

# Technical Bulletin

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## Effect of METASTIM<sup>®</sup> adjuvant on the immune response in horses vaccinated with killed A/equi/Kentucky/97 equine influenza virus and challenged with the A/equi/Kentucky/07 strain

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### Key Points

- An experimental killed virus (KV) vaccine containing the Kentucky/97 (KY97) strain of equine influenza virus (EIV) combined with the proprietary METASTIM<sup>®</sup> adjuvant (KV-KY97 + METASTIM) was protective for at least 3 months against the KY07 subtype currently circulating in the North American horse population.<sup>1</sup>
- Following challenge with KY07 3 months after vaccination, 6 of 6 non-vaccinated control horses developed clinical disease versus 1 of 7 horses vaccinated with KV-KY97 + METASTIM and 2 of 7 horses vaccinated with KV-KY97 vaccine formulated with a conventional aluminum gel adjuvant (KV-KY97 + alum gel).
- Compared to non-vaccinated control horses, both vaccinated groups had significantly lower ( $p \leq 0.0045$ ) levels of EIV shedding at post-challenge days 3 through 8.
- The KV-KY97 + METASTIM group had lower levels of EIV shedding than the KV-KY97 + alum gel group from PC days 2 through 8.
- Since the antigen content of the two vaccines was the same, these results indicate that the adjuvant may be responsible for improved protection in horses vaccinated with KV-KY97 + METASTIM.

*Results of a study conducted at:  
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Killed-virus (KV) vaccines in general as well as all commercial KV equine influenza

vaccines require an adjuvant to elicit a protective and prolonged immune response.<sup>2,3</sup> Various adjuvants are available, with some proving more effective than others.<sup>3</sup> METASTIM, a proprietary oil emulsion adjuvant, is used in a commercial equine influenza vaccine containing the KV A/equi/Kentucky/97 (KY97) strain

(FLUVAC INNOVATOR<sup>®</sup>, Zoetis). A challenge-of-immunity study was conducted to compare the immune responses of horses vaccinated with an experimental formulation of KV-KY97 vaccine adjuvanted with METASTIM and a conventional aluminum gel adjuvant. Except for the adjuvant, the two vaccines were identical in all respects. The challenge agent was an A/equi/Kentucky/07 (KY07) isolate, an American-lineage H3N8 subtype currently prevalent as a cause of clinical disease in North American horses. The study's objectives were to evaluate whether there was any difference in the immune response to a METASTIM adjuvanted vaccine versus an identical counterpart adjuvanted with aluminum gel, and whether the KV-KY97 strain was cross-protective against the contemporary KY07 strain of equine influenza virus (EIV).

## Study Design

Twenty EIV-seronegative yearling horses were randomly assigned to one of three treatment groups. Group 1 (n=6) served as non-vaccinated controls. Group 2 (n=7) was vaccinated with KV-KY97 + METASTIM, formulated according to the outline of production for the commercial product. Group 3 (n=7) was vaccinated with KV-KY97 at the same potency as the Group 2 vaccine, plus an aluminum gel adjuvant (KV-KY97 + alum gel). The investigators were blinded to the identity of the vaccines, which were identical in appearance.

Vaccine was administered in two 1 mL IM doses given 26 days apart (days 0 and 26). Three months after the second vaccination (on day 118), all horses were challenged with virulent KY07 administered in a dose of  $10^7$  egg-infectious units per  $m^3$ , as previously described.<sup>4</sup> Horses were exposed to the aerosolized challenge agent for 45 minutes in a tented stall.

Blood samples were obtained at the time of each vaccination, on the day of challenge, and at 7 and 14 days after challenge for serologic assays. Daily post-challenge (PC) clinical examinations were performed for 8 days using a clinical disease scoring system that evaluated nasal discharge, respiration, demeanor, and lung abnormalities as determined by auscultation. A score of zero was given if any of the clinical signs was absent. Fever was determined by

a rectal temperature  $\geq 103.5^\circ\text{F}$ , well above the generally accepted threshold of  $102^\circ\text{F}$  for equine pyrexia. Nasopharyngeal swabs were obtained on the day of challenge and for 8 days thereafter to evaluate the incidence and extent of EIV shedding. Viral shedding was measured by quantitative reverse transcription PCR analysis calculated by the velocity of genomic amplification.<sup>5</sup> A Least Squares Mean (LSM) for each test group was calculated for each outcome and time point. Statistical significance of quantitative values was determined by repeated measures ANOVA at the 10% level of significance ( $p < 0.10$ ).

