PORCINE NEONATAL CYSTOISOSPOROSIS: TOWARDS ALTERNATIVE CONTROL STRATEGIES
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„Fang nie an aufzuhören,
hör nie auf anzufangen.“

*Marcus Tullius Cicero*
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1. PUBLICATIONS AS PART OF THE THESIS AND AUTHORS’ CONTRIBUTIONS


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**Authors’ contributions:** Joachim A, Freudenschuss B and Pagès M designed the study and Shrestha A provided the recombinant merozoite protein. Abd-Elfattah A and Freudenschuss B designed and tested the PCR primers; Ruttkowski B provided oocysts, sporozoites and merozoites. Joachim A, Freudenschuss B and Abd-Elfattah A carried out the trials; Freudenschuss B and Ruttkowski B processed the samples. Freudenschuss B analyzed the samples, conducted the statistical analyses and drafted the manuscript. Ladinig A revised and edited the manuscript and contributed to FMIA data generation and interpretation. All authors read and approved of the final version of the manuscript.

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2. DECLARATION

I declare that this thesis and all work associated with it were performed by following the rules of Good Scientific Practice in every aspect.
The thesis was submitted to the University of Veterinary Medicine Vienna in fulfilment of all requirements for the degree of Doctor medicinae veterinariae.

Barbara Freudenschuss
Vienna, June 2018
3. INTRODUCTION

3.1. Cystoisospora suis

The protozoan parasite *Cystoisospora suis* (syn. *Isospora suis*) is a coccidian species exclusively infecting the small intestines of swine (Stuart et al. 1980, Vitovec and Koudela 1990). It completes its life cycle within one host after infection via the fecal-oral route (Lindsay et al. 1997). Thereafter, sporozoites released from the ingested oocysts enter the small intestines’ (particularly the mid-jejunum’s) epithelial cells and undergo asexual replication (merogony) inside a parasitophorus vacuole, resulting in the emergence of motile merozoites which destroy their host cells to penetrate further cells. Asexual stages are categorized into types according to their number of nuclei, dimensions, size and the time when they appear (Lindsay et al. 1980, Harleman and Meyer 1984, Vitovec and Koudela 1987). After several cycles of merogony, merozoites enter the phase of sexual reproduction (gamogony) where they either develop into microgamonts or macrogamonts from which subsequently micro- and macrogametes, respectively, emerge (Lindsay et al. 1980, Harleman and Meyer 1984). Upon fusion of a macrogamete with a microgamete a zygote is formed that further develops into an unsporulated oocyst and is excreted with the faeces (Harleman and Meyer 1983, Lindsay et al. 1997). Sporulation, and thus the development into the infective stage, takes place in the surroundings and can be accomplished within 12 hours when environmental conditions are optimal (Lindsay et al. 1982). The developmental cycle of *C. suis* is usually finalized within five to six days (Lindsay et al. 1980, Harleman and Meyer 1984, Vitovec and Koudela 1990). Oocysts cannot be eradicated by commonly used disinfectants but have to be inactivated with specific, cresol-based chemicals (Lindsay et al. 1997, Mundt et al. 2005, Straberg and Daugschies 2007), and can survive for up to several months under suitable conditions (Langkjaer and Roepstorff 2008).

The existence of extraintestinal stages in paratenic as well as in definitive hosts has been described in various species of *Cystoisospora*. Thereby sporozoites form monozoic cysts in different tissues and organs outside the intestinal tract (Pinckney et al. 1993, Lindsay et al. 1997,
Lindsay et al. 2014). However, evidence of such stages in *C. suis* infected piglets or mice as possible paratenic hosts has not been provided until now (Lindsay et al. 1980, Pinckney et al. 1993, Stuart et al. 1982a), although gnotobiotic piglets did shed oocysts after they were intraperitoneally injected with homogenized lymph nodes, spleen and liver of piglets experimentally infected with *C. suis* (Harleman and Meyer 1984).

A cell culture system representing the whole life cycle of *C. suis* is available (Worliczek et al. 2013), making more detailed studies on its characteristics, dynamics and on host-parasite interactions possible. Apart from that, it allows for the isolation of single developmental stages which can be exploited for diagnostic tools or further studied for their biological functions, morphology, the presence of immunogenic components or their suitability as vaccine candidates.

### 3.2. Porcine cystoisosporosis

Infection with *C. suis* and its high-frequent reproduction in the host wreaks severe damage on the intestinal epithelium characterized by necrotic or shortened and fused villi as well as detachment of the *lamina epithelialis mucosae* (Stuart et al. 1980, Vitovec and Koudela 1987, Bach et al. 2003, Mundt et al. 2006, Mundt et al. 2007). Clinical porcine cystoisosporosis is consequently dominated by non-haemorrhagic, yellowish, pasty to watery diarrhoea and dehydration, resulting in lowered body weight gain and subsequently reduced weaning weights (Lindsay et al. 1985, 1992, Vitovec and Koudela 1990, Mundt et al. 2006, 2007). Neonates and suckling piglets are particularly susceptible to disease, potentially due to their poorly developed immune system in the first weeks of their lives (Becker and Misfeldt 1993, Schwager and Schulze 1997, Stepanova et al. 2007) that is not able to effectively fight the infection. In older pigs, by contrast, clinical signs are generally absent after infection, with no or only low levels of oocyst excretion (Stuart et al. 1982b, Koudela and Kučerová 1999). This reduced susceptibility might be attributed to age resistance resulting from the maturation of the immune system and/or the alteration of the gut physiology (for example, a faster epithelial turnover) in older animals; alternatively, it might be
attributed to immunity following a primary infection (Lindsay et al. 1985, Koudela and Kučerová 1999, Worliczek et al. 2009a).

Although mortality rates are mostly low, cystoisosporosis is associated with a remarkably high morbidity (Lindsay et al. 1985, Mundt et al. 2007, Worliczek and Joachim 2011). This, together with the high prevalence of the parasite in countries worldwide (Roepstorff et al. 1998, Meyer et al. 1999, Mundt et al. 2005, Hamadejová and Vítovec 2005), makes *C. suis* one of the most frequent causes of diarrhoea in piglet production with serious economic damage (Harleman and Meyer 1984, Mundt et al. 2006, 2007, Worliczek and Joachim 2011). Kreiner et al. (2011) calculated a loss of income of more than 5,000 Euros per year for an average Austrian pig producing farm due to reduced profit and additional expenses when affected by coccidiosis. Likewise, Scala et al. (2009) determined a financial benefit of 0.915 Euros per treated animal in comparison to untreated piglets.

