Isospora suis

Haematological parameters and antibody-development during porcine coccidiosis in suckling piglets

Bron: http://jahrgang1964-goetzis.at/aktuelles.htm

Name	Drs. M. Schlepers
Studentnumber	0352721
Date	16-06-2009
Research	05-01-2009 till 05-04-2009
Department	Infectieziekten en Immunologie
Supervisors:	Mag. H. L. Worliczek (Veterinärmedizinische Universität Wien)
	Prof. Dr. A. Joachim (Veterinärmedizinische Universität Wien)
	Dr. Ir. H.W. Ploeger (Utrecht University)

Table of contents

Abstract

Isospora suis is economically one of the most important infections of young piglets and causes diarrhoea (Mundt et al., 2006a), which lasts for three till seven days (Mundt et al., 2003). Histologically, villous necrosis and villous atrophy is found in the jejunum and ileum (Mundt et al., 2006a; Taylor, 2006).
Treatment with Toltrazuril can lower the prevalence of diarrhoea and the number of diarrhoea days. It also will achieve a lower oocyst excretion and a higher weight gain than in infected pigs (Mundt et al., 2006b).

In this experiment there were sixteen piglets used, who were orally infected with 1000 oocysts per piglet on study day 3. On study day 5 eight of the piglets were treated with Toltrazuril and the other eight piglets remained untreated.
The following parameters were determined:

- Counting oocysts; study day 7 – 20
- Faecal consistency; study day 3 and day 7 – 20
  1 = normal, 2 = pasty, 3 = semi-liquid, 4 = liquid
- Weighing; study day 3, 5, 7, 14, 21 and 28
- Blood samples; study day 7, 14, 21 and 28
  1 Hematology
    Erythrocytes, haemoglobin, haematocrit, MCV, MCH, MCHC, leukocytes and white blood cell differentiation
  2 Indirect immunofluorescence assay

The oocyst excretion of the non-treated piglets showed a biphasic pattern with a first peak on day 9 and a second peak on day 16. The treated piglets did not shed oocysts. The faecal score was significantly higher in the non-treated group.
The weight was not significant different between the groups, which could be the result of the breed used for this experiment (Landrace x Duroc x Pietrain). The piglets of this breed had no bad diarrhoea and started shedding oocysts quite late in the infection.
Haematology showed that the eosinophils, erythrocytes, lymphocytes, monocytes and mean cell volume (MCV) were significantly different for the parameter study day. The eosinophils, erythrocytes, lymphocytes and monocytes increased during the study period. An explanation could be that this increase is due to the normal development of the piglet immune system.
The mean cell volume increased significantly during the study period by the replacement of foetal erythrocytes by normal ‘adult’ erythrocytes.

Finally, the indirect immunofluorescence assay showed that there was a significant difference in antibody titer between treated and non-treated piglets for study day 21 and 28. This difference could be due to the decrease in maternal antibodies in the treated piglets and the production of own antibodies in the non-treated group. The overlap for the production of own antibodies and the decrease of maternal antibodies could lie somewhere between study day 14 and study day 21. Also, due to gut damage in non-treated piglets and therefore creating a decreased barrier against incoming pathogens, a result could be, a prolonged infection. This longer infection period could cause the sustained high antibody titer of non-treated piglets.

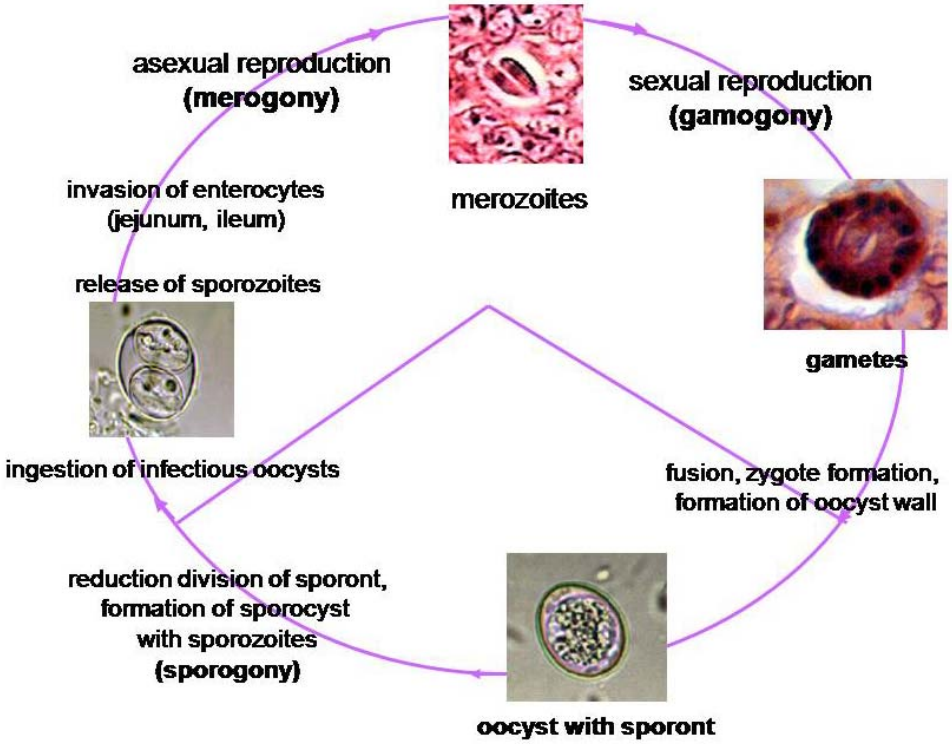
The presence of maternal antibodies against *Isospora suis* in piglets was proven by an indirect immunofluorescence assay on colostrum and precolostral serum. The colostrum tested positive for the presence of maternal antibodies and the precolostral serum tested negative for these antibodies. However, the maternal antibodies do not protect against an infection with *Isospora suis*.

Introduction

Isospora suis is economically one of the most important infections of young piglets (Mundt et al., 2006a). The infection is present in most countries worldwide where pigs are reared, but the real prevalence in the different countries is not exactly known because it is underreported in the field (Mundt et al., 2006a; Taylor 2006). The probable reason for this is the special excretion pattern and age-related susceptibility of pigs for *Isospora suis* (Mundt et al., 2006a).
A study in Germany, Austria and Switzerland showed that the prevalence of *Isospora suis* on the 324 farms tested was 76.2% (Mundt et al., 2005). A study in the Netherlands in 1990 showed a prevalence of 56% (Eysker et al., 1994).
The economic losses are caused by decreased weight gain and unthriftness as well as treatment of diseased pigs (Mundt et al., 2006b).

Life cycle

Isospora suis is a parasite belonging to the family Eimeriidae (Mundt et al., 2006a). Piglets are infected with sporulated oocysts of *Isospora suis* by ingestion of infected faeces or contaminated food. The sporulated oocysts become sporozoites and infect epithelial cells in the cranial jejunum. These sporozoites become merozoites, which will invade the epithelial cells again. This process will continue till a third generation of merozoites is reached about four to five day's post-infection. These merozoites invade cells in the distal small intestine and give rise to microgamonts and macrogamonts. The gamonts mature and become micro gametes and macro gametes. When a microgamete fuses with a macrogamete, an oocyst will be formed and subsequently excreted with the faeces (Taylor, 2006; Worliczek, 2007).
From day five till seven post-infection the first peak of oocyst excretion starts (Mundt et al., 2006a; Taylor, 2006; Worliczek, 2007), which last for four days (Mundt et al., 2006a). On day ten and eleven post-infection there is a second peak of oocyst excretion, due to the second-generation merozoites in the epithelial intestinal cells, which return to the gut and finish their development (Mundt et al., 2006a; Taylor, 2006).
The prepatent period is, as mentioned above, about seven days (Mundt et al., 2006a; Taylor, 2006). However, pigs can already show clinical signs during the prepatent period. At the onset of oocyst excretion there are already tissue alterations in the gut, which causes clinical signs (Mundt et al., 2006b; Worliczek et al., 2007).



Bron: H.L. Worliczek

Epidemiology

Infection is most common in the first three weeks of life, but the younger the infected pig, the higher the excretion rate of oocysts and more pronounced diarrhoea will occur. So, there is an age-related resistance to the infection (Mundt et al., 2003). It seems that this resistance is mainly based on the cellular immune response, which is immature in the first three till four weeks of life. Other explanations are that the maturation of the innate immunity is more important than specific immune mechanisms, the involvement of the local immune system and an increased epithelial cell turnover in older piglets. However, there is no evidence for either one of these explanations (Worliczek et al., 2007).

The role of the initial infection is not completely clear. On one hand, a high initial dose results in a fast and wide distribution of the infection among piglets, whereas a low infection dose initiates a slower and less wide spread (Mundt et al., 2003). On the other hand, there was no dose correlation between increasing infection doses and prevalence of excretion and diarrhoea (Mundt et al., 2003, Worliczek et al., 2007). It seems that age is the most important factor in infection (Worliczek et al., 2007).

Immunity and immune response

The sow has an epitheliochorial placenta, which allows no transport of immunoglobulines from the maternal to the foetal side (Noakes et al., 2001; Salmon et al., 2009; Senger, 2003). The consequence of this is that the piglets are born without immunoglobulines in their blood; they are agammaglobulinemic at birth (Salmon et al., 2009).

After birth the piglets will ingest colostrum derived from the sow, which is rich in maternal antibodies (IgA, IgG, IgM). This lactogenic immunity consists mainly of IgG in sow colostrum and IgA in sow milk (Salmon et al., 2009; Xu et al., 2003).

As described in the previous section, there is an age-related resistance to infection with *Isospora suis*. It is assumed that maternal antibodies play only a minor role in the resistance to *Isospora suis* and that the resistance is mainly based on the cellular immune response. It seems that the porcine immune system is not fully developed at birth and that the piglets are during the first three to four weeks of life very susceptible to infection. Components of the specific cellular immune response remain then immature. Thus, maturation of the cellular immune system is very important for development of resistance to infection (Worliczek et al., 2007). In protozoan infections T_H1 cells play a major role (Rommel et al., 2000).
After a pig has been infected by *Isospora suis*, it will develop a resistance to re-infection.
The local immune response in the gut might also play a role in the immune response. *Isospora suis* remains within the superficial cell layers of the intestine, so contact with cells of the host immune system may be limited. Second, the number of T-cells is different in newborn piglets. In the villi of adult pigs dendritic cells and T-cells can be found, cytolytic $CD8^+$ cells superficial and $CD4^+$ cells deep in the core of the villus. B-cells and plasma cells can be found in the crypts. At birth, there are almost no T-cells in the villi. At an age of six weeks the numbers of cells are the same as in an adult pig.
Finally, the Peyer's patches are not fully developed at birth, which would be populated with B-cells. They are complete at twelve days of age (Worliczek et al., 2007).