## Results

### Serologic results

Hemagglutination-inhibition (HI) titers were not significantly different between Groups 2 and 3 vaccinates at any time point (Figure 1). Vaccinated horses had LSM-HI titers greater than Group 1 control horses at every time point except the day of first vaccination and day of challenge. Group 2 vaccinates had a significantly greater Least Square mean (LSM-) HI titers than those for Group 1 controls at 7 and 15 days after challenge ( $p=0.0174$  and  $0.0023$ , respectively).

LSM total IgG titers as determined by ELISA were significantly greater ( $p < 0.001$ ) in Groups 2 and 3 versus Group 1 controls at 7 days following vaccination. The significant difference in LSM total IgG versus Group 1 controls persisted for Group 2 horses ( $p=0.0015$ ) through the day of challenge, but not for Group 3 ( $p=0.2183$ ). On the day of challenge, the LSM total IgG value for Group 3 was greater than that for Group 1 controls, but the difference was not significant (1493.3 vs. 892.3;  $p=0.2183$ ). LSM total IgG values for Group 2 were consistently greater than Group 3 values, with the difference being significant on the day of challenge (3521.8 vs. 1493.3;  $p=0.0347$ ). LSM IgGa and IgGb post-vaccination responses in Group 2 and Group 3 vaccinates generally corresponded to those for total IgG. On the day of challenge, the LSM IgGa for Group 2 was greater than the corresponding value for group 3 (1334.1 vs. 371.0;  $p=0.0600$ ).

## ***Clinical Protection***

Figure 2 shows the post-challenge clinical disease outcomes for the three treatment groups. Compared to the Group 1 controls, both vaccinated groups had a significantly lower ( $p \leq 0.0027$ ) LSM clinical disease incidence. Every control horse (6/6) was clinically affected versus 1 of 7 (14.3%) Group 2 vaccinates and 2 of 7 (28.6%) Group 3 vaccinates. The severity of challenge and the extent of protection were indicated by the number of horse-days that test animals were affected by fever (pyrexia  $\geq 103.5^\circ\text{F}$ ), coughing, and mucopurulent nasal discharge. The number of horse-days that Group 1 controls were affected by each of these measures was at least two-fold higher than the number for either vaccinated group. Group 3 vaccinated horses had twice as many horse-days that were febrile (5 vs. 2) and with mucopurulent discharge (14 vs. 7) compared to Group 2 vaccinates.

LSM rectal temperatures (Figure 3) were significantly greater ( $p \leq 0.0045$ ) in Group 1 control horses versus Groups 2 and 3 vaccinates on post-challenge days 2 through 7. Every Group 1 control horse had a rectal temperature  $\geq 103.5^\circ\text{F}$  for at least 2 days. Rectal temperatures  $\geq 103.5^\circ\text{F}$  occurred in only 2 of 7 Group 2 horses and 4 of 7 Group 3 horses, for one day each. The difference in LSM rectal temperatures between the two vaccinated groups was not significant following challenge. The  $103.5^\circ\text{F}$  threshold for pyrexia was a relatively high standard that applied to clinical disease scoring. However, it had no bearing on the significant post-challenge temperature differences that occurred between the two vaccinated groups and control horses.

## ***Viral Shedding***

LSM viral shedding as indicated by PCR values were significantly greater for Group 1 controls than those for Groups 2 or 3 vaccinates on days 3 through 8 ( $p \leq 0.0452$ ) following challenge. Viral shedding in both vaccinated groups declined faster and to a greater extent in the two vaccinated groups (Table 1), indicating transient local infection. LSM viral shedding values in the vaccinated horses favored Group 2, and were lower compared to Group 3 values at post-challenge days 5 through 7 ( $p \leq 0.0938$ ).

