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RESEARCH ARTICLE

Detection of Botulinum Neurotoxin Serotypes C and D, and Their Effects on Expressions of SNAP-25 and Synaptobrevin in Ruminants: An Immunohistochemical Study [1,2]

Mehmet Burak ATEŞ [1,a (*)] Funda TERZİ [2,b] Mustafa Kemal ÇİFTÇİ [1,c]
Mustafa ORTATATLI [1,d] Zeynep ÇELİK [1,e]

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[1] Selçuk University, Faculty of Veterinary Medicine, Department of Pathology, TR-42130 Konya - TURKEY
[2] Kastamonu University, Faculty of Veterinary Medicine, Department of Pathology, TR-37200, Kastamonu - TURKEY
ORCIDs: [a] 0000-0003-1297-426X; [b] 0000-0002-6184-5408; [c] 0000-0003-4476-2114; [d] 0000-0002-3713-813X; [e] 0000-0002-9667-5728

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Abstract

In humans and animals, botulism is a disease characterized by generalized and progressive paralysis caused by *Clostridium botulinum* neurotoxins (BoNT). BoNTs, defined in seven different antigenic types (A to G), proteolyze SNAREs (synaptosomal-associated protein/SNAP-25 and synaptobrevin) responsible for acetylcholine release in peripheral cholinergic neurons, and thus cause flaccid paralysis and death. Currently, mouse experiments are considered the reference method for definitive diagnosis. However, new diagnostic methods that are fast and accurate and would not raise ethical issues need to be developed. Therefore, using antibodies specific to the toxoid forms of BoNTs, the presence of BoNT-C and/or BoNT-D was investigated by immunohistochemical method (IHC) in the study. The tissues of thirty ruminants (twenty cattle, seven sheep, three goats), which had the clinical and pathological findings of botulism and a herd history of the disease, were used as material. BoNTs were detected with IHC in sixteen of the thirty ruminants as three BoNT-C, eleven BoNT-D, and two BoNT C+D. In the mouse experiments, BoNT was isolated in only three cases (two BoNT-D, one BoNT-C). Additionally, being responsible for the clinical findings of botulism, the interaction of BoNTs with SNAP-25 and synaptobrevin was investigated using IHC. It was determined that BoNT-C specifically reduces the expression of SNAP-25, and BoNT-D reduces the expression of synaptobrevin and partially SNAP-25. It was concluded that additional studies may be valuable to investigate the use of IHC in the diagnosis of botulism.

Keywords: *BoNT-C, BoNT-D, SNAP-25, Synaptobrevin, Immunohistochemistry*

Ruminantlarda Botulinum Nörotoksin Serotip C ve D'nin Saptanması ve Bunların SNAP-25 ve Sinaptobrevin Ekspresyonları Üzerindeki Etkileri: İmmunohistokimyasal Bir Çalışma

Öz

Botulizm insan ve hayvanlarda *Clostridium botulinum* nörotoksinlerinin (BoNT) neden olduğu, generalize ve ilerleyici paraliz ile karakterize bir hastalıktır. Yedi farklı antijenik tipi (A dan G'ye) tanımlanan BoNT periferik kolinerjik nöronlardaki asetilkolin salınımından sorumlu olan SNARE proteinlerini (SNAP-25 ve sinaptobrevin) proteolize ederek flasid paralize ve ölüme neden olur. Günümüzde fare deneyleri hastalığın kesin teşhisi için referans yöntem olarak kabul edilmektedir. Ancak, hızlı, güvenilir ve etik problemlere yol açmayan yeni teşhis metotlarının geliştirilmesi gerekmektedir. Yapılan çalışma ile botulismus sürü öyküsü, klinik ve patolojik bulguları bulunan 30 adet ruminanta (20 adet sığır, 7 adet koyun ve 3 adet keçi) ait çeşitli dokular BoNT-C ve BoNT-D yönünden immunohistokimyasal (IHC) yöntemle BoNT'un toksoid formuna spesifik antikorlar kullanılarak incelenmiştir. Bu kapsamda 30 ruminantın 16 (3 BoNT-C, 11 BoNT-D ve 2 BoNT C+D)'sında IHC metodu ile BoNT tespit edildi. Fare deneylerinde ise yalnızca 3 vakada BoNT (2; BoNT-D, 1; BoNT-C) izole edildi. Ayrıca, botulizm klinik bulgularından sorumlu olan SNAP-25 ve sinaptobrevinin BoNT ile olan etkileşimi IHC ile araştırılmış ve BoNT-C'nin ruminantlarda spesifik olarak SNAP-25'i, BoNT-D'nin ise spesifik olarak sinaptobrevini kısmi olarak da SNAP-25'i proteolize ettiği saptanmıştır. Botulizm tanısında IHC kullanımını araştırmak için ek çalışmaların yapılmasının değerli olabileceği sonucuna varıldı.

Anahtar sözcükler: *BoNT-C, BoNT-D, SNAP-25, Sinaptobrevin, Immunohistokimya*

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[(*)] **Corresponding Author**

Tel: +90 332 223 2734 Fax: +90 332 241 0063
E-mail: mehmetburakates@selcuk.edu.tr (M.B. Ateş)

Introduction

Botulism is a neuroparalytic disease characterized by muscle laxity and generalized and progressive paralysis arising from the specific effects of the botulinum neurotoxins (BoNT) produced by *Clostridium botulinum (C. botulinum*), which occurs in most mammals, birds, and fish [1,2]. Apart from *C. botulinum*, some BoNT isoforms have been reported to be produced by *C. baratii*, *C. butyricum* and *C. argentinense* [3-5]. Although botulism is seen sporadically throughout the world, it often occurs in herds as outbreaks and causes significant economic losses, especially in farm animals, because the disease has a high mortality rate [1,6]. *C. botulinum* is a gram-positive, anaerobic bacterium with a spore form. Seven different antigenic types of neurotoxins, from A to G, belonging to *C. botulinum* have been described [7]. Then, a new type of BoNT (BoNT-H), responsible for infant botulism, was defined as the eighth isoform [3,8]. BoNT-A, B, E, and F primarily induced botulism in humans, while BoNT-C and D were only harmful to animals [4]. Mosaic recombinant toxins (both type C and D weak chains) defined as BoNT-C+D and BoNT-D+C can also be seen in cases of botulism in ruminants [4].