Clinical signs and pathology

After infection the faecal consistency becomes semi-liquid to liquid. Five till six days post infection most of the animals become diarrhoeic (Mundt et al., 2006a), which lasts for three till seven days and is pasty to watery (Mundt et al., 2003). The colour can vary from white to yellow (Taylor, 2006). There is also a decreased body weight gain. On histological examination of the jejunum and ileum there are villous necrosis and villous atrophy. Five days post-infection the alterations are mainly situated in the cranial part of the jejunum (first generation merozoites). On eleven days post-infection the alterations were mainly situated in the distal parts of the small intestine (third generation merozoites). Because of the villous atrophy there is a decreased absorption surface in the gut, which explains the decreased weight gain (Mundt et al., 2006a; Taylor, 2006).

Treatment and prevention

There is no real treatment against an outbreak of isosporosis, so prevention is very important. When a pig shows clinical signs of *Isospora suis*, there are already tissue alterations, so a treatment is not effective any more. *Isospora suis* can be made more controllable by prophylactic treatment with Toltrazuril or hygiene measures.
Toltrazuril. The exact mode of action of Toltrazuril is still not fully known (Mundt et al., 2006b), but it seems that Toltrazuril interferes with the enzymes of the respiratory chain of *Isospora suis* and that it inhibits the pyrimidine synthesis (Mundt et al., 2006b; Harder, 1989). The coccidia will still get intracellular in the host, so there is development of immunity (Greif, 2000).

Toltrazuril lowers the prevalence of diarrhoea and also lowers the number of diarrhoea days. Further, the oocyst excretion will be lower and the weight gain is higher than in infected pigs. Histological, the epithelium of the distal jejunum appears normal compared to the villous necrosis and villous atrophy seen in infected pigs. This can be the reason for the better weight gain.
It is recommended to use Toltrazuril in a single treatment with 20 mg/kg of body weight on days three, four or five of life (Mundt et al., 2006b).
Hygiene measures. Piglets mainly become infected in farrowing pens contaminated with oocysts, because oocysts are very resistant against dehydration and disinfectants. One infected piglet can infect all its littermates. So, an improved hygiene helps to decrease the exposure and infection rates (Meyer et al., 1999). Cleaning with steam (Eysker et al., 1994) or flambing the floors will lower the infection pressure. Another possibility is the use of Neopredisan 135-1®, which induces lysis of the oocysts of *Isospora suis*. Before using Neopredisan 135-1® the stable has to be properly cleaned, otherwise the disinfection will fail (Straberg et al., 2007).

Isospora suis has a sporulation time of twelve hours at a temperature of >30°C (Mundt et al., 2006b). So, there are in a relatively short time a lot of infectious oocysts in a stable, which is nice and warm (like a farrowing pen).

Aim of the study

Since there is not much known about the immune response to *Isospora suis*, as described in 'Immunity and immune response', the antibody response will be investigated after infection with *Isospora suis*. More precisely, the difference in antibody response between piglets treated with Toltrazuril and non-treated piglets.
The haematologic parameters will also be investigated during the infection. This is very interesting, because there is no literature about haematologic parameters in piglets. What will happen with the haematologic parameters during an infection with *Isospora suis* in piglets?

Materials and methods

This research was carried out as an experimental, longitudinal cohort study.

Animals

For this experiment two Landrace x Duroc x Pietrain sows were used and one Landrace sow, housed on straw. They were bought from a farm, which was not free of any specific pathogens. The sows arrived two weeks before parturition and were tested for parasites. They tested positive for *Trichuris suis* and consequently treated with Panacur® for five days. After a second test they were all negative for *Trichuris suis*.

The farrowing crates were cleaned and disinfected before the sows arrived. The sows got a standard diet, without any antibiotics and coccidiostatics, and had free access to drinking water.

Two days before and two days after parturition the sows got a diet with bran and water, which has been shown to be readily eaten and easily digested by sows.

The piglets were raised under standard conditions until weaning. The pigs for the trial were of three litters. Half of the piglets in a litter were treated with Toltrazuril.

Experimental design

The following actions are done (the table can also be found in appendix I):

- Day 2: vaccination against *Mycoplasma hyopneumoniae* (Stellamun® 2 * 1 millilitres intramuscular) and an iron injection (Vanafer® 2 * 1 millilitres subcutaneous).
- Day 3: tattoo in the ear and oral infection with 1000 oocysts per piglet with a single use plastic Pasteur pipette. Further weighing and determining the faecal consistency on a scale of four (1 = firm/normal, 2 = pasty, 3 = semi-liquid and 4 = liquid).
- Day 5: piglets at random divided in two groups (treated and non-treated). Half of the piglets treated with Toltrazuril (Baycox® 1 millilitre). In one litter, half of the piglets was treated and half non-treated. So the study was done with a negative control group and done double-blinded.
- Day 7: viral and bacteriological examination of all animals.
- Day 3, 5, 7, 14, 21 and 28: weighting the piglets.
- Day 7, 14, 21 and 28: taking blood samples. One tube was for haematology and one tube was for the immunofluorescence test and titration.
  The haematology was done by the laboratory of the Veterinärmedizinische Universität Wien and the following parameters were determined: erythrocytes, haemoglobin, haematocrit, MCV, MCH, MCHC, leukocytes and the differentiation of the white blood cells.
  The blood used for the immunofluorescence test and titration was centrifuged for ten minutes, 4600 RPM. The serum was put in Eppendorf cups and stored in the refrigerator.
- Day 7 till day 28: faeces examination (see below). The faeces were collected by putting the piglets in crates, one piglet per crate. When the piglets produced a faecal sample, they were putted back to the sow. Of the remaining piglets, which produced no faecal sample, a rectal sample was taken. This was not done routinely, because almost all pigs produced a faecal sample by itself, it takes much more time and it is more stressful.

Isolation of oocysts from piglet faeces

Isolation of oocysts. The oocysts used for the infection and the indirect fluorescence test were derived from the faeces of piglets of the Institute of Parasitology, which were shedding oocysts. The faeces was collected and suspended in water, so there appeared a dilution of water: faeces of 1:10. It was mixed with a magnetic stirrer and magnet. Then it was transferred to a centrifuge container using a metal sieve and centrifugated for five minutes, 1700 x *g*. The supernatant was put in a new centrifuge container and centrifuged again five minutes, 1700 x *g*. The pellets were resuspended in 25% Percoll® and twice centrifuged as described above. Percoll® separates the fatty supernatant from the rather clean pellet, which contains the oocysts. The pellet was washed in tap water twice as above.

Sporulation. After the last washing step with water, the supernatants were thrown away and the pellets were put in a Petri dish. This oocyst suspension was mixed with an equal volume of potassiumdichromate 4%, which gave a final concentration of 2%.

Now the suspension was aerated twice a day with a Pasteur pipette for about five seconds. It is possible to automate this step by putting the solution in an Erlenmeyer and constantly slowly adding air via a machine. This option was not chosen in this setup, due to the fact aerating manually is easily done and that the machine wasn't available at this time. Evaporated water had to be replaced, to prevent the suspension from drying out. At room temperature the sporulation takes about one week. The oocysts can be stored in the dark at 10°C, where they will remain viable and infectious for about twelve months.

Infection dose. The *Isospora suis* oocysts were stored in a 2% potassiumdichromate solution and this had to be washed out by centrifugation. It was mixed in the Vortex and filled further with tapped water. Then it was centrifuged at 1700 x *g* for five minutes and the supernatant was thrown away. This process of washing with tapped water was repeated until the solution was clear. Next a dilution was made with 0.9 millilitres of flotation solution and 0.1 millilitres of the oocyst suspension in an eppendorfcup. Two McMaster chambers were filled, left to flotate and the sporulated oocysts were counted. From this number of sporulated oocysts the required concentration was calculated following the formula ($V_1 * C_1 = V_2 * C_2$ in which V is volume and C is concentration). After preparing the required concentration by adding water to a certain amount of oocyst suspension, again 0.9 millilitres of flotation solution and 0.1 millilitres of the new oocyst suspension were put in an eppendorf to verify if the concentration was right. Subsequently, the required numbers of doses were prepared in eppendorfs.

Oocyst count

Autofluorescence. At first the faecal samples were investigated under the microscope for autofluorescence, because oocysts of *Isospora suis* are autofluorescent. This was an effective and efficient manner to check if a piglet was shedding oocysts.

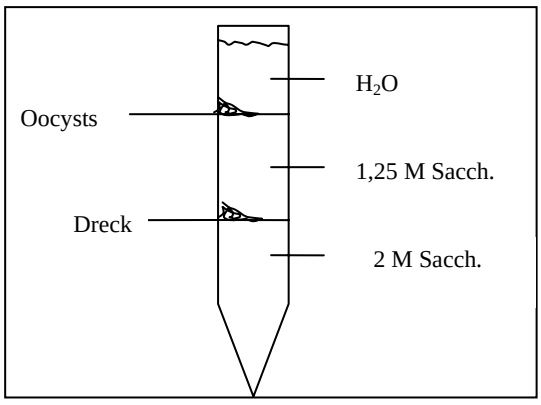
When there was an oocyst found, the sample was counted. Unfortunately this method resulted in some false positive samples (an oocyst under the autofluorescence microscope, but no oocysts while counting.)

The slide was prepared by putting a little drop of faeces (around 0.3 gram) on a slide and adding two drops of water. Because the faeces and water were not weighted, this method is not really reproducible. The faeces and water were mixed with a spatula and a cover sheath was put on it.

Oocyst count. When a faecal sample was positive for autofluorescence, the number of oocysts was counted in a McMaster chamber.
First 0.5 gram of faeces and 4.5 gram of flotation solution were mixed with a wooden spatula. Then the suspension was put in a tube. A two-layered gauze was laid on the opening of the tube and with a wire-öse the gauze was pushed into the tube. In this way the large faecal material was pushed down and a watery suspension came above the gauze.
An eppendorf was filled with 1800 µl flotationsolution and 200 µl of the suspension was added to this. Then after mixing two McMaster chambers were filled with the suspension, which were left to flotate for a couple of minutes. Finally the oocysts were counted in the two McMaster chambers.
The number of oocysts in the two chambers is the number in 300 µl of the diluted suspension (dilution 1:100). The number of oocysts per gram of faeces (OPG) can be calculated by the formula OPG = number of counted oocysts * 333.

