משרד החקלאות - דו"ח לתוכניות מחקר
לקרן המדען הראשי

קוד זיהוי	א. נושא המחקר (בעברית)
705 - 0007	בדיקת התגובה החיסונית ויעילות החיסון בטוקסואיד משופר כנגד בוטוליזם בעגלות חלב

ב. צוות החוקרים

	שם משפחה	שם פרטי
חוקר ראשי	שפיגל	נחום
חוקרים משניים		
1	אמיר	שטיינמן
2		
3		
4		
5		
6		
7		

ג. כללי

מוסד מחקר של החוקר הראשי

האוניברסיטה העברית, פקולטה לחקלאות, ביה"ס לרפואה וטרינרית

סוג הדו"ח	תאריכים		
	תקופת המחקר עבורה מוגש הדו"ח		תאריך משלוח הדו"ח למקורות המימון
	התחלה	סיום	
מסכם	שנה חודש 2006 / 01	שנה חודש 2007 / 12	שנה חודש /

ד. מקורות מימון עבורם מיועד הדו"ח

שם מקור המימון	קוד מקור מימון	סכום שאושר למחקר בשנת תיקצוב הדו"ח
		בשקלים
מועצת החלב		

ה. תקציר שים לב - על התקציר להיכתב בעברית לפי סעיף ה' שבהנחיות לכתיבת דיווחים

ו. אישורים

הנני מאשר שקראתי את ההנחיות להגשת דיווחים לקרן המדען הראשי והדו"ח המצ"ב מוגש לפיהן

חוקר ראשי	מנהל המחלקה	מנהל המכון (פקולטה)	אמרכלות (רשות המחקר)	רשות המחקר	תאריך (שנה) (חודש) (יום)

דוח מסכם לתוכנית מחקר מספר 7050007

לתקופה 2006-2007

בדיקת התגובה החיסונית ויעילות החיסון בטוקסואיד כנגד בוטוליזם בעגלות חלב

מוגש לקרן המדען הראשי במשרד החקלאות ולהנהלת ענף הבקר

ע"י

נחום שפיגל - ביה"ס לרפואה וטרינרית ע"ש קורט, הפקולטה לחקלאות, האוניברסיטה העברית בירושלים.

אמיר שטיינמן - ביה"ס לרפואה וטרינרית ע"ש קורט, הפקולטה לחקלאות, האוניברסיטה העברית בירושלים.

Nahum Shpigel, Koret school of veterinary medicine, faculty of agricultural food &environmental sciences, the Hebrew university of Jerusalem, P.O.B. 12, Rehovot 76100.

shpigeln@ari.huji.ac.il

Amir Steinman, Koret school of veterinary medicine, faculty of agricultural food &environmental sciences, the Hebrew university of Jerusalem, P.O.B. 12, Rehovot 76100.

הממצאים בדו"ח זה הינם תוצאות מחקר

המחקר מהווה המלצות לחקלאים: כן/

חתימת החוקר

תקציר

בוטוליזם הינה מחלה קטלנית המאופיינת בשיתוק שרירים הנגרם בהשפעת רעלן של החיידק *קלוסטרידיום בוטולינום*. רעלן הבוטולינום, הרעלן הביולוגי הפוטנטי ביותר בטבע, פוגע ביונקים, עופות ודגים. ארבעה מהסרוטיפים (A-D) גורמים להרעלה בבקר. הרעלות בוטוליזם בבקר הינן בעיה כלל עולמית הגורמת נזק כלכלי רב. בשנים האחרונות אירעו מקרים ספורדיים והתפרצויות גדולות באירופה, צפון אמריקה, דרום אמריקה, אוסטרליה וישראל. דרכי ההתמודדות עם המחלה כוללות שיטות יצור נאותות של המספוא למניעת שגשוג החיידק ויצור הרעלן, ובנוסף חיסון הבקר והצאן כנגד הרעלן. תוכניות החיסון המקיפות אשר הופעלו בישראל בעקבות התפרצות חמורה של המחלה בסוף שנות השבעים, נחשבו במשך שנים רבות כמוצלחות ביותר. הופעת התפרצויות, בחלקן חמורות ביותר בשנים האחרונות בבקר מחוסן, היא מקור לדאגה רבה משום אופייה הקטלני של המחלה והעלו חשש לגבי יעילות רמת החיסון.

