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Molecular characterization and Phylogenetic Analysis of *Clostridium botulinum* Mosaic Type D/C Isolated from Sudan

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Abstract *Clostridium botulinum* types C and D are related to animal botulism. The disease has been reported in sheep in western Sudan causing economic losses. However, the BoNTs that cause sheep botulism in Sudan have not yet subjected to genetic characterization. The aim of this study is to perform genetic analysis for sheep botulism–related isolates from recent outbreak between January to May 2013 at western Sudan in order to improve the efficiency of control strategies and vaccine development. In this study isolation of *Clostridium botulinum* from sheep samples was obtained by culture methods and mouse bioassay. Positive samples were confirmed by PCR and DNA sequencing. PCR was used to amplify the BoNTs gene using three sets of primers to differentiate the gene of the mosaic type from the conserved genes of type C and D. The results of polymerase chain reaction with these primers indicated that sheep botulism–related isolates possess the gene for the mosaic form of the neurotoxin. PCR products were sequenced and subjected to genetic analysis. The results provided evidence for close relationships and genetic variation of the isolates and reference strains published on the GenBank. Multiple sequence alignments showed numerous substitutions occurred in heavy chain in the most homologous regions of BoNT/CD and in the light chain of toxin type C and D, respectively. Following sequencing, isolates were compared phylogenetically with reference strains. The close genetic relationship to the strain 193_09 and strain: OFD16 suggests that neurotoxins produced from sheep botulism is BoNT type DC. The present study provides information on genetic classification of BoNTs related to sheep botulism isolates in Sudan.

Keywords: *Clostridium botulinum, sheep botulism, PCR, DNA sequencing, Sudan*

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1. Introduction

Botulism is a rapid onset, usually fatal disease caused by the botulinum toxin produced by the bacterium *Clostridium botulinum*. The disease occurs more frequently in animals than it does in humans [2]. A large number of animals may be affected, with both environmental and economic impact [3,9]. Animal botulism has specific clinical symptoms such as abnormal posture of the head, weakness, loss of tongue tone, dilated pupils, and flaccid paralysis [26]. Seven immunologically distinct types of botulinum neurotoxin (BoNT/A to G) cause botulism in humans and animals [12,15,22]. Botulinum neurotoxin (BoNT) serotypes C and D and their mosaic variants CD and DC cause severe cases of botulism in animal husbandry and wildlife [7]. The neurotoxin genes of *C. botulinum* type C and D are carried by bacteriophages, which express unstable lysogeny and are frequently lost during cultivation [8,19,20,26]. BoNT is produced as single-chain peptides with a molecular mass of about 150 kDa which are proteolytically activated into a light chain (approximately 50 kDa) and a heavy chain (approximately 100 kDa) linked by a disulfide bond. The heavy chain is further divided into two domains, an amino-terminal (H_N) and a carboxyl-terminal (H_C) domain [15,23]. Diagnosis of botulism is obtained by detecting the neurotoxin and/or *C. botulinum* in the suspected animal. The mouse lethality assay has remained the standard test for the detection of neurotoxins [6,11]. Moreover a rapid diagnosis of botulism is a prerequisite and PCR has already proven its usefulness as a rapid detection method [6,11,16]. Many outbreaks of animal botulism have previously been recorded in Sudan [1,10]. All occurred during dry season while lower rainfall, poor soils and water shortage cause the deaths of various living organisms and consequently the proliferation of anaerobic bacteria. This study was conducted to detect and characterize *C. botulinum* isolates from recent outbreaks in Sudan.

2. Material and Methods

2.1. Test Samples

The samples investigated in this study were collected from different provinces of North Kordofan state at western Sudan during dry season from January to May 2013. Twenty field and clinical samples collected from suspected sheep (tissue, carcasses), water, soil and feed samples were grown in various media using good aseptic culturing technique, at biosafety level 2 cabinet. *C. botulinum* type C 2300 (kindly donated by Dr. Frank Gesslar, *Institute of Tropical Animal Health, Germany)* was used as positive control in this study.

2.2. Optimization of Isolation Procedure

Different media were used to choose the best medium to work with. Five broth media (Cooked meat medium (CMM), Nutrient broth (NB), Reinforced Clostridial Medium (RCM), Brain-heart infusion medium (BHIM) and Trypticase peptone yeast extract glucose (TPYG) medium and four agar media (Fastidious anaerobe agar (FAA) Lab M, Egg yolk agar (EYA), Reinforced Clostridial agar (RCA) and Blood agar (BA) were used. Also different incubation periods were tested with different media including 48h, 72h, 4 days and five days to find the optimum incubation period for toxin production. Single recommended temperature for growth (37°C) was used [11]. The pH for all used media was adjusted to 7.0 and all media were autoclaved at 121°C for 15 min.

