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Protective vaccination in the horse against *Streptococcus equi* with recombinant antigens

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***Streptococcus equi* subspecies *equi* (*S. equi*) is a clonal, equine host-adapted pathogen of global importance that causes a highly contagious suppurative lymphodendopathy of the head and neck, more commonly known as Strangles. The disease is highly prevalent, can be severe and spread easily by visibly infected animals or by carrier animals that show no clinical signs of disease. Antibiotic treatment is usually ineffective. However, the majority of horses develop immunity to re-infection, suggesting that vaccination should be a feasible way to prevent the infection. Live attenuated vaccine strains of *S. equi* are available but adverse reactions have been reported and they suffer from a short duration of immunity. Thus, a safe and effective vaccine against *S. equi* is highly desirable. In this report, Welsh mountain ponies vaccinated with a combination of seven recombinant *S. equi* proteins, were significantly protected from experimental infection by *S. equi*, resembling the spontaneous disease. The protective antigens consisted of five surface localized proteins and two IgG endopeptidases. The results from a second vaccination trial indicate that the endopeptidases were important for good protection. The similarity of *S. equi* to other pyogenic streptococci suggests that our findings have broader implications for the prevention of streptococcal infections.**

Strangles is characterized by abscessation of the lymph nodes of the head and neck and is of significant welfare and economic importance. The development of effective preventative vaccines has been slow. A non-encapsulated strain of *S. equi* (Pinnacle IN™) has been used as a nasal vaccine against strangles, but has not been licensed for sale in Europe due to safety concerns. A second live attenuated vaccine was marketed in Europe [1] (Equilis StrepE), but was withdrawn in 2007. Safety concerns have also been raised over the use of Equilis StrepE [2,3]. A safe and effective vaccine is thus highly desired.

S. equi evolved from an ancestral strain of *S. equi* subsp. *zooepidemicus* (*S. zooepidemicus*). The population of the *S. zooepidemicus* group is extremely diverse and consists of at least 219 sequence types, whereas isolates of *S. equi* are either ST-179 or a single locus variant, ST-151 that are characteristic of *S. equi* isolates from the USA, Canada, Australia and Europe [4] (http://pubmlst.org/szooepidemicus/). The limited genetic diversity of *S. equi* suggests that an effective vaccine could confer broad protection to horses throughout the world.

Access to the genome sequence data of bacterial pathogens permitting the identification of surface exposed and secreted proteins has long been anticipated to revolutionize vaccine design, referred to as reverse vaccinology [5,6]. However, few vaccines have been taken beyond studies in mouse model systems and shown to confer protection against challenge infection in the natural host.

We have demonstrated previously that vaccination of Welsh mountain ponies with EAG [7,8], SclC [9] and CNE [10] (Trivacc) conferred partial protection against challenge by *S. equi* [11]. The amount of nasal discharge, the number of bacteria recovered from nasal washes and the occurrence of abscess material (empyema) in the guttural pouch, following rupture of abscesses formed in the retropharyngeal lymph nodes, differed significantly between the vaccinated group and a non-vaccinated control group. However, clinical scoring and mean rectal temperatures were not significantly different. This experiment thus showed that parameters of importance for spreading disease between horses were significantly reduced, but that the level of protection in individual horses was limited [11].

We report here the use of a combination of seven antigens in a vaccine, Septavacc, to prevent *S. equi* infection. Five of the antigens in the Septavacc composition are predicted to be localized on the surface of *S. equi* (EAG [7,8], CNE [10], SclC [12], SEQ0256 and SEQ0402[13]) through sortase-mediated attachment to the peptidoglycan cell wall. EAG binds to albumin, α-2 macroglobulin (A2M) and IgG [8,14]. CNE binds to collagen [10], and is located within the FimI pilus locus of *S. equi* and *S. zooepidemicus* [13,15]. SclC is a member of a collagen-like protein family, which in *S. equi* consists of seven members, each with a unique N-terminal domain of unknown function [12]. The proteins encoded by SEQ0256 and SEQ0402 contain features typical of cell surface anchored proteins and an N-terminal non-repetitive domain. The N-terminal domains were used in this study, the functions of which are unknown. Neither of them show homology to any characterized protein. The two additional antigens in Septavacc, IdeE and IdeE2 are IgG endopeptidases where IdeE2 has greater activity towards horse IgG. Both IdeE and IdeE2 are predicted to be secreted [16,17] and IdeE has an antiphagocytic activity by binding directly to neutrophils [17]. These antigens were selected from a larger antigen pool based on the level of protection conferred in an experimental mouse model of strangles. Mice were immunized with recombinant antigens either individually, or in combination, experimentally infected with *S. equi* and the effectiveness of each antigen ranked (Supplementary Table 1).

