‘Experimental vaccination with GP300 (thrombospondin-like protein) against the bovine lungworm *Dictyocaulus viviparus’.*

Investigation internship K.L.J.Moonen, student number 3259609

Supervisors: Dr. F.N.J. Kooijman
Dr. ir. H.W. Ploeger

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- **Abstract**

GP300 is a thrombospondin-like immunodominant and phosphorylcholine (PC) containing glycoprotein of *Dictyocaulus viviparus* and related nematodes. Antibodies induced by a primary *D. viviparus* infection or vaccination are mainly directed against the PC moiety of GP300 (Kooyman et al., 2009). IgE antibodies specific for GP300 correlate with protection against infection (Kooyman et al., 2002) In the following experiment, protection against a challenge infection with 500 L3 *Dictyocaulus viviparus* larvae was measured after administration of a vaccine consisting of 100 µg purified thrombospondin-like protein and Quill A. The vaccine does elicit an antibody response consisting of IgE, IgG1 and IgG2, but differences in protection between a vaccinated and an adjuvant control group could not be demonstrated. Clinical signs did not improve in the vaccinated group. A strong positive correlation between IgG2 and total worm counts was demonstrated. First, this positive correlation consisted between the IgG2 antibody reaction against the native GP300 and total worm counts. Later this correlation consisted between the IgG2 antibody reaction against protein backbone of GP300 and total worm counts. In general, immune reactions against the native GP300 are more abundant than those against the protein backbone of GP300 alone. More detailed investigation of the antibody response is needed in order to draw a final conclusion about the suitability of GP300 as a vaccine against parasitic bronchitis caused by *Dictyocaulus viviparus*.

Keywords: GP300, thrombospondin, *Dictyocaulus viviparus*, ELISA, LPG, worm counts.

- **Introduction**

The nematode *Dictyocaulus viviparus* causes parasitic bronchitis in cattle. Symptoms in calves range between a mild to severe harsh cough, tachypneu, hyperpneu, weight loss and sporadically, death. In susceptible adult lactating cows, a reduction in milk yield occurs on top of the signs seen in calves. (Holzhauer et al., 2003; Taylor et al., 2007; Wapenaar et al., 2007)

Historically, it was assumed only calves in their first grazing season were clinically affected. One believed older animals had acquired a strong immunity against *D. viviparus*. Nowadays, it seems that Dictyocaulosis is causing clinical problems in adult dairy cattle too. (Holzhauer et al., 2003; Wapenaar et al., 2007)

Prevention against lungworm disease is based on (a combination of) anthelmintic treatment, pasture management and vaccination with irradiated larvae. There are several downsides considering these methods of prophylaxis. The resistance of nematodes against anthelmintics is growing (Jackson et al., 2006; Stafford & Coles, 1999). Recently, research of Molento et al., (2006) suggested development of resistance of *D. viviparus* against macrocyclic lactones. Strategic grazing to prevent lungworm disease is not practical, because of the different epidemiology of *D. viviparus* compared to gastrointestinal helminths. The use of anthelminthics in

combination with strategic grazing may limit the exposure of calves to *D. viviparus* during their first grazing season. This may incorporate a risk, because this exposure is needed for the build up of immunity for the second grazing season (Kooyman, 2008). The only commercially distributed vaccine against lungworm disease (Huskvac, Intervet) consists of 2 doses of 1000 irradiated L3 larvae that are administered at a four week interval (Eysker, 1994). These attenuated larvae survive for approximately two weeks in the host. In this period, the larvae do elicit an immune response, but they die before they can cause disease. Four weeks after the second vaccine dose, calves are protected against infection. Unfortunately, Huskvac possesses some major drawbacks. Firstly, the vaccine does not induce long lasting immunity without a natural booster infection with *D. viviparus* on a regular basis (Eysker, 1994; Holzhauer et al., 2003). In other words, the vaccine induces a poor memory response. Secondly, infected donor calves are needed for the production of Huskvac. Besides these two major negative aspects, the composition of Huskvac is undefined and the nature of the vaccine antigens that provide protection against infection are still unknown (Kooyman, 2008).

In search of a new synthetic vaccine against lungworm disease (that would induce a memory response without the need for donor animals) glycoproteine 300 was presented by Kooyman et al, (2009) as a candidate vaccine antigen.

Glycoproteine 300 (GP300) is a high molecular weight protein of the bovine lungworm. The protein backbone of GP300 consists of seven thrombospondin domains with a 'papilin cassette' and six highly allergenic Kunitz domains. Because of the presence of the thrombospondin domains, GP300 is also known as 'thrombospondin-like protein'. To the protein backbone of GP300, phosphorylcholine-substituted N linked glycans are attached (Kooyman et al., 2007b; Kooyman et al., 2009). The protein backbone of GP300 is capable of inducing a memory response, while the N linked glycans elicit only a short lived (but strong) immune response. GP300 elicits broad isotype response, consisting of IgA, IgE, IgG1, IgG2 and IgM (Kooyman et al., 2007a). Specific IgE antibody titres against GP300 are correlated with protection against lungworm disease. (Kooyman et al., 2002)

Deglycolysation of GP300 with PNGase F strongly reduces the immune-reactivity of the protein (Kooyman et al., 2007a). This suggests that the glycans, or moieties linked to the glycans, are responsible for the immunodominance of GP300. The phosporylcholine (PC) unit that is attached to the N glycans turns out to be the immunodominant component of GP300 (Kooyman et al., 2007b). Antibodies directed against the phosphorylcholine – moiety of GP300 cross-react with platelet-activating factor (PAF) (Kooyman et al., 2007b). PAF is a proinflammatory mediator that is produced by a large number of inflammatory cells, like macrophages, neutrophils, basophils and eosinophils. PAF effectuates a broad range of functions considering inflammatory processes. Among these are macrophage and eosinophil activation and chemotaxis during infections with parasites (Negrao-Correa et al., 2004). In human peripheral blood mononuclear cells, PAF stimulates IgG2 production (T helper 1 response) but not IgG1 production (T helper 2 response) (Ishihara et al., 2000). It is possible that the demonstrated cross reaction of GP300 specific antibodies with the PC-moiety on PAF has physiological consequences in vivo too. The cross reaction may cause neutralisation of PAF, leading to less macrophage and eosinophil

activation, less chemotaxis and less IgG2 production (down regulation of the inflammatory reaction). This could conduct a shift of the inflammatory response towards a T helper 2 response. Less symptoms of illness for the host may be a result of this process. The idea of an immunomodulatory function of PC is not new. Harnett et al., (1999) reported about ES-62, a PC-substituted glycoprotein of the rodent filarial nematode *Acanthocheilonema viteae,* with the ability to set off polyclonal inhibition of B cell proliferation. ES-62 induces a T helper 2 type antibody response as well. The immunomodulatory effect of the phosphorylcholine- moiety of ES-62 is however not caused by cross reaction of antibodies, but by direct modulation via ligand-receptor interactions.

In order to test the suitability of GP300 as a vaccine, a vaccination experiment was conducted. The vaccine consisted 100 µg purified complete GP300 and Quill A as an adjuvant. Hypothetically, two different possible mechanisms of protection were expected:

Vaccination with thrombospondin leads to the production of thrombospondin specific antibodies that…

1) reduce the number of worms.
2) reduce the inflammation reactions caused by an infection with *D. viviparus*.

- **Materials and Methods**

Experimental design

Fourteen male Holstein-Friesian calves were randomly divided in two groups, a vaccinated group (n=7) and a control group (n=7). On day 0 and 21, the vaccinated group received a vaccination that consisted of 100 µg purified thrombospondin and 750 µg Quill A in a total of 2 ml TBS. The control group received on the same days only 750 µg Quill A. The vaccination was given intramuscular in the triangular area in the neck, cranial to the shoulder blade and well above the vertebral column. Subsequently, both groups were orally infected with 500 L3 on day 42. These L3 were brought into the mouth by using a plastic 20 ml syringe without needle. The syringes were emptied against the inner wall of the cheek near the base of the tongue. It was checked if the calves swallowed properly. On day 72, the control group and the vaccinated group were slaughtered. Lungs and heart of each animal were collected for worm counts.

Animals

Fourteen male worm-free Holstein-Friesian calves were used for the thrombospondin vaccination experiment. At the start of the experiment, the fourteen calves were all approximately 4 months old.

Housing conditions

The calves were housed indoors in groups of three or four calves. The four boxes were embedded with straw and each box was provided with a brush. The measurements of the boxes were 9 x 3 metres.

The calves had ad lib access to fresh water and hay. Twice a day, they were fed a mixture of maize and concentrate sufficient for growth and maintenance. The offered maize load was 31 kilograms twice a day for all 14 calves throughout the experiment. The offered concentrate load was adjusted throughout the experiment according to the growth of the calves. On day 59, the offered concentrate load was 35 kg twice a day for all 14 calves.

L3 larvae for challenge infection

L3 larvae used for challenge infection were obtained from faecal cultures from two donor calves from the Faculty of Veterinary Medicine, Utrecht University. The faecal cultures were kept at 15 °C for one week. Subsequently, by submersing the faeces in water, the L3 larvae were collected. Larvae were approximately 3-4 months old when used. Viability of the L3 larvae was tested by Bile Agar Migration test (Hussaini, 1990).