The anaerobic conditions were presented by using anaerobic jar and Oxoid anaerobic generation system (AnaeroGen™ sachets OXOID). The active component within each sachet is ascorbic acid. Heat treatment was performed for the test samples to kill the vegetative form of contaminants. It was conducted at 70°C for 15 min. Eight tissue samples showed positive toxicity by mouse bioassay were tested by PCR.

2.3. Genotypic Detection

Genomic DNA was extracted with the DNA extraction Kit GenElute™ Bacterial Genomic DNA Kit (Sigma Aldrish, Belgium).

Three sets of primers were used to targets both the heavy chain and light chain parts of the neurotoxin genes, allowing detection and also distinction between mosaic and non-mosaic type C and D BoNT gene.

General set situated in the heavy chain for detection of mosaic *C. botulinum* types C and D (primer pair pM) with expected size 327bp, the second sets of primers localized in the light chain of BoNT/C (primer pair pC) with expected size169bp and the third set (primer pair pD) with expected size 264bp was localized in the light chain of BoNT/D, (Table 1). The primer sequence were selected from the published sequence described by Prevot et al., [16].

Lyophilized Ready PCR Mix (Maxime PCR Premix, iNtRON Biotechnology, Geonggi-do, Korea) was used. It included all reagents at optimal concentrations for efficient amplification except dissolving water, template and primers. The final reaction mixture of 20µl contained 2.5 µl of each primer, 5µl of DNA template and 10µl of nuclease-free water. The final concentration of primers in the reaction mixture was 0.5 µl.

The PCR was carried out using thermal cycler (TechneTC-512 Thermal Cycler, UK). The cycling began with an initial step at 95°C for 15 min, followed by 40 cycles of denaturation at 94°C for 30s, primer annealing at 55°C for 30 s, and extension at 72°C for 30 s. A final step at 72°C for 10 min followed to complete extension. Positive control with DNA from reference strains (*C.botulinum* type C 2300) and a negative control containing all reagents except DNA template were included during amplification. PCR products was analyzed by electrophoresis in a 1.8% agarose gel in TBE 1X that contain 2.5 µl of (1 µg/ml) ethidium bromide at 100V for 40 min. The products was visualized and photographed with the gel documentation system (Syngene InGenius3, San Diego, USA).

2.4. Nucleotide Sequencing and Phylogenetic Analysis

Sequencing of positive PCR amplicons was performed for both strands by Macrogen Company (Seoul, Korea). The sequences chromatogram was viewed using Finch TV software (www.geospiza.com/Products/finchtv.shtml). The nucleotides sequences of the BoNT gene were searched for sequences similarity using nucleotide BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi.). Highly similar sequences were retrieved from NCBI (shown in Table 2) and subjected to multiple sequence alignment using BioEdit software. Phylogenetic tree of BoNT C&D gene and their evolutionary relationship with those obtained from database was done online by ClustalW (www.genome.jp/tools/clustalw/).

Table 1. Location of primers within specified BoNT genes

Target type	Primer set [A]	Sequence [B] 5' - 3'	Target toxin chain [C]	Size of amplicons
C	**p**C ^{F}	TCCTCGAGTTACAAGCC	Lc	169 bp
	pC ^{R}	CAGGAAAGGGTATATCTG		
D	pD ^{F}	TTAGACTATACAGCATCCC	Lc	264 bp
	pD ^{R}	TAACTTGTGGACGAATCC		
Mosaic C-D	pM ^{F}	TTTATACGAGAATGTTCYG	Hc	327 bp
	pM ^{R}	CATTATATCCTGATGTATCC		

[A] three pairs of primers (pC^{F}-pC^{R}, pD^{F}-pD^{R}, pM^{F}-pM^{R}) were used to differentiate BoNT genes, [B] is the sequence of oligonucleotides, Y was substituted for C and T, [C] Lc is light chain and Hc is heavy chain.

Table 2. Referential *C.botulinum* strains used for phylogenetic analysis

No	Strain	Country	BoNT Type	Accession Number
1.	Strain 193_09	Germany	DC	KT025245
2.	Strain:OFD16	Japan	DC	AB745669
3.	Strain-BVD/3	Germany	D	X54254
4.	Strain 1873	South Africa	D	AB012112
5.	Strain:TW/2003	Taiwan	C	AY251553
6.	Strain:003-9	Japan	C	AB200360
7.	Strain S19	Sweden	CD	FN436022
8.	Strain OTZ07	Japan	CD	AB745659

3. Results

3.1. Purification and PCR Results

Best growth and more toxic isolates were shown by CMM and RCM, which were then used for culture and isolation. Also, EYA was found better than BA, RCA and FAA. The period of 48h incubation was found suitable for EYA and five days incubation was found suitable for RCM and CMM.

