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Neutralization of Tetanus Neurotoxin with Various Botulinum Antitoxins (A-F) in a Study to Isolate *Clostridium botulinum* from Field Samples

[1,2]E.M.A. Saeed, [1]H. Bohnel and [3]F. Gessler
[1]Institute for Tropical Animal Health, Georg-August University, Gottingen, Germany
[2]Department of Microbiology, Faculty of Veterinary Medicine,
University of Khartoum, Khartoum, Sudan
[3]Institute for Applied Biotechnology in the Tropics at the Georg-August University,
Gottingen, Germany

Abstract: In a study to isolate *Clostridium botulinum* from field samples, 53 *C. botulinum*-like isolates were found neutralizable by botulinum antitoxins using the standard mouse bioassay. The neutralization results of 14 of them were found false-positive due to contamination with *C. tetani* which was separated from these isolates and found neutralizable by the same botulinum antitoxin types. Due to this result, *C. tetani* was isolated from other five field samples to test their toxins by botulinum antitoxins. They were also found neutralizable by certain botulinum antitoxins. Supernatants of *C. tetani* isolates were confirmed by being neutralized by tetanus antitoxin. Tetani toxins were found neutralizable by different botulinum antitoxins (A-F) but mainly by polyvalent type ABE (Aventis Behring, Marburg) and type CD mixture (Onderstepoort Veterinary Institute, Onderstepoort). These results were mainly attributed to the non-specificity of the proteins of some (at least ABE and CD) botulinum antitoxin hyperimmune sera which may contain antibodies against *C. tetani* as well.

Key words: Neutralization, tetanus neurotoxin, botulinum antitoxins, mouse bioassay, antibodies, Germany

INTRODUCTION

C. botulinum, the cause of botulism, encompasses a heterogeneous group of obligate anaerobic, spore-forming, Gram-positive rods that produces the most potent biologic toxin known (9). It produces seven neurotoxins that are pharmacologically similar but serologically distinct from each other and designated as types A through G (Rocke, 1993). Isolation of *C. botulinum* from samples of botulism-suspected cases is one of the important methods to diagnose this fatal disease in the laboratory (Smith and Sugiyama, 1988).

However, the isolation is usually difficult due to several reasons including the inhibitory effect of some microflora. *C. tetani*, a drum stick-shaped Gram-positive anaerobic bacillus is one of the important organisms that can inhibit the growth of *C. botulinum* (Graham, 1978) or make the isolation difficult by the swarming character (Sharma and Anand, 2002; Jousimies-Somer *et al*., 2002). Several *Clostridium* species are known with their swarming growth (Sharma and Anand, 2002) and very recently even *C. botulinum* was found swarming (Saeed, 2005) but *C. tetani* is considered as the most swarming *Clostridium* and it is just second to *Proteus mirabilis* in the rate of surface translocation (Sharma and Anand, 2002). The critical factors that determine whether the cells swarm or form regular colonies are the concentration of agar, surface moisture and viscosity (Sharma and Anand, 2002). Plates with 4% agar were used to have discrete colonies of *C. tetani* (Hernandez-Chavarria *et al*., 2001). *Clostridium tetani* can swarm on 3% agar plates if not well dried and sometimes not on 1.4% agar plates if well dried (Saeed, 2005). So, it seems the degree of dryness is more critical than percentage of agar. However, reduced surface moisture is not very reliable as the dryness required to inhibit swarming would completely suppress the growth of fastidious organisms (Sharma and Anand, 2002).