### 3.3. Treatment

At present, toltrazuril is the only effective compound licensed to control porcine coccidiosis (Mundt et al. 2007, Joachim and Mundt 2011, Rypula et al. 2012). The triazinetrione is, in form of an oral suspension, licensed as single metaphylactic treatment (20 mg/kg bodyweight) to be given within the third and fifth day after the piglet’s birth and acts against all endogenous *C. suis* stages (Bach et al. 2003, Mundt et al. 2007, Mehlhorn and Greif 2016). However, the long-term blanket use of a single drug compound is likely to cause the development of resistance, a problem already existing in avian coccidia where resistance to all classes of anticoccidials, including triazinetrones, is described (Chapman 1984, Chapman 1997, Stephan et al. 1997, Shirley et al. 2007, Peek and Landman 2011). In fact, resistance of a *C. suis* field isolate against toltrazuril was lately reported for the first time (Shrestha et al. 2017a). Moreover, the possible threat of drug residues in the environment or in products of animal origin is of increasing public interest (Olsen et al. 2012, Beyene 2016). In 2005, for example, the proceedings for the submission of Baycox® (5 % oral suspension, Bayer Animal Health GmbH, Leverkusen, Germany) as treatment of
porcine coccidiosis were discontinued in Canada because toltrazuril was not unequivocally shown to be free of carcinogenic ingredients (Government of Canada 2005), and farmers were left without effective alternatives. This underpins the necessity to develop alternatives to the current treatment options and explore new, sustainable ways to combat this parasitic disease, including targeting the pig’s immune system.

3.4. Immune response to coccidia

As with all members of the Apicomplexa, most of the development of *C. suis* takes place inside the host cell. The elimination of any intracellular pathogen is generally accepted to be largely dependent on cell-mediated immune mechanisms and T helper 1 (Th1) pathways (Casadevall 2003). This is accomplished by a complex interplay of specialized T cell subsets and phagocytic cells which either directly kill infected cells or secrete different chemokines or cytokines that in turn attract or stimulate effector cells (Mackaness 1971, Stenger and Röllinghoff 2001). Likewise, for the successful control of infections with avian or mammalian coccidia the cellular immune system, particularly T cells of various types [CD4⁺ and CD8⁺ cells, cytotoxic T lymphocytes (CTL)] and dendritic cells, is generally considered essential. Specifically Th1-associated and inflammatory cytokines such as interleukin (IL)-2, IL-12, interferon (IFN)-γ and Tumor Necrosis Factor (TNF)-α either directly inhibit the invasion of and survival and replication in host cells or promote killing of extracellular parasite stages or infected cells by activation of macrophages, natural killer (NK) cells or CTL (Rose 1987, Bliss et al., 1999, Hermosilla et al. 1999, Yap and Sher 1999, Daugschies and Najdrowski, 2005, Worliczek et al. 2007, Frölich et al. 2012, Verhelst et al. 2015). This has been described in detail for several apicomplexan parasites related to *C. suis*. In *Neospora caninum* infected cattle a predominant Th1 response, particularly in conjunction with the production of IFN-γ, is critical for the establishment of a protective immunity, and is associated with lower abortion rates and limited parasite growth (Regidor-Cerrillo et al. 2014, Almería and López-Gatius 2015). Also, its local production was shown to be essential for the elimination of *Cryptosporidium muris* in mice (Culshaw et al. 1997). On the other hand, during acute *Toxoplasma gondii* infections, IL-10 was
shown to balance the clearly important and dominant Th1 cytokine production to avoid critical immunopathology (Yap and Sher 1999).

Until now, the contribution of humoral and cellular immune components in effectively controlling infections with *C. suis* and their respective roles in developing a protective immunity is not quite resolved. As with other apicomplexan parasites, a cellular immune participation was also suggested for piglets with coccidiosis. The increase of T-cell receptor-γδ+ (TcR-γδ+) T cells in the small intestinal mucosa of *C. suis* infected piglets pointed towards an involvement of this subset in the immune response to the parasite (Worliczek et al. 2009b, 2010a, Gabner et al. 2014) as already shown for other coccidia (Hermosilla et al. 1999); however, their precise role in the case of *C. suis* needs further investigation. Generally, TcR-γδ+ T cells make up a large proportion of total peripheral lymphocytes in swine but are only present in low numbers in the gut mucosa of healthy pigs. They are supposedly involved in the protection against both intra- and extracellular pathogens and are, among various other relevant functions, important producers of IFN-γ, TNF-α and IL-17 (Sedlak et al. 2014). Also, numbers of cytotoxic T cells and expression levels of TNF-α were shown to be increased in the small intestines of *C. suis* infected piglets, but IFN-γ or other potentially relevant cytokines have not been investigated so far (Gabner et al. 2014).

On the other hand, production of specific immunoglobulins (Ig) has been demonstrated in response to *C. suis* (Schwarz et al. 2013, 2014) and to other coccidia of various hosts (Faber et al. 2002, Reeg et al. 2005, Wallach et al. 2010, Frölich et al. 2012, Matos et al. 2017), although it is discussed controversially whether they contribute to the elimination of the parasite and/or to protection or whether they simply reflect infection (Rose et al. 1987, Faber et al. 2002, Daugschies and Najdrowski 2005, Worliczek et al. 2007, Wallach et al. 2010, Frölich et al. 2012, Matos et al. 2017). Schwarz et al. (2014) suggested IgA as potential marker correlating with protection against *C. suis* but a direct contribution of this or another immunoglobulin to protection could so far not be unequivocally proven. However, some authors nowadays do suggest a protective role of - either passively received or endogenously produced - antibodies against intracellular pathogens in general and coccidia in particular (Casadevall 2003, Wallach et al. 2010). A maternal vaccine against *Eimeria maxima* conferring protection to broiler chicks via
passive transfer of antibodies is available (Sharman et al. 2010), and several studies suggest an ability of specific antibodies to inhibit the invasion of host cells by parasites and their intracellular development (Mack and McLeod 1992, Jahn et al. 2009, Wallach et al. 2010). Moreover, antibodies might as well indirectly contribute to parasite elimination and immunoprotection through modulation or stimulation of the cell-mediated immune response as was shown for *E. bovis* (Behrendt et al. 2008, Taubert et al. 2009).

### 3.5. Vaccination

In poultry, live vaccination of chicks against *Eimeria*, either with attenuated or with virulent vaccine strains, has been proven a successful alternative to anticoccidial treatments (Chapman et al. 2002, Crouch et al. 2003, Sharman et al. 2010). Piglets, however, cannot be actively vaccinated straight after birth - when protection is needed the most - since their immature immune system and maternal antibodies acquired with the colostrum would impede an adequate immune response (Becker and Misfeldt 1993, Schwager and Schulze 1997, Morein et al. 2002, Stepanova et al. 2007, Chase et al. 2008). Passively transferred immunity through maternal immunization might represent an attractive alternative to active immunization. Hereby immune components, either humoral or cellular or both, are taken up with colostrum and milk and mediate protection until the piglet’s own immune system can take over (Salmon et al. 2009). Indeed, in chicken *Eimeria*, maternal immunization with the parasite has been shown to induce specific antibody production and to successfully confer passive protection against infection to the offspring (Sharman et al. 2010, Wallach et al. 2010). Moreover, Jenkins et al. (1999) found mice to be partially protected against *Cr. parvum* infections by cow hyperimmune colostrum specific against a recombinant antigen of the abovementioned.