C.botulinum type C strain 2300 tested positive for the amplification of a 327 bp and 169 bp DNA fragments with primers pM and pC only, see Figure 1. Two samples were showing positive bands at 327 bp, 264 bp and 169 bp with pairs of primers pM, pD and pC, respectively see Figure 2. The results were also confirmed by sequencing.

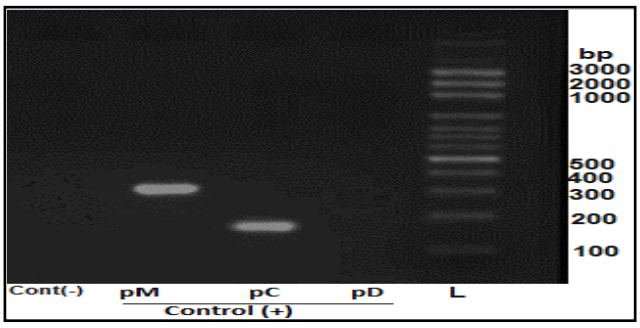

Figure 1. Amplification of *C. botulinum* type C strain 2300 with primer set pM and pC, (L) molecular size standards (100 bp DNA ladder; Vivantis)

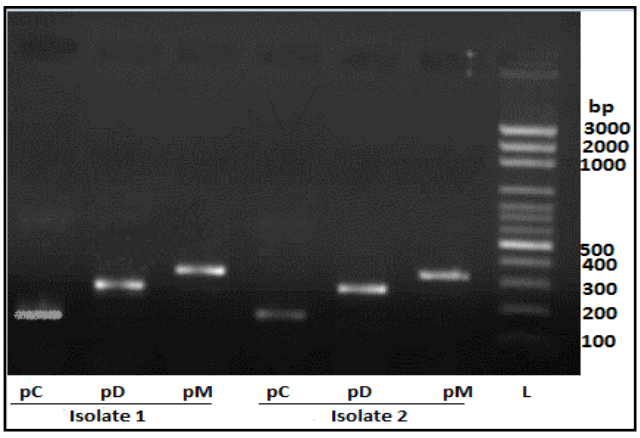

Figure 2. Amplification of sample 1 and 2 with primer set pM, pD and pC. (L) molecular size standards (100 bp DNA ladder; Vivantis)

3.2. BLAST Search and Phylogenetic Analysis of Partial Gene

Nucleotide sequences for Isolate.1.pC (partial sequence of the light chain) revealed 97% identity with strain TW/2003 (BoNT C) gene, 94% with strain:003-9 (BoNT C) gene, 94% with strain:OTZ07 (BoNT CD) and 94% with strain: S19 (BoNT CD) while Isolate.2.pC shows 99% identity with strain TW/2003, 94% identity with strain: 003-9, 93% with strain:OTZ07 and 93% with strain:S19.

Isolate 1.pD (partial sequence of the light chain) demonstrated 99% nucleotide identity with strain193_09 (BoNT DC) gene, 99% nucleotide identity with strain: OFD16 (BoNT DC), 99% with strain 1873 (BoNT D) and 99% with strain-BVD/-3 (BoNT D) gene. Same reference strains were found identical with 98% identity with Isolate 2pD.

The mosaic sequence of Isolate 1pM (partial sequence at binding/translocation domain of heavy chain) shows 99% nucleotide identity with strain 193_09 (BoNT DC) gene, 99% identity with strain: OFD16 (BoNT D/C) gene, 99% with strain-BVD/-3 (BoNT D) gene , 99% identity with strain 1873(BoNT D) gene, 97% with strain: TW/2003 (BoNT C), 97% with strain:003-9 (BoNT C), 96% with strain:S19 ((BoNT CD) and 96% with strain: OTZ07 (BoNT CD). While Isolate 2pM revealed 100% identity with strain 193_09, 100% identity with strain: OFD16, 99% with strain-BVD/-3, 99% with strain 1873, 95% with strain: TW/2003, 95 % with strain: 003-9, 96% with strain: S19 and 96% with strain: OTZ07.