## **Conclusions and Clinical Relevance**

Although the test population was small, study results showed a consistent pattern of better serologic response, improved protection from clinical disease, and reduced viral shedding in Group 2 (KV-KY97 + METASTIM) and Group 3 (KV-KY97 + alum gel) vaccinated horses compared to non-vaccinated Group 1 controls. These outcomes were determined by objective measures (serologic assays, rectal temperatures, and viral shedding) as well as by subjective evaluation (clinical disease scores). All laboratory and clinical evaluations and the statistical analyses were made by administrators blinded to the treatment identity of the test animals, ensuring the validity of results.

Although the differences were not statistically significant, Group 2 horses vaccinated with KV-KY97 + METASTIM had a consistent pattern of better post-challenge clinical outcomes (Figure 2) and less viral shedding (Table 1) than Group 3 horses vaccinated with KV-KY97 + aluminum gel adjuvant. Moreover, Group 2 horses had a better mean IgG<sub>a</sub> level on the day of challenge compared to Group 3 horses. Collectively, these results may indicate that the METASTIM adjuvant has immuno-potentiating properties at least as good if not better than an aluminum gel adjuvant. The study indicates that choice of an adjuvant is not an incidental consideration, but can positively affect immunogenicity of KV-EIV vaccines.

As reflected by viral shedding on post-challenge days 3 through 8 and clinical disease outcomes (fever, coughing, nasal discharge), horses vaccinated with either of the KV-KY97 vaccines realized significant cross-protection ( $p \leq 0.05$ ) against virulent EIV KY07 challenge. Pyrexia in vaccinated horses lasted  $\leq 24$  hours, in contrast to protracted fever spikes in non-vaccinated Group 1 control horses (Figure 3). The increased fever, clinical disease scores, and viral shedding lasting from post-challenge days 2 through 7 in Group 1 control horses indicated that the test animals were exposed to a severe EIV challenge. The protection realized by vaccinated horses against the EIV challenge that affected 100% of non-vaccinated control horses demonstrated that EIV KY97 continues to be an effective, clinically relevant immunizing agent against contemporary

EIV strains dominant in North America, including KY07.

Not included in this preliminary report are results of post-vaccination CD4 and CD8 T-cell proliferation assays. A positive CD8 response would be an indication of cytotoxic, cell-mediated immunity (CMI) that limits local influenza virus infection prior to the onset of humoral immunity.<sup>6</sup> CD4 proliferation would indicate an antigen-specific humoral immune response. The positive IgGa and IgGb responses we observed in vaccinated horses is noteworthy because these serum immunoglobulins are associated with viral clearance and relatively long-lasting protective immunity, such as that which occurs following natural infection and what we observed in the 3-month challenge in our study.<sup>7-9</sup>

Whether the IgGa and IgGb responses to KV-KY97 + METASTIM are correlates of CMI remains to be determined. However, the antigen-distribution properties of the METASTIM oil-emulsion adjuvant may enable KV-KY97 to stimulate both a cellular and humoral immune response. To illustrate, recent studies have shown that immunotherapeutic vaccines with lipid-based delivery systems have enhanced immunostimulatory properties due to uptake by antigen presenting cells (APCs) following systemic administration.<sup>10,11</sup> In other words, the possibility of enhanced phagocytosis of lipid-embedded KY97 particles by macrophages may result in a more complete immune response, including CD4 and CD8 T-cell proliferation, than aluminum-gel adjuvanted KV-EIV vaccines. This could explain the improved serologic response, viral shedding results, and clinical disease outcomes in Group 2 versus Group 3 horses.