To date, the immune response to *C. suis* was predominantly investigated in suckling piglets since they are most affected by disease while only few attempts were made to characterize the response of older, immune-competent animals. Yet, studying and potentially manipulating the immune mechanisms of immunologically mature pigs is an important first step towards the development
of a passive immunization strategy for piglets. The antigen-specific IFN-γ response of splenocytes from six challenged weaner pigs (Worliczek et al. 2010b) and the increase of specific antibodies in blood and colostrum of pregnant sows after high-dose infections with oocysts of C. suis (Schwarz et al. 2014) suggest that an infection of immune-competent animals can induce an immune response despite the absence of oocyst excretion. Also, the experimentally infected offspring of sows superinfected during late pregnancy showed a less severe course of disease (Schwarz et al. 2014). However, protection against neonatal coccidiosis was not sufficient to consequently prevent clinical disease, and also the routine administration of oocysts to sows in such high doses is not feasible. Therefore, other sow immunization protocols should be investigated for their feasibility and for their ability to achieve a better passive protection of piglets.
4. AIMS AND HYPOTHESES

It could previously be shown that *C. suis*-specific antibodies are passively transferred from mother sows to their offspring via colostrum and milk and that a high-dose infection with *C. suis* of sows before birth can at least relieve the symptoms of disease in their piglets (Schwarz et al. 2013, 2014). It was hypothesized that the mode of infection has an impact on the intensity and character of the immune response of immune-competent animals and that therefore a better passive protection of piglets by immunizing sows is generally achievable if the sow’s immune system is sufficiently stimulated.

Thus, the main objectives of this project were:

- to gain deeper knowledge on the immune-competent host’s response to *C. suis* by investigating antibody- and cytokine-related aspects of the immune response of immunologically mature pigs
- to identify a potential immunization model for sows by comparing the immune responses that different oral infection protocols induce
- to identify markers associated with protection or the intensity of immune response

It was furthermore hypothesized that the indiscriminate, long-term use of toltrazuril will result in the development of resistance in *C. suis*, which can be confirmed by the experimental evaluation of field isolates for their toltrazuril sensitivity.
5. PUBLICATIONS

5.1. Publication 1

Antibody and cytokine response to *Cystoisospora suis* infections in immune-competent young pigs

Barbara Freudentshuss, Bartel Rustocki, Aruna Shrestha, Ahmed Abd-Elsatar, Marc Pagès, Andrea Lading and Anja Joachim

**Abstract**

**Background:** To date, investigations on the immune response to *Cystoisospora suis* infections focused on suckling piglets, the age group clinically most affected. Activating-immunizing piglets is unfeasible due to their immature immune system and the typically early infection in the first days after birth. Therefore, understanding and possibly enhancing the immune response of immune-competent animals is the prerequisite to develop a passive immunization strategy for piglets which currently rely on very limited treatment options.

**Methods:** To investigate antibody and cytokine responses of immune-competent animals and the impact of the oral immunization protocol on their immune response, groups with unknown previous exposure to *C. suis* (10–11 weeks-old) were infected once or three times with different doses (500 and 1000) or 200 and 3000, respectively, of *C. suis* oocysts and compared to uninfected controls. Oocyst excretion was evaluated, and blood and intestinal mucus antibody titers were determined by IFAT. Systemic production of Th1, Th2, inflammatory and regulatory cytokines was determined in different immune compartments at mRNA and (after stimulation with a recombinant mercoxite-protein) at protein level by PCR and multiplex fluorescent immunomassay, respectively.

**Results:** Infection generated significantly increased serum IgA and IgG levels against *C. suis* sporozoites and mercoxites, irrespective of infection mode, with IgG against mercoxites showing the strongest increase. No clinical signs and only occasional excretion were observed. The systemic cytokine response to *C. suis* was only weak. Nonetheless, in white blood cells, IL-4, IL-6 and IL-10 mRNA-levels significantly increased after infection, whereas IFN-γ, IL-2 and TGF-β expression tended to decrease. In mesenteric lymph nodes (MLN), IL-10 and TNF-α levels were elevated while splenic cytokine expression was unaltered upon infection. Stimulated MLN-derived lymphocytes from infected pigs produced slightly more IL-12 and less IFN-α than controls.

**Conclusions:** An infection and a subsequent systemic immune response can be induced in immune-competent animals by all evaluated infection models and growers can be used as models to mimic sow immunizations. The immune response to *C. suis*, although mild and with considerable variation in cytokine expression, was characterized by a Th2-associated and regulatory cytokine profile and antibody production. However, none of the parameters clearly stood out as a potential marker associated with protection. Antibody titers were significantly positively related with oocyst excretion and might thus serve as correlates for parasite replication or severity of infection.

**Keywords:** Coccioidosis, Pig, Immunity, Antibody, Cytokine, Immunization, Stimulation, T helper 2
Background
The coccidian species *Cystoisospora suis* (syn. *Isospora suis*) occurs worldwide at high prevalences, with neonates and suckling piglets being specifically susceptible to clinical disease [1–4]. Oocysts as resistant environmental stages, together with a high morbidity rate, make neonatal porcine coccidiosis one of the main causes of diarrhea in piglet production, resulting in impaired weight gain and subsequently reduced, uneven weaning weights [5, 6], and thus inflicting serious financial losses to the farming industry [7–9].

Toltrazuril is currently the only compound available to successfully contain coccidiosis [10–12]. However, toltrazuril resistance has recently been reported for the first time in a field isolate of *C. suis* [13], and the emergence of more widespread resistance as described in avian coccidia [14, 15] has to be expected. Moreover, the potential hazard of drug residues in animal products and environment is of growing concern to the public [16–18]. Canada, for example, rescinded the submission process of Bayroc® in 2006 because carcinogenic properties of toltrazuril could not be ruled out [19], leaving farmers without satisfying alternatives. Consequently, the necessity of new strategies to combat this parasitic disease, possibly targeting the immune system, has dramatically increased.

The involvement of humoral or cellular immune components and their protective role in the case of *C. suis* are not yet fully understood. The cellular immune system is considered a key player in controlling infections with mammalian and avian coccidia [4, 20–24] and an involvement has also been shown in piglets infected with *C. suis* [25–27], although only limited data on cytokine responses of the involved cell populations are available. On the other hand, humoral immune activity has been demonstrated in *C. suis* [28, 29] and other coccidia [30–33] but its contribution to the development of protective immunity remains to be resolved [4, 9, 20, 22, 23, 34, 35].

Until now, investigations on the immune response to *C. suis* focused primarily on suckling piglets as the most affected age group. Yet the immune mechanisms of older, immune-competent pigs are of interest, particularly with regard to the development of a passive immunization strategy for piglets. Immunizing neonates with a live vaccine similar to poultry [34, 35] is not possible as their immature immune system [36–38] and interfering maternal immunity would not allow for an adequate immune response at this early stage of life [39, 40] when the infection is most relevant [1, 7]. To date, only few data are available on the immune response of immunologically mature animals. Wodicka et al. [41] demonstrated an antigen-specific IFN-γ response of splenocytes from weaners upon challenge, and Schwarz et al. [29] showed an increase of specific antibodies in blood serum and colostrum of pregnant sows after high-dosed infections, indicating that the infection of immune-competent animals can induce an immune response, despite the absence of oocyst shedding [29, 41]. Moreover, superinfection of pregnant sows was followed by a milder course of disease in their experimentally infected offspring. However, a sufficient protection of piglets against clinical coccidiosis was not achieved, and also the routine administration of oocysts to sows in such high doses is not practical.