3.3. Multiple Sequence Alignment

The multiple sequence alignment of the Sudanese isolates with similar nucleotide sequences that obtained from BLASTn was carried out to find the homology and evolutionary relation between these sequences. BioEdit software shows transversions and transitions at different regions on the light chain (pC, pD) and heavy chain (pM), see Figure 3(B), Figure 5(B) and Figure 7(B1&B2).

A phylogeny, or evolutionary tree, represents the evolutionary relationships between reference strains and *C. botulinum* type DC Sudanese isolates. The clustering was performed by the unweighted pair group method. The tips of the tree represent groups of descendent taxa and the nodes on the tree represent the common ancestors of those descendants. Two descendants that split from the same node are called sister groups. The horizontal lines are branches and represent evolutionary lineages changing over time, see Figure 4, Figure 6, Figure 8.

Nucleotide sequence of partial pC in isolates 1, 2 showed transversion of Guanine(G) to Thymine(T) (TGC >TTC) and transition of Adenine(A) to Guanine(G) (CAA>CGA).

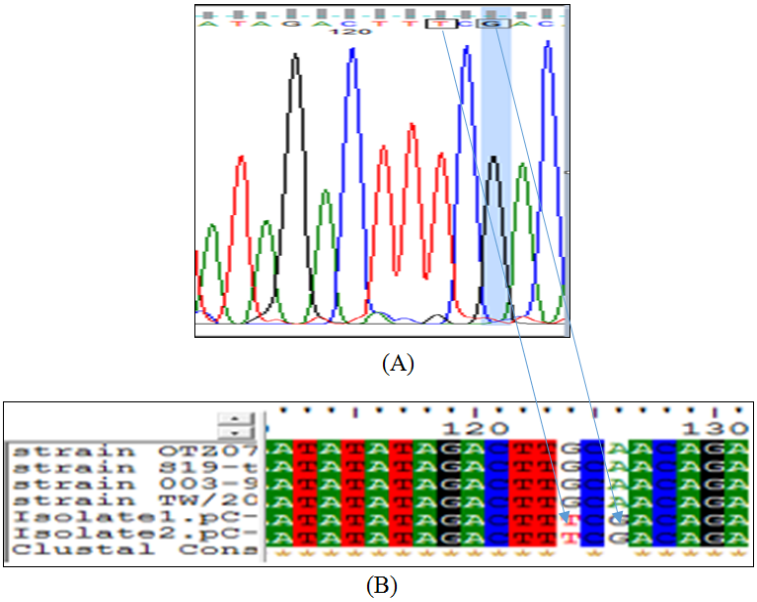

Figure 3. (A) Sequence chromatogram of the amplified segments produced with primer pC shown by Finch TV software, (B) BioEdit multiple sequence alignment shows the homology and evolutionary relation between Sudanese isolates (the amplified segments produced with primer pC) and reference strains. Differences were illustrated by arrows

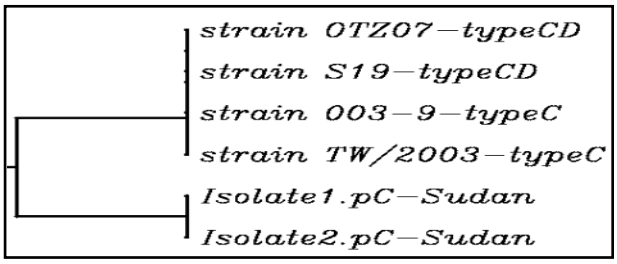

Figure 4. Dendrogram showing the genetic relationships of *C.botulinum* isolate in Sudan (the amplified segments produced with primer pC) and different controls retrieved from database. The phylogram shows that sequence set pC for the two isolates are the closest