Purification of Thrombospondin-like vaccine

Purification of GP300 was performed as described by Kooyman et al., (2007b), with one minor modification: precipitation with trichloroacetic acid acetone was not performed.

The gel was checked for purity (5 µl/lane Ag-stain). Protein contents were measured using Bradford.

Deglycolysation of GP300

GP300 was degycolysated by incubating a mixture of 100 µl GP300 (3 µg/µl), 100 µl 1% SDS, 120 µl H_2O, 25 µl 10% β Mecapethanol and 50 µl deglycolysation buffer for 5 minutes at 100 °C. After cooling to room temperature, 100 µl 10% NP-40 and 5 µl PNGaseF (peptide-N-glycosidase, Roche cat nr. 1365177) was added. This final fusion was incubated overnight at 37 °C. Incubation was stopped by heating for 3 minutes at 100 °C. Mock treated samples (all except the PNGase F) were incubated simultaneously.

SDS-PAGE

Purification and deglycolysation of GP300 was verified by SDS-PAGE (7,5% gel). 0,1 µg antigen was loaded into each lane under reduced conditions (10% ß -Mecapethanol). Gels were stained with silver (supplementary data).

Collection of blood serum

From one week before the start of the experiment (week -1) on, blood was collected from each calf twice a week. This was carried out by a puncture in the vena jugularis. The blood was collected in a BD Vacutainer ® 8,5 ml. After collection the blood was kept in the refrigerator (4 °C) for 24 hours. Subsequently, the blood was put in at 37 °C for one hour, after which it was centrifuged for 10 minutes at 3000 rpm. The serum was collected and stored at - 20 °C until needed.

Serology

Enzyme Linked Immunosorbent Assay (ELISAs)

For all ELISA's, Greiner Bio One high binding plates were coated overnight with antigen in 0,06 M Na_2CO_3, pH 9,6. All incubations were for one hour at 37 °C with 100 µl per well. In between incubation steps, plates were washed with 0,05% Tween in distilled water. Dilutions of sera and antisera were made in Phosphate Buffered Saline containing 0,1% of gelatine and 0,05% of Tween-20 (PBS-GT). The utilised ELISA reader was a Ceres UV 900 C.

IgG1 ELISA

Different plates were coated with GP300/Mock treated and GP300/PNGase F treated (0,02 µg/ml). The test sera were diluted 1:2000. Standard sera were administered in duplo using the following dilutions: 1:500, 1:1000, 1:2000, 1:4000, 0. IgG1 specific monoclonal antibody (mca 627, serotec, 1:1000 solution) was applied, followed by 1:2000 goat antirabbit Ig/AP (DAKO cat nr. D0487). PNPP (Pierce, cat nr. 37620ZZ) was used as a substrate. The plates were read after half an hour incubation at RT and after overnight incubation at 4 °C. The reading was performed at 405 nm.

All obtained ODs from the test sera were deducted with the blank (PBS-GT instead of serum). A standard sera dilution with a clear measurement (OD between 0,5 and 1) was deducted with the blank too. This $OD_{\text{standard serum}} - OD_{\text{blank}}$ was set at 100%. All the $OD_{\text{test sera}} - OD_{\text{blank}}$ were expressed as a percentage of the $OD_{\text{standard serum}} - OD_{\text{blank}}$. This procedure was performed for each incubated plate individually, to correct for differences between plates. These transformed data were used in statistical analysis.

IgG2 ELISA

The same protocol as described for IgG1 was used, with the only exception of the isotype specific monoclonal antibody (mca 626, serotec, 1:500).

*Total IgE ELIS*A

Plates were coated with purified monoclonal antisheep IgE (IE7, Kooyman et al., 2002) in coatbuffer at 3 µg/ml. Sera were heat treated before use (1 hour at 56 °C). Subsequently, the sera were diluted 1:5. Standard sera were heat treated in the same way as the test sera. The standard sera were diluted as follows: 1:5, 1:10, 1:20, 1:100, 1:200, 0. Standard sera were administered to the plates in duplo. Polyclonal rabbit anti cattle IgE 1:1000 was utilised as second antibody solution. Then, plates were incubated with 1:4000 Goat antirabbit Ig/AP conjugate (DAKO, cat nr. D 0487). P-Nitrophenyl phosphate (PNPP, Pierce, cat nr. 37620ZZ) was used as substrate. The plates were read twice, after half an hour incubation at RT and after overnight incubation at 4 °C, at 405 nm.

Obtained ODs were expressed in mUnits of standard serum.

Clinical observations

From day 35 on, respiration rate and coughing score were determined three times a week. Respiration rate and coughing score were always recorded between 12.30 and 13.00 pm, without fixation of the calves. Respiration rate was determined by counting respiratory drafts for half a minute; by doubling this figure, respiration rates per minute were established. Coughing was evaluated on frequency (merely, regularly, frequently) and type (dry, harsh, productive etc.). Lung auscultation was performed three times a week. Rectal temperature was determined twice a week. On day 38 and 64 of the experiment, body weight of the calves was determined using a chest size-body weight measuring tape for cattle.

Faecal samples

On day 0 and from day 59 on rectal faecal samples were collected from each calf. Faecal larval counts were carried out at day 0, 59, 62, 64, 66, 68, 70 and 72 by means of the Baermann method using 30 grams of faeces. Results were expressed as larvae per gram faeces (LPG).

Post-mortem investigation

In order to determine protection, worms in the lungs of the slaughtered calves were counted. The worms were collected by means of the modified Inderbitzin method, as described by Eysker et al., (1990), with some minor modifications. Each pair of lungs was washed with 10 liters of saline containing 2 mM EDTA instead of water. A tube attached to a pump was inserted in the arteria pulmonalis. By applying moderate pressure (10 liters of saline in 15 minutes), the alveoli collapsed and the worms were washed with the fluid out of the trachea. Worms were collected on a 150 µm sieve. The rinsed lungs were chopped into pieces and were subjected to a modified Baermann-method, in order to collect as much worms as possible. This modified Baermann-method was performed as described by Eysker et al., (1990). The only minor modification was the time allowed for 'Baermannisation'. In our experiment the contents of the buckets were sieved after 4 hours. Collected worms were preserved in 70% alcohol, to be sexed and counted at a later time.

Statistical analysis

In this vaccination experiment, 'protection' against infection is defined as: resistance against a primary infection with *D. viviparus* in animals vaccinated with the thrombospondin-vaccin compared to an age-matched non-vaccinated control group.

Protection is determined on the basis of LPG and worm counts, as these are the only parameters that are a direct reflection of protection. The used 0 hypothesis is: H_0: protection vaccinated group = protection control group. H_1: protection vaccinated group ≠ protection control group.

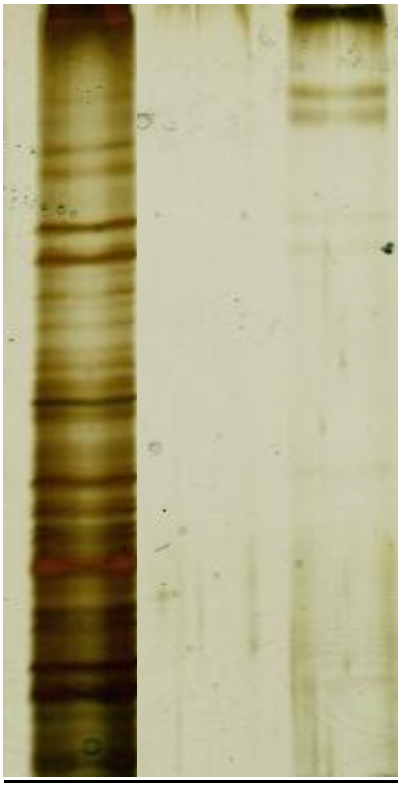
Temperature, breathing rate, coughing score and antibody titres are indirect reflections of protection. In case of significant differences between the control group and the vaccinated group regarding these parameters, correlation of these

parameters with LPG or total worm counts was investigated. Temperature, breathing rate and coughing score were used to monitor clinical condition of the calves.

Statistical analysis was carried out using SPSS software package (version 16.0). The Kolmogorov-Smirnov Test was used to verify normality of the data. Significance of difference in means between groups was calculated using the Independent Samples Test. Significance of difference in means within groups was determined using the Paired Samples Test. Significance of correlation was calculated by the parametric Pearson's correlation test. Data that were not normally distributed were analysed using a Mann-Whitney U non-parametric test. Transformed data (as described for the IgG1 ELISA) were used fore the calculations instead of the rough data. Correlations and differences were considered to be significant when $P < 0.05$.

- **Results**

Purification of the vaccine



Ureum WGA

Figure 1. SDS-PAGE showing the results of purification of GP300 by WGA lectin affinity chromatography.

Figure 1 shows the results of one-step purification of GP300 by WGA lectin affinity chromatography. The first lane contains water insoluble protein of *Dicytocaulus viviparus* dissolved in ureum. In the second lane, only the protein doublet characteristic for GP300 is visible, indicating that the purification of GP300 by WGA lectin affinity chromatography has been successful.

Clinical signs

According to the literature, the reference for the respiration rate of healthy calves differs between 30-50 respiratory drafts per minute (Kuiper & van Nieuwstadt, 2008) and 20-50 respiratory drafts per minute (Rosenberger, 1979). Figure 2 shows the average breathing rates from calves used in this experiment, before infection but after vaccination. These higher average respiratory values were not caused by a few calves influencing the mean. The higher respiratory rates were observed in all fourteen calves, and ranged from 32 to 74 respiratory drafts per minute.