Seven Welsh mountain ponies were vaccinated with Septavacc and seven were given adjuvant only as control via both the subcutaneous and intranasal routes, followed by experimental infection with 1 x 10^8 colony forming units (cfu) of *S. equi* strain 4047. Serum samples and nasal washes were analyzed by ELISA to quantify the antibody responses against all antigens (Supplementary Figures 1 a and b). All ponies responded well and it was noted that responses in nasal washes had low correlation with responses in sera (R^2 from 0.01 to 0.28), implying the generation of independent immune responses in mucosa and sera, possibly as a result of the two routes of immunization employed.

The swelling and abscessation of submandibular lymph nodes is a typical clinical sign of infection by *S. equi*. Figure 1 shows that the mean lymph node scores differed

between the groups and that the number of days where an individual pony's score exceeded 2 was significantly different (p= 0.0013) with two vaccinated ponies and four control ponies exceeding this level.

The normal rectal temperature of Welsh mountain ponies is 37-38^{o}C and a pony with a rectal temperature of 39^{o}C or higher is considered pyrexic. All ponies in the control group became pyrexic at some stage during challenge compared to only one pony in the vaccinated group. The accumulated number of days that individual ponies in the vaccinated or control groups were pyrexic was 5 and 30 days, respectively (p=0.0001) (Figure 2).

Infection by *S. equi* leads to an increase in blood fibrinogen and neutrophil levels. As shown in Figure 3 a and b, fibrinogen and neutrophil levels of vaccinated ponies remained normal, whereas the non-vaccinated group had significantly higher mean values.

To minimize suffering and in accordance with our strict ethical and welfare code, ponies were euthanized as soon as clinical signs of *S. equi* infection became apparent. All of the control ponies were euthanized between 8 to 12 days post challenge. Vaccinated horses, however, had reduced clinical signs and all ponies reached the end of the study, 21 days post challenge. Following euthanasia, all of the ponies were subject to post mortem examination to quantify the level of pathology observed using a scoring system as described in Methods.

In a separate study, seven ponies were immunized with a Pentavacc formulation, containing the same antigens as in Septavacc with the exception of IdeE and IdeE2, and then challenged *S. equi*. Although the Pentavacc ponies differed from the corresponding control group in terms of elevated temperature, fibrinogen levels and nasal discharge, this was not statistically significant. One pony was fully protected.

Figure 4 summarizes the individual post mortem scores of ponies vaccinated with Trivacc, Pentavacc and Septavacc, which contain three, five and seven antigens respectively. Increasing the number of antigens comprising each vaccine reduced the post mortem score. However, the large improvement in efficacy between Pentavacc

and Septavacc (p=0.036 for post mortem scoring), suggests that inclusion of one or both of the endopeptidases IdeE and IdeE2 is important for protection in the natural host. Only one of the ponies vaccinated with Septavacc had lymph node abscesses, compared with abscesses in all seven non-vaccinated ponies. To confirm these gross pathological findings, samples from ponies vaccinated with Septavacc were examined histopathologically and scored using a system as described in Methods. Again, significant differences were seen between the Septavacc and control groups (p=0.006) (Supplementary Figure 2). Histopathological examination of the left and right retropharyngeal and submandibular lymph nodes identified 19 lymph node abscesses in the control ponies and 3 lymph node abscesses in a single pony in the Septavacc group (p=0.00001). Seventy-eight and 10 % of all lymph nodes were positive for *S. equi* in the control and vaccine groups respectively (p=0.004).

Taking all of the results together, vaccination with Septavacc resulted in 85% protection from disease, with only one vaccinated pony out of seven being infected.

In a study by Timoney et al, [18] two combinations of recombinant extracellular proteins derived from *S. equi* (SzPSe, CNE, Se51.9, Se44.2 and Se46.8 or SeM, Se44.2, Se75.3, Se42.0, Se110.0 and Se18.9) were tested as vaccines against strangles. However, neither combination protected horses from infection by *S. equi* [18]. Two of these proteins CNE and IdeE2 (Se44.2) are included in the Septavacc vaccine, suggesting that the additional components of Septavacc are important in generating a protective immune response. It was also suggested that an effective strangles vaccine should result in immune-mediated tonsillar clearance since tonsillar adherence is a crucial early step in the pathogenesis of strangles [18],[19]. Thus, the route of immunization and choice of adjuvant, which differ between these studies, might be of utmost importance, an issue to be further addressed in our future studies. It should be noted that mice immunized by the intranasal route are far better protected than those immunized subcutaneously (data not shown).

