Title: Duration of tetanus IgG titres following basic immunisation of horses

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Abstract

Reasons for performing study: Recommendations for prophylactic vaccination against tetanus in horses vary greatly between countries and have scarce scientific support in the peer-reviewed literature. In human medicine, recommended booster vaccination intervals are also very variable, but are considerably longer than for horses. More information is needed about the duration of immunity induced by modern vaccines.

Objectives: To investigate if the duration of antibody titres previously determined to be protective against tetanus differ from what is indicated by recommended vaccination intervals for horses.

Study design: Prospective seroconversion study.

Methods: Thirty-four horses were enrolled for basic immunisation with an ISCOM Matrix-combination vaccine (Equilis® Prequenza Te). Horses received the first vaccination at 5-11 months of age, and the second dose 4 weeks later. A third vaccine dose was given 15-17 months after the second dose. Serum tetanus antibody titres were analysed by ToBi ELISA 2 weeks as well as 14-16 months after the second dose. After the third vaccine dose, titres were checked once yearly for 3 years. Results were described by age and level of antibody titre at first sampling.

Results: Two weeks after the second dose all horses (34/34) had antibody levels that exceeded the limit of detection, 0.04 IU/ml. After 16 months the levels were above 0.04 IU/ml in 28/33 horses, the remaining 5 horses potentially had suboptimal protection against tetanus. After the third vaccine dose antibody levels remained above 0.04 IU/ml in 25/26 horses for 1 year, 16/16 horses for 2 years, and 8/8 horses for 3 years.

Conclusions: Horses that undergo basic immunisation with 3 doses of vaccine after the age of 5 months are likely to have serum antibody titres consistent with protection against tetanus for more than 3 years. Current guidelines for tetanus prophylaxis should be revised.

Introduction

Tetanus prophylaxis is part of routine veterinary care for horses in the industrialised world, but recommendations for best practice vary widely between countries. For example, the AAEP guidelines recommend annual boosters after basic immunisation, but state that protective titres may persist for longer [1]. In Sweden, the general recommendation for practitioners is to give a tetanus vaccination booster once every 3 years, whereas in the UK it is generally recommended to give the booster every 2 years. In New Zealand, tetanus vaccines are registered for boosters at 5-year intervals after basic immunisation (http://www.ivsonline.co.nz). The situation is similar in human practice, where the recommended booster intervals after basic immunisation vary between countries. However, all intervals are considerably longer than postulated for horses with at least 10-20 years between boosters being the norm.

Horses are one of the more susceptible species to tetanus based on relative amount of toxin per weight required to produce lethal disease [2]. This is coupled with the fact that horses may often be exposed to environments containing spores of *C. tetani*, increasing the risk of contamination of wounds. These factors warrant good prophylaxis, however, more evidence-based knowledge is needed on the duration of immunity. Previous studies have examined long-term duration of titres [3-9], and these consistently show that what is thought to be protective titres (>0.01 IU/ml) are well maintained for several years, but the vaccines used in these studies often contain adjuvants that are no longer in use such as water in oil emulsions, and results may not be able to be extrapolated to vaccines currently available.

The aim of this 3-year longitudinal study was to determine the development and duration of tetanus antibody titres after basic immunisation of horses, using a combined tetanus and influenza vaccine[1] with ISCOM matrix.

Material and Methods

Horses

Thirty-four privately owned horses were enrolled at the start of the study. Horses were identified through convenience sampling; owners known to the researchers were approached and offered to participate based on likely availability for follow-up for the length of the study period. Horses were eligible if they were between 5 and 11 months of age, had not previously been vaccinated and were in good health as reported by the owners. Horses were managed and housed according to the owners' routine at 6 different facilities. In addition to the study protocol the horses were only vaccinated against influenza, according to the owners´ management procedures. Once enrolled, exclusion criteria were tetanus vaccination for other reasons than the study (for example at the treating veterinarian's discretion if the horse sustained a wound) or steroid treatment. Owners could remove horses from the study at any point should they wish to do so.

