Pakistan Veterinary Journal

ISSN: 0253-8318 (PRINT), 2074-7764 (ONLINE)
Accessible at: www.pvj.com.pk

RESEARCH ARTICLE

Immune Response of Cattle to Botulinum Type C and D Toxoid Administered on Three Occasions

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ARTICLE HISTORY (14-223)

Received: May 06, 2014
Revised: January 06, 2015
Accepted: February 10, 2015

Key words:
Antibody
BoNT
Botulism
ELISA
Vaccination

A B S T R A C T

The aims of the present study were to investigate the antibody response of cows from an outbreak region to vaccination with a bivalent botulinum toxoid (Type C and D) on three occasions and to investigate the antibody response to vaccination with the botulinum toxoid in the presence of anti-BoNT antibodies. Twenty-two cows from an outbreak region and 10 cows from a farm without a history of botulism were used in this study. Blood samples were collected before each vaccination and three weeks after the third vaccination (days 0, 21, 42 and 63). The level of anti-BoNT/C antibodies steadily increased after each vaccination (0.471±0.04, 0.566±0.03 and 0.663±0.04, respectively); however, the levels of anti-BoNT/D antibodies were not significantly different after the second and third vaccinations (0.377±0.03, 0.493±0.03 and 0.465±0.03, respectively). Post vaccination antibody responses of animals found positive and negative for anti-BoNT antibodies at the beginning of the study were similar. The results of the present study indicated that vaccination of cattle with botulinum toxoid on three occasions is recommended, particularly in outbreaks that are suspected to be caused by BoNT/C and that presence of naturally acquired antibodies against BoNT did not interfere with post vaccination immune response.

To Cite This Article: Mecitoglu Z, EM Temizel, O Ozyigit, G Akgul and E Kennerman, 2015. Immune response of cattle to botulinum type C and D toxoid administered on three occasions. Pak Vet J, 35(2): 231-235.

INTRODUCTION

Botulism is a rapid and fatal disease manifested by flaccid muscular paralysis that is caused by the ingestion of the exogenously produced botulinum neurotoxin (BoNT) of *Clostridium botulinum* (Radostits *et al.*, 2004).There are seven known serotypes of neurotoxins produced by the clostridial species (A-G) (Woodward *et al.*, 2003; Popoff, 2014). C. botulinumis capable of producing all seven types of neurotoxins, whereas *C. barati*, *C. butyricum* and *C. argentinense* produce only one: the F, E and G serotypes, respectively (Simpson, 2004). By inhibiting the release of acetylcholine, BoNT causes pre-synaptic blockage at nerve motor end plates resulting in flaccid paralysis (Cunha *et al*., 2014). The disease is clinically characterized by findings such as progressive flaccid paralysis affecting the limb, jaw and throat muscles. In most cases, the tongue is paralyzed and hangs from the mouth or the animal cannot retract its tongue when it is pulled out (Radostits *et al.*, 2004). Outbreaks of cattle botulism have been reported worldwide, and these outbreaks in cattle are reported to be caused by serotypes B, C and D (Galey *et al.*, 2000; Otter *et al.*, 2006, Senturk and Cihan, 2007). The disease is generally caused by eating carrion, contamination of feedstuff with carcasses and in particular, feeding cattle poultry litter; improper use of poultry litter in pastures is also among the most frequently reported sources of BoNT in the major outbreaks (Galey *et al.*, 2000; Kennedy and Ball, 2011). In addition, outbreaks of botulism have occurred in which silage is the important etiological factor (Yeruham *et al.*, 2003). When contaminated plants are used to prepare silage, C. botulinum can use the plant's proteins for their growth and production of BoNT type B (Whitlock and Buckley, 1997). *C. botulinum* can proliferate in environments with pH values higher than 4.5 and the high moisture content of silage provides optimal conditions for BoNT production (Whitlock, 1990). Definitive diagnosis of botulism is not possible in every case because the source of the toxin may be absent and the circulating toxin levels are often extremely low. The mouse bioassay for the detection of BoNT in serum or the

abomasal, ruminal and intestinal contents of a suspected animal is the gold standard test for the diagnosis of botulism (Scarlatos *et al.*, 2005; Wilson *et al*., 1999). In addition to the mouse bioassay, detection of BoNT using a monoclonal antibody-based sandwich ELISA is a method used for diagnosis of the disease, also detection of BoNT antibodies in non-affected or survived animals from an outbreak region was evaluated as retrospective toll for diagnosis of the disease on herd level, however the diagnostic value of this method is questionable (Brooks *et al.*, 2011; Mawhinney *et al.*, 2012). Vaccination with a type-specific or combined toxoid is practiced to control botulism, particularly in the outbreak regions. Vaccination is recommended to be performed in two doses given at two or three week intervals (Radostits *et al.*, 2004). Steinman *et al.* (2007) reported that administering a six-month booster dose, instead of an annual booster, results in continuous protective levels of anti-BoNT antibodies in cattle. However, the main problem in botulism is that the source of the toxin cannot be identified in most outbreaks. Therefore, vaccination on three occasions is recommended in outbreak areas, especially where the source of the toxin is unknown (Smith, 2009).