Indirect immunofluorescence assay: oocyst preparation

Cleaning of the oocysts. As mentioned above, the *Isospora suis* oocysts were stored in a 2% potassiumdichromate solution. First the fluid in the tube was mixed, followed by taking a sample for counting the number of oocysts under the microscope. The fluid was filtered over a 100 µl sievesilk to remove the large particles. A second sample was taken from the new solution for counting the number of oocysts under the microscope. Now empty tubes were filled with 5 ml 2 M saccharose (δ = 1.25 gram/litre), 5 ml 1.25 M saccharose δ = 1.15 gram/litre) on it and finally as top layer 2.5 ml of the oocyst suspension. The tubes were centrifugated for seven minutes, 1500 x *g*.
The result of this centrifugation step is represented in the picture. There are five layers, namely 2 M saccharose, muck, 1.25 M saccharose, the oocysts and a layer of water on top of it. The oocysts of the different tubes were sucked up with a 2.5 ml eppendorf-pipette and put in one fifty millilitre tube. At last it was diluted with water, where the density must stay under 1.02 g/l. From this solution a third sample was taken for counting the number of oocysts under the microscope. The clean oocysts can be stored in the refrigerator.



The obtained three samples had to be counted in a McMaster chamber. Therefore an Eppendorf was filled with 1800 µl flotationsolution and 200 µl of the suspension was added to this. Then after mixing two McMaster chambers were filled with the suspension, which were left to flotate for a couple of minutes. Finally the numbers of sporulated oocysts were counted in the two McMaster chambers. The number of sporulated oocysts in the two chambers is the number in 300 µl of the diluted suspension (dilution 1:10). The number of sporulated oocysts per millilitre = number of counted sporulated oocysts * 33.33. The initial tube had a capacity of fifty millilitres, so the number of sporulated oocysts per millilitre can be multiplied by fifty to calculate the amount of sporulated oocysts in the tube. The purpose of counting the three samples is to determine the loss of oocysts during the cleaning process.

The sugar solution used above for the centrifugation was prepared as follows:

- 2 M saccharose: 500 ml double destiled water added to 342.3 grams of sugar. First the sugar was mixed in 200 ml water for half an hour and after this the rest of the water was added and mixed for another half an hour.
- 1.25 M saccharose: 187.5 ml 2 M saccharose added to 112.5 ml double destiled water.

Excystation. The excystation of the cleaned oocysts was done in three steps:

1. The needed amount of sporulated oocysts was calculated and these volumes of cleaned oocysts were put in two 15 ml tubes. They were centrifuged 1700 x *g*, five minutes. The supernatant was thrown away. The pellet was resuspended in 5 ml ddH_2O and 100 µl NaOCl, which was used for desinfection. The tubes were closed, mixed and put in the refrigerator (4°C) for 10 minutes. After this time period, ddH_2O was added to the tubes until 14 ml and centrifuged, 1700 x *g*, five minutes. This washing step with centrifugating and resuspending the pellet in ddH_2O was repeated five times. Before the last washing step, the oocysts suspensions were put thru a 50 µm sieve in a new 15 ml tube. For this the supernatant was thrown away and the pellet resuspended in 5 ml ddH_2O. This solution was put thru the sieve and now there is only one tube left.
   After the last washing step the pellet was resuspended in ddH_2O and the tube stored in the incubator, ready for further use.
2. The oocyst suspension from step one was centrifuged 1700 x *g*, five minutes. The supernatant was thrown away and the pellet resuspended in 5 ml L-Cystein-$NaHCO_3$. This was put in a new 50 ml tube. Another 5 ml L-Cystein-$NaHCO_3$ was put in the used tube to clear it from all oocysts and put in the 50 ml tube. It was gassed with CO_2 for one to two minutes. Now the tube was immediately closed, using a piece of parafilm. Incubation over night at 37°C.
   The L-Cystein-$NaHCO_3$ solution was made by combining 31.52 mg L-Cystein-HCl, 168.02 mg $NaHCO_3$ and 10 ml ddH_2O. It was mixed (with the vortex) and sterile filtrated. L-Cystein-$NaHCO_3$ destabalizes the oocyst wall.
3. The oocyst suspension from step two was centrifuged 1700 x *g*, five minutes and the supernatant thrown away. The pellet was resuspended in 5 ml bile-trypsine and put in a new tube. This was repeated with 5 ml bile-trypsine. The tube was stored in the incubator (37°C, 5% CO_2) for three to four hours. Every thirty minutes a microscopic control.

After three to four hours of incubation, the suspension was put in a 15 ml tube, filled up with medium and centrifugated 3200 RPM for duration of five minutes. The supernatant was thrown away and the pellet was resuspended in 1 ml medium. The sporozoites were counted in a Bürcker chamber and the oocysts in a McMaster chamber. From the number of sporozoites the needed volume for infection could be calculated (100000 sporozoites/chamber). The solution was now ready for further use to infect the cell culture.

The bile-trypsine was made by adding 10 ml HBSS to 800 µl pig bile and 0.04 gram trypsine. It was mixed and sterile filtrated thru a 0.22 µm sterile filter. The trypsine breaks down the oocyst wall. Bile stimulates the sporozoites and merozoietes to come out of the oocyst, because bile imitates the small intestine where bile is secreted.

Indirect immunofluorescence assay: cell culture

A porcine intestinal epithelial cell line (IPEC), infected with *Isospora suis* sporozoites, was used.
To the medium 50 ml 5% foetal calf serum, 5 ml Penicilline-Streptomycine (10000 units penicilline/millilitre and 10 mg streptomycine/millilitre) and 5 ml L-glutamine were added. Foetal calf serum was chosen for its high fructose-content and glutamin for metabolism.
Splitting the cell culture. The medium was put out and 4 ml of PBS inserted into the little flask to wash the medium away. The PBS was taken out of the flask. Then 4 ml of accutase was inserted in the flask. Accutase is a mixture of proteolytic and collagenolytic enzymes and lets the cells detach from each other and the bottom of the flask. After ten minutes in the incubater, there was 10 ml medium added to the accutase-solution and mixed. For splitting the cells 1:7 for example, 2 ml was put in a new flask and 3 ml of medium added. The cells were stored in the incubator and the medium was changed between two and three times a week until the cells were ready for a new splitting.
Chamber slides. The medium was put out and 4 ml of PBS inserted into the little flask to wash the medium away. The PBS was taken out of the flask. Then 4 ml of accutase was inserted into the flask. After ten minutes in the incubater, there was 10 ml medium added to the accutase-solution and mixed. The solution was put into a 15 ml tube and centrifugated 300 x *g*, 5 minutes. The supernatant was thrown away and the pellet was resuspended in 5 ml medium.
Per chamber slide were 4 * 10^5 cells needed, so the number of cells in the solution had to be counted in a Bürcker chamber twice. For one counting session the number of cells per millilitre was the number of counted cells/4. From this number the needed amount of millilitres in each chamber could be calculated. Finally per chamberslide was added 2 ml medium. The cells were stored in the incubator overnight.
The next day the cell culture could be infected with sporozoites (100000 sporozoites/chamber). For this the medium was put out and a new medium given in for washing the cells in the chamber slide. This medium was also put out and a volume of sporozoites was inserted in the chamber slide. The chamber was further filled with medium, so there was approximately 3 ml in the chamber.
The day after infection of the cell culture, the medium was changed three times.

Glass slide with ten marked dots. There was added 1 ml of trypsine to the chamber slide. This was mixed in the chamber slide and thrown away. Again 1 ml of trypsine was inserted in the chamber slide and this was incubated for 30 minutes in the incubator. After 30 minutes the fluid was transferred to a 15 ml tube. The chamber was washed with 2 ml medium, which was added the tube too. Again 2 ml of medium was added to the chamber slide and this time the bottom of the slide was cleaned with a cell scraper. The medium was inserted in the tube. For the third and last time the chamber slide was washed with 2 ml of medium. The 15 ml tube with the infected cells was centrifuged 3200 RPM, five minutes. The supernatant was thrown away and the cells were resuspended in 500 µl PBS. The number of merozoites was counted in a Bürcker chamber; the number of merozoites per millilitre was the number of counted merozoites/4. The aim was to get 0.02 – 0.1 * 10^4 merozoites/ml. When the suspension was diluted to the desired concentration of merozoites, 25 µl was put on each dot of the slide.

Indirect immunofluorescence assay

A microwell plate was used to make different dilutions of the sera. There were four sera per piglet, taken from the piglet on different time points (Appendix I). The positive and negative controls were made in a dilution 1:40. The microwell plate was filled as follow:

1	2	3	4	
○	○	○	○	A
○	○	○	○	B
○	○	○	○	C
○	○	○	○	D
○	○	○	○	E
○	○	○	○	F
○	○	○	○	G
○	○	○	○	H

1 = first serum piglet
2 = second serum piglet
3 = third serum piglet
4 = fourth serum piglet

A = dilution 1:20	190 µl PBS + 10 µl serum
B = dilution 1:40	100 µl PBS + 100 µl of A
C = dilution 1:80	100 µl PBS + 100 µl of B
D = dilution 1:160	100 µl PBS + 100 µl of C
E = dilution 1:320	100 µl PBS + 100 µl of D
F = dilution 1:640	100 µl PBS + 100 µl of E
G = dilution 1:1280	100 µl PBS + 100 µl of F
H = dilution 1:2560	100 µl PBS + 100 µl of G

Four glass slides with ten marked dots with the infected cell culture were used, for each serum one slide. Eight of the dots were filled with 10 µl of dilutions B till H and the last two dots with a 1:40 dilution of the positive- and negative control. This was done for the four sera.
The glass slide with ten marked dots was filled as follows:

B	C	D	E	F
○	○	○	○	○
○	○	○	○	○
+	-		H	G

After this, the slides were laid in a water bath for thirty minutes, to make sure the dots wouldn't dry out. Next, two times washing in PBS during seven minutes each.
The conjugate (goat anti-swine IgG) was made in a dilution 1:50. On each dot of the slide was put one drop of conjugate with a syringe and needle. Now again the slides were put in a water bath for thirty minutes and two times washed in PBS for seven minutes each. Finally, three drops of PBS-glycerin mixture were added on the slide and the slide was covered with a cover-slide.

Statistical evaluation

The statistical evaluation was performed with SPSS 14.0 for Windows. The haematology was evaluated using the Kolmogorov-Smirnov test to see if the data were normally distributed. When the data were not normally distributed, the Mann-Whitney U test was used. Then the Levene's test was used to check if the ANOVA-test (analysis of variance) could be used. When the Levene's test was significant and thus the ANOVA-test could not be used, a t-test was done.
When any significance in the data of the ANOVA-test was found, a supplementary Bonferroni-test and t-test were done. A supplementary t-test was also done when the Mann-Whitney U test was significant.

Results

Haematology: blood cells

The values of eosinophils, erythrocytes, lymphocytes and monocytes were significantly different for study day (Table 1). Also the mean cell volume was significantly different.

Eosinophils. There was an increase in eosinophils during the study period (Figure 1). The ANOVA-test proved that there was a significant difference for the parameter study day (P = 0.000). This significance did not appear in the Bonferroni-test or the t-test. There is no significant difference between treated and non-treated animals (P = 0.973).