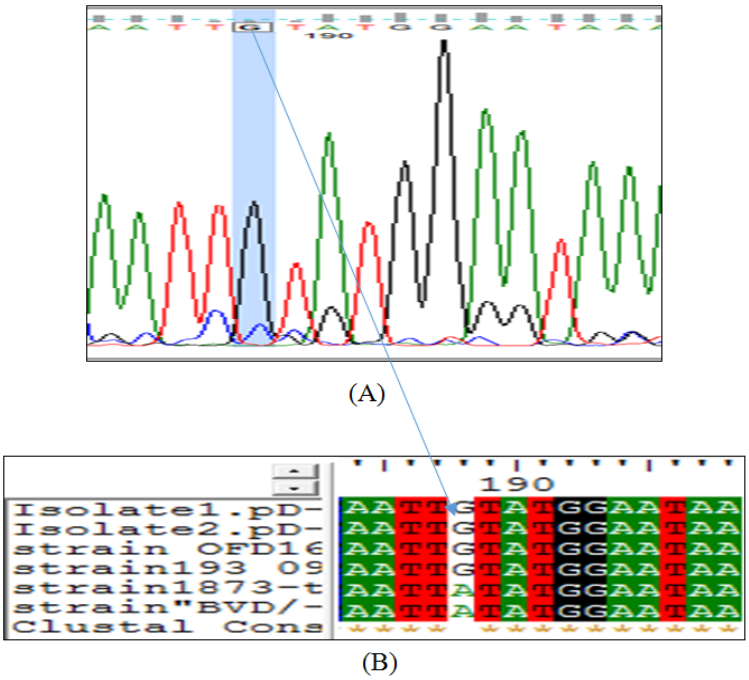

Figure 5. (A) Sequence chromatogram of the amplified segments produced with primer pD shown by Finch TV software, (B) BioEdit multiple sequence alignment shows the homology and evolutionary relation between Sudanese isolates (the amplified segments produced with primer pD) and reference strains. Differences were illustrated by arrows

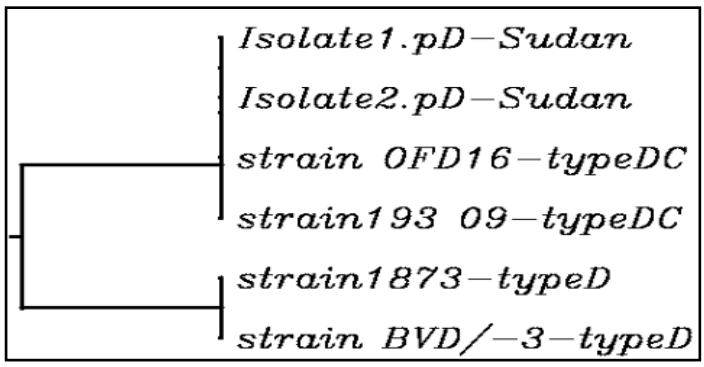

Figure 6. Dendrogram showing the genetic relationships of *C.botulinum* isolate in Sudan (the amplified segments produced with primer pD) and different controls retrieved from database. The phylogram shows that sequence set pD and BoNT/DC are the closest

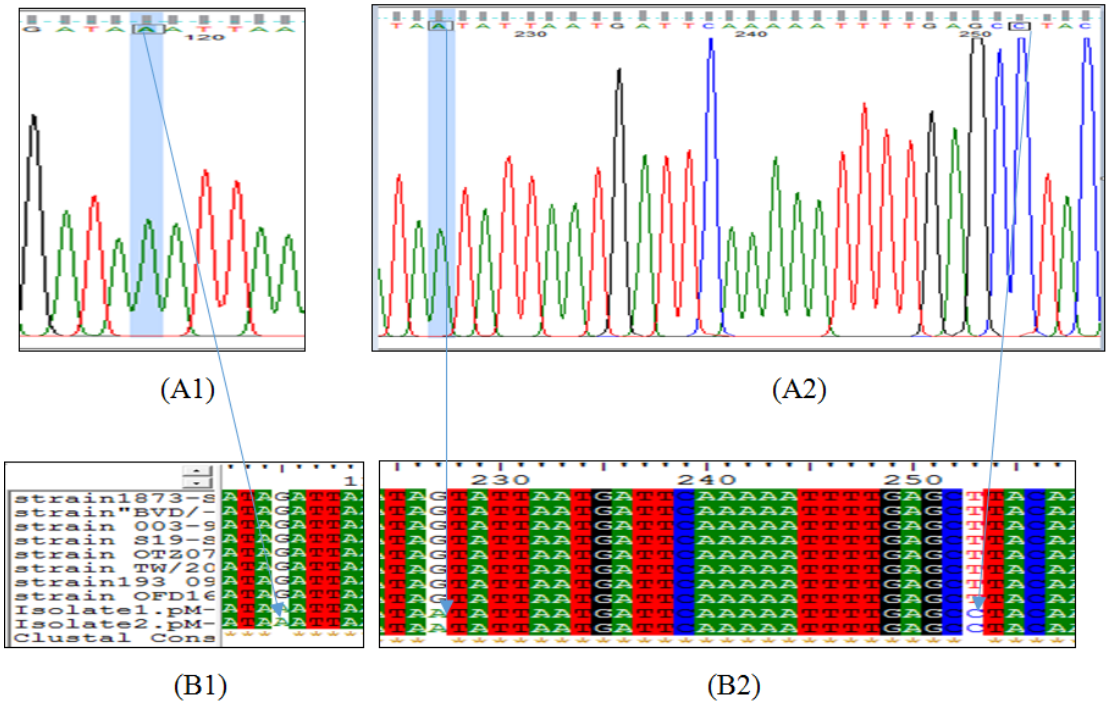

Figure 7. (A1) & (A1) Sequence chromatogram of the amplified segments produced with primer pM shown by Finch TV software, (B1)& (B1) BioEdit multiple sequence alignment shows the homology and evolutionary relation between Sudanese isolates (the amplified segments produced with primer pM) and reference strains