בעבודות קודמות שנעשו במעבדתנו הראנו שרמת ההגנה המוקנית לעגלות בעקבות חיסון בפרוטוקול הנוכחי (חיסון ראשון בגיל שמונה שבועות, חיסון שני בגיל 12 שבועות וחיסון שנתי) אינה מקנה הגנה מספקת בפני הרעלה. אנחנו משערים שחיסון בפרוטוקולים משופרים יקנה הגנה טובה יותר בפני הרעלת בוטוליזם. על מנת לבדוק את רמת ההגנה המוקנית בעזרת החיסון אנו משתמשים בערך סף של רמת נוגדנים שנקבע בעזרת מערכת אליזה. ערך סף זה נקבע על ידנו בעבר ע"י ניתוח נתונים שנאספו במהלך התפרצויות בוטוליזם מסוג D במספר עדרי בקר בתקופה האחרונה והוא מצוי בקשר ישר עם הגנה כנגד הרעלה.

מהלך ושיטות העבודה: מטרת העבודה היתה לבדוק את רמת ההגנה המוקנית לעגלות בעקבות חיסון בטוקסואיד דו-ערכי? (C, D) תוך שימוש בפרוטוקולים שונים. לצורך כך נבחרו שלוש קבוצות עגלות בנות 10 פרטים בכל קבוצה שחוסנו בשלושה פרוטוקולים שונים של חיסון. כל העגלות חוסנו לראשונה בגיל שמונה שבועות. זריקות נוספות ניתנו לעגלות לאחר ארבעה ו-52 שבועות (קבוצה 1), ארבעה, שנים עשר ו-52 שבועות (קבוצה 2), ולאחר שבועיים, שנים עשר, עשרים וארבע ו-52 שבועות (קבוצה 3) ממועד הזריקה הראשונה. דגימות דם נלקחו בצורה סדירה במהלך הניסוי עד שבועיים לאחר החסון השנתי. רמת הנוגדנים הספציפיים כנגד רעלן בוטולינום מסוג D נקבע בעזרת מערכת ELISA שפותחה במעבדתנו והושוותה לערך סף מגן שנקבע בעבודה קודמת.

תוצאות עיקריות: בדיקות רמות הנוגדנים של קבוצות העגלות המחוסנות הראו שכל העגלות, משלוש הקבוצות היו מחוסנות ברמה הנדרשת בגיל שישה חודשים, לאחר תום סדרת החיסונים הראשונה. עגלות מהקבוצה הראשונה שחוסנו לפי הפרוטוקול המקובל כיום הראו ירידה ברמת הנוגדנים שיצרה חלון שארך כשלושה חודשים לפני החיסון השנתי בו פרטים רבים היו עם רמת נוגדנים נמוכה מהרצוי. לעומתם מרבית הפרטים בקבוצה השלישית היו עדיין מוגנים במועד החיסון השנתי. למרות שעגלות בקבוצות שתיים ושלוש חוסנו באחד או בשני חיסונים יותר מעגלות בקבוצת הביקורת, במספר פרטים נמצאה רמת נוגדנים נמוכה לכל אורך הניסוי והם לא הראו תגובה חיסונית יעילה לטוקסואיד.

מסקנות והמלצות: כתוצאה מניסוי זה אנו ממליצים על ישום פרוטוקול חיסונים חדש שיכלול תוספת של זריקה שלישית לפרוטוקול המקובל כיום, שישה חודשים לאחר הזריקה הראשונה. שימוש בפרוטוקול זה ידחה את הירידה ברמת הנוגדנים ויקנה הגנה טובה יותר עד מועד החיסון השנתי. יחד עם זאת, בעקבות השונות הרבה בתגובה החיסונית בין הפרטים השונים, והירידה המהירה ברמת הנוגדנים יש צורך בפיתוח חיסונים חדשים בכדי להחליף את החיסונים הקיימים כיום בשוק.