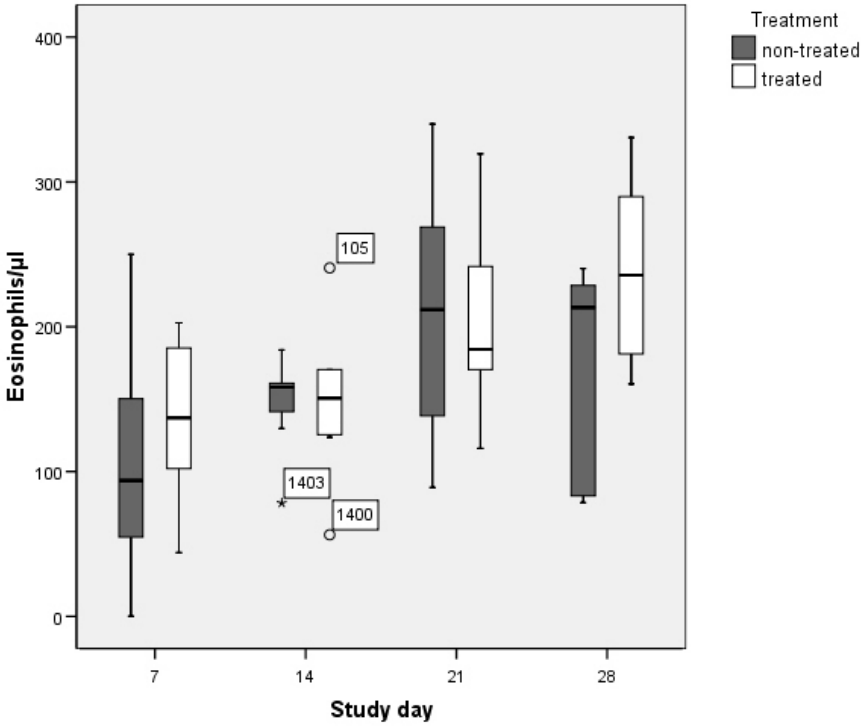


Figure 1: Eosinophils/µl on study day 7, 14, 21 and 28. Number 105, 1400 and 1403 are outliers.

Erythrocytes. The amount of erythrocytes increased with age (Figure 2). In the ANOVA-test was a significant difference for the parameter study day (P = 0.000). There is no significant difference between treated and non-treated animals (P = 0.633). The significant difference of study day also appeared in the Bonferroni-test for the following studying days:

- Study day 7 non-treated + study day 21 non-treated (P = 0.001)
- Study day 7 non-treated + study day 28 non-treated (P = 0.012)
- Study day 7 treated + study day 21 treated (P = 0.010)
- Study day 7 treated + study day 28 treated (P = 0.000)

The t-test was not significant.

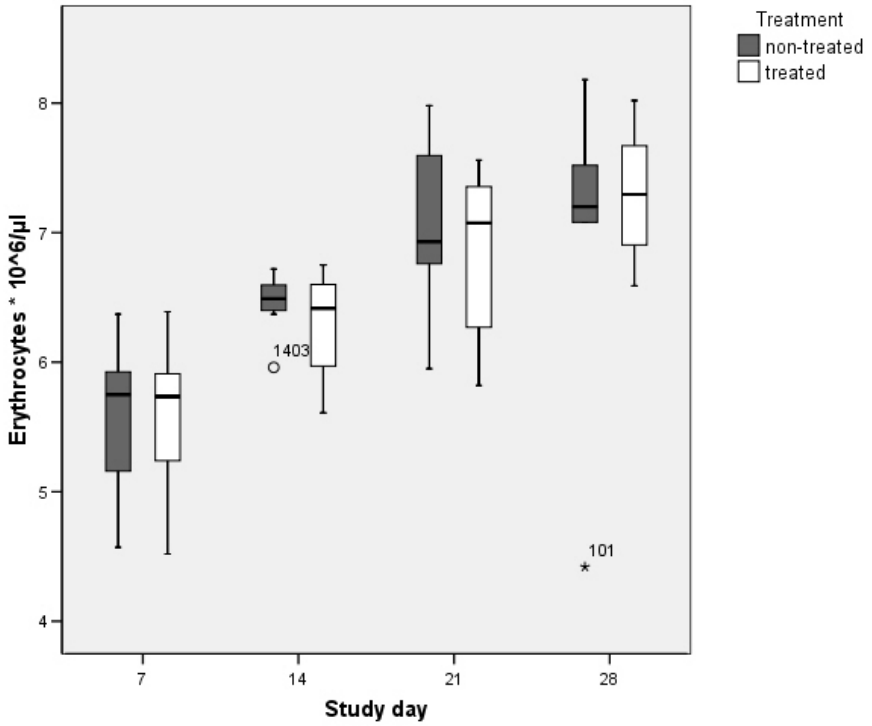


Figure 2: Erythrocytes * 10^6/µl on study day 7, 14, 21 and 28. Number 101 and 1403 are outliers.

Lymphocytes. The lymphocytes increased with age (Figure 3). The ANOVA-test showed a significant difference for the parameter study day (P = 0.000). This significance did not appear in the Bonferroni-test or the t-test. There is no significant difference between treated and non-treated animals (P = 0.769).

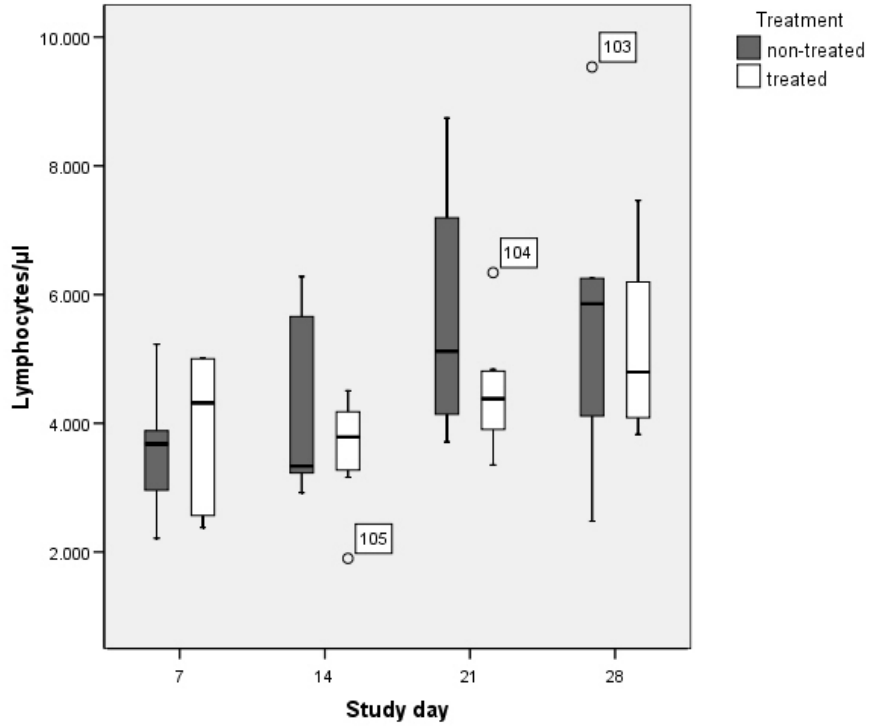


Figure 3: Lymphocytes/µl on study day 7, 14, 21 and 28. Number 103, 104 and 105 are outliers.

Monocytes. The monocytes increased during the study period (Figure 4). The ANOVA-test proved that there was a significant difference for the parameter study day (P = 0.005). This significance did not appear in the Bonferroni-test or the t-test. There is no significant difference between treated and non-treated animals (P = 0.999).

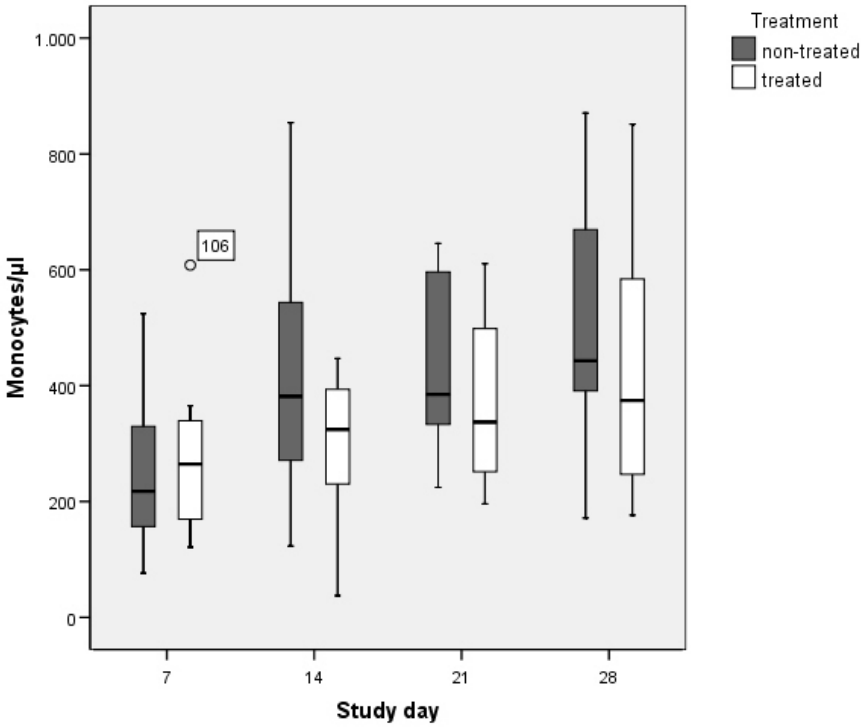


Figure 4: Monocytes/µl on study day 7, 14, 21 and 28. Number 106 is an outlier.

Haematology: erythrocyte related

Haematocrit. The ANOVA-test showed no significant difference for the parameter study day (P = 0.844) or treatment (P = 0.828) (Table 2).

Haemoglobin. The haemoglobin concentration was not normally distributed, so a Mann-Whitney U Test was done. The haemoglobin concentration was not significantly different (P = 0.947) in the Mann-Whitney U Test (Table 3).

Mean cell haemoglobin. The mean cell haemoglobin was not normally distributed, so a Mann-Whitney U Test was done. The mean cell haemoglobin was not significantly different (P = 0.423) in the Mann-Whitney U Test (Table 3).

Mean cell haemoglobin concentration. The mean cell haemoglobin concentration was not normally distributed, so a Mann-Whitney U Test was done. The mean cell haemoglobin concentration was not significantly different (P = 0.436) in the Mann-Whitney U Test (Table 3).