Notwithstanding the differences in the amino acid sequence and immunological variations, all BoNTs are synthesized in their inactive form and activated later by tissue proteases [2]. BoNTs, absorbed from the intestines, are attached to the presynaptic cholinergic nerve terminals and especially the somatic neuromuscular nerve endings, after arriving via the hematogenous route. The tissue proteases in these areas activate the toxin by breaking down the ligaments of the light (LC) and heavy (HC) chains [9]. The HC bind to target cells, helping LC to enter the cell cytoplasm [10]. LC is released into the cytosol, where it inactivates the soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complex and thence prevents the clamping and fusion of synaptic vesicles [10,11]. Therefore, neurotransmitter release from vesicles containing acetyl-choline in the neuromuscular junction is prevented [7]. SNARE proteins include vesicle-associated membrane proteins (vamp/synaptobrevin), plasma proteins (synaptosomal-associated protein/SNAP-25), and syntaxin [12]. It has been reported that BoNT-C proteolyzes both SNAP-25 and syntaxin, BoNT-A and E only break down SNAP-25, and BoNT-B, D, F, and G proteolyze synaptobrevin and thereby inhibit acetylcholine synthesis [5]. As a result of this inhibition, clinical findings are observed in animals and death occur due to subsequent paralysis of the diaphragm muscles [13,14].

Botulism is an important intoxication that affects both humans and animals on a global scale and is particularly difficult to diagnose owing to difficulties in toxin isolation. The most important problems today in overlooking the botulism cases are the mouse experiments, which are not sensitive enough, and the inadequate interpretation of clinical and necropsy findings in field conditions. Besides, the selection of the sample to be sent to the laboratory, the lack of knowledge of the transfer conditions of the samples, the shortage of equipped laboratories, and the scarcity of rapid and reliable methods for diagnosis are other important factors [15,16]. However, a rapid decision-making mechanism for herd and animal health should be operated without wasting time on such problems. Currently, the mouse inoculation test is still used as a reference method to determine the presence of toxins necessary for the definitive diagnosis of botulism [15-18]. However, this test is not sensitive enough and useful because of having some disadvantages such as the scarcity of accredited laboratories where mouse experiments are performed, the amount of time needed for the tests, and ethical problems related to working with living organisms [15,18-20]. A study showed that cattle are 12.88 times more susceptible to BoNT-C compared to mice relative to their weight [21]. Another limiting factor in ruminant animals is the selection of the sample to be used for testing is uncertain. These situations cause a serious time lag in the definitive diagnosis of the disease, which sometimes may remain hidden because of the inability to determine the toxin. Therefore, in recent reports, some notifications have been made that immunological tests such as enzyme-linked immunosorbent assay (ELISA) and immuno-PCR for the diagnosis of the disease may be an alternative to mouse experiments [22]. After a detailed review of the literature, no studies have been identified in which BoNT toxins were investigated by immunohistochemical (IHC) method based on a similar immunological basis to these tests.

For all these reasons, firstly we aimed to determine the presence and the distribution of BoNT-C and D using the IHC method for the first time in the tissues of thirty ruminants (twenty cattle, seven sheep, and three goats) had clinical findings and a herd history of botulism. In addition, we secondly intended to investigate the interactions of these neurotoxins with SNAP-25 and synaptobrevin receptors, which are responsible for the development of clinical findings, in the ruminant neuropil tissue. In light of all these assessments, we finally aimed to analyse every detail of the disease from anamnesis information, environmental, feeding and sheltering observations to clinical, necropsy, and microscopic findings to make a preliminary diagnosis of the disease and take necessary protection and control measures immediately.

Material and Methods

Animals and Ethic Statements

The animal material of the study consisted of thirty ruminants (twenty cattle, seven sheep, and three goats), which were brought to the Faculty of Veterinary Medicine of Selçuk University, showing clinical botulism symptoms, and having a herd history of the disease. Each of these animals

represented a separate herd. While some animals were brought dead, others were sent to our laboratory for necropsy with the consent of their owners after necessary examinations in faculty clinics.

It was decided that the study was suitable in terms of research ethics by the Selçuk University Veterinary Faculty Experimental Animal Production and Research Center Ethics Committee (No: 2016/117).

Mouse Experiments

The liver, intestine, kidney, spleen, lung, heart, and brain (cerebrum, cerebellum, and brain stem) samples and rumen contents taken from the animals were directed to the local Veterinary Control and Research Institute authorized by the Ministry of Agriculture and Forestry. But here, mouse inoculation and toxin neutralization experiments were performed from only rumen contents according to the routine official protocol [23]. The rumen content is suspended with physiological saline, centrifuged at 3000 rpm for 15 min, passed through the filter (45 nm) and then administered intraperitoneally (0.5 mL) to the mice. The mice were monitored for 72-120 h in terms of botulism symptoms (respiratory paralysis, hump breathing, death). After the presence of BoNT was confirmed, typing was performed using specific BoNT antitoxins [23].

Histopathological Method

In the systemic necropsy of animals, samples were taken from liver, kidney, spleen, lung, heart, brain, and intestines and fixed in 10% neutral buffered formalin solution. After routine histopathological tissue processing, tissues were embedded in paraffin. Five-µm-thick sections were taken from the paraffin blocks with microtome and were stained with hematoxylin-eosin (HE) before being examined under a binocular light microscope (Olympus BX51, Tokyo, Japan). Photographs were also taken from typical lesions identified in microscopic examinations (Olympus DP12, Tokyo, Japan).

Immunohistochemical Method

For immunohistochemical staining, 5-µm-thick sections from target organs were stained in the immunohistochemistry staining device (Leica, Bondmax) according to the Bond™ polymer refine detection (Leica DS9800) kit procedure. First, all tissues were deparaffinized with heat and dewax solution (Bond™, Leica AR9222) and then rehydrated in graded alcohols (Merck). The sections were washed at least three times with washing solution (Bond™, Leica, AR9590) and deionized water after each step. Then, according to the primary antibody feature used, these sections were applied heat-induced epitope retrieval (epitope 1 antigen retrieval solution, Bond ™, Leica, AR9961, citrate buffer pH: 6.0, 100°C, 20 min). To remove peroxidase activity and prevent nonspecific binding, peroxidase block and protein block were applied at different times. After the reaction with each primary antibody (anti-*C. botulinum* C toxoid antibody [1:400, Abcam, ab27165], anti-*C. botulinum* D toxoid antibody [1:100, Abcam, ab64402], anti-SNAP25 antibody [1:400, Mybiosource, MBS395111], anti-synaptobrevin antibody [1:400, Mybiosource, MBS500033]) at room temperature, post-primary and polymer applications were performed. Afterward, all sections were incubated with 3,3′-Diaminobenzidine (DAB) for 5 min at room temperature. After the sections were washed with distilled water, contrast staining was performed with Mayer's hematoxylin. Sections were examined by binocular light microscope (Olympus BX51, Tokyo, Japan) and photographed (Olympus DP12, Tokyo, Japan).