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Cattle immune response to botulinum type D toxoid: Results of a vaccination study

A. Steinman [a], N. Galon [b], A. Arazi [a], Y. Bar-Giora [a], N.Y. Shpigel [a,*]

[a] *Koret School of Veterinary Medicine, Faculty of Agricultural, Food and Environmental Quality Sciences, The Hebrew University of Jerusalem, P.O. Box 12, Rehovot 76100, Israel*
[b] *Hachaklait, Mutual Society for Insurance and Veterinary Services in Israel, P.O. Box 3039, Qesaria 38900, Israel*

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Abstract

Cattle botulism is a food-borne intoxication caused by the ingestion of preformed botulinum neurotoxins (BoNT) of serotypes B, C, or D. Protection in cattle against botulinum intoxication is based on the presence of specific serum neutralizing antibodies upon exposure. Outbreaks in vaccinated cattle have raised concerns about vaccine quality and efficacy. To this end, three different immunization protocols and the effect of maternal anti-BoNT/D antibodies, at the priming dose, were analyzed in 2-month-old dairy calves. Based on previously determined protective anti-BoNT/D antibody levels analyzed in field outbreaks, the immune response to type D toxoids was analyzed using an in-house ELISA system. Here we show that using the current vaccination strategy of using a priming dose in 2-month-old calves followed by booster doses after 4 weeks and annually thereafter, did not result in continuous protective levels of anti-BoNT/D antibodies. As a result of this vaccination protocol, only 15–31% of cattle in parities 1–3 were protected at the time of the annual booster. Vaccination study in calves indicated that adding a 6-month booster dose to the current protocol resulted in continuous protective levels of anti-BoNT/D antibodies well above the cut-off protective levels. The presence of maternally derived anti-BoNT/D antibodies did not interfere with the immune response to toxoids that can be administered to 2-month-old calves.

Keywords: Botulinum toxoid; Vaccination protocol; ELISA; Maternal immunity

1. Introduction

Botulism is a fatal disease manifested by muscular paralysis caused by the effect of neurotoxins produced by the bacteria *Clostridium botulinum, Clostridium baratii,* and *Clostridium butyricum*. *C. botulinum* produces all seven known neurotoxin serotypes (A–G), whereas *C. baratii* and *C. butyricum* produce only one serotype each (F and E, respectively) [1]. Botulinum neurotoxin (BoNT), the most lethal substance known, affects all mammals, birds, and fish. Worldwide, field outbreaks of cattle botulism were caused by serotypes B, C, and D, whereas in Israel the most prevalent serotype is D [2].

In recent years, sporadic cases and massive outbreaks of cattle botulism occurred in Europe, North America, South America, Australia, and Israel [3–14]. Most notable is the marked increase in reported incidents of suspected botulism in cattle in the UK since 2003 [15] and in Israel since 2002 [2]. However, the current situation in these two countries is very different. For example, in Israel all livestock have been routinely vaccinated since the late 1970s after massive outbreaks, resulting from introducing unprocessed chicken manure as a feed additive [16]. For two decades, botulism vaccination with various bivalent type C and D toxoids was considered highly successful in Israel. However, in June 2002, a large type D botulism outbreak occurred in southern Israel, involving 28 dairy farms, killing more than 600 dairy cattle [17]. All animals were routinely vaccinated with a bivalent toxoid, using the currently recommended vaccination protocol: with a priming dose at the age of 2 months, followed

* Corresponding author. Tel.: +972 3 9680534; fax: +972 3 9604079.
E-mail address: shpigeln@agri.huji.ac.il (N.Y. Shpigel).

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4 weeks later and annually by booster doses. In this outbreak, botulism occurred 10–12 months after the last annual booster and affected mainly calves and heifers. This unexpected outbreak in vaccinated animals is in contrast with previously reported data from Australia [18] and South Africa [19] where similar botulinum toxoids (albeit with different protocols) were used raised concerns about the cattle vaccine's quality, efficacy and protocols in use. Cattle botulism outbreaks in Israel are characterized by rapid onset and high morbidity and mortality [2]. Therefore, protection is entirely dependent on the presence of specific serum neutralizing antibodies upon ingestion of the preformed toxin. We have previously used field outbreaks to determine the protective levels of anti-BoNT/D antibodies following vaccination with the commercially available bivalent C and D toxoids. Here we report the analysis of anti-BoNT/D antibody levels following routine vaccination of cows on a commercial dairy farm using the predetermined cut-off level as a measure of vaccination efficacy. Furthermore, the result of our previous [2] and this initial study casted doubts on our current vaccination protocols. To this end, three different immunization protocols and the effect of maternal anti-BoNT/D antibodies at priming dose were analyzed in 2-month-old dairy calves.