It was recommended to use Phenyl Ethanol (PE) at a concentration of 0.25% to combat the swarming growth of some clostridia (Jousimies-Somer *et al*., 2002). However, it was found that the swarming character of *C. tetani* was not prevented by 0.25 and 0.375% and prevented by 0.5%, a percentage that was shown to completely inhibit the growth of *C. botulinum* (Saeed, 2005). The swarming growth may not appear in the primary plates due to less moisture or it may be a very thin layer that could not be seen by naked eye but appears in the subsequent plates during the purification process or only after an apparently pure colony of another bacterium being grown into liquid

Corresponding Author: E.M.A. Saeed, Institute for Tropical Animal Health, Georg-August University, Gottingen, Germany

medium and then cultured on solid medium (Saeed, 2005). The mouse bioassay has been used for the detection of botulinum toxins and identification of toxigenic *C.botulinum* (Smith and Sugiyama, 1988; CDC, 1998). The method was described as the standard method for detecting, identifying and typing of BoNTs. However, the method is not suitable for examination of test samples containing other lethal substances (Dezfulian and Bartlett, 1985). *Clostridium tetani* is widely distributed in nature especially in soil in form of spores so, it is recommended to pre-inject mice with tetanus antitoxin in mouse bioassay used for BoNT detection to avoid the non-specific death due to TeNT (Smith and Sugiyama, 1988). BoNT and TeNT are closely related in structure and function (Brin *et al*., 2002). Both of them are produced as an inactive polypeptide chain of approximately 150 kDa which is activated by proteases to generate the active dichain form; the heavy chain (H, 100 kDa) and the light chain (L, 50 kDa).

The proteins remain linked through a highly conserved disulfide bond (Pellizzari *et al*., 1999; Brin *et al*., 2002). Both toxins act by cleaving the proteins associated with the synaptic vesicle exocytosis leading to inhibition of the release of the neurotransmitter acetylcholine. In spite of this similarity in structure and function, BoNT and TeNT cause drastically different clinical syndromes. BoNT acts peripherally at the cholinergic nerve endings producing flaccid paralysis while the TeNT acts centrally at the inhibitory nerve endings in the spinal cord producing persistent contraction of the affected muscles (Brin *et al*., 2002). Due to the similarity between BoNT and TeNT, cross-reaction between them is expected however, it is rarely found reported. Dolimbek *et al*. (2002) reported a cross-reactivity between TeNT and BoNT types B and A. The aim of this manuscript is to present and discuss the results of neutralizations of TeNT with various botulinum antitoxins that were obtained during a study to isolate *C. botulinum* from field samples and to show the effect of *C. tetani* on isolation and identification of *C. botulinum* by mouse bioassay.

MATERIALS AND METHODS

Samples and isolates: The samples from which the *C. tetani* like isolates were obtained were from environment and tissues of botulism suspected cases of horses and cattle. The *C. tetani* like isolates (drum stick-shaped Gram-positive or negative anaerobic bacilli) were mainly found as contaminants with other isolates and some of them were isolated as *C. tetani* from other different samples. Culture and isolation methods used were mentioned elsewhere (Saeed, 2005).

Botulinum antitoxins and neutralization tests: Polyvalent antitoxin type ABE (Aventis Behring, Marburg), monovalent antitoxin types C and D (Onderstepoort Veterinary Institute (OVI), Onderstepoort), monovalent antitoxin types A to E (ID-DLO, Lelystad), types B and E (Institute for Applied Biotechnology (IBT), University of Gottingen) type A from Russia and type F from U.S.A. were used. Antitoxins were rehydrated and used according to manufacturer's instructions. *C. tetani* culture supernatants were tested to see if they can be neutralized by botulinum antitoxins according to the mouse bioassay used for BoNT detection (CDC, 1998). White mice of the institute's breeding station weighing 18-25 g were used. Mice were observed for 4 days for signs of tetanus especially the spastic paralysis. The *C. tetani* isolates were mainly tested by polyvalent type ABE and mixture of C and D (OVI) and some of them were also tested by some single toxin types. The original suspected *C. botulinum* isolates were tested by types ABE and CD mixture (OVI). The *C. tetani* isolates were also tested by tetanus antitoxin (Tetagam®N, Aventis Behring). The *C. tetani* isolates were also tested by PCR using primers for *C. botulinum* (Saeed, 2005).