The first objective of the present study was to investigate the antibody responses of cows vaccinated with bivalent (C, D) botulism toxoid on three occasions in an outbreak area in northwest Turkey and in a herd without a history of botulism. The second objective of this study was to investigate the immune response of cattle to botulinum toxoid in the presence of anti-BoNT antibodies acquired following natural exposure to subclinical doses of BoNT.

MATERIALS AND METHODS

Two groups of animals were used in this study. The outbreak group consisted of 22 cows from an outbreak area in Northwestern Turkey and the non-outbreak group consisted of 10 cows from a herd without a history of botulism from the southeastern Marmara region, Turkey. The outbreak involved herds from five villages in which a total of 168 cattle had died within 3 months. All five villages were beside the same river with a distance of few kilometers between each other and intensive poultry industry was present in the region. The presence of BoNT/C was determined using the mouse bioassay in two cattle from the outbreak region that showed clinical signs and died. The animals selected for the outbreak group were the herd mates of documented botulism cases and all animals were randomly selected. None of the cattle were showing clinical signs of botulism. All of the cows used in the study were Holstein-Friesian, with a mean age of 3.7(±0.9) years, and all of the cows were in the mid-lactation phase. All 32 cows used in the study had never been vaccinated against botulism.

The 32 cows were vaccinated by subcutaneous injection of bivalent botulinum toxoid (Type C and D) (Botupen®, Pendik Veterinary Research and Control Institute, Istanbul, Turkey) on three occasions at three-week intervals. Along with 22 cows from outbreak region used in the study all herds from the five villages were also vaccinated on three occasions. Three cows from two different herds which were not included to the study, showing typical signs of botulism died till the eight day after the first vaccination. After the eight day of the study there were not any reported deaths associated with botulism. Blood samples were collected in plain tubes by jugular venipuncture before each vaccination, on days 0, 21 and 42 and at three weeks after the third vaccination, on day 63 of the study. The sera were separated by centrifugation (2000 rpm, 15 minutes) and stored at -80°C until analysis.

The content of anti-BoNT antibodies in the blood samples was determined in Germany using an antibody ELISA that utilized purified C and D BoNT antigens (Miprolab GmBH, Göttingen, Germany). The antigens were coated on microtiter plates at +4°C overnight at a concentration of 0.5µg/ml. The wells were washed and then blocked with casein-based blocking buffer (mipro BLOCK C). After a second washing, the serum samples as well as the positive and negative control samples were diluted 1:100 in casein-based blocking buffer and were pipetted into the wells. The plates were incubated for 30 minutes at room temperature and then washed. Peroxidase-labeled anti-bovine IgG diluted in casein-based blocking buffer was used as the secondary antibody and tetramethylbenzidine 3,3, 5,5 (TMB) was used as the substrate for the conjugated peroxidase. The plates were then read spectrophotometrically (450/620 nm) to obtain the absorbance values. The cut-off level for ELISA absorbance values was 0,251 as established by reference to positive control serum by the laboratory, so samples with absorbance levels higher than 0,251 were designated as positive.

The ELISA absorbance values for the samples from the 32 animals tested for BoNT/C and D were evaluated and compared, regardless of the source of the animals or their anti-BoNT antibody levels at the beginning of the study. The anti-BoNT antibody levels of animals from the outbreak region (n:22) after vaccination were compared with those of the animals from the control farm (n:10), and the anti-BoNT antibody levels of animals discovered to be positive on day 0 were compared with those of animals that were negative on day 0.

Statistical analyses of the results were performed by using Sigma Plot 12 software (Systat Software Inc., San Jose, CA). The mean values of the antibody levels within the vaccination groups were compared using a one-way repeated measures analysis of variance (one-way RM ANOVA). The mean values of the antibody levels between the vaccination groups were compared using Student t-test.