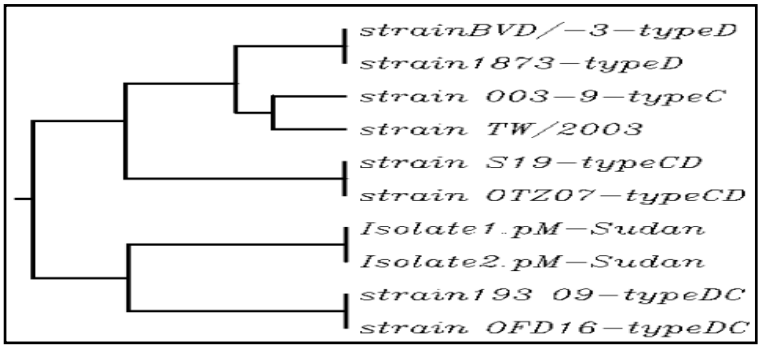

Figure 8. Dendrogram showing the genetic relationships of *C.botulinum* isolate in Sudan (the amplified segments produced with primer pM) and different controls retrieved from database. The phylogram shows that sequence set pM and BoNT/DC are the closest

Nucleotide sequence of partial pD in isolates 1, 2 showed substitution of Adenine (A) to Guanine(G) (TAT >TGT).

Figure 7(B1) showed transitions of Guanine (G) to Adenine (A) (AGA >AAA), Figure 7(B2) showed transitions of Guanine (G) to Adenine (A) (AGT>AAT) and from Thymine(T) to Cytosine(C)(CTT>CCT).

4. Discussion

Animal botulism is a worldwide problem that causes large economic losses since it affects livestock [10]. In the present study we attempt to isolate and characterize the causative agent of sheep botulism in Sudan. According to our results, best growth and more toxic isolates were

shown by CMM and RCM, which were then used for culture and isolation. Also, EYA was found better than BA, RCA and FAA. The period of 48h was found suitable for EYA and five days period was found suitable RCM and CMM. Fach et al., [5]) reported that there is no efficient selective media for isolation of *C. botulinum* available, there for sample treatment as well as culture method, depend on sample nature and conservation status [4]. Saeed [17] reported that the best growth and toxicity of suspected colonies are the two important criteria for the selection of media, so different media were used and the best broth and agar media were chosen and other cultural procedures were optimized.

Pure cultures were then submitted for confirmation by PCR as it is considered as highly sensitive method when used for detection of BoNT genes in pure cultures in contrast to the detection directly in crude samples [21].

PCR results showed that the isolates from sheep botulism exhibited three PCR-positive bands, one of which was derived from the mosaic type C/D BNTs gene at binding /translocation domain (heavy chain) and two bands from the light chain of type C and D BoNTs gene respectively, indicating that their BoNTs constitute a mosaic of parts of type C and D BoNTs.

This finding agrees with Moriishi *et al.*, 1996 [22] and Prevot *et al.*, (2007) [15] as they suggested that BoNTs produced from *C. botulinum* types C and D organisms are composed in mosaic-like fashion. Nakamura et al., 2010 [5] also reported that in type C and D, some strains possess the C-D mosaic BoNT that comprises two thirds of one type and one third of the other gene.

In subsequent analysis the BLAST results unveiled that the isolates have high identity to corresponding regions of BoNT type C, BoNT type D, BoNT type CD and DC genes respectively. We also found that the core region (pM) is common in four toxin types BoNT C (strain TW/2003, strain:003-9), BoNT D (Strain 1873, Strain-BVD/3), BoNT CD (strain S19, strain OTZ07) and BoNT DC(Strain 193_09, Strain:OFD16).

This agrees with Moura *et al.*, (2011) [23] who shows that analysis of the different toxin domains revealed that the region of highest similarity among all group III-producing BoNTs studied is located at the translocation domain.

Moreover, Webb *et al.*, (2007) [24] reported that all sequenced BoNT C or D strains have shown remarkable conservation to either the standard or mosaic toxins while amino acid alignments of BoNT C, BoNT C/D, BoNT D/C and BoNT D showed mosaic nature of the C/D or D/C toxins.