S. equi shares >80% sequence identity with *Streptococcus pyogenes* [13] and several components utilized in our studies have similarities to *S. pyogenes* antigens, either by homology or function. The *S. pyogenes* gene encoding the collagen binding protein Cpa is located in the variable FCT region (fibronectin- and collagen-binding T-

antigen) and is part of a pilus-like structure [20]. Similarly, *cne* is located in a pilus locus (FimI) that includes genes encoding SrtC.1 and a putative backbone pilus subunit suggesting that also CNE is attached to a pilus-like structure [13]. EAG, like GRAB from *S. pyogenes*, binds the proteinase inhibitor A2M [8,21,22]. SclC is one of seven collagen-like surface proteins in *S. equi*, whilst *S. pyogenes* genomes contain two such putative proteins, SclA and SclB [9]. The IgG-specific endopeptidases used here, IdeE and IdeE2, are similar both in function and amino acid sequence to IdeS/Mac/sib35 of *S. pyogenes* [16,17,23,24]. Antibodies against IdeS in convalescent patients were able to neutralize its function [25]. Interestingly, Cpa (plus other pili components), GRAB and Sib35 have been identified as protective antigens in mouse models of *S. pyogenes* infection. The Cpa combination and Sib35 gave good protection whereas vaccination with GRAB was only effective against capsule-deficient mutants of *S. pyogenes* [26-28]. Thus, it is conceivable that vaccination of humans with a combination of *S. pyogenes* antigens similar to the ones used in Septavacc, could prove effective against this important human pathogen.

This study is one of only a few demonstrations of protection in a natural host from streptococcal infection conferred by a recombinant multi-component subunit vaccine. A protective immune response against *S. equi* infection can be obtained by immunization using recombinant antigens and does not necessarily require previous infection or a subclinical infection, a strategy taken by vaccines based on attenuated live vaccines. No adverse effects were seen in any of the vaccinated horses, demonstrating that both the recombinant antigens and the adjuvant were safe. It is also clear that the antiphagocytic capsule did not prevent successful vaccination with the recombinant proteins used here. The approach taken here is likely to be significantly safer than live attenuated strains of *S. equi*.

METHODS

Cloning and production of recombinant antigens.

Antigens used in the vaccination studies were cloned and expressed in *Escherichia coli* (Supplementary methods)**.**

Mouse model of strangles

Mice were immunized with recombinant antigens followed by experimental infection with *S. equi*. Infection was assessed by nasal colonization and weight loss [29, 7] (Supplementary Methods).

Immunization of ponies

Healthy Welsh Mountain Ponies (n=7) were vaccinated with Septavacc via administration of 1 ml subcutaneous (s.c.) injections bilaterally close to the retropharyngeal lymph nodes and 2 ml intranasally (i.n.) by spraying into each nostril on days 4, 60, and 74. The Septavacc vaccine doses contained 150 µg for i.n. and 50 µg for s.c. injections of each antigen (EAG, CNE, SclC, SEQ0256, SEQ0402, IdeE, and IdeE2). Abisco 300 (Isconova, Uppsala, Sweden) (500 µg per i.n. dose) and Abisco 200 (375 µg per s.c. dose) were used as adjuvants. Septavacc ponies were challenged on day 88. Pentavacc vaccinated ponies (n=7) followed the same vaccination protocol as above, but were given an additional booster vaccination on day 270 and challenged on day 284. Negative control ponies were given adjuvant only, mixed with PBS (n=7). Sera and nasal washes were taken regularly to quantify antibody responses by ELISA [11].

Experimental infection of ponies

Ponies were transferred to a containment unit three days before challenge. Two weeks after the final booster immunization, each pony was challenged with *S. equi* strain 4047 administered via the spraying of a 2 ml culture containing $5x10^7$ cfu into each nostril. Bacteria were grown overnight in Todd Hewitt broth and 10% foetal calf serum (THBS) in a 5% carbon dioxide enriched atmosphere at 37^{o}C, diluted 40-fold in fresh pre-warmed THBS, further cultivated and harvested at an OD=0.3. This

infection dose has been shown to optimize the infection rate, whilst avoiding overwhelming the host immune response, as determined in previous studies [11,30].