Mean cell volume. The mean cell volume decreased during the study period (Figure 5). The ANOVA-test showed a significant difference for the parameter study day (P = 0.000). There was no significant difference between treated and non-treated animals (P = 0.507). The significant difference of study day also appeared in the Bonferroni-test for the following studying days:

- Study day 7 non-treated + study day 14 non-treated (P = 0.004)
- Study day 7 non-treated + study day 21 non-treated (P = 0.000)
- Study day 7 non-treated + study day 28 non-treated (P = 0.000)
- Study day 7 treated + study day 14 treated (P = 0.038)
- Study day 7 treated + study day 21 treated (P = 0.000)
- Study day 7 treated + study day 28 treated (P = 0.000)

The t-test was not significant.

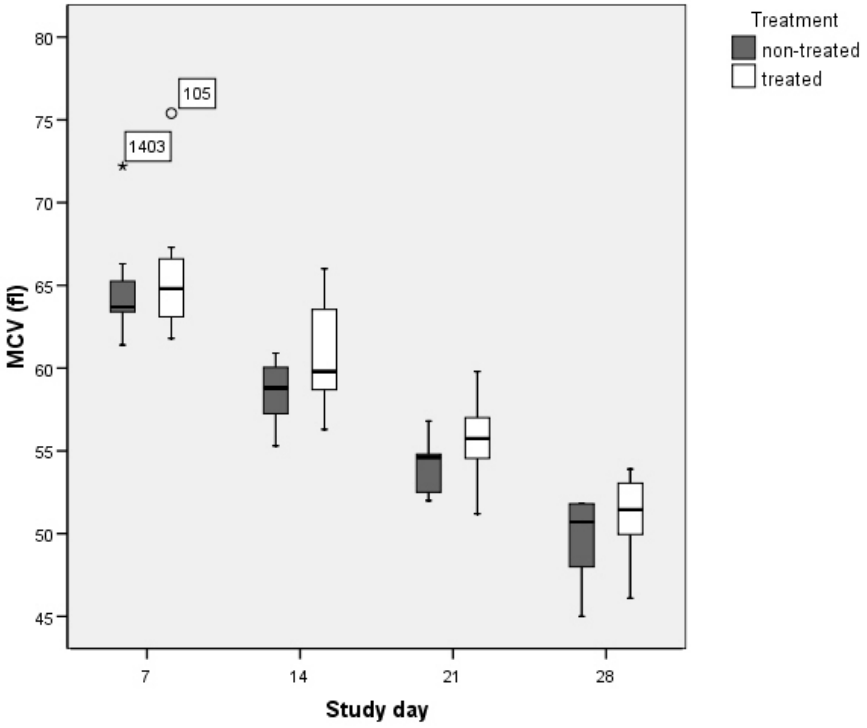


Figure 5: Mean cell volume (MCV) on study day 7, 14, 21 and 28. Number 105 and 1403 are outliers.

Table 1: Distribution of different cell types in the blood and between-subjects effects of ANOVA comparing treated and non-treated piglets with study day as covariate.

Phenotype	Treatment	*n*	Mean cells/µl (S.D.)		Levene´s test			ANOVA between-subjects effects					
								Study day			Treatment		
					df	F	P	df	F	P	df	F	P
Band neutrophils	-	28	0.10	(0.03)	1, 58	0.013	0.909	1, 56	1.454	0.233	1, 56	0.097	0.757
	+	32	0.10	(0.03)									
Eosinophils	-	28	160.83	(81.02)	1, 58	1.535	0.220	1, 56	16.763	**0.000**	1, 56	0.001	0.973
	+	32	181.83	(69.44)									
Erythrocytes	-	28	$6.50 * 10^6$	$(0.95 * 10^6)$	1, 58	0.290	0.592	1, 56	46.268	**0.000**	1, 56	0.230	0.633
	+	32	6.50	(0.83)									
Juveniles	-	28	0.10	(0.03)	1, 58	0.013	0.909	1, 56	1.454	0.233	1, 56	0.097	0.757
	+	32	0.10	(0.03)									
Large unstained cells	-	28	219.49	(183.79)	1, 58	1.049	0.310	1, 56	2.116	0.151	1, 56	0.117	0.734
	+	32	206.90	(135.66)									
Leukocytes	-	28	10460.54	(2790.04)	1, 58	0.026	0.874	1, 56	1.627	0.207	1, 56	0.129	0.721
	+	32	10097.47	(2888.50)									
Lymphoblasts	-	28	0.10	(0.03)	1, 58	0.013	0.909	1, 56	1.454	0.233	1, 56	0.097	0.757
	+	32	0.10	(0.03)									
Lymphocytes	-	28	4806.11	(1894.97)	1, 58	2.834	0.098	1, 56	14.366	**0.000**	1, 56	0.087	0.769
	+	32	4293.51	(1222.94)									
Monocytes	-	27	402.98	(217.34)	1, 57	1.465	0.231	1, 55	8.429	**0.005**	1, 55	0.000	0.999
	+	32	346.13	(174.13)									
Segmented neutrophils	-	28	4752.66	(1686.61)	1, 58	3.363	0.072	1, 56	2.245	0.140	1, 56	0.559	0.458
	+	32	4973.71	(2240.07)									
Basophils	-	28	112.39	(71.83)	1, 58	6.423	**0.014**	-	-	-	-	-	0.381[a]
	+	32	98.73	(41.54)									

[a] Results of Student's t-test since Levene's test for equality of variances was significant, and therefore ANOVA could not be performed.

Significant results ($P \leq 0,05$) are shown in bold characters.

Table 2: Haematocrit percentage in the blood and between-subjects effects of ANOVA comparing treated and non-treated piglets with study day as covariate.

Phenotype	Treatment	*n*	Mean (S.D.)		Levene´s test			ANOVA between-subjects effects					
								Study day			Treatment		
					df	F	P	df	F	P	df	F	P
Haematocrit (%)	-	28	36.68	(3.94)	1, 58	1.647	0.205	1, 56	0.039	0.844	1, 56	0.048	0.828
	+	32	37.50	(2.25)									

Table 3: Blood parameters for treated and non-treated piglets.

Parameter	Treatment	*n*	Mean (S.D.)		Kolmogorov-Smirnov test	Mann-Whitney U Test
					P	P
Haemoglobin (g/dl)	-	28	11.59	(2.41)	0.000	0.947
	+	32	11.78	(2.10)		
MCH (pg)	-	28	17.77	(3.41)	0.003	0.423
	+	32	18.25	(3.47)		
MCHC (g/dl)	-	28	31.56	(5.80)	0.000	0.436
	+	32	31.44	(5.47)		

Indirect immunofluorescence test

Piglets treated with Toltrazuril had a lower antibody titer on study day 21 and 28 compared to the non-treated piglets (Figure 7, Figure 8 and Figure 8). For study day 21 and 28 there was a significant difference between treated and non-treated animals in the Mann-Whitney U test (P = 0.038 and P = 0.038).

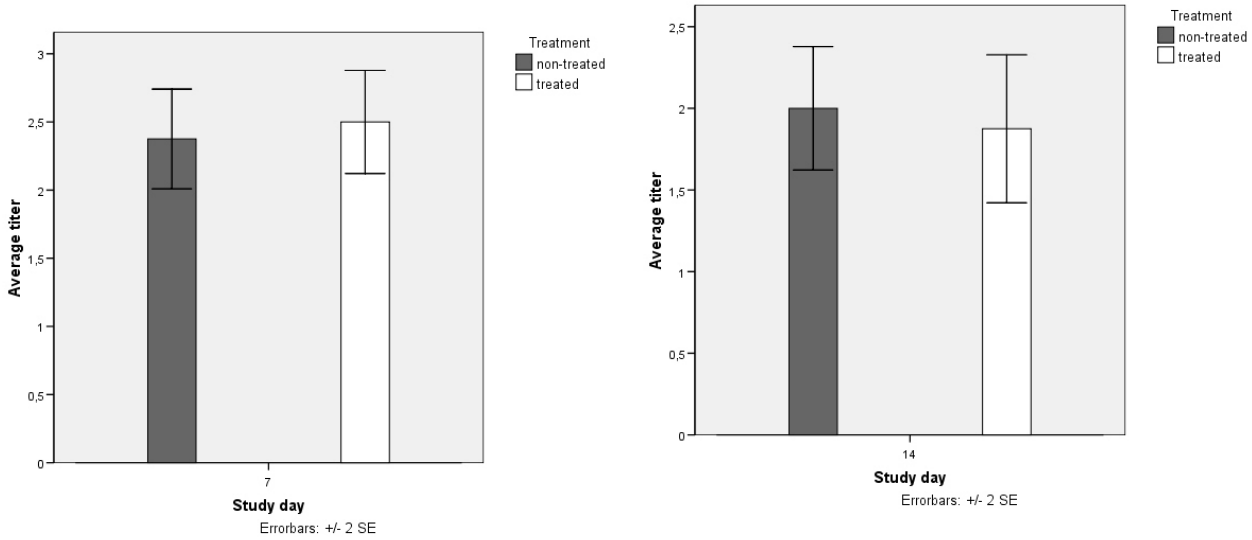


Figure 6: average antibody titer of treated and non-treated piglets on study day 7 and 14. Titer 1 is dilution 1:320, titer 2 is dilution 1:640 and dilution 3 is 1:1280. Both study days had no significant difference between treated and non-treated piglets.

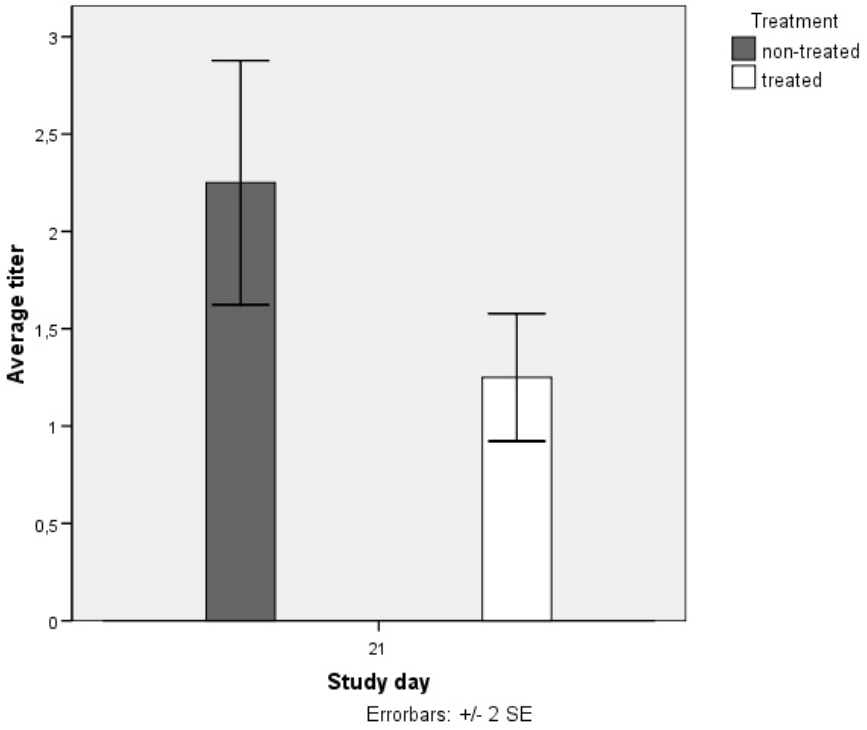


Figure 7: average antibody titer of treated and non-treated piglets on study day 21. Titer 1 is dilution 1:320, titer 2 is dilution 1:640 and dilution 3 is 1:1280.

