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Changes in Serum Antibody Levels after Vaccination for Strangles and after Intranasal Challenge with *Streptococcus equi* subsp. *equi* in Horses

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In this study, to evaluate the influence of strangles vaccination on serological test results, we investigated the changes in strangles serum antibody levels in horses after vaccination and subsequent intranasal challenge with S. equi. The horses were vaccinated for strangles with either a component vaccine (Group C) or a live vaccine (Group L). We measured changes in strangles serum antibody levels weekly for 20 weeks after vaccinating horses twice for strangles over a 3-week interval, and for 7 weeks after intranasal challenge with S. equi in the same horses. Serum antibody responses to the proline-glutamic acid-proline-lysine (PEPK) antigen with five repetitions (PEPK-5R) were higher at all times (up to 2.4-fold) following vaccination in Group C than in Group L, and the value peaked at 2.9-fold above the initial value after the second vaccination in Group C horses. However, the value was lower than that in horses infected with S. equi, and it gradually decreased, reaching the initial (week 0) value by the 15th week. Serum antibody responses to PEPK-5R after challenge with S. equi increased in both groups of horses, but the value tended to be lower than that reported for unvaccinated horses. In addition, the average value in Group C was 2.6-fold higher than that of Group L. These results suggest the serum antibody responses of horses infected with S. equi varies according to the type of vaccine with which they have been vaccinated. Although the serological diagnostic test for strangles in which PEPK-5R is used as an antigen is effective for the investigation of serum antibodies to strangles in vaccinated horses, the present data suggest it is necessary to consider the vaccination history when interpreting the results.

Key words: *horse, PEPK antigen with five repetitions, serological diagnostic test, strangles, Streptococcus equi*

Strangles is a contagious disease of the upper respiratory tract characterized by the abrupt onset of fever followed by upper respiratory tract catarrh. In addition, strangles is characterized by a mucopurulent nasal discharge and acute swelling with subsequent abscess formation in the submandibular and retropharyngeal lymph nodes [4, 8, 9, 11]. Strangles is a commonly diagnosed and important infectious disease of horses worldwide [6].

A serological diagnostic test that could specifically diagnose strangles would be extremely helpful for its diagnosis and for undertaking epidemiological surveys. However, there has not been a sufficiently specific serological diagnostic assay [3, 12, 13], because sera from *Streptococcus equi* subsp. *equi* (*S. equi*)-infected horses can not be definitively distinguished from sera from *Streptococcus equi* subsp. *zooepidemicus* (*S. zooepidemicus*)-infected horses. Recently, it has been reported that an enzyme-linked immunosorbent assay (ELISA) method using the proline-glutamic acid-proline-lysine antigen with five repetitions (PEPK-5R) has proven useful as a serological diagnostic test for strangles (PEPK-5R-ELISA) [5], and that sera from *S. equi*-infected horses

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are clearly distinguishable from sera from *S. zooepidemicus*-infected horses. However, that report did not mention the influence of strangles vaccination on the serological diagnostic test. Because strangles vaccines are used relatively frequently in many countries, it is important to distinguish in a clinical application of the serological diagnostic test whether increases in serum antibody levels are caused by strangles infection or by prior vaccination against strangles. Moreover, it is important for the diagnosis and epidemiological study of strangles to determine if and how serum antibody levels change in vaccinated horses after *S. equi* infection.

In this study, to evaluate the influence of strangles vaccination on the serological test results, we investigated changes in strangles serum antibody levels in horses after vaccination and subsequent intranasal challenge with *S. equi*.

Materials and Methods

Vaccine

This study employed a component vaccine (Strepguard®, Intervet, U.S.A.) and a live vaccine (Pinnacle™ I.N., Fort Dodge Animal Health, U.S.A.). Detailed information on the elements of the component vaccine is unavailable.