The 3-month interval between vaccination and challenge exceeds the 2 to 4 week interval normally used in vaccine licensing studies. Our results suggest that there was evidence of protection for at least 3 months after vaccination with the KV-KY97 + METASTIM vaccine, a post-vaccination interval that commonly occurs in field settings. In contrast, other investigators have reported that EIV challenge given 100 days after vaccination with two doses of an aluminum gel-adjuvanted KV-EIV vaccine resulted in poor efficacy characterized by clinical signs of

infection, viral shedding, and a limited antibody response that did not include IgGa or IgGb.<sup>8</sup> This earlier research corroborates our results indicating that adjuvant composition can be a critical factor in completeness of the immune response to KV-EIV vaccines.

Results of our study support the value of METASTIM as an adjuvant for a KV-KY97 vaccine, and indicate that the METASTIM-adjuvanted KY97 vaccine strain was effective for at least 3 months against one of the most common contemporary EIV strains circulating in North America. The cross-protection provided by the KY97 strain against the KY07 subtype may be due in part to the reasonably close homology between these two American lineage, clade 1 strains.<sup>2</sup> However, another factor that may be at least as important is the ability of the METASTIM-adjuvanted vaccine to stimulate the IgGa and IgGb immunoglobulins associated with viral clearance and long-term protection.

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## References

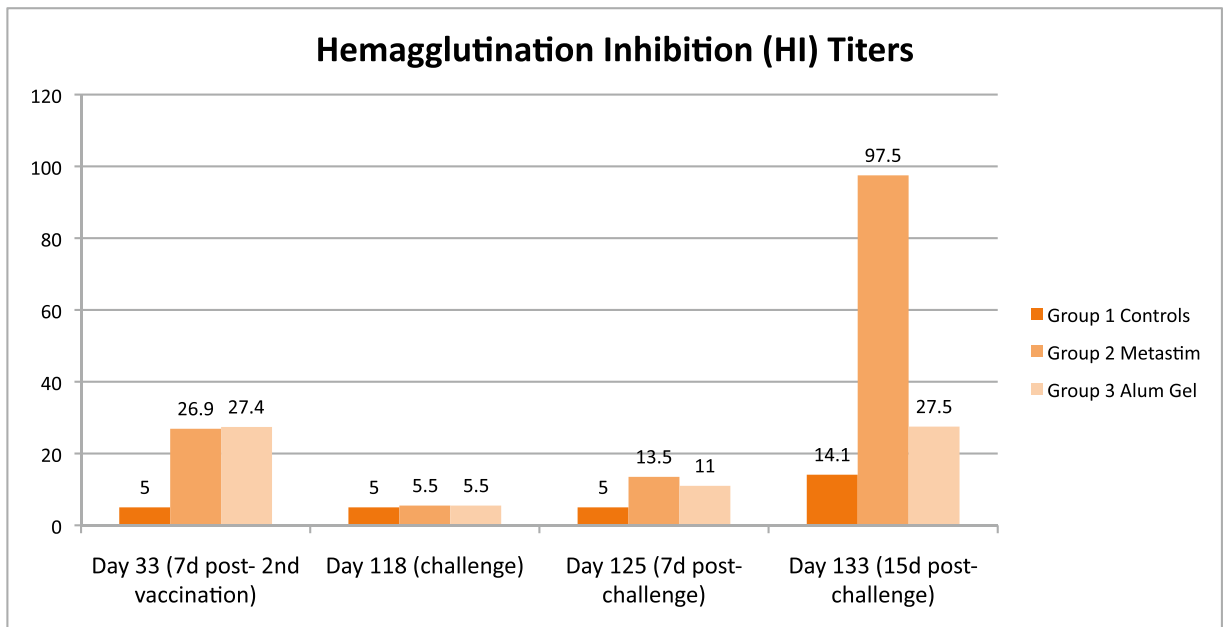
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**Table 1 – PCR values for viral shedding following challenge with A/equi/Kentucky/07 strain of equine influenza virus**

Test Group	Post-challenge day and LSM-PCR values								
	0	1	2	3	4	5	6	7	8
1 - Controls	0.3	90.6	82,947.6	14,884.3	7893.1	17,034.1	10,365.1	71.6	18.0
2 - METASTIM	2.2	4.1	20,974.0	379.2	109.4	48.5	4.7	0.6	0.7
3 - Alum gel	0.0	3.2	52,162.1	1899.5	405.9	348.2	63.0	4.5	1.3