Vaccine

The vaccine consisted of an aqueous suspension of purified haemagglutinin and neuraminidase proteins of equine influenza virus together with 40 Lf/dose of tetanus toxoid. Each dose of 1 ml contained 375 µg ISCOM matrix as adjuvant.

Vaccination and sampling

Horses received the first vaccination with Equilis® Prequenza Te[1] at the start of the study and the second dose 4 weeks later. A third vaccine dose was given 15-17 months after the second dose. This protocol was based on the recommendations for basic immunization against tetanus with the used vaccine. Serum samples were obtained by jugular venepuncture prior to the first vaccination, 2 weeks after the second vaccination, 14-16 months after the second vaccination and once yearly for 3 years after the third vaccination (Figure 1). The blood was

centrifuged on site after sampling, and serum was frozen as soon as possible prior to transportation to a -80°C freezer. Samples were kept at -80°C until the time of analysis (1-2 years) and samples were analysed consecutively on 3 separate occasions throughout the study.

Samples were transported to the laboratory on dry ice, ensuring that the samples were kept frozen until analysis.

Analysis

Antibody levels were determined by tetanus toxin-binding ELISA (ToBi ELISA) as previously described [8]. Twofold serial dilution of serum samples were made in a microtiter plate. After addition of a fixed dose of tetanus toxoid, the plates were incubated. During incubation the neutralizing tetanus antibodies in the serum are bound to the toxoid. The following day, the content of the plates was transferred to a novel microtiter plate coated with tetanus toxoid specific antibodies to determine the amount of non-neutralized (unbound) tetanus toxoid still remaining in the serum sample. Biotinylated tetanus specific antibodies and avidin-peroxidase were used to visualize the captured tetanus toxoid in an ELISA, thereafter the antibody titres in the samples were calculated. The WHO International Standard for tetanus antitoxin was used as a standard in each test. The limit of detection was 0.04 IU/ml.

Data analysis

The non-normally distributed antibody titre levels, at each time point, were described as medians and interquartile range (IQR). Categorical variables were described as counts and percentages. Where appropriate, variables were stratified by age, sex and breed. An outcome variable of detectable titre level at the start of the study (<0.04 IU/ml) was created as a binary variable (0=<0.04 IU/ml, 1=>0.04 IU/ml). The Wilcoxon Mann Whitney test was used to compare the outcome of detectable titre level and the median age of horses at the start of the study and the titre level of horses at time points 1, 2, 3, 4 and 5. Median and IQR antibody levels were represented graphically, stratified by detectable titre level at the start of the study (Figure 2). Other than for the categorical outcome of detectable titre level, antibody levels below the detectable limit were excluded from the statistical analyses. All analyses were conducted using Stata version 11.

Results

Titres were obtained for 34 horses after the first vaccination but numbers declined throughout the study for reasons unrelated to this project and 8 horses (24%) remained at the end of the experimental study period (Table 1). Titres for individual horses are provided as supplementary material. Horses received their first vaccination (V1) at a median age of 7 months (IQR 6 to 8 months). Age at the start of the study was missing for one horse.

At the first serum sampling, 13 horses (38%) had antibody levels below the limit of detection (<0,04 IU/ml). Horses with no detectable antibodies had a median age of 7.5 months (IQR 6 to 8 months), compared to horses with detectable antibody levels with a median age of 6 months (IQR 6 to 7 months; P<0.02). Two weeks after the second vaccine dose (V2) horses

with no detectable antibodies at time point 0 had a median titre of 8.23 IU/ml (IQR 4.61 to 13.98 IU/ml), compared to a titre of 2.16 IU/ml (IQR 1.10 to 4.73 IU/ml; P<0.01) for horses that did have detectable antibodies at time point 0 (Figure 2). There was no significant difference between horses with detectable and no detectable antibodies at time point 2 (P<0.25), time point 3 (P<0.17), time point 4 (P<0.08) or time point 5 (P<0.12).