RESULTS AND DISCUSSION

In the study to isolate *C. botulinum* from botulism suspected cases, 53 isolates were found neutralizable by botulinum antitoxins. However, when these isolates were checked in verification tests, the results turned out to be non-specific. The results of 14 of them were considered due to contamination with *C. tetani* which was separated from them and found neutralizable by the same botulinum antitoxin types which gave the previous results as well as by tetanus antitoxin. Five other *C. tetani* isolates isolated from other different specimens were also found neutralizable by certain botulinum antitoxins as well as by tetanus antitoxin. Twelve of these 19 *C. tetani* isolates were from samples from animal environment and seven from animal tissues.

All the isolates tested with tetanus antitoxin (12 isolates) were positive. All the isolates tested by the polyvalent botulinum antitoxin type ABE (except one) and mixture of types C and D (OVI) were positive using different dilution rates of the culture supernatants. One isolate was tested negative for ABE at a dilution of the culture supernatant of 1:4. In higher dilutions the agent was neutralized by ABE and CD antitoxins. Similarly, two isolates were tested by type A from Russia and both of them were positive.

However, only few were found neutralizable by the botulinum antitoxin types from other manufacturers and

no one was found neutralizable by type B (IBT) (Table 1). The *C. tetani* found contaminating the 14 *C. botulinum*-suspected isolates was found as a thin or very thin layer of swarming growth which was not observed during the subculturing of these isolates by plating. It appears only after these isolates being subcultured in a liquid medium and then on agar plates. The swarming growth of some of them was not seen by naked eye but only by hand lens. Also, the swarming character was encouraged by using plates with less agar (1%) instead of the normal agar percentage (1.4) in the agar medium used or by using less dried plates. Discrete colonies of *C. tetani* were obtained by using 0.5% Phenyl Ethanol (PE) (Sigma-Aldrich Chemicals). Swarming and discrete colonies and smears of *C. tetani* are shown in Fig. 1. None of the *C. tetani* isolates were positive for *C. botulinum* by PCR. In spite of the similarity between BoNT and TeNT in structure and function (Brin *et al*., 2002), cross-reactivity between BoNTs and TeNT was rarely reported. A cross-reactivity between TeNT and BoNT types B and A was reported (Dolimbek *et al*., 2002). In this study TeNT was found neutralizable by various botulinum antitoxins (A-F) with various degrees depending on the source of the botulinum antitoxin.

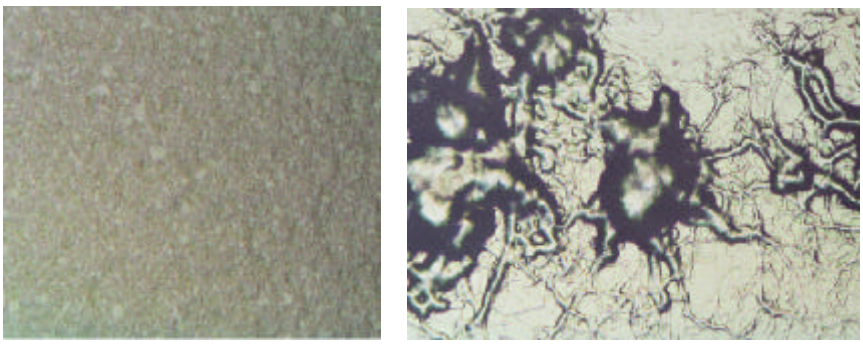
Table 1: Neutralization results of *C. tetani* isolates by botulinum antitoxins

	No. of isolates		
Type of botulinum antitoxin	Tested	Positive	Negative
CD (OVI)	10	10	0
ABE	10	9	1
CD (NL)	5	1	4
A (NL)	6	3	3
B (NL)	6	1	5
E (NL)	5	1	4
A (Russia)	2	2	0
B (IBT)	7	0	7
E (IBT)	5	1	4
F (USA)	6	2	4