Throughout the experiment, no significant differences were observed between the vaccinated group and the control group regarding breathing rate. Mean breathing rate after infection dispersed between 47 and 76 respiratory drafts per minute. Peak breathing rates were observed between day 56 and day 60.

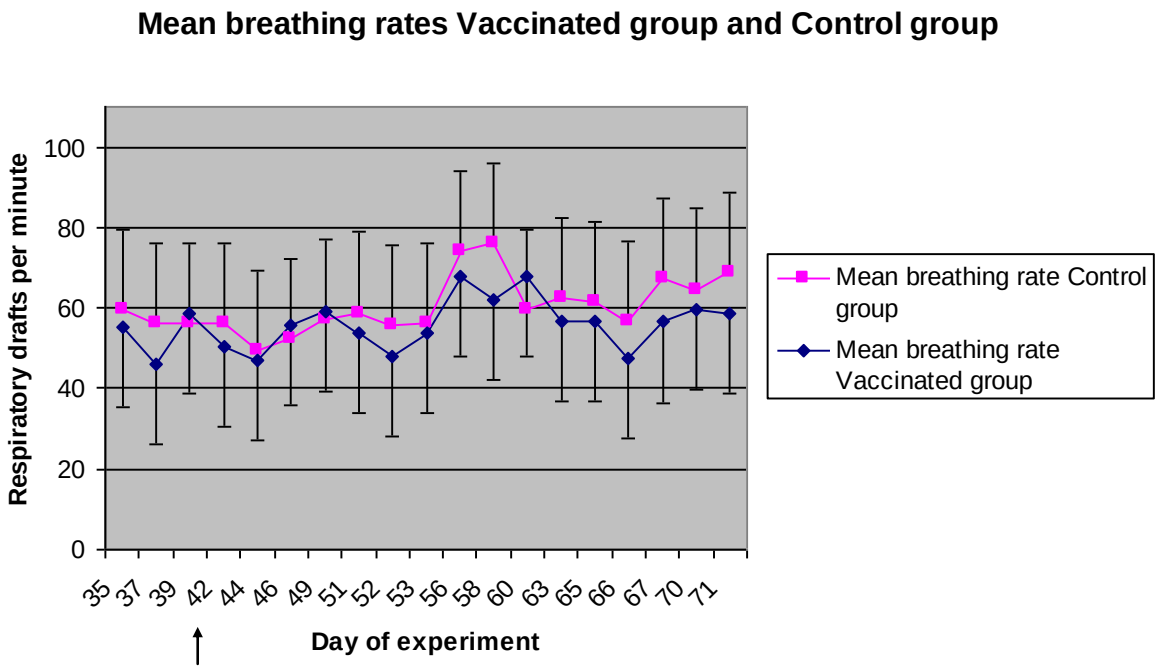


Figure 2. Mean breathing rate and standard deviation of the Control group and the Vaccinated group, before and after challenge infection. Day of challenge infection is marked with an arrow. No difference was observed between both groups concerning breathing rate. Notice the relatively high mean breathing rates before challenge infection.

Reference for rectal temperature of calves < 1 year is 38,5-39,5 °C (Kuiper & van Nieuwstadt, 2008). The vaccinated group contained calf 4549, that was diagnosed with bronchopneumonia on day 43. The control group enclosed calf 7180, that was diagnosed with pneumonia on day 31. Both calves were treated with antibiotics and NSAIDs. Only calf 4549 showed temperatures higher than the reference on day 42. Mean group temperature stayed within the reference throughout the whole experiment. No significant differences in mean rectal temperature were observed between the control group and the vaccinated group.

Unlike lung auscultation of adult cows with parasitic bronchitis, lung auscultation of calves with parasitic bronchitis can provide useful information. Wheezes, squeaks,

crackles and ronchi can be noticed, mainly on the posterior lung lobes (Holzhauer et al., 2003; Taylor et al., 2007). In this experiment, few abnormal lung sounds were heard in the vaccinated group before day 56. Calf 4549 was an exception to this, due to his diagnosed bronchopneumonia. The control group showed 3 calves with enhanced breathing and ronchi on day 49, but also in the control group, auscultation became convincingly positive from day 56 on. Most noticed lung sounds were squeaks under front legs, ronchi and enhanced breathing, in both groups. No striking differences were observed between the control group and the vaccinated group considering lung auscultation.

The Kolmogorov-Smirnov Test showed that coughing was not normally distributed. Non-parametric testing of coughing score revealed a significant difference between the control group and the vaccinated group on day 63 ($p = 0.038$). However, considering the fashion of determination of the coughing score, together with the fact that the difference on the other days is clearly not significant, makes this figure biologically seen of low importance. Coughing type was not used in statistical testing. Results of the coughing assessment are not shown. The most abundant coughing type was a dry, harsh cough.

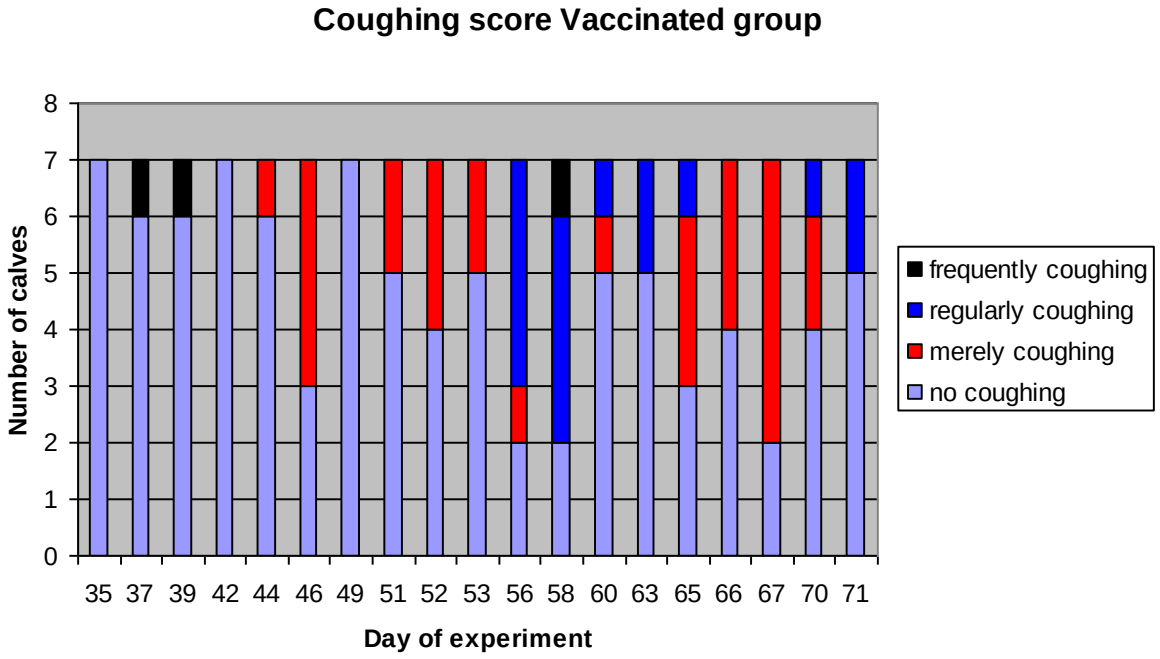


Figure 3. Coughing score of the Vaccinated group (n=7).

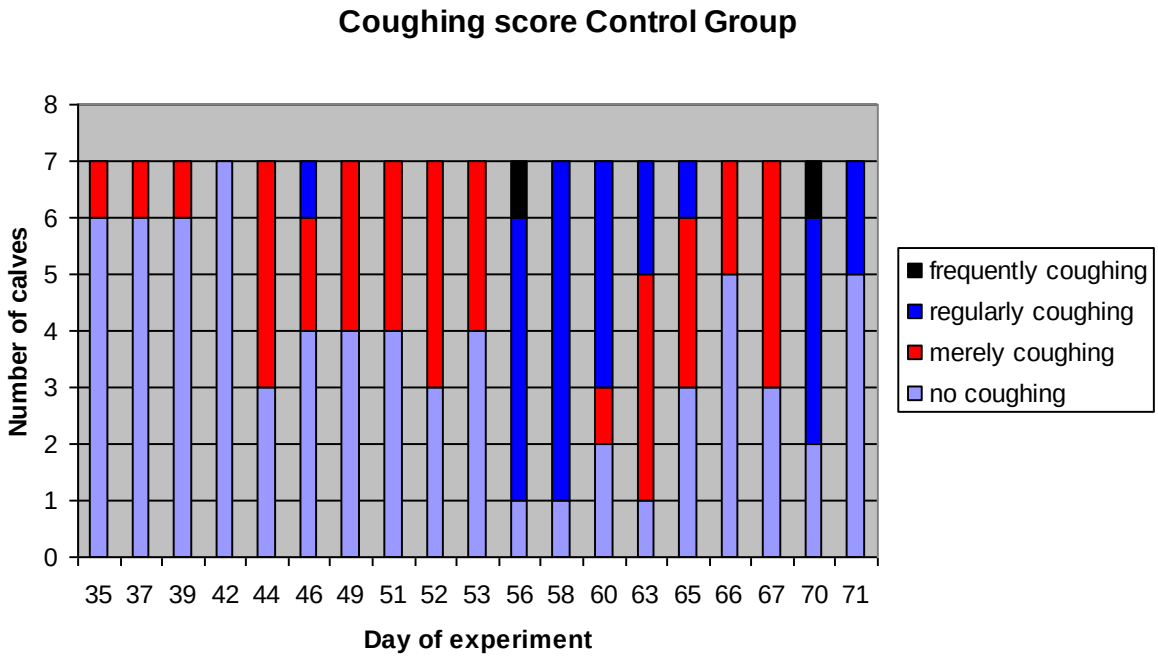


Figure 4. Coughing score of the control group (n=7).