Two weeks after the second dose, all 34 horses had antibody levels that exceeded 0.04 IU/ml. After 16 months the levels were above 0.04 IU/ml in 28/33 horses (85%). After the third vaccine dose antibody levels remained above 0.04 IU/ml in 25/26 horses (96%) for 1 year, all 16/16 horses for 2 years, and all 8/8 horses for 3 years.

Discussion

This study suggests that horses that undergo basic immunisation with 3 doses of tetanus vaccine after the age of 5 months are likely to have serum antibody titres consistent with protection against tetanus for more than 3 years. Long term studies of adult horses have shown that most horses have titres above 0.01 IU/ml for 5-8 years after basic immunisation [3,4,9]. However, adult horses likely mount a stronger immune response than horses < 1 year old [7]. Also, horses that have undergone previous immunisations may not be comparable to naïve, not previously vaccinated individuals. The minimum IgG titre level for protection of horses has been set to 0.01 IU/ml. This is likely a direct extrapolation from the human recommendations for protective titres, which in turn are based on studies in guinea pigs [5,10-12], and is to the best of the authors' knowledge not based on experimental evidence that a slightly lower titre would put horses at risk of disease after intoxication. In fact, in one study a horse with a serum IgG level as low as 0.0025 IU/ml failed to develop signs of tetanus

after subcutaneous injection of 3 times the lethal dose of tetanus toxin [3]. The ToBi ELISA used in this study is comparable to the mouse inoculation test [13] and was chosen for ethical and animal welfare reasons in order to decrease the use of lab animals. Unfortunately the limit of detection for this method of analysis was 0.04 IU/ml, which is above the suggested limit for protection. Therefore, the horses that were below the limit of detection may or may not have been above the least accepted IgG level of 0.01 IU/ml. In the present study, 13 horses had IgG titres below 0.04 IU/ml before the first vaccination. Horses developed a strong antibody response after the two initial vaccinations despite the presence of maternal antibodies, confirming results from a previous study [9]. Within 2 weeks all horses had high titres (Figure 2), but horses with maternal antibodies present had a significantly lower response than horses with no detectable antibodies at the start of the study, indicating that the maternal antibodies may interfere with the immune response to tetanus vaccination. Maternal antibodies have previously been suggested to interfere with the response to tetanus vaccination [7], but that study may have been biased by the young age of the foals (3 months) as Jansen and Knoetze (1979) have shown that foals less than 3 months of age are unable to respond to vaccination, even in the absence of maternal tetanus antibodies. The fact that all horses had high antibody titres 2 weeks after the second dose of vaccine suggests that elective surgical procedures could safely be done at this time. Fourteen to 16 months after the two basic immunisations, 5/33 (15%) horses had antibody titres below 0.04 IU/ml. As it was unknown if these horses were below the proposed limit of protection, a third vaccination was included in the immunisation protocol.

Recommendations for tetanus vaccination boosters vary widely between different countries. It is not always possible to find the scientific basis for these recommendations but some hypotheses can be made. The AAEP guidelines from 1995 [1] state that protective titres may

be attained for up to 5 years, but recommend yearly boosters for all horses and additional vaccination if a horse sustains a wound more than 6 months after the last booster. This is supported by a case series [14] where the prognosis for survival was better if horses had been vaccinated within one year. However, when looking more closely at this data only 4/20 horses in this data set were known to be vaccinated. Three of these 4 horses survived. It is not specified how many doses of vaccine these horses had been given, however, judging by the age of the horses and the information given, only one of the vaccinated horses could have received 3 tetanus vaccinations as a basic immunisation (in this text further referred to as "complete basic immunisation"). The Swedish recommendation of a 3 year booster interval was merely "decided" in 1991 at the time of product registration for one of the tetanus vaccines in the country (Agneta Gustafsson, pers com 2014). There is one recent study [8] showing that 7/7 horses had tetanus IgG titres above 0.04 IU/ml for two years after complete basic immunisation with Equilis Prequenza Te, indicating that yearly boosters are excessive. The New Zealand recommendation of a 5-yearly booster interval may be based on a paper by Liefman (1980) where the author recommends this booster interval in the discussion. Unfortunately, enquiries to the pharmaceutical companies responsible for these products in New Zealand have failed to yield information to confirm this. Comparison between immunisation studies is complicated by the use of different vaccines and adjuvants which may have some impact, especially when comparing more recent work to older experiments. Also, the early toxicity studies use different modes of inoculation (subcutaneous vs intramuscular vs inoculation by introduction of foreign material laced with toxin) [3,15] which is likely to influence the antibody titre required for protection. The distance from the port of entry to the central nervous system (CNS) and the dose of toxin is likely to impact [15,16] as introduction of spores or toxin closer to the CNS may warrant higher IgG levels for protection