IHC results for BoNT-C and BoNT-D were evaluated as positive or negative. IHC scoring methods for SNAP-25 and synaptobrevin were adapted from a previous study [12]. Accordingly, AnalySIS LS starter software (Soft Imaging System GmbH, Olympus, Germany) was used to measure and compare the dye intensity from images obtained from IHC stained brain sections. The areas from each section were selected and a grey scale was obtained in which absolute numbers of minimum and maximum staining density were determined for the regions of interest. SNAP-25 staining intensity was found as maximum 70.20 and minimum 171.05. For synaptobrevin 67.20-169.70 (maximum-minimum density values) were determined. Density values for each marker (SNAP-25 and synaptobrevin) were divided into four categories equally. Then, each case was scored as negative (0, no staining); mild (+1), (SNAP-25; 145.85-171.05, synaptobrevin; 144.09-169,70); moderate (+2), (SNAP-25; 120.63-145.84, synaptobrevin; 118.46-144.08); severe (+3), (SNAP-25; 95.42-120.62, synaptobrevin; 92.83-118.45); or very severe (+4), (SNAP-25; 70.20-95.41, synaptobrevin; 67.20-92.82).

In this study, animals of the same species (four cattle, one sheep and one goat) without any neural, toxic, or paralysis symptoms were used as the control group. We determined that there were no macroscopic or microscopic signs of toxicity in these animals. The organs of the cases that were found to be BoNT positive according to the mouse inoculation test results (1; BoNT-D, 1; BoNT-C) were used as positive control for IHC. Furthermore, the cases with suspected botulism in the study but found negative according to the mouse test and IHC staining results (14 of 30 ruminants) were evaluated as negative groups for SNAP-25 and synaptobrevin analyses.

Statistical Analyses

Statistical analysis of IHC findings was performed with the Statistical Package for the Social Sciences (SPSS for Windows® version 25.0) program. Distribution analyses were performed to check the variation of the data obtained using the Kolmogorov-Smirnov test. In addition, the homogeneity of variances was checked by Levene test. Normally distributed, these data were evaluated with one-way

ANOVA test, and then post-hoc Duncan analysis was performed to determine the differences between the groups. Results were given as mean±standard error (X±Sx), and $P<0.05$ was considered significant. In addition, the correlation among these data was examined by Pearson test (Minitab® version 18.1).

Results

Anamnesis, Clinical and Macroscopic Findings

In the history of suspected botulism cases, each of which represents a separate herd, it was recorded that the owners encountered snake, tortoise, or poultry carcasses in bait bales such as straw, clover, silage, and sugar beet pulp. Common clinical findings observed in preliminary reports by local veterinarians and animal caregivers are as follows: no fever, gait disturbances, tremors, paralysis in the hind legs, sternal lying position, head resting on the shoulder, anorexia, dehydration, hypersalivation, pouring of unswallowed feed and liquids. It was stated that in sheep and goats, paralysis and swallowing difficulties were less pronounced or death occurred without any symptoms. The widely observed macroscopic findings after necropsy were: accumulation of fluid in the chest and to a lesser extent in the abdominal cavity; foam in the trachea, pale lungs, and fluid leakage in the airways; endo-epicardial petechiae; multifocal hemorrhage and congestion in the liver; dilatation of the rumen, especially induration of omasum content; coffee grounds-like content in the abomasum; catarrhal enteritis or constipation; presence of skull imprint in the hemispheres; severe hyperaemia, edema, thickened and/or opaque appearance in meninges; and pushing of the cerebellum toward the occipital foramen *(Fig. 1)*.

Mouse Experiments Results

According to the results of mouse experiments performed from rumen contents in the authorized local laboratory, the presences of BoNT-D and BoNT-C in two and one cattle respectively were reported. In other cases, existence of toxin could not be detected.

Histopathological Findings

The main histopathological findings observed in visceral organs were pulmonary edema and emphysema, hydropic degeneration and focal steatosis in hepatocytes, hydropic degeneration in kidney tubules epithelium, and catarrhal enteritis. Meningeal changes consisted primarily of hyperemia, perivascular hemorrhage and edema in the cerebrum and cerebellum. Pronounced hyperemia, edematous dilatation in the perivascular space and hemorrhages were determined in the neuropil tissue of cerebrum, cerebellum, and medulla oblongata. Embolism or thrombosis was not detected. In the grey matter of cerebrum, cerebellum and brain stem, degeneration, chromatolysis and necrosis in neurons,