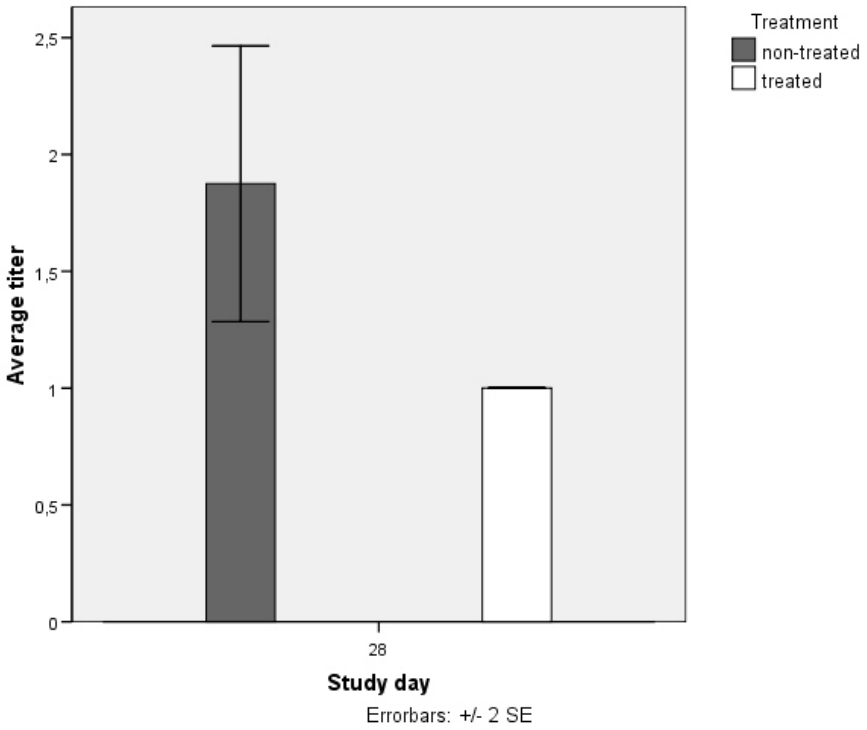


Figure 8: Average antibody titer of treated and non-treated piglets on study day 28. Titer 1 is dilution 1:320, titer 2 is dilution 1:640 and dilution 3 is 1:1280.

Oocyst excretion

Piglets treated with Toltrazuril excreted less oocysts than non-treated piglets and had a lower faecal score (Figure **10**9 and Figure 10). On study day nine there is a small peak in the graph of treated piglets, caused by only one excreting piglet in the treated group.

The faecal score was not normally distributed, so the Mann-Whitney U test was done. There was a significant difference found (P = 0.037) between the faecal scores of the treated and non-treated animals. The animals treated with Toltrazuril had a lower faecal score than the non-treated animals.

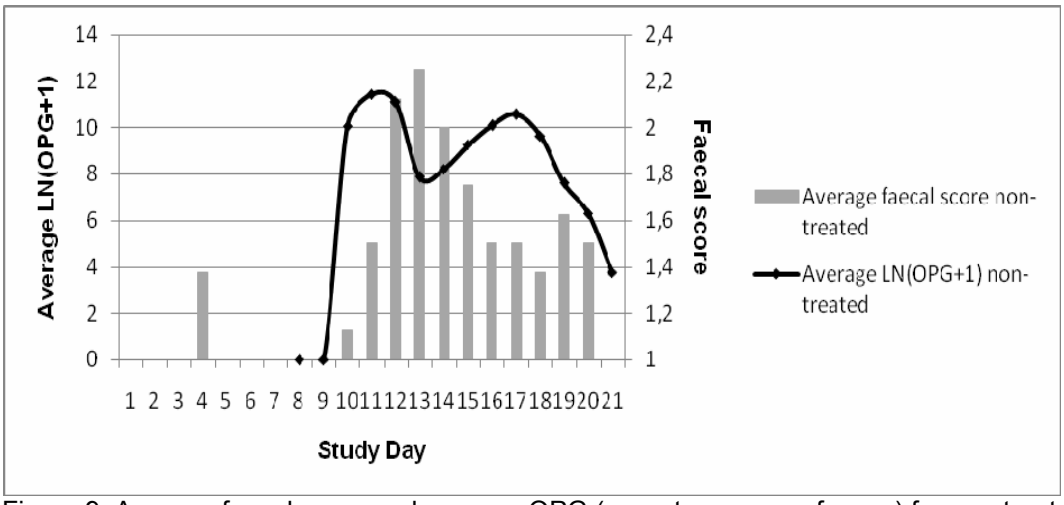


Figure 9: Average faecel score and average OPG (oocysts per gram faeces) for non-treated piglets

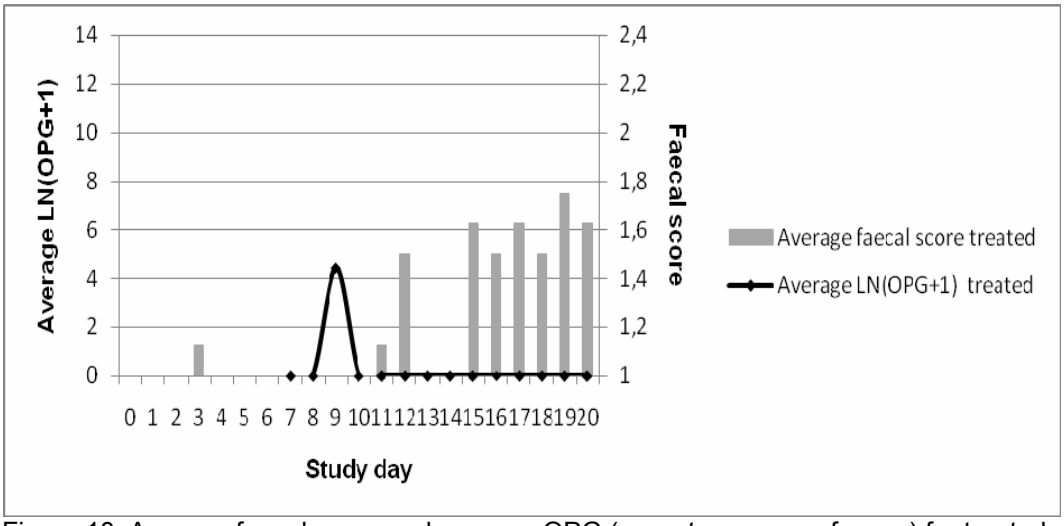


Figure 10: Average faecel score and average OPG (oocysts per gram faeces) for treated piglets

Weight

There was no significant difference in weight between the treated and non-treated group (Table 4 and Figure 11). For the first seven study days, the weight of the non-treated group was a little higher than those of the treated group. However, after the first week the situation turns around and the treated animals became heavier till day 28 (Figure 12). But as said before, there was no significant difference in weight between treated and non-treated piglets.

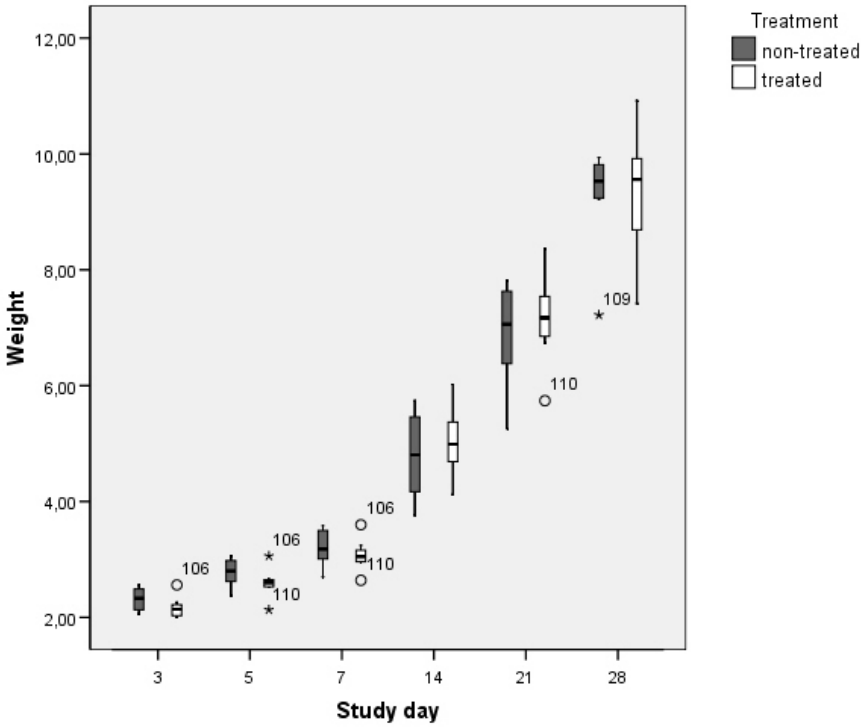


Figure 11: Weight of the piglets in kilogram on study day 3, 5, 7, 14, 21 and 28. Number 106, 109 and 110 are outliers.