Experimental study

The Animal Use and Care Committee and Animal Welfare and Ethics Committee of the Equine Research Institute, Japan Racing Association, approved the protocols for this study. Six thoroughbreds (1 year old; five female and one male) were used for this study. These horses had no history of strangles, and were clinically confirmed to have been in good health for at least six months. The six thoroughbreds were randomly divided into two groups, horses inoculated intramuscularly with the component vaccine (horses 1–3, Group C) and horses inoculated intranasally with the live vaccine (horses 4–6, Group L). Both groups were vaccinated twice, once each at weeks 0 and 3. Clinical signs were assessed and nasal cavity swabs and serum samples were drawn just before and every week following the vaccination. The horses were examined twice a day for clinical signs, such as reduced levels of appetite, rhinorrhea, coughing, enlarged lymph nodes, spontaneous rupture of the lymph nodes, abnormal lung sounds or increased rectal temperature. Sera and nasal cavity swabs were obtained from these horses every week for up to 30 weeks after the initial vaccination.

Intranasal challenge with S. equi

The challenge with *S. equi* was performed by intranasal inoculation of 1×10^8 colony forming units (CFU) of an overnight broth culture of *S. equi* strain CF32 at 23 weeks after the initial vaccination. Clinical signs were monitored and sampling was performed as described above for 7 weeks. All the horses' clinical signs were treated with antipyretics and the horses were rehydrated if necessary. They were euthanized by an intravenous injection of a mixture of sodium thiopental (Ravonal, Tanabe Pharmaceutical, Osaka, Japan) and suxamethonium chloride (Relaxin, Kyorin Pharmaceutical, Tokyo, Japan) 7 weeks after the challenge, and necropsied .

Isolation and PCR examination of S. equi

The nasal cavity swab was suspended in 500 μ*l* of normal saline solution. *S. equi* was isolated by inoculating Colombian agar containing 5% horse blood with 50 μ*l* of the suspension, and *S. equi* was identified using API Strep 20 (SYSMEX bioMerieux, Tokyo, Japan). Total DNA was extracted from the suspension using InstaGene Matrix (BIO-RAD, U.S.A.), and the *S. equi seM* gene was detected with a semi-nested PCR method [1]. Additionally, the *seM* genotype of the isolated *S. equi* was analyzed as described in a previous report [2].

Detection of serum antibody to S. equi

The serum antibody to *S. equi* was detected as described in a previous report by using PEPK-5R [5]. Briefly, microtitre plates (MAXISORP, NUNC, Denmark) were coated with 50 μ*l*/well (in 10 μg/m*l* of 10 mM phosphate-buffered saline solution: a large volume of phosphate-buffered saline (25X®), Lab Vision, U.S.A.) of the synthesized PEPK-5R (>95% purity). After blocking (1:4 diluted blocking buffer: BlockAce, Dainippon Pharmaceutical), 50 μ*l* samples of diluted (1:100) test sera (in 1:10 diluted blocking buffer) were added to the wells in triplicate. Bound proteins were reacted with horseradish peroxidase-conjugated anti-horse IgG (H+L) (Goat Anti-Horse IgG (H+L)-HRP, Southern Biotechnology Associates, U.S.A.) in 1:10 diluted blocking buffer. Color was detected by adding a substrate solution (Horseradish Peroxidase Substrate Kit, BIO-RAD, U.S.A.) and the reaction was stopped by adding 2% oxalic acid (50 μ*l*/well). The absorbance at 415 nm was measured with an ELISA reader (Spectrophotometer DU 800, Beckman Coulter, U.S.A.). Data are expressed as optical density (OD).

Table 1. Clinical examination and postmortem examination findings after vaccination for strangles and intranasal challenge with *S. equi* in 6 horses

Group	Horse no.	Clinical signs		Postmortem examination	
		after vaccination	after challenge	abnormal findings	isolation of *S. equi*
C	1	none	EL	EL	–
C	2	none	Fever (39.3°C), RAc, RAp, Rh, EL	EL	–
C	3	none	Fever (39.3°C), RAc, RAp, Rh, EL, SRL	EL, SRL	+
L	4	none	EL	EL	–
L	5	none	Fever (38.5°C), RAc, RAp, Rh, EL, SRL	EL, SRL	+
L	6	none	Fever (40.2°C), RAc, RAp, Rh, EL, SRL	EL, SRL	+

Group C (horses 1–3) were inoculated the component vaccine. Group L (horses 4–6) were inoculated the live vaccine. Fever: increased rectal temperature (≥38.5°C); RAc: reduced activity; RAp: reduced appetite; Rh: rhinorrhea; Co: coughing; EL: enlarged lymph nodes; SRL: spontaneous rupture of lymph nodes; ALS: abnormal lung sounds.