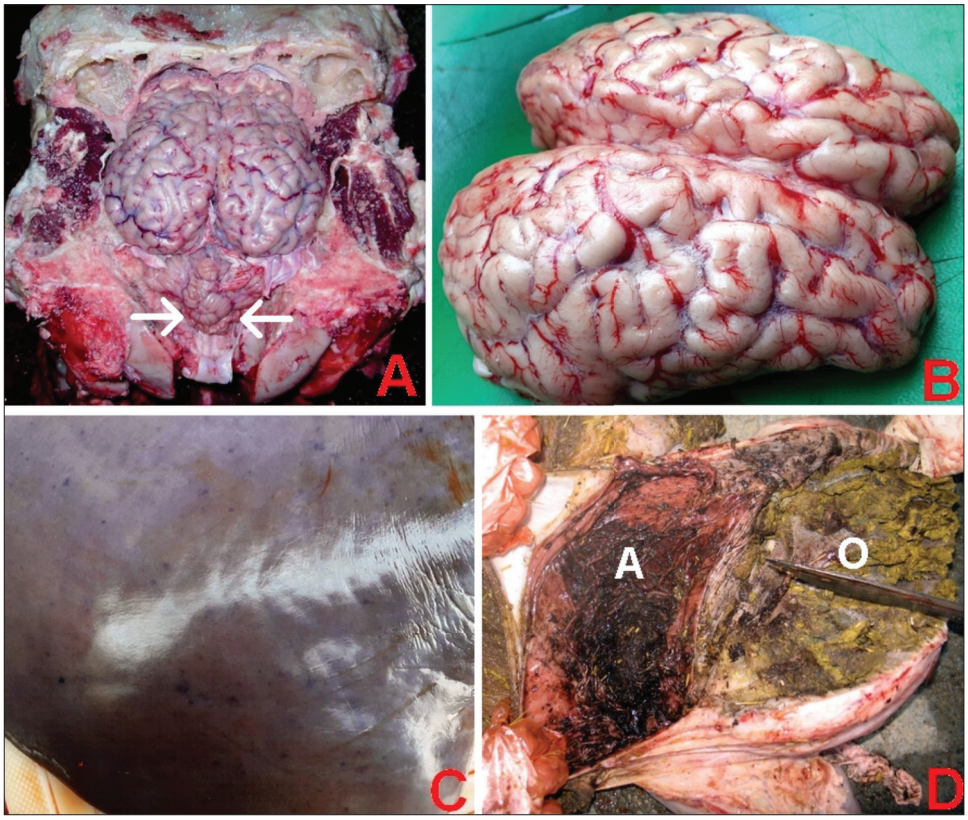

Fig 1. **(A)** Thickened and opaque meninges and hyperaemia in the brain and compression of the cerebellum to the occipital foramen *(arrows),* **(B)** Oedema and hyperaemia in meninges, **(C)** Multifocal hemorrhages in liver, **(D)** Induration and dryness of the omasum content **(O)**, a coffee grounds-like content in the abomasum **(A)**

perineuronal edema and gliosis were observed. Irregularly vacuoles and rarely demyelination were seen in the white matter of the whole central nervous system *(Fig. 2)*. To a lesser extent, hyperemia in the abomasum mucosa and depletion in lymphoid foci in the spleen were determined. The distribution of histopathological findings observed according to the BoNT type determined is presented in *Table 1* in detail. In addition, in the histopathological examination of two animals (one sheep, one goat), a micro-abscess and perivascular cuffing were found in the brain stem, and it was determined that these cases were listeriosis as a result of IHC staining.

Immunohistochemical Findings

The presence of BoNT type C and type D, and the density of SNAP-25 and synaptobrevin, were determined immunohistochemically in the various tissues of research animals. No immunoreactivity was found in animals used as negative control in the study. The positive reaction was obtained in two cases whose mouse test result was found to be positive and was used as positive control in the study. BoNT-C was detected in two of twenty cattle and one of seven sheep. BoNT-C immunoreactivity was not observed in goats. BoNT-D was determined in seven cases of cattle, two of sheep, and two of goats. BoNT-C+D immunoreactivity was observed in two cases of cattle. In summary, BoNT immunopositivity was achieved in a total of sixteen of thirty ruminants *(Table 2)*. Immunoreactivity was found in the cytoplasm and sometimes in the nucleus of the parenchymal cells *(Fig. 3)*. When the distribution of neurotoxins by organs was examined, no positive staining in the heart tissue was observed in any of the animal species. The detailed distribution of BoNT by organs and animal species is presented in *Table 3*.

The IHC scores for SNAP-25 and synaptobrevin are summarized in *Table 4*. SNAP-25 immunoreactivity in BoNT-C- and BoNT-C+D-positive cases were found to be significantly reduced compared to the control group, toxin-undetectable cases (negative animals), and BoNT-D-positive cases ($P<0.05$). In BoNT-D-positive cases, this effect

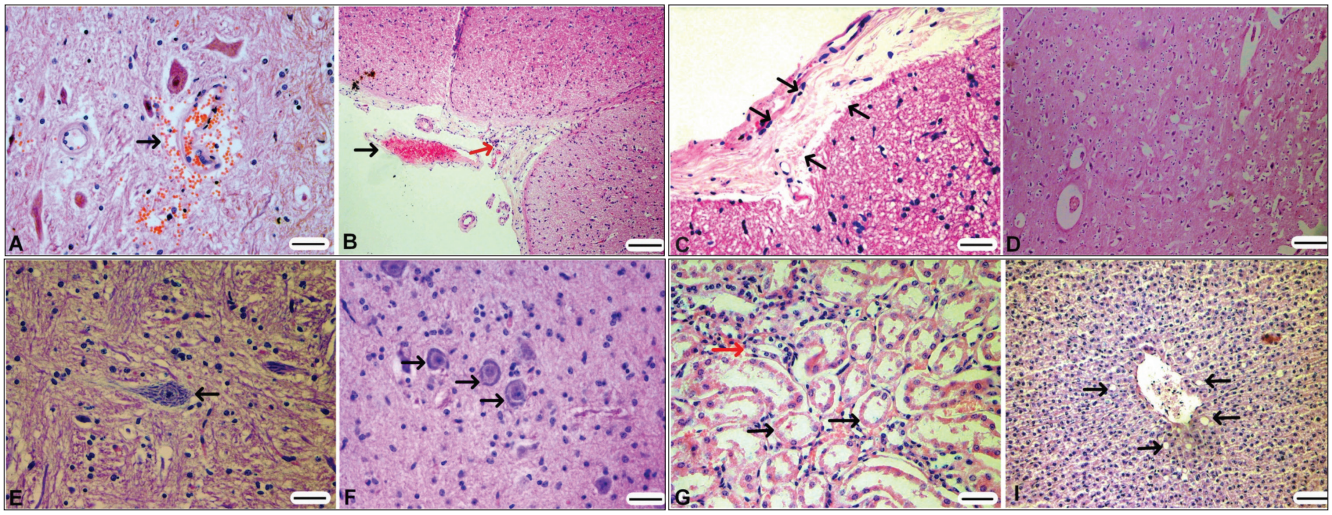

Fig 2. Botulism microscopic images (A) Bleeding in neuropil tissue (arrow), (B) Hyperemia (black arrow) and edema (red arrow) in meninges, (C) Edema and thickening in meninges (arrows), (D) Perineuronal oedema and gliosis, (E) Central chromatolysis in neurons (arrow), (F) Degeneration in neurons (arrows), (G) Degeneration (red arrow) and necrosis (black arrows) in kidney tubular epithelium, (I) Steatosis (arrows) in hepatocytes. HE, Scale bars: 50 µm (A, C, E, F, G), 100 µm (B, I) and 200 µm (D)