Webb *et al.*, (2007) [24] at the same study provides another indication of such cross-reactivity when bivalent vaccines of recombinant BoNT/C1 and /D heavy chains used. In that study, mice challenged with BoNT/C1, /D and mosaic/CD were completely protected, while those challenged with BoNT/DC mosaic were only partially protected.

BioEdit multiple sequence alignment revealed DNA substitutions (transversions and transitions) at different positions on the analyzed regions. Sagermann M, *et al.* (2006) [25] reveal that amino acid substitutions, deletions, and insertions are generally well tolerated in the loops of the proteins and it may have an effect on the stability and speed of folding. His results illustrate how sequence insertions can facilitate protein evolution through both local and nonlocal changes in structure. In this light, we recommend that more researches should be conducted to determine whether the differences in these sequence alignment reflect the substitutions in the functional genes which may have an effect on the structural and functional diversity of proteins.

The phylogenetic analysis showed that the Sudanese isolates were more closely related to type DC BoNT. These results suggest that neurotoxins produced from sheep botulism is BoNT type DC. Our results were agree with "Woudstra C, *et al.* (2012) [4] and Nakamura *et al.* (2010) [5]" as they reported that DC types were highly represented among the bovine isolates, whereas CD types were highly represented among isolates from several avian species.

The tree also showed that the two isolates have a closer common ancestor with each other than they do with any other strains. However, the unique structure of DC mosaic BoNT associated with sheep botulism in Sudan should contribute to the development of a vaccine and immunologic methods to devise a countermeasure for the disease [26].

5. Conclusion

It can be concluded that DC mosaic BoNT is associated with sheep botulism in Sudan. The detected gene revealed DNA substitutions at different positions on the analyzed regions. To the best of our knowledge, this is the first report on genetic characterization of animal botulism in Sudan. More researches should be conducted for further phylogenetic analysis to enable a full view on the evolving status of Botulism in Sudan and to improve the disease control efforts.

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Competing Interests

The authors declare that they have no conflict of interests.

References

[1] Busharah, I. A., and M. T. Musa (2012). Botulism in Livestock in North Darfur State. Resilience of agricultural systems against crises.

[2] Critchley, E. M. R. (1991). A comparison of human and animal botulism: a review. *Journal of the Royal Society of Medicine*, 84: 295-298.

[3] Espelund, M. and D. Klaveness, (2014). Botulism outbreaks in natural environments–an update. *Frontiers in Microbiology*, 5: 287.

[4] Fach, P., D. Hauser, J. P Guillou, and M.R. Popoff, (1993). Polymerase chain reaction for the rapid identification of Clostridium botulinum type A strains and detection in food samples. *Journal of Applied Microbiology*, 75(3): 234-239.

[5] Fach, P., S. Perelle, F. Dilasser, J. Grout, C. Dargaignaratz, L. Botella, and V. Broussolle (2002). Detection by PCR-enzyme-linked immunosorbent assay of Clostridium botulinum in fish and environmental samples from a coastal area in northern France. *Applied and environmental microbiology*, 68(12): 5870-5876.

[6] Grenda T., E. Kukier and K. Kwiatek (2014). Methods and difficulties in detection of *Clostridium botulinum* and its toxins, *Review*. *Polish Journal of Veterinary Sciences*, 17(1):195-205.

[7] Hansbauer E, M. Skiba, T. Endermann, J. Weisemann, D. Stern, M. Dorner, F. Finkenwirth, J. Wolf, W. Luginbühl, U. Messelhäußer, L. Bellanger, C. Woudstra, A. Rummel, P. Fach, and B. Dorner., (2016). "Detection, differentiation, and identification of botulinum neurotoxin serotypes C, CD, D, and DC by highly specific immunoassays and mass spectrometry. *Analyst*, 141: 5281-97.

[8] Hariharan H, and WR. Mitchell (1976). Observations on bacteriophages of Clostridium botulinum type C isolates from different sources and the role of certain phages in toxigenicity. *Applied and Environmental Microbiology*, 32: 145-158.

[9] Hoc, A. (2007). Advisory Committee on the Microbiological Safety of Food. https://acmsf.food.gov.uk/sites/default/files/acmsf-virus-report.pdf.

[10] Ibrahim, A. A. and M. T. A. Shigidi (2014). An outbreak of botulism among sheep and goats in northern localities of north Kordofan state, Sudan. *Journal of Veterinary Medicine and Animal Production*, 5: (1).