RESULTS

Among the 22 samples collected in the outbreak region, 7 tested positive for anti-BoNT type C antibodies at the beginning of the study. Anti-BoNT type D and anti-BoNT type C antibodies were detected in 3 of the 7 samples. Fifteen cows from the outbreak region and 10 cows in the control group were negative for anti-BoNT antibodies. The mean ELISA absorbance values for the anti-BoNT/C antibodies of the 32 animals increased significantly on days 21, 42 and 63 of the study (Table 1). The increases of the ELISA absorbance values levels for the anti-BoNT/D antibodies of the 32 animals after the

Table 1: Comparison of ELISA absorbance values (450-620nm) for anti-BoNT/C and D antibodies of animals from outbreak region and the control farm

Experimental Day	Anti-BoNT/C antibodies		Anti-BoNT/D antibodies	
	Animals from outbreak region (n=22)	Animals from control farm (n=10)	Animals from outbreak region (n=22)	Animals from control farm (n=10)
0	0.200±0.04[a]	0.077±0.01[a]	0.170±0.03[a]	0.069±0.01[a]
21	0.472±0.05[b]	0.468±0.09[b]	0.375±0.04[b]	0.380±0.07[b]
42	0.588±0.04[c]	0.523±0.07[b]	0.508±0.05[c]	0.466±0.04[b]
63	0.696±0.05[d]	0.606±0.08[b]	0.478±0.04[c]	0.422±0.06[b]

Values (mean±SEM) bearing different small letters in a column (P<0.05) and capital letters in a row differ significantly (P<0.001).

Table 2: Comparison of ELISA absorbance values (450-620nm) for anti-BoNT/C and D antibodies of animals positive and negative for anti BoNT antibodies on day 0 of the study

Experimental Day	Anti-BoNT/C antibodies		Anti-BoNT/D antibodies	
	Positive (n=7)	Negative (n=25)	Positive (n=3)	Negative (n=29)
0	0.423±0.07[aA]	0.078±0.01[aB]	0.342±0.06[A]	0.073±0.01[aB]
21	0.572±0.09[a]	0.440±0.05[b]	0.417±0.04	0.365±0.04[b]
42	0.601±0.04[b]	0.555±0.04[c]	0.543±0.08	0.479±0.03[c]
63	0.707±0.04[b]	0.655±0.05[c]	0.554±0.02	0.449±0.04[c]

Values (mean±SEM) bearing different small letters in a column (P<0.05) and capital letters in a row differ significantly (P<0.001).

first and second vaccinations were statistically significant (P<0.05); however, the slight decrease observed on day 63 was not statistically significant. The ELISA absorbance values were significantly higher in animals that were positive for anti-BoNT/C antibodies on day 0 of the study as compared to the animals that tested negative (Table 2). The post-vaccination anti-BoNT/C antibody response of the negative animals was marked after the first vaccination (day 21); however, the antibody levels of the animals that were positive on day 0 were significantly elevated on day 42 compared to levels on day 21. Statistically significant differences were not detected in either group of animals between days 42 and 63 (Table 2). Comparison of the anti-BoNT/C antibody levels of the animals from the outbreak region and the control farm did not reveal a statistically significant difference between the groups on any day of the study (Table 1). The ELISA absorbance values after vaccination were significantly different on days 0, 21, 42 and 63 in the outbreak group; however, although the antibody levels steadily increased after vaccination, no statistically significant difference was found on days 21, 42 and 63 with the values for animals from the control farm (Table 1). The ELISA absorbance value of the animals positive for anti-BoNT/D antibodies on day 0 did not show any statistically significant elevation after the vaccinations had been given; however the absorbance values were significantly elevated after the first and the second vaccinations in the animals that were negative for pre-existing antibodies on day 0. The ELISA absorbance values for anti-BoNT/D antibodies were likewise significantly elevated after the first and the second vaccinations but did not differ significantly after the third vaccination in the animals from the outbreak region (Table 1). The ELISA absorbance values for the anti-BoNT/D antibodies were elevated after the first vaccination, on day 21, but did not differ significantly between days 21, 42 and 63 of the study. Although the exact source of the toxin could not be determined, outbreak stopped following the vaccination schedule.

DISCUSSION

Vaccination for botulism plays a key role in the prevention of the disease because the neutralizing antibodies elicited by vaccination provide protection against exotoxins by preventing them from binding their target receptors and promoting their elimination by phagocytes (Steinman *et al.*, 2006). Steinman *et al.* (2006) reported that the level of anti-BoNT antibodies obtained by vaccination are positively correlated with the protection against botulism in cattle and that by monitoring the anti-BoNT antibody levels, the vaccination protocols to protect against botulism could be optimized. Krüger *et al.* (2013) reported that vaccination of cattle with botulinum types C and D toxoid resulted with elevation of anti-BoNT antibodies and also vaccination significantly reduced the amount of BoNT and C. botulinum spores in the feces. ELISA systems are used to determine specific anti-BoNT antibodies and are considered to be effective for monitoring the immunological responses to botulinum toxins (Lindsey *et al.*, 2003). The ELISA system used in the present study has been employed also in several investigations (Mawhinney *et al.*, 2012; Kümmel *et al.*, 2012).