Table 1. *Histopathological findings according to the type of toxin*

Histopathological Findings	Toxin Types			
	Negative (n:14)	BoNT-C (n:3)	BoNT-D (n:11)	BoNT C+D (n:2)
Lung oedema and emphysema	5/14	3/3	9/11	2/2
Hydropic degeneration/fatty changes in hepatosit	4/14	2/3	8/11	1/2
Hydropic degeneration in kidney tubules epithelium	3/14	2/3	6/11	2/2
Catarrhal enteritis	4/14	2/3	7/11	1/2
Degeneration/necrosis and gliosis in neurons	6/14	3/3	8/11	2/2
Hyperaemia in brain vessels	6/14	3/3	8/11	2/2
Oedema in neuropil tissue	5/14	2/3	7/11	2/2
Haemorrhage in neuropil tissue	-/14	2/3	5/11	1/2

Table 2. *Distribution of BoNTs detected by IHC method and mouse test according to animal species*

Animal Species	IHC				Mouse Test	
	BoNT-C	BoNT-D	BoNT-C+D	Total	BoNT-D	BoNT-C
Cattle (n:20)	2	7	2	11 (55 %)	2	1
Sheep (n:7)	1	2	0	3 (42.85 %)	0	0
Goat (n:3)	0	2	0	2 (66.66 %)	0	0
Total	3	11	2	16 (53.33 %)	2	1

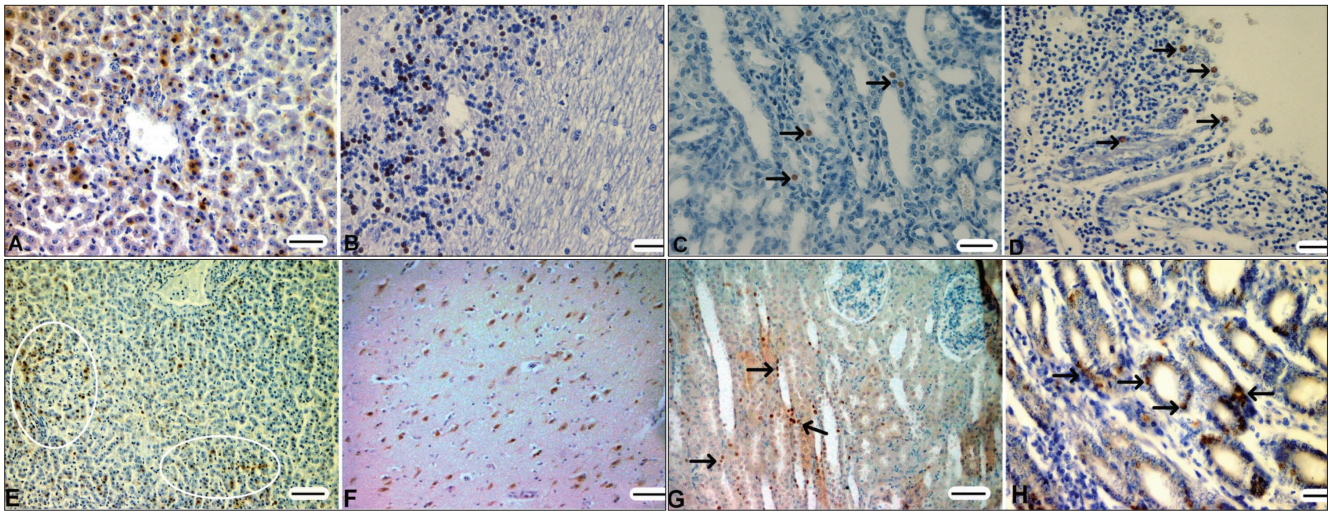

Fig 3. BoNT-C **(A-D)** and BoNT-D **(E-H)** immunoreactivity. Immunoreactivities in hepatocytes **(A)**, stratum granulosum of cerebellum **(B)**, tubular epithelial cells *(arrows)* **(C)**, intestinal epithelial cells *(arrows)* **(D)**, hepatocytes *(rings)* **(E)**, neurons in cerebrum **(F)**, tubular epithelial cells *(arrows)* **(G)**, intestinal gland epithelial cells *(arrows)* **(H)**. IHC staining, DAB chromogen, Mayer haematoxylin, Scale bars: 50 µm (A-D, H) and 100 µm (E-G)

Table 3. *Distribution of BoNT according to organs and animal species*

Tissue/Toxin Type	Cattle		Sheep		Goat		Total	
	BoNT-C	BoNT-D	BoNT-C	BoNT-D	BoNT- C	BoNT-D	BoNT- C	BoNT-D
Liver	1	3		1		2	1	6
Intestines	1	5	1				2	5
Brain	1	2	1	2		1	2	5
Kidney	2	1				1	2	2
Spleen						1		1

Table 4. *IHC staining scores of SNAP-25 and synaptobrevin by BoNT types**

SNARE Protein Type	Toxin Type				
	Control	BoNT-C (+)	BoNT-D (+)	BoNT-C+D (+)	Neg (-)
SNAP-25	3.75±0.25^{a}	1.33±0.33^{c}	2.63±0.24^{b}	1.50±0.50^{c}	3.35±0.17^{ab}
Synaptobrevin	3.50±0.28^{a}	3.33±0.33^{a}	1.18±0.12^{b}	1.00±0.00^{b}	3.28±0.24^{a}

$^{a-c}$ Values in columns with no common superscripts are significantly different according to the post-hoc Duncan test after the one-way ANOVA (P<0.01).
** The numbers represent the average values of IHC staining intensity scores for SNAP-25 and synaptobrevin according to BoNT types. Scores: Negative (0), mild (+1), moderate (+2), severe (+3), very severe (+4)*

was more partial. The immunoreactivity of synaptobrevin was found to be significantly reduced in BoNT-D- and BoNT-C+D-positive cases compared to other groups (P<0.05). There was no statistically significant difference in BoNT-C-positive cases (P>0.05). In addition, a moderately negative correlation (r:-0.50, P<0.005) between BoNT-C and SNAP-25 and a stronger negative correlation (r:-0.75, P<0.000) between BoNT-D and synaptobrevin were

Table 5. *Correlation relationship between BoNT types and SNAP-25 and synaptobrevin**

SNARE Protein Type	Toxin Type			
	BoNT-C (+)	**BoNT-D (+)**	**BoNT-C+D (+)**	**Neg (-)**
SNAP-25	r:-0.50 **P<0.005**	r:-0.104 P<0.585	r:-0.354 P<0.055	r:+0.578 **P< 0.001**
Synaptobrevin	r:+0.269 P<0.150	r:-0.75 **P<0.000**	r:-0.305 P<0.101	r:+0.718 **P<0.000**