than more peripheral injuries. In 104 reported equine cases of tetanus [14,17-19] none of the horses were known to have been completely vaccinated according to any of the current guidelines. There are cases with complete tetanus immunisations that have shown clinical signs of tetanus (Gaby van Galen, pers com 2014), but to the best of the authors´ knowledge, there are currently no reports of a horse with proven complete basic immunisation dying of or being euthanized due to severe tetanus.

The fact that one horse had IgG levels below 0.04 IU/ml a year after complete basic immunisation may be of concern as it was not possible to distinguish if the horse was above the traditional cut-off of 0.01 IU/ml. This horse was excluded from the study and vaccinated at this time, and responded well with high serum titres found on testing the following year (5.84 IU/ml), data is not shown graphically or in the supplementary material as this horse was excluded from further analysis. It is unclear why this individual did not respond like the other horses. Several causes for failure are possible. Inherent individual low response is possible but unlikely in this case as the horse responded well to the first two vaccinations, and had a good response to the booster vaccination once removed from the study. Vaccine failure is possible due to incorrect storage or injection, however, other horses in this study were vaccinated at the same time and showed an appropriate IgG response. Some horses in the study showed an increase in anti-tetanus antibodies at time points when they had not received vaccinations. The reason for this is not known, but several mechanisms are possible. Firstly, this difference could be due to expected level of error for the serum ELISA. Variation in the method of analysis could account for some of the difference and ideally all the samples should have been analysed at the same time. However, this was not possible as the titres had to be assessed during the study in order to ensure that horses had acceptable levels of anti-

tetanus antibodies for protection. Acquired immunity is also possible as horses may have experienced subclinical infection with tetanus and a subsequent rise in titres.

Although vaccinating often may pose little risk to the patient, veterinarians should strive to practice evidence based medicine. In countries where an annual vaccination against equine influenza is warranted, clients may elect to use a combination vaccine and thereby give a yearly booster of tetanus vaccine. However, in countries where influenza is not endemic, or in individuals that are not routinely vaccinated for reasons such as previous anaphylaxis, optimal recommendations for booster vaccination against tetanus is imperative. Tetanus is best prevented by prophylaxis, but the proposed titre limit of 0.01 IU/ml may be higher than needed for protection against disease. Current guidelines for tetanus vaccination are not based on sound scientific evidence and should be revised.

Manufacturer's details

[1]Equilis Prequenza Te, Intervet AB, Stockholm, Sweden

242 **Table 1**

243 The number and percentage of horses remaining in the study at each time point.

Variable	Level	Time point 0 Number (%) n=34	Time point 1 Number (% remaining)	Time point 2 Number (% remaining)	Time point 3 Number (% remaining)	Time point 4 Number (% remaining)	Time point 5 Number (% remaining)
Age at start of study*	5 months	2 (6)	2 (100)	2 (100)	1 (50)	2 (100)	1 (50)
	6 months	14 (41)	14 (100)	13 (93)	10 (71)	4 (29)	1 (7)
	7 months	6 (18)	6 (100)	5 (83)	5 (83)	5 (83)	2 (33)
	8 months	8 (24)	7 (88)	8 (100)	8 (100)	3 (38)	2 (25)
	9 months	2 (6)	2 (100)	2 (100)	1 (50)	1 (50)	1 (50)
	11 months	1 (3)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)
Sex	Colt	18 (53)	17 (94)	17 (94)	14 (78)	9 (50)	5 (28)
	Filly	16 (47)	16 (100)	15 (94)	12 (75)	7 (44)	3 (19)
Breed	Connemara	4 (12)	4 (100)	4 (100)	3 (75)	2 (50)	2 (50)
	Swedish Warmblood	30 (88)	29 (97)	28 (93)	23 (77)	14 (47)	6 (20)