The most distinct exhibition of clinical signs of the respiratory apparatus (breathing rate, coughing score and lung auscultation) seems to be concentrated around day 56.

Body weight of the calves was determined on day 38 and 64 (results not shown). No striking differences between both groups were observed concerning weight gain.

Larval excretion

LPG was approximately normally distributed according to the normality tests. No significant differences were found between the groups. From day 70 on, LPGs start to rise swiftly in both groups (figure 5 and 6).

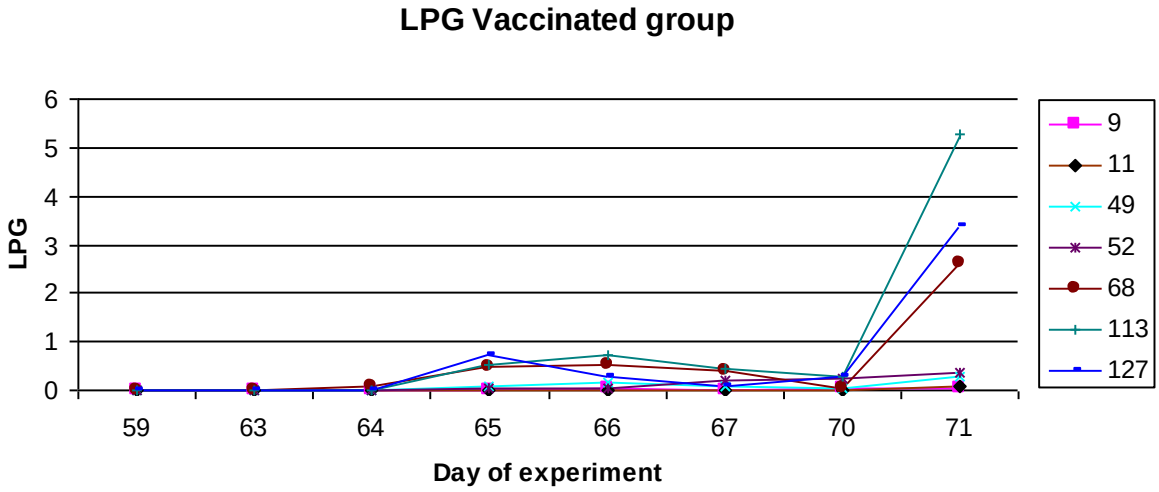


Figure 5. LPG of the Vaccinated group. Calves are named according their total worm burden.

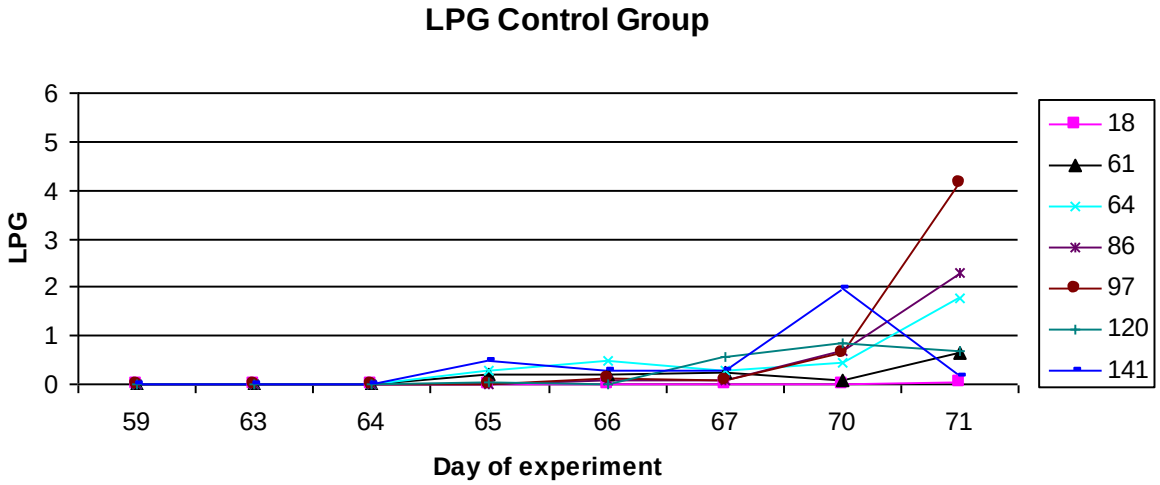


Figure 6. LPG of the Control group. Calves are named according their total worm burden.

Deglycolysation of GP300 and SDS PAGE

Figure 7 shows the results of SDS-PAGE of PNGase F and Mock treated GP300. PNGase F treated molecules have migrated faster compared to Mock treated molecules, indicating a reduced molecular weight caused by deglycolysation. Size difference of GP300 before and after deglycolysation is ~ 70 kDa (markers not shown). These GP300 fractions were used for serology.

Figure 7 SDS-PAGE of GP300 Mock and GP300 PNGase F treated.

Serology

IgG1 and IgG2 antibody responses were measured against GP300 after PNGase F treatment and against GP300 after Mock treatment. Deglycolysation was confirmed.

IgG1 ELISA

Figure 8 (anti-GP300/Mock antibody response) clearly shows the IgG1 response to the thrombospondin vaccination. One week after the first vaccination (day 7) IgG1 levels start to rise in the vaccinated group. A peak in antibody levels against Mock treated GP300 was observed two weeks after the first vaccination. One week after the second vaccination (day 28) a larger peak appeared in the anti-GP300 Mock response. After challenge infection a further increase of anti-GP300 antibody levels was shown. Responses against GP300/PNGase F only started after the second vaccination and increased slightly after challenge infection (figure 9). Barely any

immune response is seen in the control group until challenge infection. This indicates that the thrombospondin vaccine indeed provokes antibody production, at least of the IgG1 isotype.

When comparing the anti GP300/PNGase F response of the vaccinated group and the control group, significant differences are observed from day 14 to 70 ($p<0.01$). This indicates that in the vaccinated group the protein backbone of GP300 is recognised and that it leads to a stronger immune response compared to the control group.

Significant differences were observed as well when comparing the anti GP300/Mock response between both groups. These differences were encountered on the same days as for the anti GP300/PNGase F response (day 14 to 70, $p<0.01$). Together these results indicate that both the protein backbone and the native GP300 (i.e. GP300 with protein backbone, N glycans and PC) are recognised in the vaccinated group, but that there is no difference regarding the moment of presentation.

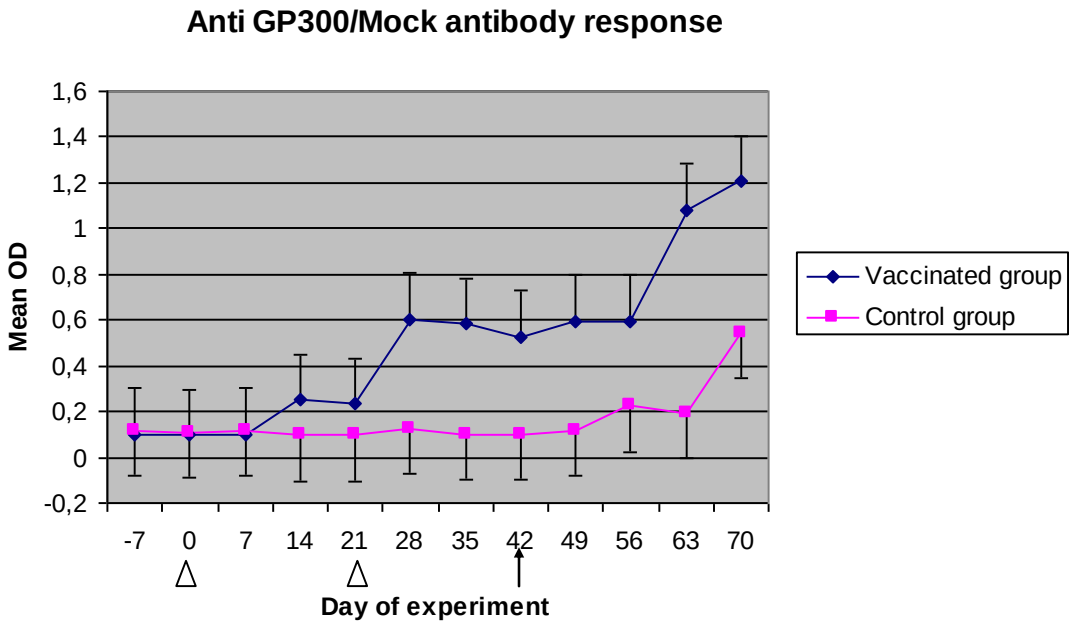


Figure 8. Mean production of IgG1 antibodies against GP300/Mock in the vaccinated group and the control group. Days of vaccination are marked with a ∆. Day of challenge infection is marked with an arrow. Notice the distinct reaction to the thrombospondin vaccine in the vaccinated group. Significant differences between the vaccinated group and the control group were observed on day 14 (p=0.002), 28 (p=0.000), 35 (p=0.001), 42 (p=0.003), 49 (p=0.000), 56 (p=0.003), 63 (p=0.000) and day 70 (p=0.007).