Statistical analysis

Optical density values were compared with Student's *t*-test between Group C and L at weeks 0 and 23 to determine if differences between the groups existed at the start of each treatment (vaccination or inoculation). Differences between pre-vaccination (week 0) and pre-inoculation (week 23) OD were evaluated with the paired *t*-test to determine if effects of the vaccinations persisted at the time of inoculation. Optical density values were analyzed with 2-way ANOVA for repeated measures (RMANOVA) between Group C horses and Group L horses with vaccine and time as factors for both the post-vaccination interval (weeks 1–23) and the post-innoculation interval (weeks 24–30). Values of $p<0.05$ were considered significant. The data are expressed as mean ± SD.

Results

Clinical observation after vaccination

In the six tested horses, no abnormal clinical findings were observed following vaccination (Table 1). In addition, *S. equi* was not isolated and the *S. equi seM* gene was not detected a week after the vaccinations in the horses (4–6) inoculated with live vaccine.

Clinical observation after intranasal challenge with S. equi

Although pyrexia was observed in all horses challenged with *S. equi*, only horses 3, 5 and 6 suffered spontaneous rupture of lymph node (submandibular lymph node) abscesses (Table 1).

Postmortem examination

Although swelling of the cranial lymph nodes (especially the submandibular and retropharyngeal nodes) was found in all of the tested horses, *S. equi* was only isolated from the spontaneously ruptured lymph nodes (submandibular lymph node) of horses 3, 5 and 6 (Table 1). In addition, *seM* gene analyses confirmed that the isolated *S. equi* was the inoculated strain, CF32.

OD values of test sera

Remarkable increases of the antibody level were not seen in either of the vaccination groups, but changes in antibody levels were different between them. Initial (week 0) OD values for both groups of horses were comparable: 0.112 ± 0.015 vs. 0.122 ± 0.148 for Groups C and L, respectively, $p=0.91$. The OD of sera from Group C horses increased 2.9-fold above their initial value after the second vaccination, with a maximum OD of 0.328 ± 0.186 (Fig. 1). The OD of Group C horses decreased with time and returned to their initial (week 0) values in the 15th week ($p=0.89$). Only one horse in Group L showed a slight increase in OD following the second vaccination, and Group C OD values were higher at all times following vaccination than those of Group L, with a maximum difference of 2.4-fold. Despite these consistent differences, comparison of the OD values following the second vaccination and before inoculation (weeks 4–23) with 2-way RMANOVA detected no significant difference in the responses to the two vaccines ($p=0.376$), although time had a significant effect ($p<0.001$) and the vaccine × time interaction ($p=0.058$) bordered on significance.

Serum antibody responses to PEPK-5R after challenge with *S. equi* increased greatly in both groups of horses, and the average OD in Group C was 2.6-fold higher than that of Group L (Fig. 1). The peak OD occurred in both groups at 4 weeks after innoculation, with mean values of 0.752 ± 0.062 and 0.345 ± 0.169 for Groups C and L, respectively.