244 *One horse with a missing value for age at the start of the study

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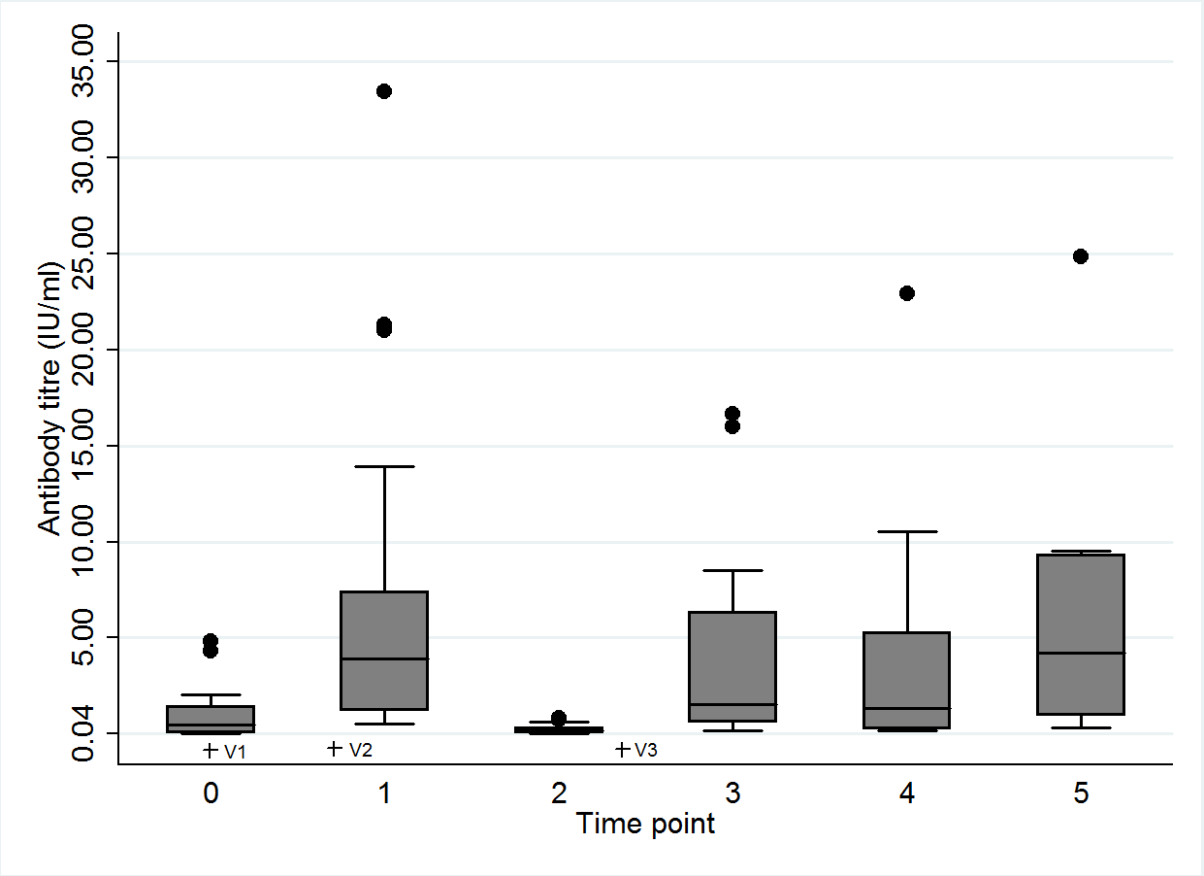
Figure legends