This study aimed to gain a better understanding of the immune-competent host’s response to *C. suis* and we therefore investigated antibody- and cytokine-related aspects of the immune response of immunologically mature grower pigs with unknown previous contact to *C. suis*. We also evaluated whether intensity and character of their immune response depend on the protocol of oral antigen delivery and whether a potential immunization model for sows can be identified which could enhance their immune response and subsequently improve passive protection of piglets. Lastly, we sought to identify potential markers associated with immunization upon experimental infection.

Methods
Study animals
A trial of 50 female crossbred growers from a conventional producer in Lower Austria with an unknown history of coccidiosis were used. Animals were 10–11 weeks-old at the beginning of the trial and clinically examined and weighed upon arrival. Their status of immunity against *C. suis* was unknown. Those animals that were going to be infected with *C. suis* oocysts were allocated to four different groups (Table 1) according to body weight ranking using a pre-set randomization scheme. Three separate infection trials with pigs from at least two litters per trial allocated to the respective infection groups were carried out to receive final group sizes of 10. Animals were kept on straw with daylight under conventional conditions at the facilities of the Institute of Parasitology, University of Veterinary Medicine Vienna, and arrived one week before the start of the trial for acclimatization. Uninfected control animals were sampled in an independent trial including pigs from two litters; they were housed in a biosafety unit of the Clinic for Swine, University of Veterinary Medicine Vienna, to prevent infections with coccidia, and arrived one day before the start of the trial. All animals had access to water ad libitum and were fed once daily with a commercial pig diet. The day of (first) infection was defined as study day (SD) 1.

Parasite material and experimental infection
Oocysts of *C. suis*, strain Wien-1 [10], obtained during previous experimental infections of piglets, were isolated...
Table 1 Groups and infection regimes for growers infected with oocytes of C. suis

<table>
<thead>
<tr>
<th>Group</th>
<th>Infection dose</th>
<th>Days of infection</th>
<th>No. of animals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Single High (SH)</td>
<td>6000 oocytes per os</td>
<td>SD 1</td>
<td>10</td>
</tr>
<tr>
<td>Single Low (SL)</td>
<td>600 oocytes per os</td>
<td>SD 1</td>
<td>10</td>
</tr>
<tr>
<td>Tackle High (TH)</td>
<td>3 x 2000 oocytes per os</td>
<td>SD 1, SD 8, SD 15</td>
<td>10</td>
</tr>
<tr>
<td>Tackle Low (TL)</td>
<td>3 x 200 oocytes per os</td>
<td>SD 1, SD 8, SD 15</td>
<td>10</td>
</tr>
<tr>
<td>Control (C)</td>
<td>1 ml tap water per os</td>
<td>SD 1</td>
<td>10</td>
</tr>
</tbody>
</table>

Abbreviation: SD study day

from feces, brought to sporulation and stored as described [28]. Oocytes were washed with tap water prior to infection. Animals were infected with different oral doses of sporulated oocytes at different frequencies (infected groups) or sham-treated with tap water (Control) on SD 1 (see Table 1 for details).

Sample collection

Individual fecal samples were taken on the day of arrival, on SD 1 and daily from SD 6–29 and examined for the presence of C. suis oocytes. Serum and whole blood samples were collected from the jugular vein on SD 1, 8, 15 and 22, and from the heart on SD 29 (after anesthesia) using a needle (18 G) and syringes (Primavette® V 10 ml, KADE Labortechnik GmbH, Nürnberg, Germany). On SD 29 animals were sacrificed by exsanguination following deep general anesthesia [1.0 mg/kg body weight (BW) of ketamine and 1.2 mg/kg BW of azaperone]. Spleen and small intestines including mesenteric lymph nodes (MLN) were isolated immediately post mortem and kept in cold, sterile PBS (Gibco®, Thermo Fisher Scientific, Waltham, MA, USA) until processing. Hematology and white blood cell counts (Additional File 1: Table S1) were performed on SD 1 and 29 by the Central Laboratory, Department of Pathobiology, University of Veterinary Medicine Vienna.

Sample processing

Blood

Serum was obtained by centrifugation of coagulated blood samples (10 min at 1500g) and stored at -20°C until further use. White blood cells were purified from 4 ml of hepatoplated blood within two hours of sampling by erythrolysis using Buffer EL (Qagen, Hilden, Germany), following the manufacturer’s instructions. Homogenized cell lytes were either stored at -80°C until further processed or directly subjected to total RNA extraction.

MLN and spleen

MLN were isolated from the small intestinal area and separated from tissue, spleen was mixed into small pieces and capsular tissue was removed. Lymphocytes were isolated from MLN under sterile conditions as described previously [36] and cells were suspended in RPMI 1640 (Gibco®, Thermo Fisher Scientific) supplemented with 10% FCS and 1% dimethyl sulfoxide and stored in liquid nitrogen. Additionally, MLN and spleen samples were snap frozen in liquid nitrogen and stored at -80°C for later extraction of nucleic acids.

Sample processing

Intestines

Small intestines were arranged in a meandering pattern, the part defined as the mid-section of the jejunum and considered most suitable for further studies [42] was sampled. Ten centimeter long segments were sampled for the retrieval of mucus (5–3 pieces) according to Schwartz et al. [28]. Supernatants containing mucus antibodies were stored at -20°C until serology was performed. One centimeter long segments were sampled for preservation in 10% buffered formalin and colorless Neg-50™ Formalin Section Medium (Richard-Allan Scientific, Kalamazoo, MI, USA), respectively. The latter were immediately snap frozen in liquid nitrogen and stored at -80°C. Transverse sections of formalin-fixed tissues were embedded in paraffin, cut and stained with hematoxylin and eosin (H&E) according to standard procedures.

Sample processing

Serology

Titters of immunoglobulin (Ig) G and IgA against C. suis sporozoites and merozoites as antigens were measured in blood serum and intestinal mucus samples using an immunofluorescence antibody test (IFAT). Sporozoites were derived by evisceration of oocysts as described by Worlick et al. [43] with minor changes. Briefly, purified oocysts were vortexed with 200 μl sodium hypochlorite (12%) and subsequently incubated at 4°C for 15 min. After being washed with PBS the resuspended pellet was vortexed with Percoll® gess bouds (peqlab, VWR International GmbH, Echingen, Germany) 3 times for 20 s. Parasites were transferred to a 15 ml tube, incubated in 2 ml of a sacrocholate/trypsin solution (Sigma-Aldrich, Vienna, Austria; 7.5 and 2.5 g/l PBS, respectively) for 60 min (37°C, 5% CO2) and subsequently washed with PBS. Merozoites were grown in intestinal porcine epithelial cells (IPEC-J2) [43] and harvested by collecting cell culture supernatants 6–10 days after infection of IPEC-J2 with sporozoites 10-dot
slides (Medco Diagnostika GmbH, Heegensberg, Germany) were coated with either of the parasite stages diluted in PBS. Slides were dried overnight at 40 °C, fixed in -20 °C cold acetone for 10 min and stored at -80 °C for later use. IFAT was performed according to Schwarz et al. [28]. In brief, slides were incubated with serum or intestinal mucosa samples in serial dilutions starting at 1:20, washed and subsequently incubated with fluorescent-labeled antibodies [goat anti-swine IgG (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA) diluted 1:150 in PBS or goat anti-swine IgA (Bethyl Laboratories, Montgomery, TX, USA) diluted 1:1000 in PBS]. Slides were washed, covered with PBS/glycerin (1:1:0) and a coverslip, and titer were assessed under fluorescence. Positive and negative controls were included in each IFAT. Titters were converted to numerics for statistical calculations, starting with 1 representing a titer of 1:20.

