.cbwinfo.com/Biological/Toxins/Cper.html). Both molecular weights (30 and 34 kDa) were recorded in our gel in Lanes 3 and 4, respectively. Ryu and Labba (1993) found a 48-kDa enterotoxin-related protein (seen in Lane 3) was found in vegetative and sporulating cell extracts of both toxin types. Their results questioned previous reports about the ability of vegetative cells and presumptive enterotoxin-negative strains of this organism to produce enterotoxin, a protein with a molecular weight of 34 kDa (Lane 4). According to Parreiras *et al.* (2002), this molecular weight corresponds to the epsilon prototoxin, produced by *C. perfringens* type D. Epsilon toxin is produced by *C. perfringens* types B and D as a relatively inactive prototoxin with a molecular weight of 32,700 (Worthington and Mulders, 1977). Proteolytic cleavage of 13 or 14 basic amino acids from the amino terminal of the prototoxin results in the production of the mature toxin with a molecular weight of 31,200 (Hunter and others 1992) (Lane 4).

The activation of the prototoxin seems to produce several isoforms with a range of specific activities between that of the prototoxin and the mature toxin (Worthington and Mulders, 1977). More recently it has

been found that the toxin itself also has several isoforms (Hunter *et al.,* 1992). The mature epsilon toxin is highly toxic with an LD_{50} in mice of <100 ng when administered intravenously (Payne *et al.,* 1994). Epsilon prototoxin produced in the gut of animals is activated by proteolytic enzymes present in intestinal fluid (Niilo, 1965) a process that could be hypothetically reproduced in the mammary gland (López-Expósito and Recio, 2008; Peng *et al.,* 2008; Wedholm *et al.,* 2008).

This study demonstrates the value of SDS-PAGE for detecting and distinguishing between toxins of *C. perfringens*, which introduces SDS-PAGE as a simple, quick, more robust, non-sophisticated methodology, reproducible and costs less than the molecular PCR and ELISA techniques (Table 1). It complements efforts to reduce the use of animals in testing which can thus be utilized in epidemiological and outbreak studies of *C. perfringens* in milk.

Table 1: Comparison of the *C. perfringens* toxins assay techniques in the bovine milk.

	Culture and Identification	Animal model	PCR	SDS-PAGE
Cost	Medium	Low	High	Low
•Ingredients	Medium	Low	High	Low
•Equipment	Low + High	Low	High	Low
•Labour	High	Low	High	Low
•/sample	Medium + High	Low	Medium + High*	Low
Speed	5 to 14 days	3 days	5 days	3.5 hr
Automation	Poor	Poor	Good	Poor
Stage of infection	Sample dependant	Early (7-10 days)	Sample dependent	Sample dependent
Sensitivity	Very Good (80%)	High (75%)	Excellent (100%)	Excellent (100%)
Specificity	Very Good	Very Good	Excellent	Excellent

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