** Correlation value (r) was calculated by Pearson test*

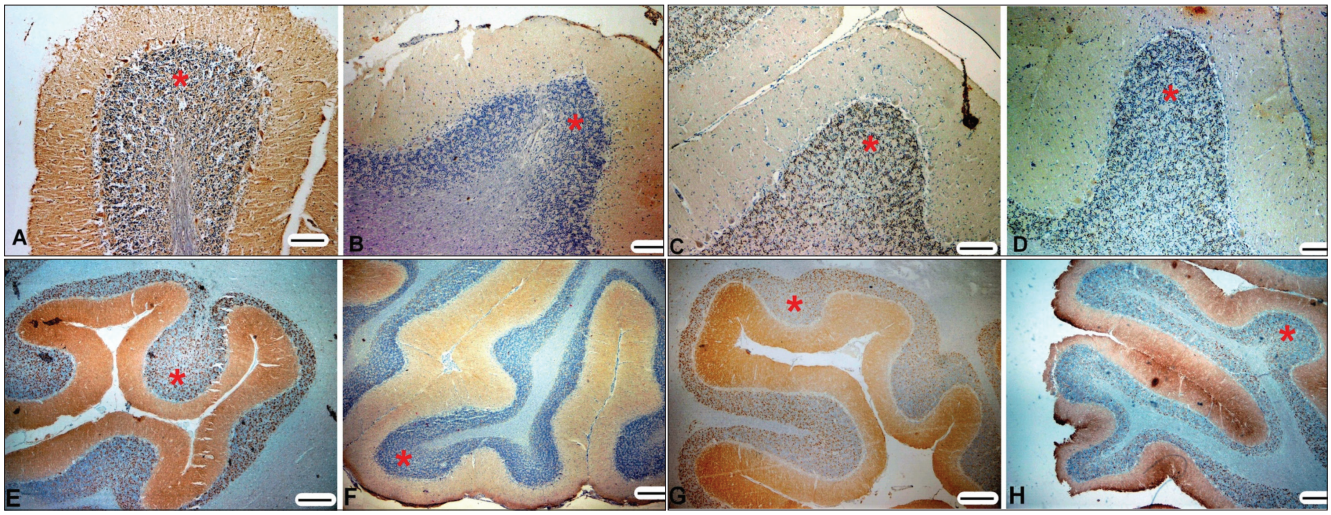

Fig 4. SNAP-25 **(A-D)** and synaptobrevin **(E-H)** immunoreactivity (Imr). **(A)** Very severe Imr in molecular, granular *(star)* and ganglionic layers, cerebellum, control group, **(B)** Mild Imr in granular layer *(star)*, cerebellum, BoNT-C positive group, **(C)** Severe Imr in granular layer (star), cerebellum, BoNT-D positive group, **(D)** Moderate Imr in granular layer *(star)*, cerebellum, BoNT-C+D positive group, **(E)** Very severe Imr in molecular, granular, and ganglionic layers, cerebellum, control group, **(F)** Mild Imr in granular layer, cerebellum, BoNT-D positive group, **(G)** Severe Imr in granular layer, cerebellum, BoNT-C positive group, **(H)** Moderate Imr in granular layer, cerebellum, BoNT-C+D positive group. IHC staining, DAB chromogen, Mayer haematoxylin, Scale bars: 500 µm (A-D), 200 µm (E-H)

observed, both of which were statistically significant. A positive correlation was found between negative cases and both SNAP-25 and synaptobrevin *(Table 5)*. Although SNAP-25 immunoreactivity was observed in neuropil tissue of the cerebrum, it was not found in neurons, glia cells, and endothelial cells. In the substantia grisea of cerebellum, intense immunoreactivity was observed in the granulosa layer and mild immunoreactivity in Purkinje cells. Substantia alba was not stained positive *(Fig. 4, A-D)*. Synaptobrevin IHC staining pattern in the cerebrum and cerebellum was detected to be exactly like the SNAP-25 *(Fig. 4, E-H)*.

Discussion

It has been reported that the most significant cause of widespread botulism outbreaks in farm animals is feed poisoning [24]. *C. botulinum* agents proliferate rapidly in deceased animal carcasses or rotted organic substances (such as improperly stored silage) and secrete neurotoxins [25]. Ingestion of feed and water contaminated with neurotoxins and, in recent years, large silage bales produced without acidification in large plastic airtight bags and the use of tight round bales of straw have been associated with increased cases of botulism in horses and cattle [25]. In the anamnesis of cases with suspected botulism that were brought to our laboratory and constitute the material of the current study, animal owners often stated that some dead rodents or other animals were found in the bales. That said, it is common for cadavers of deceased animals to be thrown into the pasture or around places where animals are housed. However, these cadaveric pieces, especially when eaten by animals that tend to eat foreign matter, are one of the important factors causing the disease. In addition, based on the technological development of agricultural machinery, the reaping of baby birds, snakes, lizards, turtles, and mice by harvesting machines during mowing while closest to the ground, and their mixing into the bales, may be another important reason for the disease. Therefore, it is understood that a good anamnesis knowledge and careful analysis of the shelter and the environment are highly important in the detection of the disease.

The clinical findings in the study are consistent with those observed in cases or outbreaks of botulism previously reported in the literature [9,13,18,20]. Although these clinical findings observed in animals vary according to the course

of the disease, they are not pathognomonic [4]. However, the most characteristic finding we observed was partial or complete paralysis in locomotor muscles. This paralysis usually starts from the back of the body and spreads forward. This condition, which occurs as a result of the inhibition in the release of acetylcholine, progresses rapidly and causes the animal to have difficulty in standing up and eating [18]. In addition, it was stated that most of the animals in our study died without any clinical signs.

The general judgment in botulism is that necropsy findings are not specific to the disease. However, systematic, and careful necropsy can provide important clues to indicate the disease. In this study, we evaluated that dilatation in the anterior stomachs, especially induration of the omasum and the dryness of its content, was caused by paralysis of the synaptic and presynaptic nerves [26]. Although these findings observed in the forestomach are also seen in some febrile diseases, they are thought to be highly important in cases where there is no fever but paralysis and toxicity symptoms are present. Despite this stagnation in the front stomachs, it is also remarkable to find watery content in the intestines. It has been interpreted that the findings such as fluid retention in the body cavities, pulmonary edema, bleeding in the visceral organs, developed because of damage caused by the toxin in the vascular endothelium. Similarly, compression of the cerebellum into the occipital foramen, the presence of skull imprints in the hemispheres, and thickened and/or opaque meninges may also be due to brain edema raised by vascular damage. Although macroscopic findings have occasionally been included in the literature [21,24], it is understood that they have not been analyzed in detail, the authors claiming that they are not specific to the disease. However, in the diagnosis of botulism, which is highly difficult to diagnose even in the most equipped laboratory conditions, the importance of evaluating and analyzing all kinds of information, especially necropsy findings, is undisputed, particularly in field conditions where resources are extremely limited.