2. Materials and methods

2.1. Animals

All animals participating in this study were part of a single Israeli Holstein commercial dairy herd consisting of 600 milking cows. Cows were housed in loose housing systems in large, completely covered open sheds, fed total mixed ration (TMR) ad lib and were milked three times per day, with an average annual milk production of 12,000 kg per cow. TMR included wheat or corn silage, concentrates and a mineral and vitamins premix. All cows and calves were identified by ear tags and/or freeze markings. Computerized dairy herd management systems were used for electronic cow identification as well as storage of all demographic data and individual vaccination records (Afimilk^{TM} and NOA, Israel Cattle Breeders Association). During the preceding 5 years no cases of botulism were diagnosed or suspected in the study farm or neighboring farms.

All animals in the herd were vaccinated by subcutaneous injection of a priming dose at the age of 2 months and received booster doses 4 weeks later and once a year thereafter, with one of the commercially available brands of type C and D bivalent toxoids (CSL Limited, Parkville, Victoria, Australia (2.5 ml per dose); Prondil S.A., Montevideo, Uruguay (2 ml per dose)). In previous experimental studies of cattle and mice, no difference could be discerned in the immune response to these two toxoids (Steinman A. and Shpigel N.Y., unpublished results [2]).

2.2. Analysis of anti-BoNT/D antibody levels in vaccinated cattle

Sera samples were collected from 129 Holstein-Friesian cows of various parities (defined as the number of calvings a cow has delivered) and replacement heifers (animals before first calving at about the age of 24 months). Sera samples were collected 1 year after the last annual booster vaccination before the annual booster was administered. Serum was separated by centrifugation ($2000 \times g$) and kept at $-80\,^{\circ}C$ until analyzed. The levels of specific anti-BoNT type D antibodies in the sera were determined using an in-house ELISA, as described previously [2].

2.3. Vaccination study

Thirty-two 2-month-old female Holstein-Friesian calves were subcutaneously immunized with a priming dose, and by use of their brand numbers they were randomly divided into three experimental groups of booster dose schedules. Group 1 calves were immunized at 4 weeks; group 2 at 4 and 12 weeks; and group 3 at 2, 12, and 25 weeks after the initial priming dose (Fig. 1). All calves were immunized again 53 weeks after the initial injection (annual booster). Blood samples were collected from all calves before the first injection and after 2, 4, 12, 17, 25, 31, 43, 53, and 55 weeks.

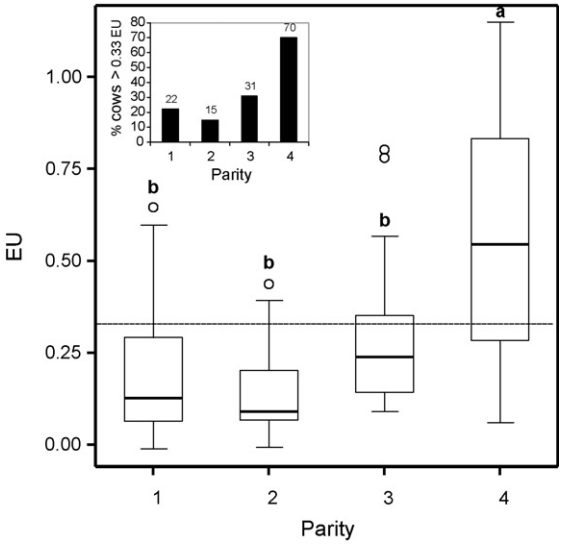

Fig. 1. Anti-BoNT/D antibody levels in cows that underwent calfhood vaccination (at 2 and 3 months of age) and annual boosters with bivalent (C and D) botulinum toxoid. Sera samples were obtained from each cow just before the annual booster and assayed individually for the presence of IgG antibodies against BoNT/D. Antibody levels in ELISA units (EU) are displayed by Box-plot diagrams. The botulism protective titer threshold (0.33 ELISA units) is denoted by a horizontal dashed line. The inset diagram displays the proportion (%) of cows in each parity group with anti-BoNT/D serum antibodies above the protective threshold. The means of antibody levels were compared among parity groups by analysis of variance; parity groups with different superscripts differed significantly when tested by one-way analysis of variance – Bonferroni comparison of means, $P < 0.05$.