Clinical evaluation of and sampling from ponies

Ponies were monitored for the onset of clinical signs of disease over a period of three weeks post challenge by daily physical examination, rectal temperature, lymph node swelling and nasal discharge scoring. Blood samples were taken for evaluation of fibrinogen concentration as described in [11] and neutrophil levels by total white blood count performed on Beckman-Coulter ACTdiff analyser with a manual differential count to calculate % neutrophils.

The level of swelling of SMLNs was defined as 0 = normal, 1 = slight swelling, 2 = moderate swelling, 3 = severe swelling and 4 = abscessated [11]. Bilateral swelling of submandibular lymph nodes was scored separately

Post mortem examination

Post mortem examination was performed on all ponies following the onset of clinical signs of infection or on reaching the study endpoint at 3 weeks post challenge. The severity of disease pathology was quantified according to a scoring system as follows: retropharyngeal or submandibular lymph node abscess (evident at gross examination) 15, retropharyngeal or submandibular lymph node microabscess (evident on microscopic examination) 10, empyema of guttural pouch (suppurative exudate in lumen on gross or microscopic examination) 5, scarring of guttural pouch (fibrosis of wall on gross or microscopic examination) 5, enlarged lymph node (showing non-specific hyperplastic changes on microscopy) 1, follicular hyperplasia of guttural pouch (lymphoid follicles in submucosa on gross or microscopic examination) 1.

Statistics

Fischer´s exact test was used for comparison of values from arbitrary scoring using a cut-off value splitting the group into “low/negative” or “high/positive”. Cut-off values were: Nasal colonization in mice 1.5; Lymph node scoring 2; Pyrexia 39^{o}C. Mann Whitney test was used for post mortem and histopathology (Supplementary Fig. 2) scoring in horses. T-test was used to compare temperatures, fibrinogen and neutrophil levels in ponies.

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Figure legends

Fig 1

Lymph node swelling over time. Lymph node swelling was monitored from three days pre-challenge (day 85) using an arbitrary scale from 0 to 4. Average values and standard error from the mean (SEM) are shown. The insert shows accumulated number of days a pony was considered positive, i.e. with a value exceeding 2. p-value in insert: **p=0.0013. Non-vaccinated (open symbols) (n=7) and Septavacc vaccinated (closed symbols) (n=7).

Fig 2

Temperature over time. Mean rectal temperature was monitored from three days pre-challenge (day 85). Average values and SEM are shown. The insert shows accumulated number of days a pony was considered pyrexic, i.e. with a temperature exceeding 39.0^{o}C. p-value in insert: ***p=0.0001. Non-vaccinated (open symbols) (n=7) and Septavacc vaccinated (closed symbols) (n=7).

Fig 3

Inflammatory markers over time. Neutrophil counts (a) and fibrinogen levels (b) were monitored from two days pre-challenge (day 86). Average values and SEM are shown. p-values: **p=0.002, ***p=0.0004 in (a), *p=0.024, **p=0.002 in (b). Non-vaccinated (open symbols) (n=7) and Septavacc vaccinated (closed symbols) (n=7).

Fig 4

Post mortem score. Ponies were vaccinated with three different antigen combinations, (Trivacc, Pentavacc and Septavacc) followed by challenge with *S. equi*. Post mortem scoring was performed using a scoring system described in Methods. Non-vaccinated (open symbols) and vaccinated (closed symbols).