As previously mentioned, vaccination on three occasions is recommended, particularly when the source of BoNT is unknown (Radostits *et al.*, 2004). However these recommendations are based on studies conducted on species other than cattle thus presented study is important for documenting the efficiency of botulism vaccination on three occasions in cattle. A study conducted on horses revealed that vaccination on three occasions elicited robust serum anti BoNT/C and D antibody responses (Stahl *et al.*, 2012). Similar findings were obtained from a study conducted on humans, ninety percent of volunteers seroconverted after two vaccinations with botulinum F toxoid, however seroconversion rate after three vaccinations was 100% in the same study (Montgomery *et al*., 1999). A different study reported that mice vaccinated on three occasions remain protected against BoNT for 12 months post vaccination (Smith, 1998). The results of the present study indicated that vaccinating cattle on three occasions with the bivalent botulinum toxoid significantly elevated the level of the anti BoNT/C antibodies; however the third vaccination did not alter the level of the anti BoNT/D antibodies as compared to the level after the second dose of vaccine was administered. The slight decrease of anti BoNT/D antibodies after the third vaccination was an unexpected finding. Brown *et al.* (1998) reported that toxin neutralization test results were coherent with ELISA results for type C antibody but not

with type D. The difference between anti BoNT/C and D antibody responses is a subject which could be clarified by further experiments.

Although, outbreaks of botulism type C have been reported, in which the source of the toxin was discovered to be carrion instead of poultry litter (Galey *et al.*, 2000). Type C botulism in cattle is usually closely related to the inappropriate disposal of poultry litter on pastures or feeding cattle poultry litter (Otter *et al.*, 2006; Senturk and Cihan, 2007). Thus, according to the data obtained in the present study, vaccination on three occasions would be particularly beneficial for the prevention of botulism outbreaks related with consumption or inappropriate use of poultry litter.

Another objective of the present study was to evaluate the effects of vaccination on animals that had acquired anti-BoNT antibodies after a natural outbreak. As the toxoid vaccines used are simply the detoxified BoNT's, theoretically anti-BoNT antibodies previously acquired by exposure to sub-lethal doses of BoNT or by vaccination could interfere with post-vaccination immune response. Interference similar to that mentioned above is reported in which tetanus maternal antibodies are reported to influence infant tetanus vaccine responses (Siegrist *et al.*, 1998; Brown *et al.*, 1999). Although, in above study, immunological immaturity could be responsible for the diminished responses to vaccination rather than maternal antibodies. Naturally acquired low dose and chronic tetanus toxin exposure in humans is reported to be associated with systemic and local immune tolerance in the gut and poor antibody responses after vaccination (Dastur *et al.*, 1981). Results of studies conducted on a different type of clostridial toxin, gave rise to question whether naturally acquired antibodies influence the immune response to botulism vaccination in cattle.

Steinman *et al*. (2006) reported that natural exposure to sub lethal doses of BoNT give rise to a specific antibody response shown by ELISA (Steinman *et al.*, 2006). Similarly high anti-BoNT antibody titers of 7 cattle used in the presented study were most likely due to exposure to sub clinical doses of BoNT during the outbreak. Steinman *et al.* (2007) also reported that the presence of maternally derived anti-BoNT antibodies did not interfere with the immune response or the antibody levels obtained due to vaccination against botulism. The similar antibody responses to vaccination of the animals that were positive and negative for anti-BoNT antibodies at the beginning of the presented study indicates that the anti-BoNT antibodies acquired following an outbreak did not interfere with the immune response to vaccination.

Conclusion: According to the data obtained in the present study, vaccinating cattle with botulinum toxoid on three occasions is recommended, particularly for protection against type C botulism and the vaccine can be administered in the presence of anti-BoNT antibodies acquired following an outbreak without the risk of an altered immune response to the vaccine. Determination of neutralizing antibodies acquired by vaccination on three occasions and comparing that data with vaccination on two occasions would be beneficial as presented study demonstrates both neutralizing and non-neutralizing antibody levels acquired following immunization. Further studies conducted on the period that the protective levels of anti-BoNT antibodies are maintained after vaccinations on three occasions would be beneficial for optimizing the vaccination protocols to protect cattle from botulism.

Author's contribution: ZM, EMT, OO and EK conceived and designed the review. ZM and EMT executed the experimental procedures. ZM, EMT and GA analyzed the data. All authors interpreted the data, Critically revised the manuscript for important intellectual contents and approved the final Version.

Acknowledgement: This study was financially supported by the East Marmara Development Agency. Authors would also like to thank to Dr. Frank GESSLER from Miprolab GmBH for his assistance.

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