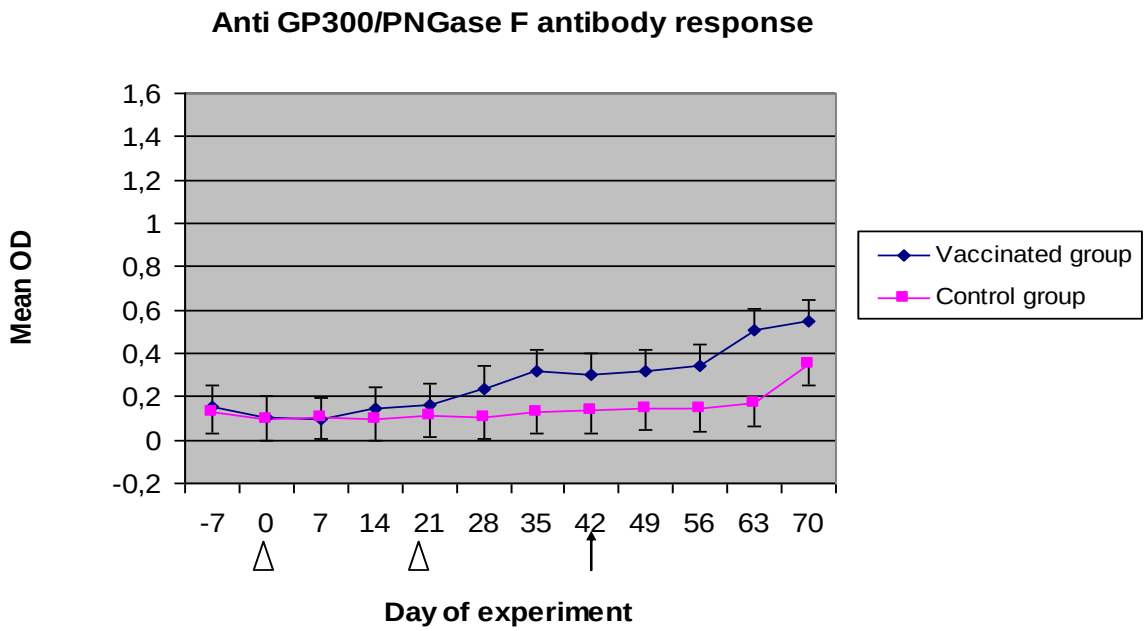


Figure 9.Mean production of IgG1 antibodies against GP300/PNGase F in the vaccinated group and the control group. Days of vaccination are marked with a ∆. Day of challenge infection is marked with an arrow. Significant differences between the control group and the vaccinated group were observed on day 14 (p=0.005), 28 (p=0.000), 35 (p=0.000), 42 (p=0.003), 49 (p=0.002), 56 (p=0.004), 63 (p=0.000) and 70 (p=0.002).

No correlation was found between IgG1 levels and protection in any group.

When comparing GP300/PNGase F responses and GP300/Mock responses within the vaccinated group, significant differences were found from day 28 to 56 ($p<0.01$). When comparing GP300/PNGase F responses and GP300/Mock responses within the control group, significant differences were found on day 28 and from day 42 to 70. In both groups, the immune reaction is directed more against the native GP300 than against the protein backbone alone, as illustrated by figure 8 and 9.

IgG2 ELISA

Figure 10 to 13 show the IgG2 response ODs against Mock treated GP300 and PNGase F treated GP300. In all those figures, the earlier rise of IgG2 in the vaccinated group compared to the control group is present. Figure 10 and 11 show that from day 7 till day 21, there are not much differences between the antibody response against GP300/Mock and the antibody response GP300/PNGase F. After the second vaccination on day 21, differences between the antibody reaction against GP300/Mock and GP300/PNGase F do arise: the antibody response against GP300/Mock rises evidently, while the antibody response against GP300/PNGase F remains at approximately the same level. Figure 10 also displays a great diversity among animals in their ability to elicit an IgG2 response. Figure 12 and 13 show the very faint antibody response in the control group. Even though, the antibody response against GP300/Mock seems to come up earlier than the antibody response against GP300/PNGase F in the control group.

Significant differences between the control group and the vaccinated group when coated with GP300/PNGase F were observed from day 28 to 70 ($p<0.05$).

When comparing both groups coated with GP300/Mock, significant differences were observed from day 14 to 63 ($p<0.05$). In the vaccinated group native GP300 is recognised by the immune system and in reaction to this, IgG2 antibody production takes place.

Comparing responses against deglycocylated GP300 and native GP300 within one group, showed a significant difference on day 28 ($p=0.037$) for the vaccinated group. On this day, the immune reaction is aimed more at the native GP300 than against the protein backbone alone. For the control group, no significant differences were observed at all.

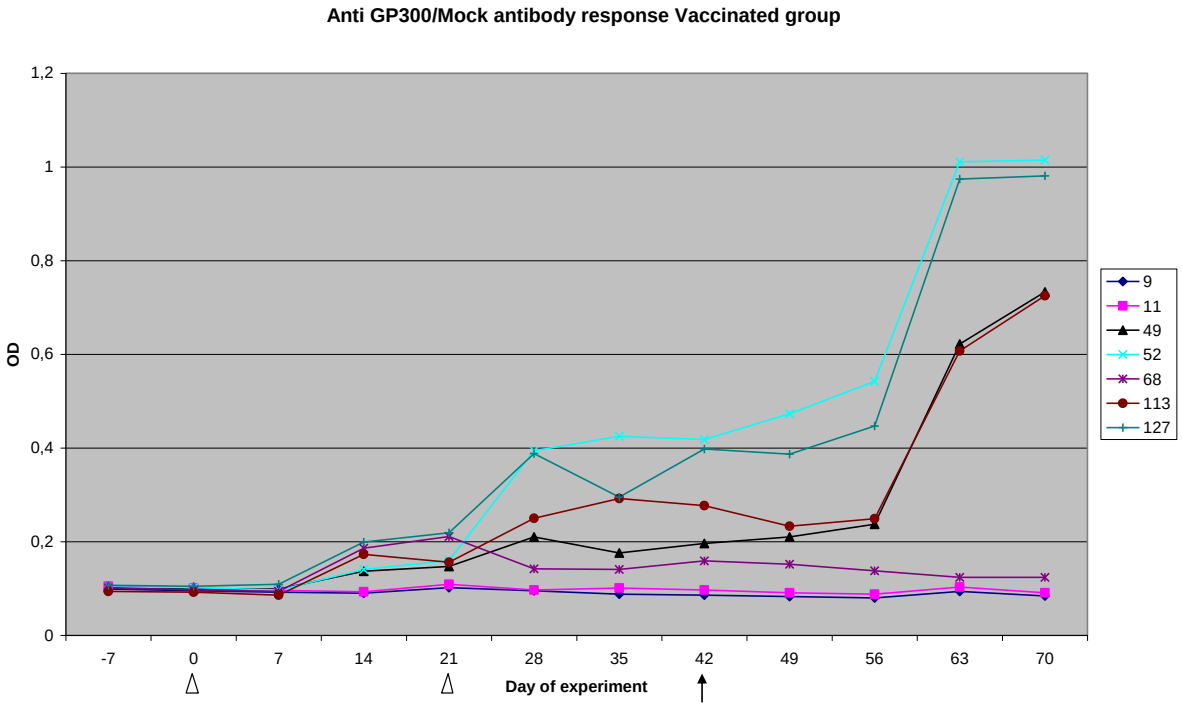


Figure 10. IgG2 antibody response of the vaccinated group against GP300/Mock. Calves are named according their total worm burden. Days of vaccination are marked with a ∆. Day of challenge infection is marked with an arrow.