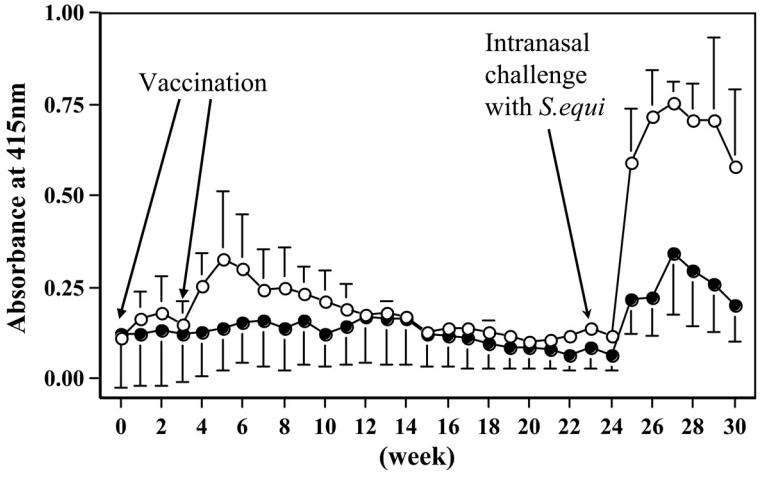

Fig. 1. Alterations in serum antibody levels (measured as optical density) for PEPK-5R in horses vaccinated for *S. equi*, and after intranasal challenge with *S. equi* five months later. Three horses in Group C (open circles) were inoculated with component vaccine and three horses in Group L (closed circles) were inoculated with live vaccine. Both groups were vaccinated at weeks 0 and 3. Horses were challenged with *S. equi* in week 23. Data are expressed as mean ± SD. The time × vaccine interaction of the post-vaccination response (weeks 4–23) was not significant when tested with 2-way RMANOVA. The serum antibody responses in the post-inoculation period (weeks 24–30) were significantly different between the two vaccines when tested with 2-way RMANOVA.

The 2-way RMANOVA detected a significant difference in the responses of the horses administered the two vaccines ($p=0.003$), but no differences due to time or time × vaccine interaction, *S. equi* isolation and the rise of serum antibody levels.

Discussion

Although serum antibody levels increased in Group C horses after the second vaccination, the increase was small, and the highest mean OD-value (0.328) was lower than the cut-off OD-value (0.427) for normal horses, the mean + 3 SD of sera from 3,106 uninfected control horses determined in a previous study [5]. In addition, the increase in serum antibody levels was transient in Group C, and it returned to the pre-vaccination value after 12 weeks. In Group L, serum antibody levels for PEPK-5R rose only minimally following vaccination, and the highest OD value (0.171 ± 0.127) was also lower than the cut-off OD value. Therefore, regarding the two vaccines tested in this study, vaccinated horses are unlikely to be misidentified as naturally infected horses by this serological diagnostic test.

There are other strangles vaccines on the market in addition to the two used in this study, and their effects and problems associated with their use have been reported [7, 10, 14]. However, the influence of other vaccines on the PEPK-5R-ELISA has not yet been investigated. Further studies using other vaccines will be necessary for accurate interpretation of the test results.

Although the challenge with *S. equi* resulted in some pathogenesis, the clinical signs were relatively slight, indicating that the vaccination was effective in suppressing the clinical signs. However, in general, vaccination did not prevent clinical strangles completely, and the development of a more effective vaccine is needed for the control of this disease.

The increases in serum antibody responses to PEPK-5R after challenge with *S. equi* in this study were slight (maxima 0.752 ± 0.062 and 0.345 ± 0.169 for Groups C and L, respectively) compared with those previously reported for unvaccinated horses challenged with *S. equi* (OD 1.707 ± 0.244) [5]. Serum antibody responses to PEPK-5R of Group C horses exceeded the cut-off value (0.427) for about 4 weeks after the challenge and the highest OD value (0.650) previously reported for a single uninfected horse [5]. However, serum antibody responses to PEPK-5R of Group L horses were even less than the cut-off value (0.427) and the highest OD value (0.650) [5], although the latter value falls within the 95% confidence intervals of both Group C

and Group L means. These results suggest that it is important to consider vaccination history when interpreting serological diagnostic results in epidemiological investigations of strangles.

There was a significant difference in the serological responses of the two vaccines following the challenge even though the horses' clinical signs and postmortem examinations were similar. We could not determine in this study what aspect of the vaccines caused this difference.

In conclusion, the results of this study suggest the possibility that the increased serum antibody responses to PEPK-5R in horses infected with *S. equi* may be markedly reduced if the animals were previously vaccinated. Accurate interpretation of the serological test results may depend on taking the vaccination history into consideration.

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