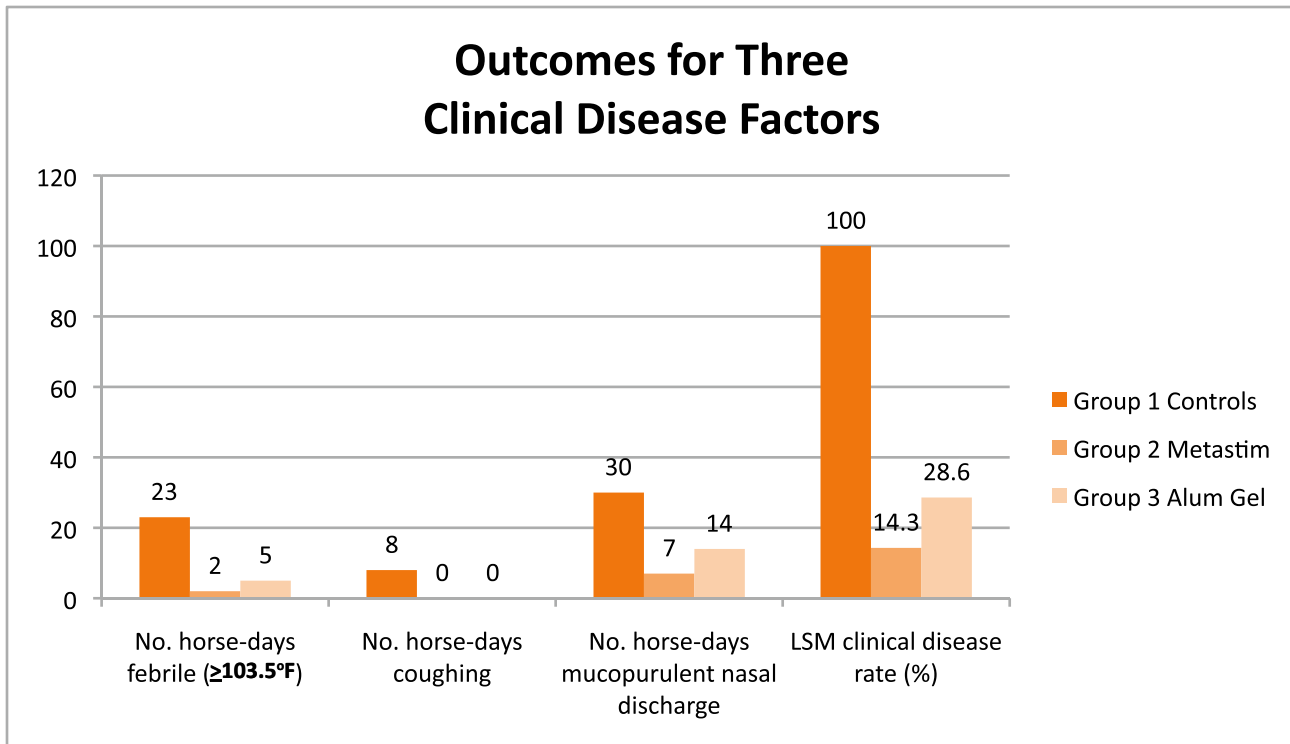
LSM = Least squares means; PCR = polymerase chain reaction.

**Figure 1 – Hemagglutination inhibition titers in horses following challenge with a A/equi/Kentucky/07 strain of equine influenza virus.**



**Figure 1 – Least squares mean (LSM) hemagglutination inhibition (HI) titers for each test group are shown following challenge with an A/equi/Kentucky/07 strain of equine influenza virus. The highest post-challenge LSM-HI titers occurred in Group 2 horses vaccinated with killed-virus KY97 + METASTIM.**