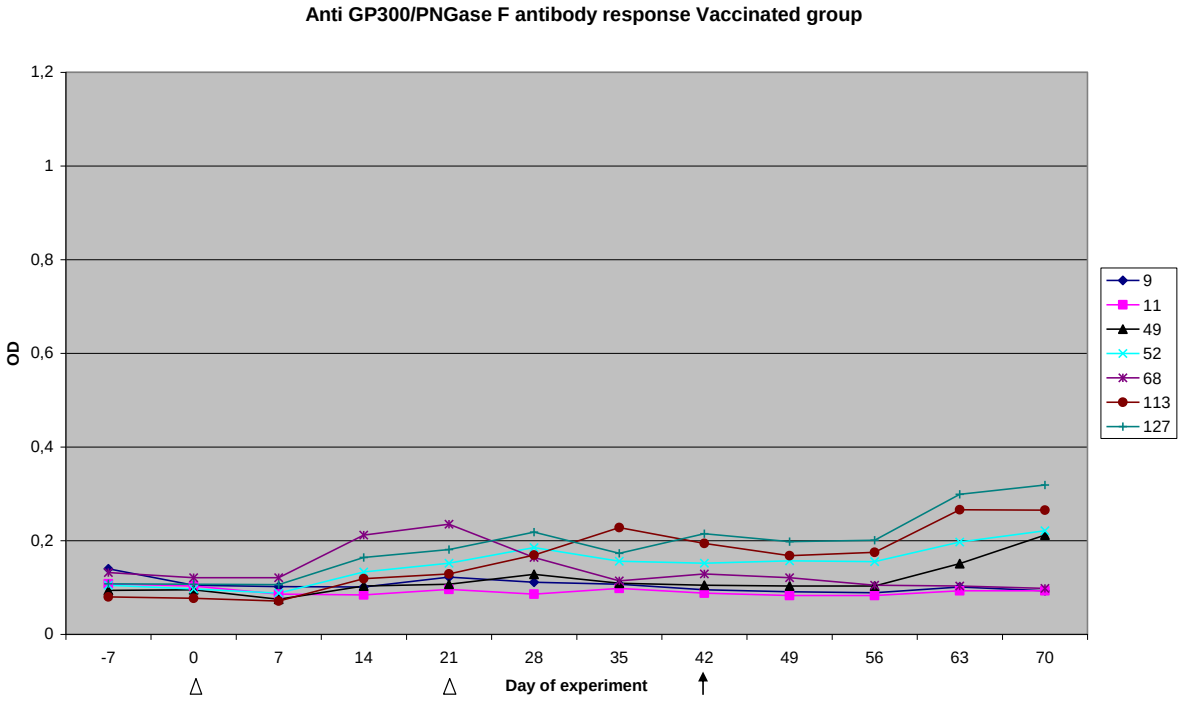


Figure 11. IgG2 antibody response of the vaccinated group against GP300/PNGase F. Calves are named according their total worm burden. Days of vaccination are marked with a ∆. Day of challenge infection is marked with an arrow.

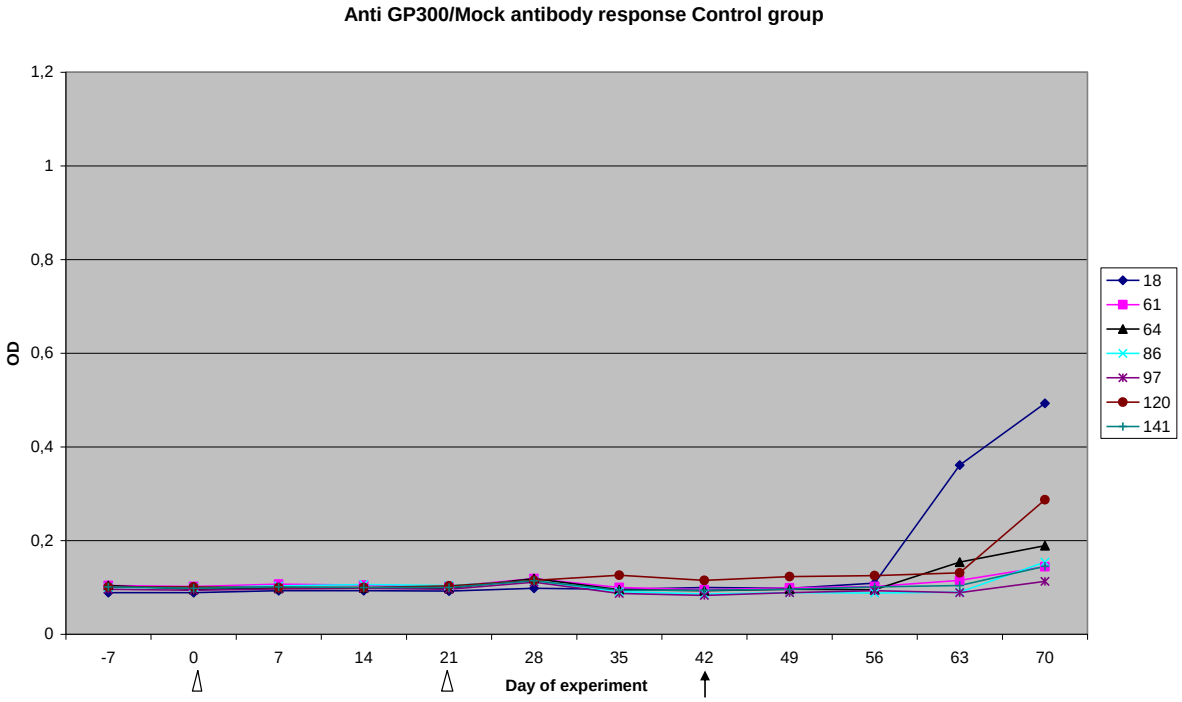


Figure 12. IgG2 antibody response of the control group against GP300/Mock.Calves are named according their total worm burden. Days of vaccination are marked with a ∆. Day of challenge infection is marked with an arrow.

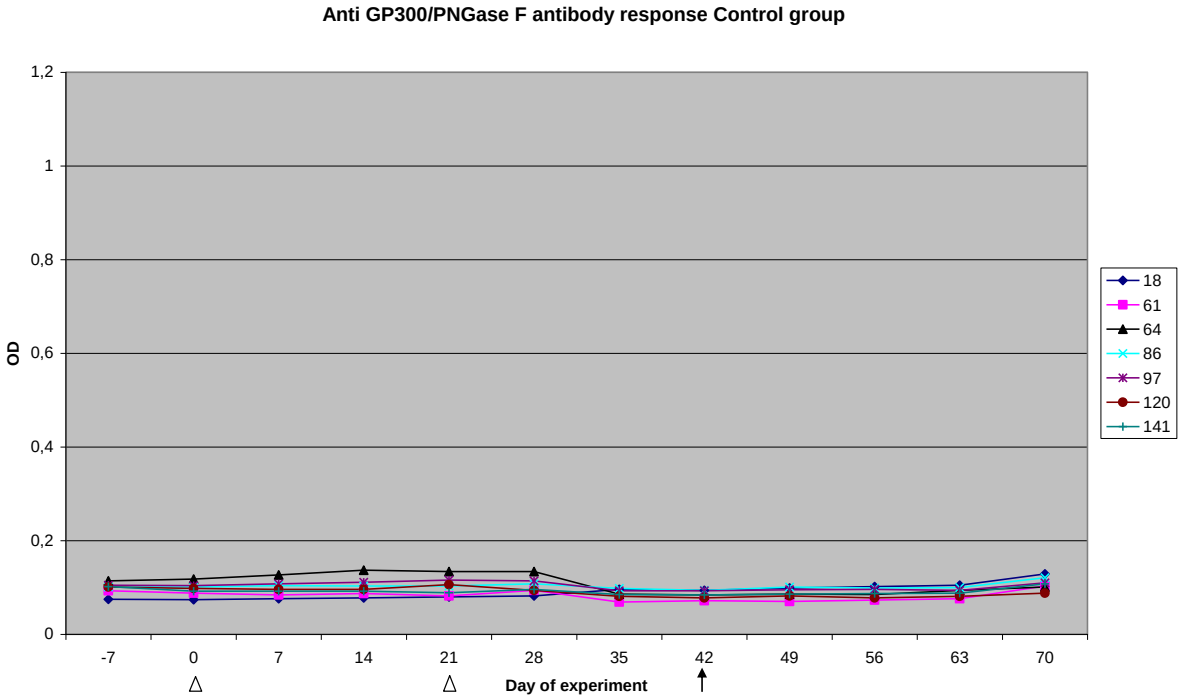


Figure 13. IgG2 antibody response of the control group against GP300/PNGase F. Calves are named according their total worm burden. Days of vaccination are marked with a ∆. Day of challenge infection is marked with an arrow.

Vaccination primed IgE response

Figure 14 shows that total IgE concentration of the vaccinated group started to rise on day 56. Total IgE concentration of the control group started to rise on day 66 (figure 15). There does not seem to be a clear reaction to vaccination as demonstrated for the IgG1 and IgG2 response. Differences between the control group and the vaccinated group were only significant on day 59 and 63. No correlations were found between total IgE concentration and parasitological parameters (total worm counts and LPG).