In the study, important histopathological findings were observed in the central nervous system. Gliosis, ischemic neuronal changes, neuronal necrosis, and chromatolysis were seen. In addition, perivascular and perineural edema, hyperemia, and bleeding, which are the causes of macroscopically hyperemic and opaque appearance of meninges, were common *(Fig. 2)*. Similar degenerative vascular findings and cell degenerations were found in visceral organs such as the liver and kidney. This strongly suggests that most of these lesions might consist of circulatory disorders caused by toxin damage to the vascular endothelium. It should also be noted that relaxation in the vascular muscles, which may occur because of inhibition of acetylcholine release by neurotoxins, can contribute these circulatory disorders.

It is noteworthy that the histopathological findings belong to botulism in the literature are very limited [21,25]. However, in this study, the presence of significant microscopic lesions in cases with BoNTs suggests that these findings may often be overlooked *(Table 1)*. We predicted that the findings detected histopathologically, especially in the central nervous system, could provide important clues about botulism when evaluated together with other data, although they were not sufficient for the definitive diagnosis of the disease. Up to this point, anamnesis, clinical findings, environmental observation, feed and shelter examination, macroscopic findings during necropsy, and general evaluation of microscopic findings have been highly important for the prediagnosis of the disease. Immediately after the findings are observed, it is vital for herd health to take necessary protection and control measures such as vaccine application, suspicious feed and material disposal, and environmental cleaning until toxin isolation by the authorities.

In the study, well-known and used routinely in many areas, the IHC staining technic was used for the first time for the detection of BoNT-C and BoNT-D through specific monoclonal and polyclonal antibodies, and the results were evaluated. As such, the presence of BoNT was detected with IHC method in eleven of twenty cattle, three of seven sheep, and two of three goats (sixteen of thirty ruminants; 53.33%) with suspected botulism *(Table 2)*.

The rapid course of most cases owing to the high toxicity of BoNT poses a major challenge both in the diagnosis of botulism and in the implementation of appropriate treatment or precautions. The gold standard method for the detection of BoNT is considered to be the mouse inoculation test that can detect BoNT at the level of 10 pg/mL [16]. This test requires observation of mice for two to four days after intraperitoneal injection with samples prepared from serum or gastrointestinal contents [18]. Furthermore, additional neutralization steps are required to identify the antigenically different serotypes of BoNT [14,27]. However, new diagnostic methods need to be developed because this test is highly laborious, time-consuming, and potentially hazardous to personnel during injection; involves ethical problems; and requires a specially equipped laboratory [22]. For this purpose, a number of immunological test formats have recently been reported for the detection of the antigenic nature of the botulism toxin or toxin complexes [27]. Compared with the mouse test, immunological tests are performed and interpreted technically, simply, and quickly [28]. Most early diagnostic tests such as radioimmunoassay [29], ELISA [16], and immuno-PCR [22,27] provided equal sensitivity to mouse experiments thanks to advances in signal amplification [19]. In the past thirty years, ELISA, a test based on antigen-antibody interaction, has been the most widely used technique for serotyping and toxin detection [19]. With the introduction of high-affinity antibodies in ELISA, lower toxin detection limits were achieved than that of possible with mouse inoculation tests (2 pg/mL-2 ng/mL) [16,30]. In a study on

the serum of botulism-suspected animals (bovine and avian), ELISA and mouse inoculation tests were compared and found that the sensitivity and specificity of ELISA were higher [31]. From all these findings, it can be interpreted that immunologically based tests are successful in determining BoNT.

In addition to the above-mentioned disadvantages of mouse tests, there are some extra factors that limit sensitivity in ruminants [4]. Firstly, in order to perform mouse tests with samples prepared from blood serum, internal organs such as liver and stomach-intestines contents, autolysis should not have occurred yet in suspicious cadavers [18]. Because, toxigenic clostridia species rapidly proliferate in decomposed organic materials and can expose BoNT through autolysis [5]. Furthermore, the neurotoxigenic *C. botulinum* found in ruminants as part of the gut microbiota can invade post-mortem cadaver [5,18]. There may also be variations between species in the sensitivity to mouse tests [18]. Cattle have been found to be 12.88 times more susceptible to BoNT-C than mice [21]. It is therefore stated that mouse tests are not sensitive enough in ruminants, especially cattle [5,18,21]. For the mouse tests, only ruminal content samples taken in accordance with the standards from animals, in this study, were accepted by the official local laboratory authorized by the Ministry of Agriculture and Forestry. Here, the presences of BoNT-D and BoNT-C in two and one cattle respectively were able to be detected with the mouse tests *(Table 2)*. As in this study, there are many studies in which insufficient results were obtained in toxin isolation and identification as a result of the mouse tests [32-34]. The consensus in these studies is that the sensitivity of mouse bioassay is insufficient to detect the presence of BoNT in ruminants due to the very low amounts of circulating toxin and the toxin is rapidly degraded. In the Republic of Ireland, it has been reported that 65 dairy cows died or euthanized in botulism outbreak and BoNT could not found in mouse experiments [34]. In France, BoNT isolation could not be obtained by mouse tests in a major botulism outbreak resulting in the death of 80 of 110 cattle, but *C. botulinum* D/C spores were detected by PCR and anaerobic cultivation in silage [32]. In another botulism outbreak that resulted in the death of 427 cattle, toxin was obtained in mouse tests in the decayed cat cadaver, which is thought to cause the epidemic, while could not be detected in the rumen content, milk, and organs of the affected animals. However, it was reported that the presence of BoNT in liver and rumen content was detected with ELISA [24]. When all these are evaluated together, it can be concluded that mouse tests have low sensitivity in ruminants, positive results highly indicate the presence of botulism, but negative results are insufficient to exclude the disease [17,18,33,34].