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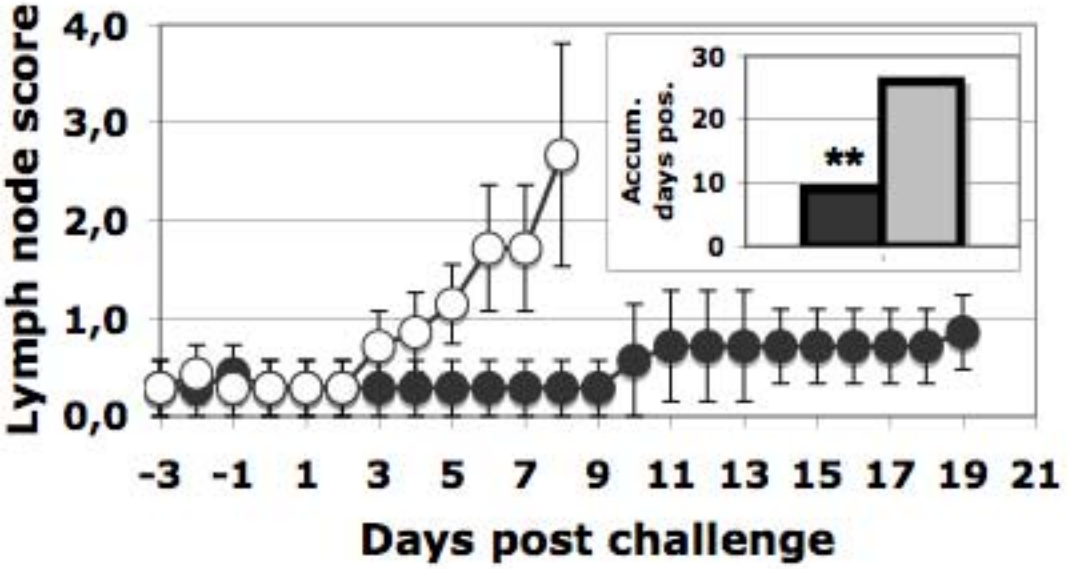
Author contribution: AW, BG, CR, and J-IF designed the study. BG and LF identified antigen candidates, cloned genes and purified the proteins. MF performed mouse experimental infection and immunological analysis. KS performed post mortem and histopathological analysis. AW and CR performed and supervised staff, and the other authors, with experimental infection of ponies. J-IF wrote the paper assisted by all other participants.