Quantification of cytokine mRNA expression in white blood cells and lymphatic tissues

Relative quantitative real-time PCR (qPCR) was performed to quantify cytokine transcription in white blood cells, MLN and spleen. For this, total RNA was isolated from homogenized leukocyte lysates (see above) and MLN and spleen tissue using a QiAamp® RNA Blood Mini Kit (Qiagen) including the optional on-column DNAse digestion (Qiagen) according to the manufacturer's instructions. The amount and integrity of extracted total RNA were evaluated using a NanoDrop® 2000 (Thermo Fisher Scientific) and agarose gel electrophoresis, respectively. For cDNA synthesis 1 µg of total RNA was reversely transcribed using an iScript® cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA). In four cases, however, less RNA (200 ng) had to be used due to low yields. qPCR was carried out on a Mx3000P thermal cycler (Agilent Technologies, Santa Clara, CA, USA) using a reaction mix with a total volume of 10 µL containing the following components: 5 µL Maxima® Universal SYBR® Green Supermix (Bio-Rad Laboratories), forward and reverse primers (see Table 2 for details on primers and targets) and 0.75 µL of 1:5 diluted cDNA. Thermal cycles composed of an initial 3 min denaturation phase at 95 °C followed by 40 cycles of 95 °C for 15 s (IL-12, 94 °C for 20 s), annealing at primer-specific temperatures (Table 2) for 30 s, and extension at 72 °C for 30 s (IL-12, IL-20 s), and a melting curve analysis afterwards. Samples were amplified in duplicates. Non-template controls were included in every run, as well as interplate calibrators (IPC) to normalize C\textsubscript{\text{t}} values measured on different plates and thus correct for inter-run efficiency of primers was determined by five-fold dilution series of a cDNA pool; agarose gel electrophoresis and melting curve characteristics were used to evaluate their specificity. Relative target gene expression was calculated applying the software GenEx 6 (MultiD. Göteborg, Sweden), including correction of qPCR data for primer efficiency, interplate calibration and normalization to the expression of reference genes OAZ1 and RPL4 (Table 2). Data were converted to a log2 scale for statistical analyses. For relative quantification, samples were expressed relative to a calibrator, either their corresponding samples from SD 1 (to analyze group differences) or the sample with the lowest expression level (to analyze changes over time), which were scaled to 0.

In vivo stimulation of lymphocytes

Cells isolated from MLN were thawed and stimulated in duplicates with a recombinant murine protein (rCSUL, Biozol) recently characterized by Shrestha et al. [44] at a final concentration of 10 ng/µL. Cells were incubated in 48-well tissue culture plates at a density of 1 × 10^6/well in a volume of 250 µL culture medium (RPMI 1640 with 10% FCS, 2 mM L-glutamine, 100 µM penicillin and 0.1 mg/ml streptomycin Gibco®, Thermo Fischer Scientific) for 30 h at 37 °C with 5% CO\textsubscript{2}. In parallel, lymphocytes stimulated with ConcanaVarin A (5 µg/ml, Sigma-Aldrich) or incubated in culture medium only served as positive and negative controls, respectively. Supernatants were analyzed for various cytokines and chemokines (IL-1β, IFN-α, IFN-γ, IL-10, IL-12p40, IL-4, IL-8, CXCL-2) by multiplex fluorescent microsphere immunoassay (FMIA) as described elsewhere [45].

Quantification of parasite DNA in tissue

To study the intestinal and extraintestinal presence of stages of C. sae, DNA was extracted from jejunum (embedded in Neg-50° Frozen Section Medium) and from snap-frozen MLN and spleen samples using a qPCR GOLD Tissue DNA Mini Kit (peqlab, VWR International GmbH, Erlangen, Germany), following the manufacturer’s instructions. Prior to DNA isolation, samples were slightly thawed on ice, several sections were excised at different locations, pooled, and the Neg-50° Frozen Section Medium was removed from intestinal tissue by washing it three times in 1 ml of LiChrosolv® Water (Merck, Darmstadt, Germany). Extracted DNA was diluted 1:10 and qPCR was performed to quantify the C. sae genome by targeting the large subunit rRNA gene (GenBank: AE003428.1) as previously described by Shrestha et al. [44]. Samples were run in duplicates together with controls.

Statistical analyses

Statistical calculations were performed with RStudio version 0.99.896 (RStudio Team, 2016). Group differences were analyzed by applying an ANOVA to data with
normal distribution and variance homogeneity or, in the opposite case, either a Wilcoxon or (when comparing more than two samples) a Kruskal-Wallis rank sum test. In case of significant omnibus tests, post-hoc tests for multiple comparisons were performed (parametric Tukey or non-parametric Conover tests), and P-values were adjusted after Bonferroni. To analyze changes of values over time, parametric ANOVA or non-parametric Friedman rank sum tests, followed by multiple pairwise comparisons (t-tests and tests after Conover, respectively), were employed. Relationships between selected parameters were calculated by computing Spearman’s rank correlation coefficients. P-values ≤ 0.05 were considered significant.

Results

Clinical and fecal examination

Low levels of oocyst excretion occurred in every group infected with C. suis while uninfected control animals did not shed oocysts throughout the study. Highest numbers of shedding and excretion were found in group SH where 70% of the pigs excreted oocysts on a total of 17 days (Table 3); however, statistics did not reveal significant differences in these parameters between infected groups. One animal (TH5) excreted oocysts before infection and was thus treated with toltrazuril once (Baycox® 5% oral suspension, 20 mg/kg BW). It remained in the study but was excluded from analyses and comparisons involving the parameter ‘oocyst excretion’. None of the pigs developed diarrhea during the study period. One animal of group SH developed a fever and general depression during the study and was treated with enrofloxacin, an antibiotic without antimicrobial effect, for three days.

Hematology and white blood cell count

Most parameters were within normal limits except for a few mild deviations observed in all five groups. Statistical comparisons were only performed for parameters which deviated from their expected reference range at least once in at least one animal. Significant differences between infected and uninfected animals and over time in the respective groups were found for several hematological (hemoglobin, hematocrit) parameters and white blood cell populations (total leucocytes, lymphocytes, monocytes and segmented neutrophils/µl whole blood) (Additional file 1: Table S2).
Table 3: Results of copropathological examinations on the day of (first) infection and from study day 5 to 29. X indicates the days of faecal excretion. Only animals with at least one day of shedding and only study days with at least one excreting animal are listed.