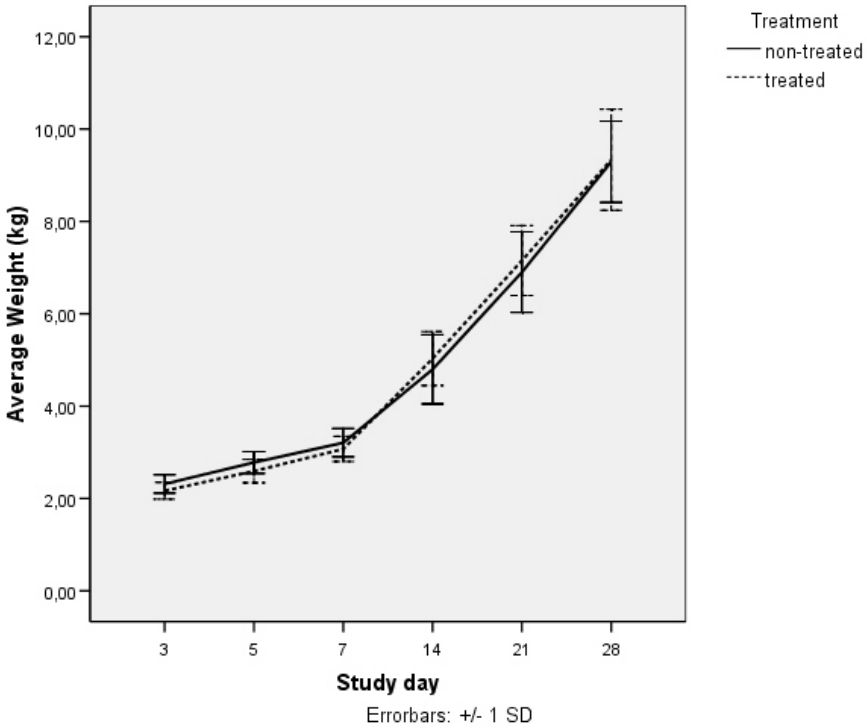


Figure 12: Weight in kilogram of the piglets on study day 3, 5, 7, 14, 21 and 28. SD: standard deviation.

Table 4: piglet body weight on study day 3, 4, 7, 14, 21 and 28

Parameter	Treatment	*n*	Mean (S.D.)		Kolmogorov-Smirnov test	Levene´s test			T-test
					P	df	F	P	
Weight day 3	-	8	2.32	(0.20)	0.864	1, 14	0.751	0.401	0.145
	+	8	2.17	(0.18)					
Weight day 5	-	8	2.78	(0.24)	0.929	1, 14	0.347	0.565	0.150
	+	8	2.59	(0.25)					
Weight day 7	-	8	3.21	(0.31)	0.890	1, 14	0.547	0.472	0.367
	+	8	3.07	(0.27)					
Weight day 14	-	8	4.80	(0.75)	0.990	1, 14	2.333	0.149	0.498
	+	8	5.03	(0.58)					
Weight day 21	-	8	6.90	(0.87)	0.991	1, 14	0.573	0.462	0.550
	+	8	7.15	(0.76)					
Weight day 28	-	8	9.29	(0.88)	0.181	1, 14	0.591	0.455	0.931
	+	8	9.34	(1.09)					

Discussion

Haematology: blood cells

Eosinophils. The major site of eosinophil production is the bone marrow. This production takes between two and six days and is stimulated by products of activated T-lymphocytes and macrophages, like granulocyte-macrophage colony stimulating factor (GM-CSF), interleukin 3 (IL-3) and interleukine 5 (IL-5). The function of eosinophils is controlling infections with metazoan parasites, the regulation of allergic and inflammatory responses and they can cause tissue injury (Jain, 1993). The parasiticidal activity is against worms. The eosinophil attaches to the surface of a parasite, which is coated with antibodies. The lysosomes of the eosinophil are discharged on the surface of the parasite so it is damaged directly (Murphy et al., 2008).

The results of haematology show that the number of eosinophils increased significantly during the study period. There was no difference between treated and non-treated animals. Since there are no standard values available for haematological parameters in piglets and no non-infected piglets were available as a negative control group, this increase in eosinophils during the infection could be explained in two ways.

First, the increase could reflect the normal development of the immune system in piglets during the first weeks of life. The reference value for eosinophils of adult pigs is <1200 eosinophils/microlitre (Veterinärmedizinische Universität Wien, Laboratoriumsmedizin), while the piglets in this study reached an average of 172.03 eosinophils/microlitre. Jain stated also that circulating eosinophil numbers change with age. Adults have often a higher eosinophil count, probably as a result of the changes in immunologic experience, like parasitism.

Second, the increasing number of eosinophils in treated and non-treated piglets could reflect the reaction of the immune system to the infection with *Isospora suis*. Treated individuals are also exposed to the parasite during the experimental infection and later on by the ingestion of oocyst shedded by their non-treated littermates. Moreover, Toltrazuril does not seem to interfere with the development of immunity (Greif, 2000). Therefore the general increase of this cell population could be caused by the infection.

However, the first explanation seems more likely because one might not expect a complete identically expansion of reactive cells during the infection in treated and non-treated piglets. Additionally, eosinophils are not known to be involved in the immune response against *Isospora suis*. It would be interesting to do the same experiment again, but then with a negative (non-infected) control group.

Erythrocytes. Erythrocytes are produced in the bone marrow and have a mean life span of sixty-three days in pigs. The main function of erythrocytes is the transport of oxygen to the tissues and carbondioxide back to the lungs (Jain, 1993).

The results of haematology show that the number of erythrocytes increased significantly during the study period. There was no difference between treated and non-treated animals. Since there are no standard values available for haematological parameters in piglets and there were no non-infected piglets available as a negative control group, this increase in erythrocytes during the infection could be explained in two ways.

First, the increase could reflect the normal development of the amount of erythrocytes in piglets during the first weeks of life. Since there are no standard values available for haematological parameters in piglets and there were no non-infected piglets available as negative control, the reference value of adult pigs is used. The reference value for erythrocytes of adult pigs is $6 – 9 * 10^6$ erythrocytes/microlitre (Veterinärmedizinische Universität Wien, Laboratoriumsmedizin).
Second, the increasing erythrocyte values could be caused by haemoconcentration. However, this is not very likely, because the piglets were in a good physical condition during the experiment and didn't show excessive diarrhoea. So the most probable explanation would be the normal development of erythrocytes during the first weeks of life.

Lymphocytes. Lymphocytes are mainly produced in the bone marrow (Jain, 1993; Murphy et al., 2008). Functions of lymphocytes are:

- Humoral immunity by production of antibodies by B-lymphocytes. Macrophages bind the antigen and present it on the cell surface. T-cells and B-cells can recognize these presented antigens and will cause the clonal proliferation of B- and T-cells. Antigen-stimulated T-cells secrete cytokines, which stimulate the lymphopoiesis. The primed progeny will recognize the same antigen, after which a second proliferation starts. Some of these second progeny become memory cells and others will express the specific immune response (humoral or cellular).
- Cellular immunity by T lymphocytes.
- Immune regulation. Participation of T_H-cells and T-suppressor cells in the humoral immune response of antibody production. T-suppressor cells and stimulating- and inhibitory from macrophages regulate the lymphopoiesis.
- Cytotoxic activity. Cytotoxic T-cells and natural killer cells are involved.
- Immune surveillance. Natural killer cells mediate in the natural resistance to tumors and certain bacterial and viral infections. Natural killer cells are poorly developed at birth.
- Secretion of cytokines (Jain, 1993).

The results of haematology show that the number of lymphocytes decreased between study day seven and fourteen and thereafter increased during the study period. The differences between days were not significant in the Bonferroni test. There was no difference between treated and non-treated animals. Since there are no standard values available for haematological parameters in piglets and there were no non-infected piglets available as negative control group, this increase in lymphocytes during the infection could be explained in two ways.

First, the increase could reflect the normal development of the immune system in piglets during the first weeks of life. The reference value for lymphocytes of adult pigs is 5000 – 16000 lymphocytes/microlitre (Veterinärmedizinische Universität Wien, Laboratoriumsmedizin) while the piglets in this study reached an average of 4532.72 lymphocytes/microlitre on study day 28.
Second, the increasing number of lymphocytes in treated and non-treated piglets could reflect the reaction of the immune system to the infection with *Isospora suis*. Treated individuals are also exposed to the parasite during the experimental infection and later on by the ingestion of oocyst shedded by their non-treated littermates. Moreover, Toltrazuril does not seem to interfere with the development of immunity (Greif, 2000). Therefore the general increase of this cell population could be caused by the infection.
However, the first explanation seems more likely because one might not expect a complete identically expansion of reactive cells during the infection in treated and non-treated piglets. Additionally, it seems that the porcine immune system is not fully developed at birth and that the piglets are very susceptible to infection during the first three to four weeks of life (Worliczek et al., 2007). The second explanation is thus less likely.

Monocytes. Monocytes are produced in the bone marrow and enter the blood stream. They migrate into tissues and body cavities and become macrophages (Jain, 1993). One of the ways in which macrophages become activated is by T_H1 cells.
Functions of macrophages are:

- Phagocytosis and microbicidal activity (Jain, 1993). Macrophages can engulf and destroy pathogens, especially intracellular organisms and those causing a granulomatous inflammatory response, like protozoa (Jain, 1993; Murphy et al., 2008).
- Regulation of the immune response. For the regulation of the immune response, macrophages induce immunity to an antigen and express cellular immunity. Macrophages induce immunity by picking up and processing an antigen for presentation to lymphocytes. This can trigger an antibody response or cellular immunity. On the other hand the cellular immunity is expressed by the possibility of macrophages to phagocytose, destruct antibody-coated pathogens or cytotoxicity (Jain, 1993).
- Scavenger role. Macrophages can remove tissue debris from areas of tissue destruction by ingestion of the material (Jain, 1993). They also continously scavenge dead or dying cells (Murphy et al., 2008).
- Secretory role. Macrophages can secrete cytokines. This secretion is normal, but increases after stimulation of the macrophage by various agents, such as bacteria (Jain, 1993). The pro-inflammatory cytokines will recruit and activate more macrophages (Murphy et al., 2008). For example, activated macrophages produce interleukine-12, which enhances the T_H1-respons (Rommel et al., 2000). T_H1-cells will produce more interferon-γ, which enhances the phagocytic abilities of macrophages (Murphy et al., 2008).

The results of haematology show that the number of monocytes increased significantly during the study period. There was no difference between treated and non-treated animals. Since there are no standard values available for haematological parameters in piglets and there were no non-infected piglets available as negative control group, this increase in monocytes during the infection could be explained in the same two ways as mentioned above for eosinophils.
First, the increase could reflect the normal development of the immune system in piglets during the first weeks of life. The reference value for monocytes of adult pigs is <1000 monocytes/microlitre (Veterinärmedizinische Universität Wien, Laboratoriumsmedizin) while the piglets in this study reached an average of 372.15 monocytes/micro litre.
Second, the increasing number of monocytes in treated and non-treated piglets could reflect the reaction of the immune system to the infection with *Isospora suis*. Treated individuals are also exposed to the parasite during the experimental infection and later on by the ingestion of oocyst shedded by their non-treated littermates. Moreover, Toltrazuril does not seem to interfere with the development of immunity (Greif, 2000). Therefore the general increase of this cell population could be caused by the infection.
However, the first explanation seems more likely because one might not expect a complete identically expansion of reactive cells during the infection in treated and non-treated piglets. It would be interesting to do the same experiment again, but then with a negative (non-infected) control group.