Competing interest statement: J-IF and BG are stockholders of Intervacc AB. The company has funded the research. J-IF, BG, LF and MF have filed a patent application covering the use of antigens for vaccination against strangles.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Days post challenge
Accum. days pos.
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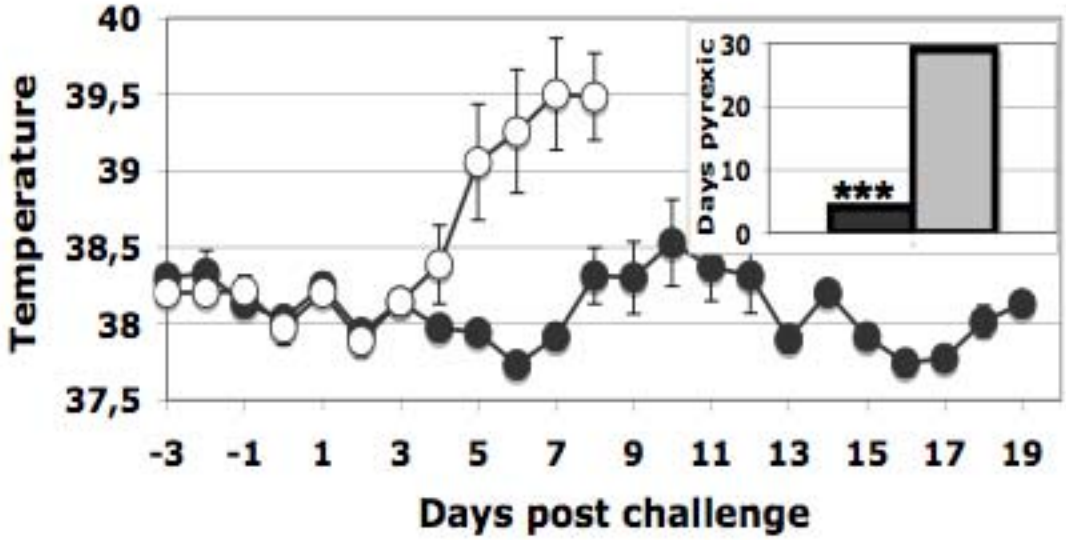
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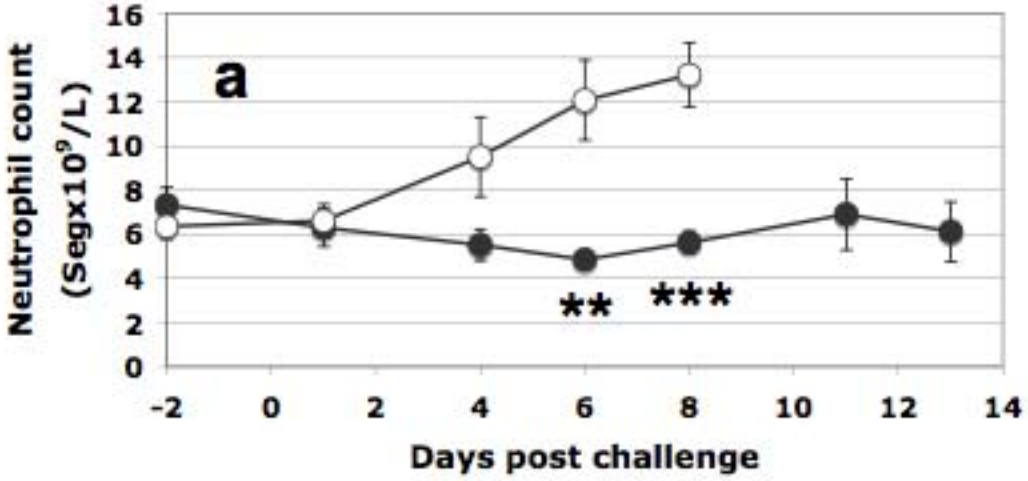
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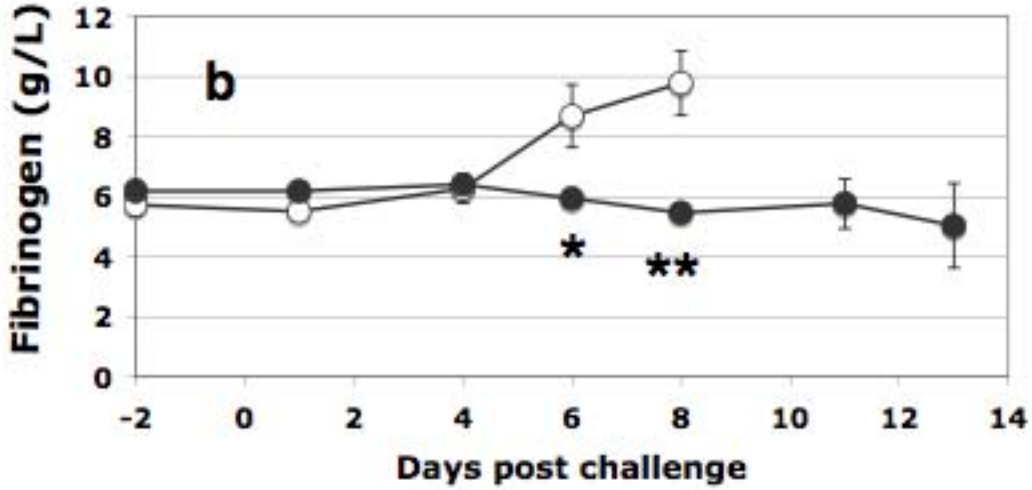
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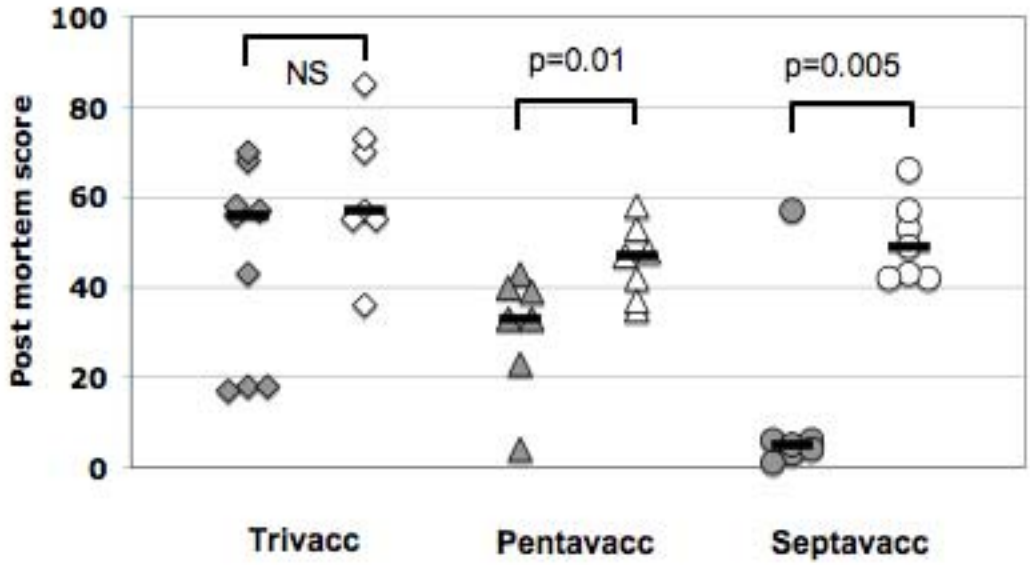
Days post challenge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Trivacc
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