Serum was separated by centrifugation (2000 × *g*) and kept at −80 °C until analyzed. The study was approved by the Ethics Committee of the Koret School of Veterinary Medicine, the Hebrew University of Jerusalem.

2.4. Statistical analysis

Serological results are presented as ELISA units, which were calculated as follows: (optical density [OD] test sample − OD negative control) divided by (OD positive control − OD negative control) [2].

The botulism protective titer threshold (0.33 ELISA units) was previously determined [2]. The proportions of animals achieving protective titer threshold were compared among parity groups using Pearson's χ^2 tests.

The means of antibody levels at various time points, within and among vaccination groups and among parity groups were compared by one-way analysis of variance (ANOVA) – Bonferroni comparison of means.

Linear regression modeling was used to analyze the effect of maternal anti-BoNT/D serum antibodies, at the time of the first immunization in calves, on the antibody response to the toxoid injections. Peak serum anti-BoNT/D antibody levels at 17 weeks after the first injection were used as the dependent variable, and the experimental group was included in the model as an independent variable.

Statistical analyses were performed using the program SPSS (SPSS Inc., version 10.0.1, 1999). *P*-values less than 0.05 were considered statistically significant.

3. Results

3.1. Anti-BoNT type D antibody levels in vaccinated cattle of various parities

At the time of the annual booster, the mean serum anti-BoNT/D antibody levels were 0.19, 0.14, 0.29, and 0.57 EU for parity 1, 2, 3, and 4, respectively (Fig. 1). The proportions of animals with serum anti-BoNT/D antibody levels above the protective threshold (0.33 EU) were 22%, 15%, 31%, and 70% for parity 1, 2, 3, and 4, respectively (Fig. 1). Only the mean serum antibody levels and the threshold proportion of parity 4 group differed significantly from other parity groups (ANOVA, $P < 0.05$ and Pearson's $\chi^2 = 22.41$, $P < 0.0001$, respectively).

3.2. Anti-BoNT type D antibody levels in experimentally vaccinated calves

During the experimental period, few calves were dropped out of the study for reasons not related to the study; at the conclusion of the study, 22 calves were sampled. After the primary series of two or three immunizations, 17 weeks after the first immunization, all calves had serum anti-BoNT/D antibody levels above the determined cut-off value. Thereafter, the levels of serum anti-BoNT/D antibodies declined and most calves in groups 1 and 2 (80% and 78%, respectively) had antibody levels below the cut-off value before the annual booster (Table 1). Immunization of calves in group 3, 24 weeks after the first immunization, resulted in a marked increase in serum anti-BoNT/D antibody levels and most (62.5%) were above the cut-off value before the annual vaccine (Fig. 2, Table 1). Two weeks after the annual vaccine, all calves had serum anti-BoNT/D antibody levels above the predetermined protective cut-off value (Fig. 2, Table 1).

3.3. Maternally derived anti-BoNT type D antibody levels and interference

The levels of maternally derived serum anti-BoNT/D antibodies in 2-month-old calves were compared to the levels of anti-BoNT/D antibodies in the same calves 3 months after the end of the initial vaccination series, before a fourth vaccine was administered to calves in group 3. At this time, 3 of the 32 calves were dropped out of the study. This comparison revealed that vaccination with botulinum toxoid at the age of 2 months did not result in interference, since no association was found between the initial level of serum anti-BoNT/D antibodies and their level thereafter ($\beta = -0.094$, $t = -0.548$, $P = 0.588$) (Fig. 3).