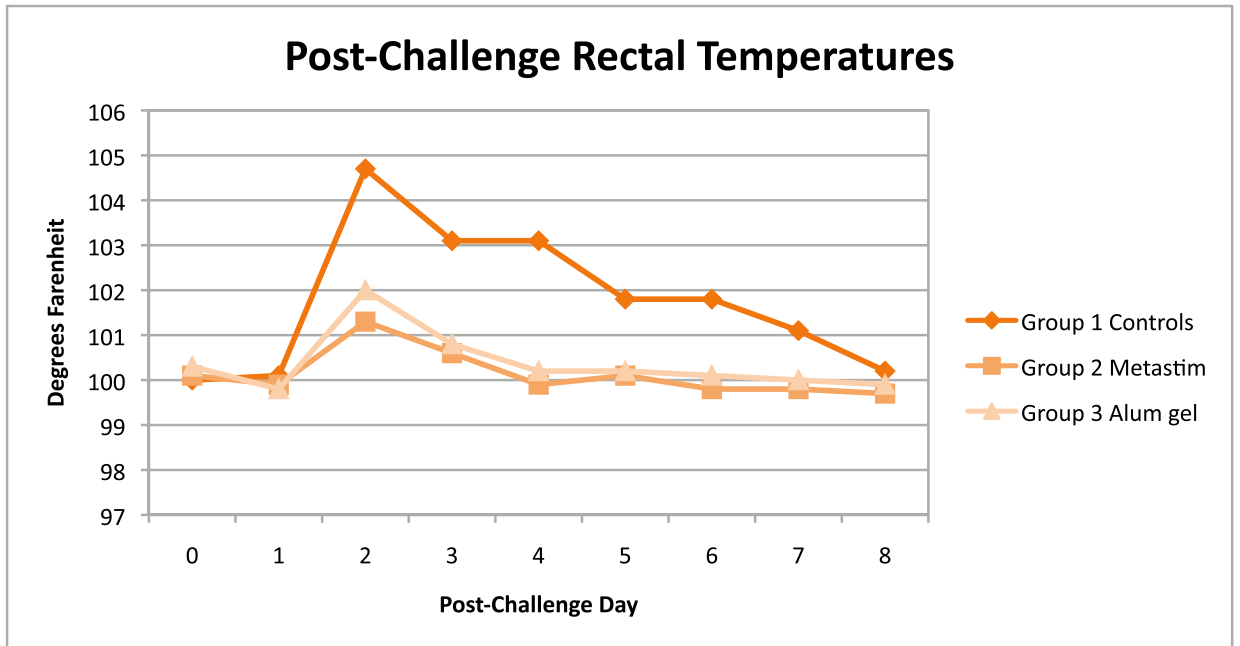
**Figure 2 – Outcome for Three Clinical Disease Factors**



**Figure 2** – Clinical disease was assessed daily for 8 days following challenge. The figure shows the outcomes for three clinical disease factors: fever  $\geq 103.5^{\circ}\text{F}$ , coughing, and mucopurulent nasal discharge. Group 2 horses vaccinated with killed-virus A/equi/Kentucky/97 + METASTIM had the fewest number of horse-days with fever  $\geq 103.5^{\circ}\text{F}$  and mucopurulent nasal discharge. The Least Squares Mean (LSM) rate of clinical disease in Group 2 horses (14.3%, 1/7) was half the rate in Group 3 horses (28.6%, 2/7) that were vaccinated with A/equi/Kentucky/97 + alum gel. The LSM clinical disease rate was significantly lower ( $p \leq 0.0027$ ) in either vaccinated groups compared to the rate in Group 1 controls (100%).



**Figure 3 – Post-Challenge Rectal Temperatures**



**Figure 3** – The graph compares Least Squares Mean post-challenge rectal temperatures (°F) for Group 1 non-vaccinated control horses versus temperatures for Group 2 horses (vaccinated with killed-virus KY97 + METASTIM) and Group 3 horses (vaccinated with killed-virus KY97 + aluminum gel). LSM temperatures were significantly higher ( $p \leq 0.0045$ ) for Group 1 non-vaccinated control horses on post-challenge days 2 through 7 versus LSM temperatures in Groups 2 and 3.