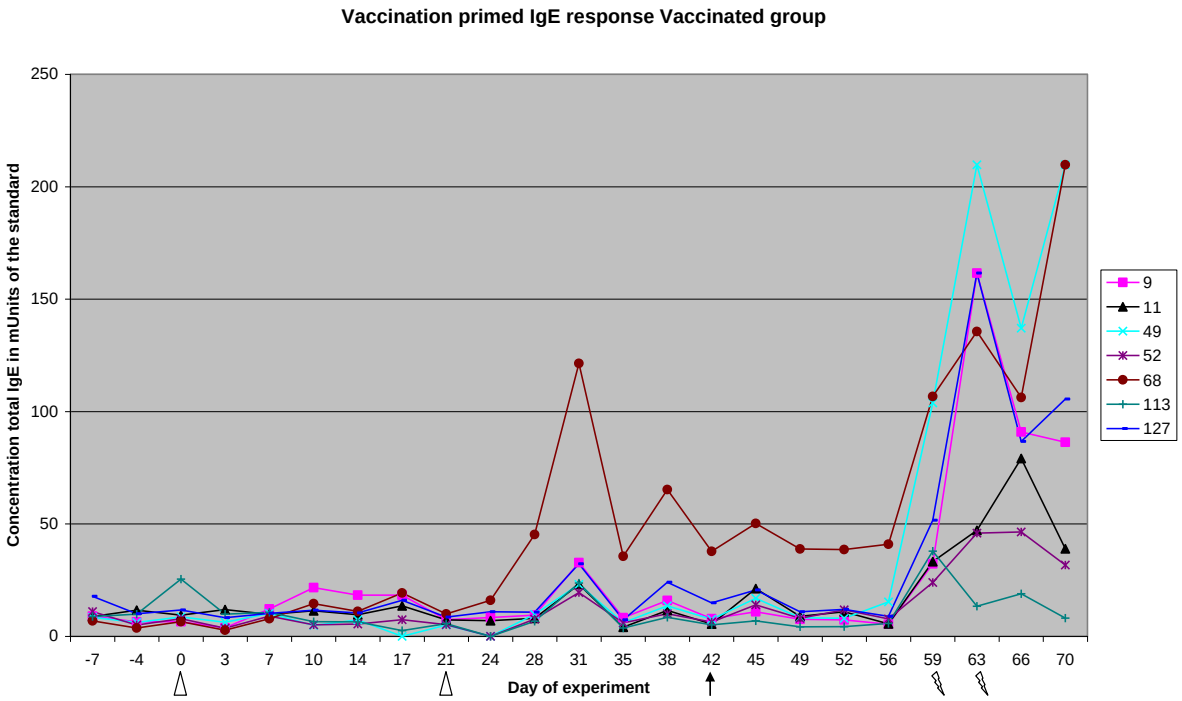


Figure 14. Total IgE Concentration in the vaccinated group given as mU of standard serum. Calves are named according their total worm burden. Days of vaccination are marked with a Δ. Day of challenge infection is marked with an arrow. Significant differences between the control group and the vaccinated group are marked with a ↯ (p=0.004).

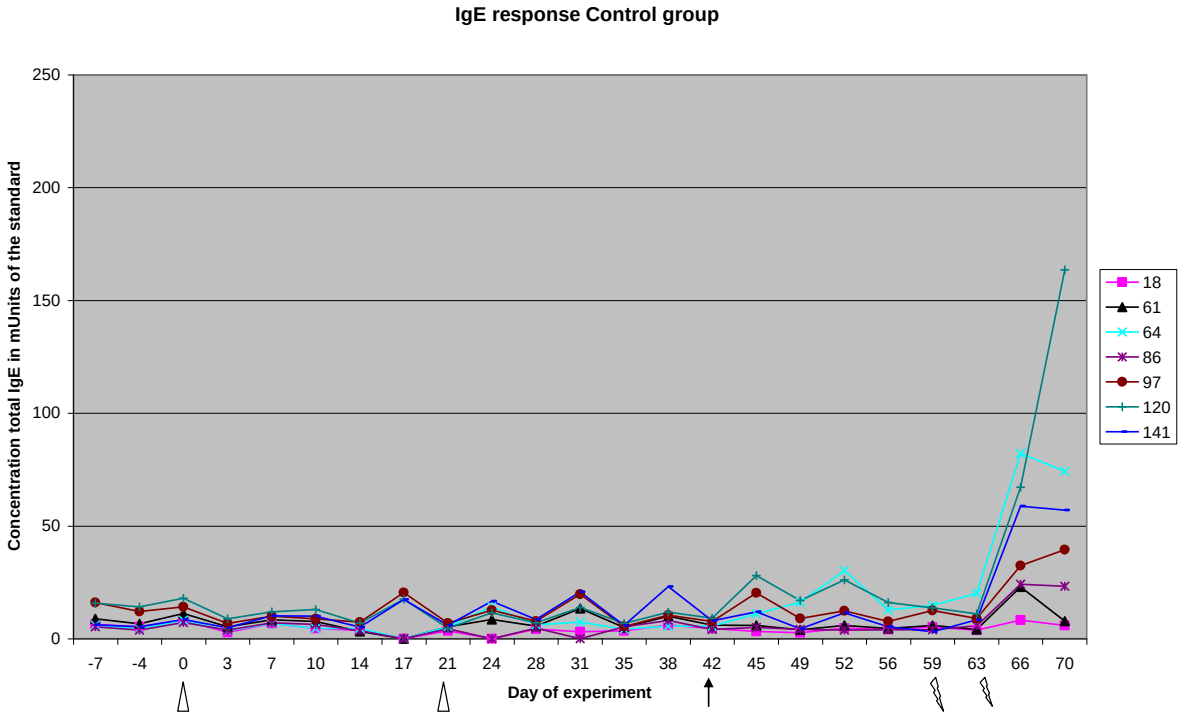


Figure 15. Total IgE Concentration of the control group given as mU of standard serum. Calves are named according their total worm burden. Days of vaccination are marked with a Δ. Day of challenge infection is marked with an arrow. Significant differences between the control group and the vaccinated group are marked with a ↯ (p=0.004).

Post-mortem worm counts

Total worm counts as determined by the modified Inderbitzin method and modified Baermann method are shown in Table 1 and 2. A difference of 27% between total worm counts of the vaccinated group and the control group was observed, but this difference was not significant. Concerning the ratio females/males, no significant differences were demonstrated between both groups as well.

Calf number	4549	6348	6042	1756	2807	4552	6501	total males	total females	Total males + females
Total worm counts	9	11	49	52	68	113	127	227	227	429

Table 1 Total worm counts (= worm counts modified Inderbitzin method + worm counts modified Baermann method) of the Vaccinated group.

Calf number	6339	6515	3836	7180	4970	3038	6577	total males	total females	Total males + females
Total worm counts	18	61	64	86	97	120	141	290	297	587

Table 2 Total worm counts (= worm counts modified Inderbitzin method + worm counts modified Baermann method) of the Control group

In order to check the reliability of the worm counts, correlation between Total worm counts and total LPGs was calculated. This correlation was significant as displayed by figure 16, suggesting not many worms had been missed during the counting process.

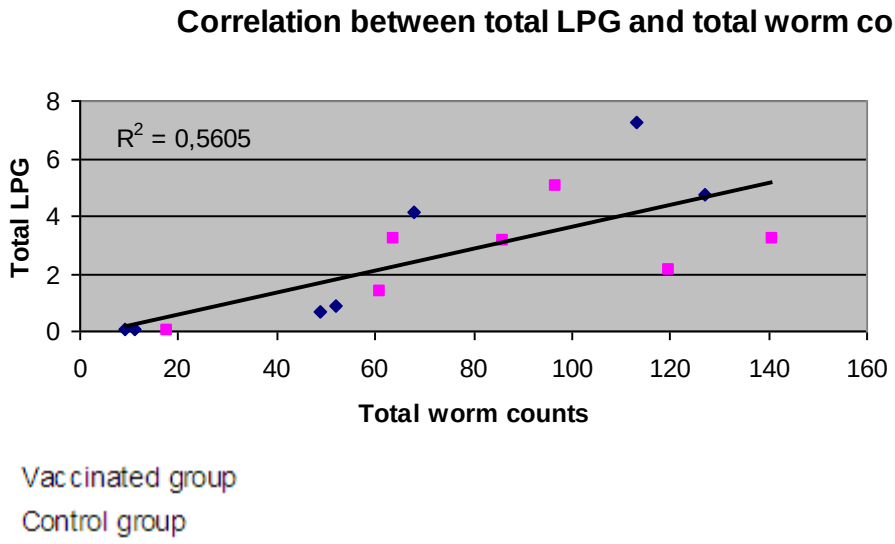


Figure 16. Correlation between total LPG and Total worm counts was significant (p<0.01).

A strong positive correlation was observed between total worm counts and IgG_2 (expressed as a percentage of standard serum). Mock treated GP300 specific IgG2 after the first vaccination correlated with total worm counts in the vaccinated group. Later on, strong positive correlations were found between total worm counts and IgG2 responses against PNGase F GP300.

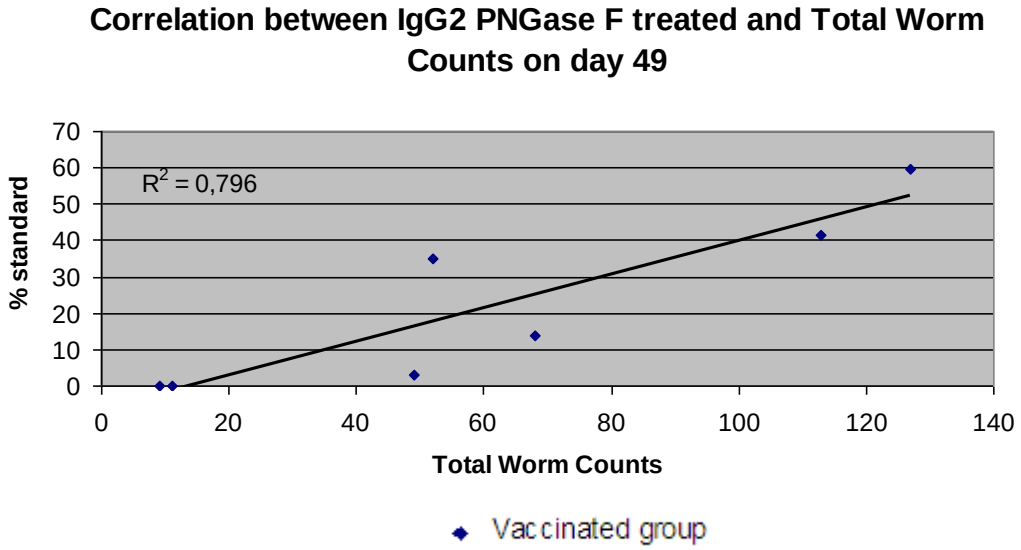


Figure 17. Example of correlation between IgG2 PNGase F treated and Total worm counts on day 49 in the Vaccinated group. IgG2 is expressed as a percentage of the standard. Significant correlations between IgG2 PNGase F treated and Total worm counts in the Vaccinated group were observed from day 28 to 70 ($p<0.05$).Significant correlations between IgG2 Mock treated and Total worm counts were observed on day 14 and 21 ($p<0.05$).