[11] Lindström M and H. Korkeala (2006). Laboratory Diagnostics Of Botulism. *Clinical Microbiology Reviews*, 19 (2): 298-314.

[12] Masuyer G, Davies JR, Moore K, Chaddock JA, Ravi Acharya K. (2015). Structural analysis of *Clostridium botulinum* neurotoxin type D as a platform for the development of targeted secretion inhibitors. *Scientific Reports*., 5:13397.

[13] Moriishi, K., M. Koura, N. Abe, N. Fujii, Y. Fujinaga, K. Inoue, and K. Ogumad (1996). Mosaic structures of neurotoxins produced from Clostridium botulinum types C and D organisms. *Biochimica et Biophysica Acta (BBA)-Gene Structure and Expression*, 1307(2): 123-126.

[14] Moura, H., R. R. Terilli, A.R. Woolfitt, M. Gallegos-Candela, L.G. McWilliams, M.I. Solano, J.L. Pirkle, and J.R. Barr, (2011), Studies on botulinum neurotoxins type /C1 and mosaic/DC using Endopep-MS and proteomics. *FEMS Immunology & Medical Microbiology*, 61: 288-300.

[15] Nakamura, K., Kohda, T., Shibata, Y., Tsukamoto, K., Arimitsu, H., Hayashi, M. and Kozaki, S. (2012). Unique biological activity of botulinum D/C mosaic neurotoxin in murine species. *Infection and immunity*, 80:2886-2893.

[16] Prevot V., F. Tweepenninckx, E. Nerom, A. Linden, J. Content and A. Kimpe (2007). Optimization of polymerase chain reaction for detection of *Clostridium botulinum* type C and D in bovine samples. *Zoonoses Public Health* 54: 320-327.

[17] Saeed, E. M. A. (2005). Studies on isolation and identification of Clostridium botulinum investigating field samples specially from equine grass sickness cases.

[18] Sagermann, M., W.A. Baase, and B.W. Matthews (2006). Sequential reorganization of β-sheet topology by insertion of a single strand. *Protein Science: A Publication of the Protein Society*, 15(5): 1085-1092.

[19] Sakaguchi Y, T. Hayashi and K. Kurokawa (2005). The genome sequence of *Clostridium botulinum* type C neurotoxin-converting phage and the molecular mechanisms of unstable lysogeny. *Proceedings of the National Academy of Sciences of the United States of America*, 102(48):17472-17477.

[20] Skarin H., T. Håfström, J. Westerberg and B. Segerman (2011). Clostridium botulinum group III: a group with dual identity shaped by plasmids, phages and mobile elements. *BMC Genomics*, 12: 185.

[21] Takeda, M., K. Tsukamoto, T. Kohda, M. Matsui, M. Mukamoto, and S. Kozaki, (2005) Characterization of the Neurotoxin Produced by Isolates Associated with Avian Botulism. *Avian Diseases*, 49: 376-381.

[22] Vidal, D., Anza, I., Taggart, M. A., Pérez-Ramírez, E., Crespo, E., Hofle, U. and Mateo, R. (2013). Environmental factors influencing the prevalence of a Clostridium botulinum type C/D mosaic strain in nonpermanent Mediterranean wetlands. *Applied and environmental microbiology*, 79: 4264-4271.

[23] Wangroongsarb P., T. Kohda, C. Jittaprasartsin, K. Suthivarakom, T. Kamthalang , K. Umeda, P. Sawanpanyalert, S. Kozaki and K. Ikuta (2014). Molecular Characterization of *Clostridium botulinum* Isolates from Foodborne Outbreaks in Thailand, 2010. *PLoS ONE.*, 9(1): e77792.

[24] Webb RP, TJ. Smith, PM. Wright, VA. Montgomery, MM. Meagher, and LA. Smith (2007) Protection with recombinant Clostridium botulinum C1 and D binding domain subunit (Hc) vaccines against C and D neurotoxins. *Vaccine*, 25: 4273-4282.

[25] Wictome, M., and C.C. Shone, (1998). Botulinum neurotoxins: mode of action and detection. *Journal of Applied Microbiology*, 84: 87S-97S.

[26] Woudstra, C., Skarin, H., Anniballi, F., Fenicia, L., Bano, L., Drigo, I., Koene, M., Bäyon-Auboyer, M.H., Buffereau, J.P., De Medici, D. and Fach, P. (2012). Neurotoxin gene profiling of Clostridium botulinum types C and D gathered from different countries within Europe. *Applied and environmental microbiology*, 78(9): 3120-3127.