<table>
<thead>
<tr>
<th>Animals</th>
<th>Study Days</th>
</tr>
</thead>
<tbody>
<tr>
<td>SL 8</td>
<td>x</td>
</tr>
<tr>
<td>SL 10</td>
<td></td>
</tr>
<tr>
<td>SH 1</td>
<td>x</td>
</tr>
<tr>
<td>SH 3</td>
<td>x</td>
</tr>
<tr>
<td>SH 4</td>
<td></td>
</tr>
<tr>
<td>SH 5</td>
<td></td>
</tr>
<tr>
<td>SH 7</td>
<td>x</td>
</tr>
<tr>
<td>SH 9</td>
<td></td>
</tr>
<tr>
<td>SH 10</td>
<td>x</td>
</tr>
<tr>
<td>TL 3</td>
<td></td>
</tr>
<tr>
<td>TL 5</td>
<td></td>
</tr>
<tr>
<td>TL 7</td>
<td>x</td>
</tr>
<tr>
<td>TL 9</td>
<td>x</td>
</tr>
<tr>
<td>TH 5</td>
<td></td>
</tr>
<tr>
<td>TH 6</td>
<td></td>
</tr>
<tr>
<td>TH 9</td>
<td>x</td>
</tr>
<tr>
<td>TH 10</td>
<td>x</td>
</tr>
</tbody>
</table>

Abbreviation: SL: Single Low, SH: Single High, TL: Tiltle Low, TH: Tiltle High

Histopathology of jejunum

H&E stained sections of the mid-jejunum were viewed for histopathological changes characteristic for C. suis infections [6, 46]. However, neither characteristic alterations nor differences between infected and control animals could be observed.

*Cytoplasma suis* specific antibodies in blood serum and intestinal mucosa

Cytoplasma suis specific IgG and IgA against both meroszoites and sporozoites could be detected in all serum samples from SD 1 onwards, with significant differences between infected animals and sham-treated controls in all antibody classes against both antigens. IgG titers of groups SL, SH, TL and TH progressively increased after the first infection and remained at significantly elevated levels until the end of the study (SD 29) when compared to baseline values prior to infection (Fig. 1 and Additional file 1: Table S3). IgG against meroszoites reached significantly elevated values at 1 (groups TL and TH), 2 (SH) and 3 (SL) week(s) post (first) infection when compared to the non-infected group C and remained significantly higher until SD 29. Differences between infected groups were only observed on SD 8 and 15 with significantly higher titers in Groups TL and TH against both classes of antibody (see Fig. 1 and Additional file 1: Table S4). By SD 8, groups SL, SH and TH exhibited significantly higher titers of IgA against sporozoites than group C while for TL significantly increased levels were first recorded on SD 15. Except for TH, titers remained significantly higher than those of group C until the end of the study (Additional file 1: Table S4 and Fig. 1).

Levels of IgA were relatively low compared to those of IgG, nevertheless titers against meroszoites significantly increased over time in all infected groups, whereas for IgA against sporozoites a statistically confirmed increase over time was only seen for groups SL and SH (Fig. 1 and Additional file 1: Table S3). When compared to group C, significantly increased levels of IgA against meroszoites were first observed in group TL from SD 8 onwards while SH and SL reached significantly elevated levels by SD 15 and 29, respectively. Titers of TH were higher than those of C, but a statistical difference was only seen on SD 15. Significant differences between infected groups were found for SL and TL on SD 8 and 15 and for SH and SL and SH and TH on SD 29 (Additional file 1: Table S4 and Fig. 1). IgA titers against sporozoites were higher in infected animals (Fig. 1) but significant differences to uninfected controls were only seen for SH and TL on SD 15 and 22 and for SH on SD 29 (Additional file 1: Table S4).

No significant differences were detected between groups SL, SH, TL and TH with regard to titer increase over time (from SD 1 to 22 or 29, respectively) in any of the evaluated antibody classes (data not shown).
In jejunal mucus, only IgG titers against merozoites were present but only in low amounts, with significantly higher levels in infected animals ($W = 330.35, P = 0.001$), specifically in groups SF and TH ($d^2 = 11.261, d = 4, P = 0.029$ and 0.034, respectively), when compared to uninfected ones.

The analysis of relationships between antibody levels and oocyst excretion revealed significant positive correlations between the number of days with excretion and the increase of serum IgG and IgA against both C. suis stages in the course of the study (Table 4). Positive correlations with the number of excretion days were also detected for serum titers of single study days: for IgG against merozoites on SD 15, for IgA against merozoites on SD 15, 22 and 29 and for both IgG and IgA against sporozoites on SD 29 (Table 4). Regarding IgG titers against merozoites in intestinal mucus, a similar trend could be observed but without statistical significance ($p = 0.192, P = 0.292$).

**Cytokine response to C. suis infections**

To investigate a potential systemic involvement of the cellular immune system in C. suis infections, the mRNA expression of selected pro-inflammatory (IFN-γ, IL-12, IL-2, TNF-α, IL-6, IL-27, IL-1E) and regulatory (IL-4, IL-10, TGF-β, IL-27) cytokines was measured in cells from three immune compartments: white blood cells, lymphocytes from MLN and splenocytes. No data could be obtained from three blood samples (T1, 7/SID 8, 14/SD 29 and 10/SD 29) due to an insufficient amount of extracted RNA.

**White blood cells**

For most cytokines, expression patterns were similar between infected groups, however relative mRNA quantities showed a high degree of variation between individuals, also within the same group. Expression of some cytokines also varied to a certain extent in
Table 4: Relationships between serum antibody titers and the number of days with copro antigen excretion. Relationships are given as Spearman rank correlation coefficients (r). (p values in parentheses. *P < 0.05; **P < 0.01; ***P < 0.001).

<table>
<thead>
<tr>
<th>Study day</th>
<th>Mean/Median</th>
<th>Spearman</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-0.175 (0.22)</td>
<td>-0.21 (1.12)</td>
<td>0.009 (0.352)</td>
</tr>
<tr>
<td>8</td>
<td>0.105 (0.55)</td>
<td>-0.16 (0.711)</td>
<td>0.052 (0.077)</td>
</tr>
<tr>
<td>15</td>
<td>0.301 (0.56)</td>
<td>0.29 (0.021)</td>
<td>0.026 (0.007)</td>
</tr>
<tr>
<td>22</td>
<td>0.247 (0.613)</td>
<td>0.316 (0.003)</td>
<td>0.674 (0.036)</td>
</tr>
<tr>
<td>29</td>
<td>0.167 (0.247)</td>
<td>0.315 (0.003)</td>
<td>0.038 (0.011)</td>
</tr>
<tr>
<td>1-22</td>
<td>0.405 (0.049)</td>
<td>0.415 (0.003)</td>
<td>0.725 (0.138)</td>
</tr>
<tr>
<td>1-29</td>
<td>0.311 (0.031)</td>
<td>0.48 (0.001)</td>
<td>0.258 (0.041)</td>
</tr>
</tbody>
</table>

Uninfected animals between study days (Fig. 2). For details on the change of expression levels over time, see Additional file 1: Table S5. Levels of TNF-α, IL-10 and IL-2 differed between groups on SD 1 already, i.e. before infection (Additional file 1: Table S6), but these discrepancies are compensated for by the paired analysis (normalization to SD 1) applied here.