No correlations between Total worm counts and IgG2 were observed for the control group..

- **Discussion**

In this experiment, calves were vaccinated with 100 µg GP300 + Quill A (2x) or with Quill A (2x) only. Three weeks after the last vaccination they were challenged with 500 L3 of *D. viviparus.* No significant protection nor down regulation of inflammatory reactions was observed.

Purification of the vaccine was carried out successfully by WGA lectin affinity chromatography, making contamination of the vaccine unlikely. One week after the first vaccination there was obviously IgG1 antibody production against native GP300. One week after the second vaccination, a booster response was visible that was stronger than the antibody response after the first vaccination. After challenge infection, the IgG1 antibody response was even more pronounced. Of course, the booster response after challenge infection commenced less fast than the booster response after the second vaccination, because the development of the larvae, and accordance to this, their immunogenicity, costs time. The IgG1 antibody response against deglycocylated GP300 commenced after the second vaccination. Probably, a booster vaccination is necessary for an IgG1 response against the protein backbone of GP300. This is in accordance with previous results presented by Kooyman et al., (2007a), reporting a protein directed memory response after re-infection. Perhaps, re-infection is mimicked by the second booster vaccination in our experiment. In conclusion, we may assume that vaccination with GP300 induces an IgG1 antibody response directed at both the native GP300 and the protein backbone alone. However, the immune reaction is directed more at the native GP300 than against the sole protein backbone. Because the native GP300 consists of a protein backbone, N glycans and PC, one can only determine the difference of the immune reaction

against the protein backbone pertaining to immune reaction against native GP300 as a whole. In other words, one cannot determine which part of the immune response against the native GP300 is caused by which part of the GP300 antigen. OD's cannot simply be deducted with one another. Consequently, one can only tell that the difference seen in antibody response against GP300/Mock and GP300/PNGase is caused by the phosphorylcholine substituted N glycans.

The booster reaction as described for the IgG1 antibody response, is also observed for the IgG2 antibody response. The IgG2 antibody response between the first and the second vaccination seems to be similar for GP300/Mock and GP300 PNGase F. Surprisingly, after the second vaccination, a booster response against the phosphorylcholine substituted N glycans arises, while no booster response against the protein backbone is observed. This is in contradiction with previous results reporting a booster response against the protein backbone of GP300 (Kooymanf et al., 2007a). Another striking detail is the great diversity among calves in their ability to elicit an IgG2 response. This is probably caused by the fact that IgG2 levels are highly heritable (Gasbarre, Leighton, & Davies, 1993; Tizard, 2004). An earlier article studying the anti-*Ostertagia* IgG2 antibody response showed even a heritability of 90% for this trait (Gasbarre et al., 1993).

One cannot observe a clear booster response in reaction to vaccination for total IgE as for IgG1 and IgG2. But the vaccination group produces an earlier and stronger IgE response compared to the control group. Apparently, vaccination conducts some sort of priming for GP300, that is not visible as a booster response, but that does create the possibility for the vaccinated group to elicit a total IgE response earlier than the control group. It would be interesting to measure the production of parasite specific IgE against GP300 too, because earlier research showed that only sera from calves protected against re-infection contained parasite specific IgE (Kooyman et al., 2002).

Referring to the two hypothetical outcomes of this thrombospondin vaccination experiment as mentioned in the introduction of this article, one may conclude neither one of these hypotheses turned out to be valid. No difference was observed between the control group and vaccinated group regarding LPGs or Total worm counts, forcing us to reject hypothesis 1: vaccination with thrombospondin does not lead to a reduction in the number of worms.

Hypothesis 2 was based on an article by Kooyman et al., (2007b). It has been clear for a longer time that parasitic helminths have evolved ways to influence the immune system of the host towards an inflammatory environment that is favourable for the parasite (Maizels et al., 2004). Kooyman et al., (2007b) revealed that the PC moiety of GP300 elicits anti-GP300 antibodies that are able to cross-react with the PC moiety of the mammalian inflammatory mediator Platelet Activating Factor (PAF). Possibly, this would lead to a shift in the IgG1/IgG2 ratio towards IgG1 (T helper 2 response) *in vivo.* This would create an advantageous environment for the parasite and lead to less inflammation for the host. In our experiment, the IgG1/IgG2 ratio does not seem to be influenced by the parasite. On top of that, we found a positive correlation between IgG2 and post mortem worm counts, indicating that calves with a high IgG2 reaction were less able to clear infection. This is in contradiction with Kooyman et al., (2007b), who postulated that IgG2 and total worm counts are negatively correlated. But Scott et al., (1996) reported a positive correlation between

IgG2 and LPG too. It should be noted, however, that to draw a final conclusion about the IgG1/IgG2 ratio in this experiment, one should measure immune reaction against an antigen that is not incorporated in the vaccine. In that way, the booster effect in the vaccinated group cannot influence the outcome. Major Sperm Protein (MSP) could serve as such an antigen (Hoglund et al., 2008). If the MSP specific IgG1/IgG2 ratio in the control group and vaccinated group is similar, it is an indication that the vaccine does not affect the IgG1/IgG2 ratio.

Considering the clinical signs of the calves in this experiment, one may conclude that vaccination did not result in less inflammation. Observation of lungs during section showed heavily affected lungs in the control group as well as in the vaccinated group. No differences were observed in breathing rate between both groups. We were unable to find an explanation for the high average breathing rates before challenge infection. Mean rectal temperature stayed within reference for both groups. According to Holzhauer et al., (2003) and Scott et al., (1996), body temperature does not rise as a result of lungworm infection. But other authors report a mild pyrexia (Taylor et al., 2007) to a fever (Kassai, 1999) during clinical husk. Either way, because mean body temperature may have stayed within reference limits for both groups, but other clinical signs typical for lungworm disease did appear, one cannot use body temperature as a indication for less inflammation.

In conclusion, hypothesis 2 can be rejected as well: vaccination with GP300 does not reduce inflammatory reactions caused by an infection with *D. viviparus.*

Our experiment indicated that the thrombospondin vaccine does provoke a total IgE immune reaction and an IgG1 reaction. IgE and IgG1 are seen as hallmarks of the Th 2 response (Tizard, 2004). In our experiment we could not measure any protection. Thus, the Th2 response that seems to take place, apparently is not immediately associated with an effector immune response. A reason for this might be, that only particular components of the Th2 response offer protection. Probably, these components are not induced by the thrombospondin vaccine. Besides that, there is an ongoing debate in parasite immunology whether the Th2 response is protective or ineffective (Maizels et al., 2004).

The question that remains is whether the thrombospondin vaccine still has a future regarding lungworm prophylaxis. This experiment showed that GP300 is clearly recognised by the immune system, and vaccination with thrombospondin induces an antibody response consisting of IgE, IgG1 and IgG2. But the vaccine does not prevent clinical parasitic bronchitis in any way and does not reduce the shedding of larvae in the faeces.

To make a final decision about the future of thrombospondin, it would be beneficial to compare the quantity and quality of the immune reaction against the thrombospondin vaccin with the immune reaction induced in calves infected with a low primary dose of *D. viviparus* larvae. A low primary dose of 30 larvae induces 70% protection against re-infection (Kooyman et al., 2002). We can compare the reactivity against GP300 of the vaccinated animals with that of the low primary infected ones. If antibody production against GP300 in the vaccinated calves is higher than in the protected primary calves, it is less likely that the antibodies raised against GP300 are important in protection. However, if antibodies against GP300 are important to harness

protection, we expect to find lower anti-GP300 levels in the vaccinated calves compared to the primary infected ones. In the latter case, it might be useful trying to increase antibody response against the vaccine. Options to increase this response comprise the use of another adjuvant, an extra booster vaccination, a higher vaccine dose, applying another route of administration or combinations of these options.

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- **Supplementary data**

Silver staining of electrophoresis gel

Fixation of the gel was acquired by the use of 200 ml 50% methanol and 5% acetic acid for 20 minutes. Following fixation the gel was rinsed twice: first with 200 ml 50% methanol for 10 minutes, then with 200 ml H_2O for 10 minutes. 200 ml 0,02 $Na_2S_2O_3$ was used for sensitization (1 minute). The gel was rinsed twice again, both times in H_20 for one minute. Under intensive shaking with 2% Na_2CO_3 and 0,04% formaldehyde, the gel was developed. After 20-30 seconds, new develop solution was added. Development was stopped by the use of 5% acetic acid (2x3 min).