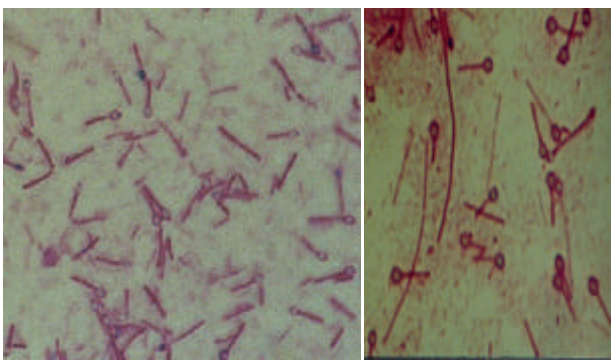
OVI = Onderstepoort Veterinary Institute; NL = Netherlands; IBT = Institute for Applied Biotechnology, Gottingen

The *C. tetani* isolates were identified by their cultural characteristics, neutralization by tetanus antitoxin, spastic paralysis in mice and negative results for *C. botulinum* by PCR. All of ten *C. tetani* isolates tested by polyvalent type ABE (Aventis Behring) and CD mixture (OVI) and two isolates by type A (Russia) were neutralized. It was not found reported that TeNT can cross-react with BoNT types C and D. Hence, these results were attributed to the non-specificity of the hyper immune sera used. It is suggest that these sera contain tetanus antibodies and consider it less likely to be cross-reactivity.

Contrarily, none of seven isolates tested by type B (IBT) was neutralized. Because type B is the BoNT that is most similar to TeNT, cross-reactivity is more likely to occur with type B than with the other BoNT types



Swarming growth | Discrete colonies (0.5% PE-containing plates



Gram-stained smears

Fig. 1: Show (a) swarming growth (b) discrete colonies (0.5% PE-containing plates gram stained smears

(Dolimbek *et al*., 2002). Thus this hyperimmune serum was considered more specific. Hyperimmune serum for BoNT/B (IBT) was raised in chickens and it is expected to contain no antibodies to *C. tetani*. A low number of neutralization results was observed with other botulinum neurotoxins from other sources.

These results or some of them are likely to be due to cross-reactivity. However, the likelihood that these neutralizations happened due to presence of low level of tetanus antibodies in these sera of botulinum antitoxins is more likely the case. We tested botulinum toxins from reference strains of *C. botulinum* types A, B and C with the tetanus antitoxin used but no cross-reaction was observed. The neutralization results of these *C. tetani* isolates indicated that the mouse bioassay might be non-specific and questionable. It recommend that the mouse bioassay result should be confirmed by another method such as PCR unless specific botulinum antitoxin is used or non-botulinal lethalities especially tetanus are considered and otherwise excluded.

Swarming growth of other bacteria is one of the problems that may contribute to the failure in isolating *C. botulinum* either preventing its growth or making the separation of the colonies on the plates very difficult. Several *Clostridium* species were reported to swarm (Hernandez-Chavarria *et al*., 2001; Sharma and Anand, 2002; Jousimies-Somer *et al*., 2002; Saeed, 2005).

The 14 *C. botulinum*-suspected isolates which were found contaminated with swarming *C. tetani* were isolated as apparently pure colonies. This swarming growth appears after these isolates were grown into liquid medium and then subcultured onto agar plates. So, it is recommended that any apparently pure colony should not be tested for *C. botulinum* toxin neutralization before being grown on moist plates or in a liquid medium with a subsequent step on moist agar plates. The usage of a hand lens helps to identify swarming growth which cannot be seen by naked eye.

CONCLUSION

In this study, the results of other hyperimmune sera or some of them may be real cross-reactivity between tetanus and botulinum neurotoxins. So, due to extreme difficulty to separate *C. tetani* from cultures intended for *C. botulinum* detection by mouse bioassay, it is crucial to use highly specific botulinum antitoxins and to confirm the mouse bioassay results by another method such as PCR. These isolates were tested by PCR and none of them was positive for *C. botulinum*.

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