The formalin-treated form of the toxin is called toxoid [27]. As is known, the tissues used in the application of the IHC method are fixed with formalin. Therefore, the use of antitoxoid primary antibodies, as in our study of toxin screening with IHC, is important to increase the affinity and specificity of staining. Rapid absorption of BoNT, which can be in minuscule amounts but with a strong toxic effect, to neuromuscular junctions also complicates toxin isolation [4,8,16,18]. This may be one of the reasons why most of the blood or fecal samples used in other diagnostic methods for live animals are often not positive. Similarly, methods such as mouse tests may give false negativity because of degradation of the toxin as a result of rapid enzymatic activity and other post-mortem changes, or false positives may arise owing to the toxins produced by the agents in the gut of dead animals [5,18]. However, in this study, thanks to formalin fixation of the tissues before the IHC method, these changes and disadvantages were prevented, and then the toxins in the tissues and organs were made visible using a toxoid form specific antibodies.

Also, visualization of toxin localization on tissues has been evaluated as another important advantage of IHC. Especially after death, there is doubt which tissue or sample to send to the laboratory for the diagnosis of botulism. Likewise, there are some contradictions about which types of samples should be used in diagnostic laboratories. In this regard, with this study we presented the organs determined to have BoNT by IHC method *(Table 3)*. But unfortunately, for the diagnosis of BoNT in Turkey, the mouse tests are performed from only gastrointestinal content, blood serum, or feed samples because of official rules [23]. This may be another reason why immunohistochemically positive cases in the study were negative in the mouse experiments (From 30 animals, a total of 16 in IHC but 3 in the mouse test; *Table 2)*.

It has been reported that the H_{CC} subdomains of BoNT-C and BoNT-D's heavy chains interact with protein receptors, gangliosides (GD1b, GT1b) and phosphatidylethanolamine, which are expressed in many cell types such as hepatocytes and crypt epithelial cells in the gut. But the high presence of both gangliosides and protein receptors in neural cells is probably the explanation for why BoNT has a particular affinity for these cells and can cause the disease even at very low concentrations [10,11,35]. Also, it is stated that BoNTs can target many neurons, though not all, and inhibit the release of various compounds by affecting non-neuronal cells at high concentrations [10,36,37]. This situation is seen as a possible cause of the presence of toxin in brain and parenchymal organs in the study. As can be understood from both the prior literature [1,14,19,24,27,31,38,39] and the findings of this study that the samples prepared from the brain and internal organs, especially the liver, which is the first station for the toxins absorbed from the intestine, should be used in the diagnostic tests.

BoNT-C and D were found to be localized in the cytoplasm and sometimes in the nucleus. There are important differences in terms of intracellular localization of BoNT types. Indeed, it has been reported that the LC of BoNT-A

and BoNT-E are localized in the plasma membrane and the cytosol respectively while the LC of BoNT-B is distributed throughout the cell including the nucleus [36,40]. Although there is no data on the intracellular localization of BoNT-C and D in literature, it can be noted from the findings of the study that they distributed throughout cell compartments as similar to BoNT-B.

BoNTs proteolyze the SNARE proteins (SNAP-25 and synaptobrevin), preventing neurotransmitter release from vesicles containing acetylcholine in the neuromuscular junction and thereby causing loose paralysis [7]. BoNT-A, C1, and E proteolyze SNAP-25, while BoNT-B, D, F, and G cause proteolysis of synaptobrevin [41]. Because the proteolysis in SNARE proteins differs according to the toxin type, the researchers have conducted studies on the usability of this feature in determining the toxin type of the disease. In hippocampal culture samples given BoNT-C, it was found that IHC staining of syntaxin and SNAP-25 were decreased [42]. It has been noted, in a study, that BoNT-A, C, and D cause significant reductions in immunoreactivity of SNAP-25, syntaxin I, and synaptobrevin II in the mouse phrenic nerve-hemidiaphragm preparation [43]. Similarly, others have reported that SNAP-25 or synaptobrevin is specifically proteolyzed by BoNTs [44,45]. However, to the best of our knowledge, there are no data in the literature on the interaction between these toxins and SNARE proteins in ruminants that are heavily exposed to BoNTs. In our study, in cases in which BoNT-C and BoNT C+D were determined, SNAP-25 expression decreased significantly compared to the control and negative groups. This situation was partially detected in cases in which BoNT-D was determined. However, only BoNT-C from these groups was found to have a significant negative correlation with SNAP-25; that is, this toxin reduced the SNAP-25. On the other hand, synaptobrevin expression was decreased in BoNT-D and BoNT-C+D groups compared to other groups, but a negative correlation was observed between only BoNT-D and synaptobrevin *(Table 4, Table 5)*. When the immunoreactivity data of SNAP-25 and synaptobrevin obtained in our study were evaluated together, it was found that BoNT-C specifically proteolyzes SNAP-25 while BoNT-D specifically proteolyzes synaptobrevin in ruminants. This indicates that the flaccid paralysis in ruminants because of BoNTs, as in other species [12,41] is a result of the proteolysis of SNAP-25 and synaptobrevin. At the same time, it can be a guide both for confirming a diagnosis of botulism made with IHC and in cases where botulism is suspected but toxins could not be detected. In addition, SNAP-25 and synaptobrevin proteolysis in the central nervous system can disrupt acetylcholine release, resulting in impaired communication between neurons. This situation may contribute to the aggravation of the observed symptoms or the increase of deaths in ruminates.

As a result, in this study, we attempted to conduct a comprehensive analysis of macroscopic and microscopic findings, which take a very limited part in the literature, as well as the diagnostic approach from the anamnesis stage to clinical findings of botulism disease. In this context, we emphasized that anamnesis, clinical symptoms, and macroscopic and microscopic findings should be evaluated together for the preliminary diagnosis of the disease. Also, we have suggested that, if necessary, preventive measures (vaccination, destruction of contaminated feed and materials, etc.) should be taken without waiting for the results of tests to reveal the presence of toxins. In addition, as an alternative to mouse inoculation tests, the effectiveness of IHC staining was questioned, and it was thought that this method might be worthy comparing with other tests. It was also determined that the proteolysis status of SNAP-25 and synaptobrevin, which are responsible for the emergence of clinical findings in patients, may contribute to the diagnosis.

Conflict of Interest

The authors have no conflict of interest.

Authors Contributions

M.B. Ateş and M.K. Çiftci made the experiment and the histological and immunohistochemical interpretation, and wrote the manuscript. F. Terzi and Z. Çelik planned methodology and investigated resources, M. Ortatatlı wrote and review and editing the manuscript. All authors discussed the results and contributed to the final manuscript.

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