The infection with C. suis induced a significant decline of IFN-γ mRNA expression from SD 1 to 20 (Fig. 2). As a result, IL-4 mRNA levels of infected animals were significantly lower on SD 10 compared to those of uninfected animals (W = 94, P = 0.009).

IL-12p35 levels significantly increased over time after infection, while they remained relatively constant in group C. Significantly more IL-12 mRNA was thus expressed in infected animals on SD 8 (W = 381, P < 0.001) (Fig. 2). Considering single- and trickle-injected infected animals separately revealed that groups which received a single infection contributed the most to this increase (r² = 32.886, df = 8, P = 0.047, compared to C).

Infected animals taken together expressed significantly less IL-2 mRNA on every study day (SD 8: W = 85, P = 0.005; SD 15: W = 60, P < 0.001; SD 29: W = 76, P = 0.007), with significantly lower levels in trickle injected animals on SD 8, 15 (r² = 65.63, df = 8, P < 0.001) and 29 (r² = 65.63, df = 8, P = 0.001) and in groups with single infections on SD 15 (r² = 65.63, df = 8, P = 0.009), when compared to the uninfected group (Fig. 2). Additionally, IL-2 was significantly less expressed in trickle than in single-injected animals on SD 8 (r² = 65.63, df = 8, P = 0.041).

TNF-α mRNA expression displayed strong variations both in infected and uninfected animals (Fig. 2). Nevertheless, it slightly increased in trickle infected animals from SD 1 to 20, resulting in significantly elevated levels compared to single-dose infected groups at the end of the study (r² = 57.271, df = 8, P = 0.018). On SD 8, TNF-α expression levels of infected animals were significantly elevated compared to controls (W = 285, P = 0.025) while they were significantly reduced on SD 15 (W = 97, P = 0.011), particularly in single infected animals (r² = 57.271, df = 8, P = 0.007).

Significantly higher IL-6 mRNA levels were detected after infections with C. suis on SD 8 (F1, 40 = 29.46, P < 0.001) and 15 (F1, 40 = 7.11, P = 0.011) when compared to the control group, as shown in Fig. 2. Among the infected groups, SL displayed the most notable increase in expression after SD 1, peaking at SD 15. SH, TL and TH showed a similar but less pronounced expression pattern. IL-6 mRNA levels of group C remained stable over time.

IL-27 mRNA levels did not differ between infected groups but were significantly higher in infected animals than in group C on SD 29 (F1, 40 = 12.61, P < 0.001). This, however, largely resulted from a significant decrease of expression in uninfected animals (Fig. 2).

Infected animals had significantly elevated IL-4 mRNA levels on SD 8 as compared to group C (W = 335, P < 0.001) while on SD 15 this was only seen in animals that received a single infection (r² = 38.792, df = 8, P = 0.039). They showed a significant increase of expression over time, with a peak on SD 15 trickle infected animals showed a similar although not significant pattern (Fig. 2).

On SD 8, IL-10 mRNA expression levels of infected animals were significantly higher than those of group C (W = 324, P < 0.001) (Fig. 2). Afterwards, particularly trickle infected animals experienced a further increase of IL-10 mRNA expression towards SD 29. Consequently, they expressed significantly higher levels at the end of the study compared to single infected (r² = 58.235, df = 8, P < 0.001) and uninfected animals (r² = 58.235, df = 8, P = 0.002).

TGF-β expression experienced a notable decrease after single infections; it reached lowest levels by SD 8, and then slowly increased to reach initial values by SD 29. Trickle infected animals showed a similar pattern but expression returned to initial values earlier. Single infected animals expressed significantly less TGF-β mRNA on SD 8 (r² = 53.346, df = 8, P = 0.005) and on SD 15 compared to control animals (r² = 53.346, df = 8, P = 0.002) and trickle infected animals (r² = 53.346, df = 8, P = 0.036) (Fig. 2). IL-1β mRNA expression was not significantly altered by infection with C. suis (Fig. 2).

Lymphatic organs: In MLN, cytokine mRNA levels were mostly unaltered upon infection, with the exception of IL-10 and TNF-α; both cytokines showed significantly elevated expression levels in infected animals (W = 255, P = 0.046 and W = 257, P = 0.046, respectively). In spleen samples, however,
Fig. 2 (See legend on next page)
no group differences were found (data not shown). IL-2 mRNA levels were too low to be measured in a large number of spleen samples; it was thus not considered for statistical analyses.

Responders
In order to avoid animals with generally low immune responses (so called low-responders) masking differences in cytokine expression between groups and over time, statistical analyses were repeated after removing those individuals from the datasets. The allocation to responders or low-responders was based on the animals’ antibody titers, and low-responders were defined as animals with no or only a mild (less than two dilution steps) increase of IgG titers against meroszoites within three weeks post-infection. Due to the reduced sizes of groups SL, SH, TL and TH in this dataset, animals were categorized in uninfected and infected and single and tickle infected groups for statistical comparisons. However, results did not differ tangibly from those analyses involving all animals, they were either identical or highly similar (IFN-γ, IL-2, IL-6, IL-27, IL-1β, IL-4, IL-10, TNF-α), or originally significant differences disappeared after removing low-responders (IL-12, TNF-α, TGF-β) (data not shown).

Correlations between cytokine and antibody response and parasitological parameters
The titer development of IgA against meroszoites from SD 1 to 22 and SD 1 to 29 showed significant positive correlations with the IL-4 mRNA expression of SD 8 and SD 15. Positive correlations were also detected between the increase of IgG titers over time and the IL-10 expression on SD 8. The increase of IgA titers against sporozoites was positively correlated with the IL-10 mRNA expression on SD 15. When comparing expressions and titers of same study days, a significant positive correlation was found for IgA against sporozoites and IL-10 on SD 15. IgG against meroszoites was positively but not significantly correlated with IL-4 mRNA levels on SD 8 (for details see Table 9). The number of days with oocyst excretion featured significant positive correlations with the IL-1β mRNA expression of SD 15 and 29 (ρ = 0.388, p = 0.006 and ρ = 0.446, p = 0.002, respectively) and the IL-4 mRNA level of SD 15 (ρ = 0.336, p = 0.018). On SD 8, a positive but not significant trend was observed for IL-1β, TNF-α and IL-10.

Stimulation of lymphocytes
Lymphocytes isolated from MLN were stimulated with a recombinant meroszoite protein [44] and concentrations of various Th1, Th2, inflammatory and regulatory cytokines and chemokines in supernatants were measured. Levels of IL-10, IL-4 and IL-1β were either not detectable or very low in both infected and uninfected animals. While a production of the type II interferon IFN-γ could not be measured after stimulation of cells from most infected and control animals, levels of type I IFN-α were decreased in 52.5% of infected animals after antigen-stimulation. Levels of IL-12 were significantly different between groups, with elevated and elevated concentrations within the same group. Nonetheless, overall mean IL-12 levels were higher after stimulation of cells from infected animals; however, differences were not statistically significant. Levels of IL-12 showed high variation between individuals, with reduced and elevated concentrations within the same group. Nevertheless, overall mean IL-12 levels were higher after stimulation of cells from infected animals; however, differences were not significant although a trend could be observed (χ² = 4.751, df = 2, P = 0.03). This effect is largely attributed to animals which received a single infection where 65% responded with an increase of IL-12 whereas levels of tickle infected animals were mostly decreased or remained unchanged (40% each) (Fig. 3b). Levels of IL-8 were not detectable in the majority of samples; nevertheless 22.5% of infected animals responded to antigen-stimulation with increased production of IL-8 (mean: 16.55 pg/ml). Stimulation of cells from infected animals induced an increase of CCL-2 production in 32.5% of the samples (mean: 152.30 pg/ml) and a mild decrease in only 10%.