4. Discussion

Field vaccination studies against cattle botulism were already undertaken in the mid-1930s and the early 1950s in Australia, using a monovalent type C toxoid produced by the Commonwealth Serum Laboratories (CSL) in Melbourne [18]. Bivalent type C and D toxoids were available in the early 1960s from the Onderstepoort Veterinary Laboratories (South Africa) and from CSL. Vaccination of cattle with

Table 1
Percentage of calves above the botulism protective titer threshold following subcutaneous immunization with bivalent (C and D) botulinum toxoid using three different injection protocols (groups 1–3), as indicated by the arrows in Fig. 2

	Week									
	0	2	4	12	17	25	31	43	53	55
Group 1	45	20	27	100	100	87.5	78	50	20	100
Group 2	45	20	27	45	100	100	82	80	22	100
Group 3	45	27	100	64	100	90	100	89	62.5	100

Sera samples were obtained from each calf over a period of 55 weeks and assayed individually for the presence of IgG antibodies against BoNT/D. The botulism protective titer threshold (0.33 ELISA units) is denoted by a horizontal dashed line in Fig. 2.

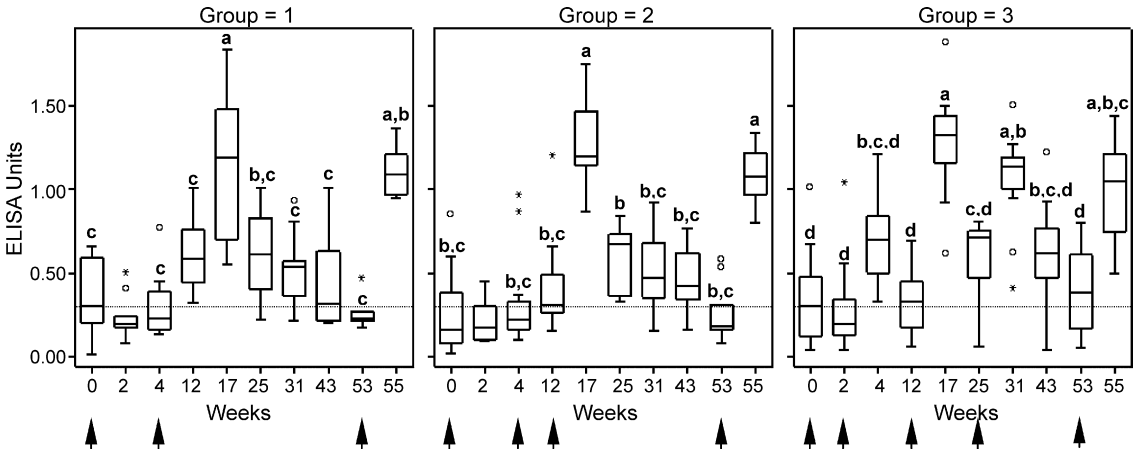

Fig. 2. Temporal response of anti-BoNT/D antibody levels in calves following subcutaneous immunization with bivalent (C and D) botulinum toxoid using three different injection protocols, as indicated by the arrows (groups 1–3). Sera samples were obtained from each calf over a period of 55 weeks and assayed individually for the presence of IgG antibodies against BoNT/D. Antibody levels in ELISA units are displayed by Box-plot diagrams. The botulism protective titer threshold (0.33 ELISA units) is denoted by a horizontal dashed line. The means of antibody levels within vaccination groups were compared by analysis of variance; the time points with different superscripts differed significantly when tested by one-way analysis of variance – Bonferroni comparison of means, $P < 0.05$.

bivalent toxoids conferred considerable immunity following exposure to BoNTs for extended time periods [18–20]. Here we show, using protective antibody levels derived from field outbreaks [2], that these botulinum toxoids do not elicit continuous protective levels following the current immunization protocol. It was initially suspected that vaccination of young calves in the presence of maternal antibodies interferes with immune response to the toxoids. Currently in Israel, calves are first vaccinated at the age of 8 weeks. At this age the levels of maternally derived antibodies vary and some still have high levels of anti-BoNT/D antibodies that were previously shown to be protective in natural outbreaks [2]. The presence of blocking levels of maternally derived antibodies might be