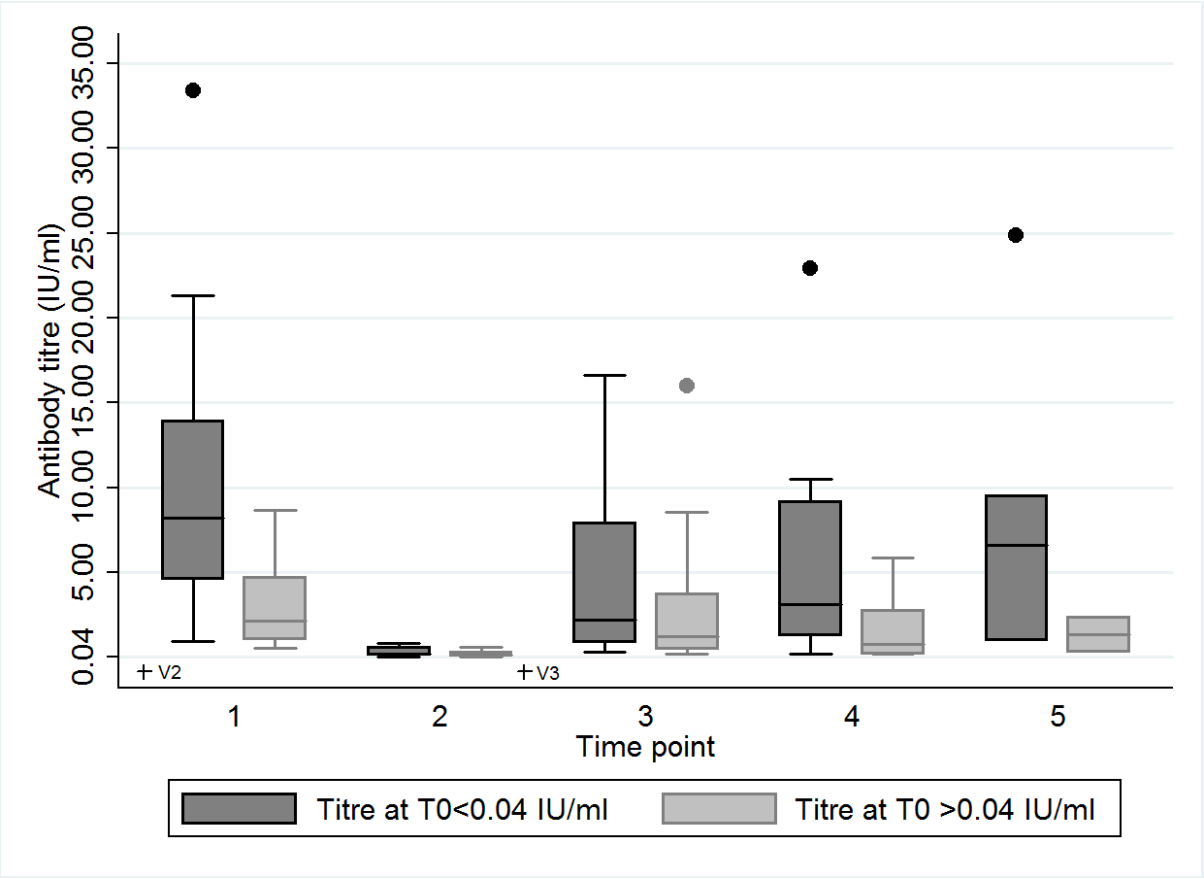
Figure 1



Median and IQR for anti-tetanus titres at the start of the study (time 0, n=20), two weeks after basic immunization with two doses of vaccine (time 1, n=34), 14-16 months after basic immunization (time 2, n=28), and yearly thereafter (time 3, n=25, time 4, n=16 and time 5, n=8). Horses with titres <0.04 were not included in the box plot.

Time points for vaccinations in relation to testing are indicated as V1-V3.

Figure 2



Box plot showing horses with and without detectable (0.04 IU/ml) antibodies at the start of the study. The groups were only significantly different (P<0.01) at time point 1, i.e. 2 weeks after basic immunization.

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Supplementary information items

Individual antibody titres for all horses