Haematology: erythrocyte related

Mean cell volume. The mean cell volume, also called mean corpuscular volume, is the average volume of erythrocytes in the blood (Jain, 1986).
The results of haematology show that the mean cell volume decreases significantly during the study period. There was no difference between treated and non-treated animals. Since there are no standard values available for haematological parameters in piglets and there were no non-infected piglets available as negative control group, this increase in mean cell volume during the infection could be explained in two ways.
First, foetal erythrocytes are larger than those of adults. These foetal erythrocytes are replaced by ones of a smaller size in the first few months of life. This means that the mean cell volume decreases during the first months of life (Jain, 1986; Jain, 1993).
Second, the decrease of mean cell volume could be caused by the infection. However, this infection dependent anemia is only seen in chronic infections (Jain, 1993). Thus the first explanation is most likely.

Indirect immunofluorescence assay

Animals treated with Toltrazuril after infection with *Isospora suis* showed lower antibody titers on study day 21 and 28 then non-treated piglets.
Treatment with Toltrazuril does not disturb the normal appearance of the epithelium of the gut. Histological, the epithelium of the distal jejunum appears normal compared to the villous necrosis and villous atrophy seen in infected pigs (Mundt et al., 2006b).

An explanation for the higher antibody titer on study day 21 and 28 in non-treated animals could be that these animals have prolonged and more severe damage to the gut. The antigen, in this case *Isospora suis* sporozoites and merozoites, could have contact with the immune system of the piglet over a longer time period and more intense. This way it could cause an antibody titer that stays longer high. In this explanation the maternal antibodies play also a role. The piglets have maternal antibodies by the intake of colostrum during the first day of life. The titer of maternal antibodies will decrease during time, which could have caused the decrease in antibody titer of the treated piglets on study day 21 and 28. In the non-treated animals the infection may still be going on at these time points, so the antibody titer could be made up of the piglet own antibodies.
That the piglets had maternal antibodies was proven by an indirect immunofluorescence assay on colostrum of one of the sows. This colostrum tested positive. Piglets who did not drink colostrum, had no antibodies in the indirect immunofluorescence assay (precolostral serum).
So, it can be stated that it seems that piglets have maternal antibodies, but that these are not protective.

Oocyst excretion

Piglets infected with *Isospora suis* start shedding oocysts on day five post infection. The excretion pattern of *Isospora suis* is biphasic and peaks at day five and day ten post infection (Mundt et al., 2006a).
In this research a biphasic pattern of excretion was also found. However, the pigs started shedding on a later date post infection; they started at day nine and had a second peak at day seventeen post infection. A reason for this delayed excretion could be the breed. Two litters Landrace x Duroc x Pietrain were used and one Landrace. This phenomenon was mentioned more often in earlier experiments with Landrace x Duroc x Pietrain sows.
Treatment with Toltrazuril among other things lowers the faecal score and oocyst excretion (Mundt et al., 2006b, Maes et al., 2005; Mundt et al., 2003). This research showed the same outcome. There is a significant difference in faecal score between treated and non-treated animals, where the non-treated animals have higher faecal scores than treated ones. The treated piglets did not excrete oocysts, except for one piglet on day nine post infection. This can be due to the uptake of oocysts from the faeces of infected littermates and shedding these out.

Weight

An infection with *Isospora suis* causes a decreased weight gain in affected piglets (Mundt et al., 2006a; Mundt et al., 2006b). When Toltrazuril is used, the animals will gain more weight than non-treated animals (Mundt et al., 2006b).

In this research no significant difference was found between the treated and non-treated group of piglets. From day seven till day twenty-eight the piglets treated with Toltrazuril had a higher average weight, but this difference was not significant. An explanation for this could be the breed. As stated in 'oocyst excretion' two litters Landrace x Duroc x Pietrain and one Landrace were used. The infection in the Landrace x Duroc x Pietrain piglets was not so severe as in the Landrace piglets, so they could gain quite normal weight and thus show less difference in weight between the treated and non-treated group.

Final conclusion

This study showed that there are no specific changes in haematological parameters during an infection with *Isospora suis*. Some parameters changed significantly during the study period. This was probably due to the normal development of the immune system (eosinophils, erythrocytes, lymphocytes, monocytes) and normal replacement of foetal erythrocytes for adult ones (MCV).

On study day 7 all piglets had high antibody titers against *Isospora suis*. Colostrum tested positive for antibodies and precolostral piglet serum tested negative. It is assumed that the antibodies at the start of the experiment are maternal antibodies and it seems that these are not protective against infection, since the non-treated group showed clinical signs and excreted oocysts.
There was a significant difference in antibody titer between treated and non-treated piglets on study day 21 and 28. The titer of maternal antibodies will decrease during time, which could have caused the decrease in antibody titer of the treated piglets on study day 21 and 28. In the non-treated animals the infection may still be going on at these time points, so the antibody titer could be made up of the piglet own antibodies.

It will be very interesting to do the same experiment again, but then with a negative control group. These piglets will stay uninfected and in this way the development of haematological parameters can be followed. Also, the maternal antibody titers can be monitored, so there becomes clear at what time point in piglet life the maternal antibodies have disappeared.

References

EYSKER, M., BOERDAM, G.A., HOLLANDERS, W., VERHEIJDEN, J.H.M. (1994) The prevalence of *Isospora suis* and *Strongyloides ransomi* in suckling piglets in the Netherlands. *Veterinary Quarterly* **16**: 203 – 205.

GREIF, G. (2000) Immunity to coccidiosis after treatment with Toltrazuril. *Parasitological research* **86**: 787 – 790.

HARDER, A., HABERKORN, A. (1989) Possible mode of action of Toltrazuril: studies on two *Eimeria* species and mammalian and *Ascaris suum* enzymes. *Parasitological research* **76**: 8 – 12.

HERMOSILLA, C., STAMM, Y. (2008) Fluorescent *Eimeria bovis* sporozoites and meront stages in vitro: a helpful tool to study parasite-host cell interactions. *Parasitology research* **102**: 777 – 786.

HERMOSILLA, C., SCHRÖPFER, E. (2008) Cytoskeletal changes in *Eimeria bovis*-infected host endothelial cells during first merogony. *Veterinary research communications* **32**: 521 – 531.

JAIN, N.C. (1993) *Essentials of veterinary hematology*. 1st ed. Philadelphia.

JAIN, N.C. (1986) *Schalm's veterinary hematology*. 4th ed. Philadelphia.

MURPHY, K., TRAVERS, P., WALPORT, M. (2008) *Janeway's immunobiology*. 7th ed. New York.

LINDL, T. (2002) *Zell- und Gewebekultur*. 1st ed. Heidelberg.

MAES, D., VYT, P, RABAEYS, P., GEVAERT, D. (2007) Effects of Toltrazuril on the growth of piglets in herds without clinical isosporosis. *The veterinary journal* **173**: 197 – 199.

MEYER, C., JOACHIM, A., DAUGSCHIES, A. (1999) Occurrence of *Isospora suis* in larger piglet production units and on specialized piglet rearing farms. *Veterinary parasitology* **82**: 277 – 284.

MORRISSETTE, N.S., SIBLEY, L.D. (2002) Cytoskeleton of apicomplexan parasites. *Microbiology and molecular biology reviews* **66**: 21 – 38.

MUNDT, H.C., JOACHIM, A,, BECKA, M., DAUGSCHIES, A. (2006a) *Isospora suis*: an experimental model for mammalian intestinal coccidiosis. *Parasitology research* **98**: 167 – 175.

MUNDT, H.C., MUNDT-WÜSTENBERG, S., DAUGSCHIES, A., JOACHIM, A. (2006b) Efficacy of various anticoccidials against experimental porcine neonatal isosporosis. *Parasitology research* **100**: 401 – 411.

MUNDT, H.C., COHNEN, A., DAUGSCHIES, A., JOACHIM, A., PROSL, H., SCHMÄSCHKE, R., WESTPHAL, B. (2005) Occurrence of *Isospora suis* in Germany, Switzerland and Austria. *Journal of Veterinary Medicine* **52**: 93 – 97.

MUNDT, H.C., JOACHIM, A., DAUGSCHIES, A., ZIMMERMANN, M. (2003) Population biology studies on *Isospora suis* in piglets. *Parasitology research* **90**: S158 – S159.

NOAKES, D.E., PARKINSON, T.J., ENGLAND, G.C.W. (2001) *Arthur's veterinary reproduction and obstetrics*. 8th ed. Edinburgh.

PATRIE, A., WATSON, P. (2003) *Statistics for veterinary and animal science*. 1st ed. Blackwell.

ROMMEL, M., ECKERT, J., KUTYER, E., KÖRTING, W., SCHNIEDER; T. (2000) *Veterinärmedizinische Parasitologie*. 5th ed. Berlin.

SALMON, H., MUSTAPHA, B., VOLKER, G., MEURENS, F. (2009) Humoral and cellular factors of maternal immunity in swine. *Developmental and comparative immunology* **33**: 384 – 393.

SCHIERACK, P., NORDHOFF. M, et al. (2006) Characterization of a porcine intestinal epithelial cell line for in vitro studies of microbial pathogenesis in swine. *Histochemistry cell biology* **125**: 293 – 305.

SENGER, P.L. (2003) *Pathways to pregnancy and parturition*. 2nd ed.

STRABERG, E., DAUGSCHIES, A. (2007) Control of piglet coccidiosis by chemical disinfection with a cresol-based product (Neopredisan 135-1®). *Parasitology research* **101**: 599 – 604.

TAYLOR, D.J. (2006) *Pig diseases*. 8th ed. Glasgow.

XU, R.J., CRANWELL, P.D. (2003) *The neonatal pig: gastrointestinal physiology and nutrition.* 1st ed. Nottingham.

WORLICZEK, H.L., BUGGELSHEIM, M., SAALMÜLLER, A., JOACHIM, A. (2007) Porcine isosporosis: Infection dynamics, pathophysiology and immunology of experimental infections. *Wiener Klinische Wochenschrift* **119**: 33 – 39.

Appendix I: Research plan

Day	Days post infection	Treatment	Weighing	Fecal Sampling	Blood Sampling	Extras
0						
1						
2		Vaccination Iron				
3	0	Infection 1000 oocysts	x	x Consistency		Tatoo
4	1					
5	2	Toltrazuril	x			Grouping
6	3					
7	4		x	x	x	Viral and bacterial examination
8	5			x		
9	6			x		
10	7			x		
11	8			x		
12	9			x		
13	10			x		
14	11		x	x	x	
15	12			x		
16	13			x		
17	14			x		
18	15			x		
19	16			x		
20	17			x		
21	18		x		x	
22	19					
23	20					
24	21					
25	22					
26	23					
27	24					
28	25		x		x	

Vaccination: Stellamun 2 ml intramuscular

Iron: Vanafer 2 ml subcutaneous

Toltrazuril: 0.4 ml/kg