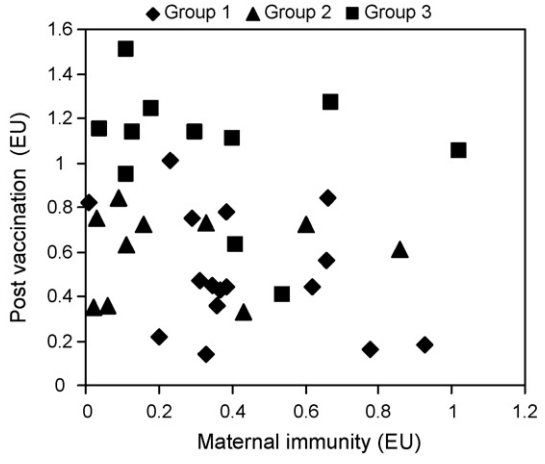

Fig. 3. Lack of interference by maternal antibodies responding to botulinum toxoid administered by subcutaneous injection to 2-month-old dairy calves. Vaccination schedules in experimental groups (1–3) are presented in Fig. 2. Antibody levels are displayed in ELISA units (EU). Sera samples were obtained from each calf just before the first injection (maternal EU) and after 17 weeks (post vaccination EU) and assayed individually for the presence of IgG antibodies against BoNT/D.

an obstacle to successful vaccination in young animals [21]. For example, French regulations do not allow rabies vaccination before 3 months of age in order to avoid any risk of interference [21]. The inhibitory influence of maternal antibodies on infant antibody responses is not restricted to live vaccines; this was also demonstrated following immunization with tetanus and diphtheria toxoids [22]. This study's results indicated that there was no association between the levels of maternally derived antibodies at priming dose and the level of antibodies after a series of booster vaccinations, thus indicating that no interference occurred following vaccination of 8-week-old calves with botulinum toxoids. Brown et al. previously came to the same conclusion [20]. They vaccinated 6–8-month-old steer weaners (first-time vaccination) and concluded that although some had high levels of maternal antibody, this had no effect on the immune response to vaccination.

Our results indicate that using the current vaccination protocol of a priming dose in 2-month-old calves, followed by booster doses after 4 weeks and annually thereafter, did not result in continuous protective levels of anti-BoNT/D antibodies. Continuous protective levels were found in most cattle in parities four or above at the time of the yearly booster, whereas most cattle in parities 1–3 were not protected at that time. These results are in agreement with epidemiological data from field outbreaks where attack rates were only 5.6% and 3% in these older cows, compared with 40.4% and 64% in younger animals [2,17]. These results differ from previously reported vaccination studies in cattle where vaccination of cattle with bivalent toxoid by either single or double immunizations resulted in protection to type D toxin for 50 weeks [19] or at least 24 months [18]. This discrepancy can be explained by the higher protective antibody levels defined by us after analyzing natural outbreaks [2]. The high attack rates, mortality rates, and nature of clinical

signs indicate that animals are exposed to very high levels of BoNT/D in botulism outbreaks in Israel. Therefore, higher levels of neutralizing serum antibodies are required. On the other hand, our results resemble those reported in humans, using similar aluminium phosphate-absorbed botulinum toxoids. The majority of humans immunized with the pentavalent (A–E) botulinum toxoid were antibody negative 52 weeks after the initial vaccination [23]. Low levels of neutralizing antibodies were also detected in humans 12 months after immunization with a botulinum tetravalent (A, B, E, F) toxoid [24]. Immediately before administering the annual first booster, 10 of 21 (48%) and 14 of 21 (67%) people lacked a detectable titer for type A and for type B BoNT, respectively [25]. Eight months after the initial vaccination with type F toxoid, about 40% of the people were antibody negative [26]. We show here that adding to the current protocol a 6-month booster dose resulted in continuous protective levels of anti-BoNT/D antibodies well above the cut-off protective levels derived from previously analyzed field outbreaks (study protocol 3). Additional booster dose 12 weeks after the priming dose did not result in continuous protective levels of anti-BoNT/D antibodies (study protocol 2) compared to the currently used protocol (study protocol 1). Another important finding was the presence of highly variable immune responses among individual animals, and the presence of a few completely non-responsive individuals. This phenomenon was also demonstrated in humans [26].

In conclusion, the current botulism vaccination protocol, using the commercially available bivalent toxoids, is inadequate and a 6-month booster dose is required after the initial priming and booster doses. The initial vaccination can be administered in the presence of maternal antibodies without compromising the immune response to these toxoids. We expect that 6 monthly vaccinations will elicit continuous protective levels of anti-BoNT/D antibodies, which most probably can be reduced to an annual vaccination after 3–4 years, though this issue will have to await future analysis of these vaccinated animals.

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