Presence of C. suis DNA in organs
In order to examine intestinal and extraintestinal tissue for stages of C. suis, small intestinal samples, MLN and spleen tissues of all 50 animals were analyzed for the presence of C. suis DNA utilizing qPCR; however, no parasite material could be detected in any of the samples.

Discussion
The aim of the present study was to further characterize the antibody and cytokine response of immune-competent
Table 5 Relationships between serum antibody titers and mRNA expression of IL-4 and IL-10. Relationships are given as Spearman’s rank correlation coefficient ρ (in parentheses, P-values). Expression on single study days was correlated with IFAT results of the same study day and with titers development over time (SD 1 to 22 and 29, respectively). Only study days with at least one significant effect are shown. Significant correlations (P ≤ 0.05) are indicated in bold.

<table>
<thead>
<tr>
<th>SD</th>
<th>IG against maerococci</th>
<th>IG against spirococci</th>
<th>IL-4</th>
<th>IG against maerococci</th>
<th>IG against spirococci</th>
<th>IL-10</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>0.259 (0.072)</td>
<td>/</td>
<td>0.181 (0.361)</td>
<td>/</td>
<td>/</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>/</td>
<td>0.222 (0.121)</td>
<td>/</td>
<td>0.000 (0.999)</td>
<td>/</td>
<td></td>
</tr>
<tr>
<td>1–22</td>
<td>0.152 (0.187)</td>
<td>0.140 (0.201)</td>
<td>0.551 (&lt; 0.01)</td>
<td>0.269 (0.059)</td>
<td>/</td>
<td></td>
</tr>
<tr>
<td>&gt;29</td>
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<td>0.008 (0.926)</td>
<td>0.185 (0.005)</td>
<td>/</td>
<td>0.002 (0.999)</td>
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</tr>
<tr>
<td>8</td>
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<td>/</td>
<td>0.150 (0.467)</td>
<td>/</td>
<td>/</td>
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<tr>
<td>15</td>
<td>/</td>
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<td>/</td>
<td>0.000 (0.701)</td>
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<tr>
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<td>0.331 (0.118)</td>
<td>/</td>
<td>/</td>
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<tr>
<td>&gt;29</td>
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<td>0.370 (0.008)</td>
<td>0.351 (0.301)</td>
<td>/</td>
<td>0.065 (0.554)</td>
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</tr>
<tr>
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<td>/</td>
<td>0.416 (0.485)</td>
<td>/</td>
<td>/</td>
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<tr>
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<td>/</td>
<td>0.152 (0.206)</td>
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</tr>
<tr>
<td>1–22</td>
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<td>0.298 (0.038)</td>
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<td>/</td>
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</tr>
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<td>0.215 (0.130)</td>
<td>/</td>
<td>0.250 (0.079)</td>
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</tr>
<tr>
<td>8</td>
<td>0.159 (0.240)</td>
<td>/</td>
<td>0.301 (0.197)</td>
<td>/</td>
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</tr>
<tr>
<td>15</td>
<td>/</td>
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<td>/</td>
<td>0.216 (0.044)</td>
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</tr>
<tr>
<td>1–22</td>
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<td>0.194 (0.411)</td>
<td>0.216 (0.074)</td>
<td>/</td>
<td>0.249 (0.013)</td>
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<td>&gt;29</td>
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<td>0.243 (0.028)</td>
<td>/</td>
<td>0.253 (0.028)</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviation: SD study day.

Animals undergoing an infection with C. suis. Understanding and eventually being able to influence the immune response of immunologically mature animals is an important step towards developing a passive immunization strategy for pigs. Previous studies indicate that the infection of immune-competent pigs induces an immune response [29, 41], but data on this age group are limited, particularly on cytokine responses. Furthermore, different protocols of oral antigen delivery were evaluated for the intensity and character of immune response they induce in order to identify a potential immunization model for sows which can improve passive protection of their offspring. Growers were chosen for this study because the pig’s immune system is fully developed at that age and immune responses should therefore be comparable to those of sows, whilst being easier to keep than sows. Since antibody levels in blood serum and colostrum of pregnant sows correlate positively [26, 29], we assume that serum titers are representative for those in colostrum and that results obtained from growers can thus be compared to sows.

Cytokine and serological findings confirmed that infection and a subsequent systemic immune response were successfully induced by all infection models, whereas infection did not lead to clinical symptoms. Oocyst excretion was observed in every group except the uninfected control group although numbers of shedding, excreted oocysts and excretion days were low compared to experimental infections in suckling piglets [13]. The absence of clinical signs and low excretion levels are a regular phenomenon in older pigs infected with C. suis [1]. This lack of susceptibility may be attributed to natural age resistance as a result of the matured immune system or to the development of immunity after an overcompensation of the immune response of immune-competent animals to C. suis, particularly when modeling the immunization of sows because an exposure of sows to C. suis prior to immunization is also most likely. Another explanation for the presence of titers could be the existence of extraintestinal stages [48]. To test this theory, we screened MLN and spleen samples for parasite DNA, but no C. suis specific DNA was detected. This does not, however, disprove the existence of extraintestinal stages. Screening of jejunum samples for C. suis specific DNA remained unsuccessful as well but, looking at the low oocyst excretion level, this is not surprising. It seems that due to age resistance or immunity, the parasite can only rarely reproduce and complete its life-cycle and infection is rapidly cleared from the intestinal tissue.
Moreover, the sampling time-point (four weeks after the first infection) was presumably too late: piglets infected with C. suis typically excrete for about two weeks with decreasing intensity [6, 47]. In histological sections, neither parasites nor typical C. suis-related tissue lesions were detected, underpinning the assumption that either the parasite cannot reproduce or complete its life-cycle in the intestines of immune-competent pigs or the infection is cleared completely afterwards, and lesions are not visible anymore.

Differences between infected and uninfected animals were found for several hematological parameters and white blood cell counts; however, these alterations could not be attributed to infection status of the animals at time course and deviations from reference ranges were minor, indicating that the infection of immune-competent animals with C. suis does not cause appreciable systemic hematological changes. This is in contrast to the study of Woidratzek et al. [26] where leukocyte and lymphocyte counts were significantly decreased in infected suckling piglets. However, these results were obtained during the acute phase of infection and from clinically affected suckling piglets.

Both IgA and IgG against sporozoites and merozoites of C. suis significantly increased after infection, which is consistent with what Schwarz et al. [29] described in sows, indicating that immune responses of growers and sows are comparable and that growers can be used to mimic infections in sows. Although all of the titers measured increased after infection, those against merozoites showed the most pronounced increase. Sporozoites are the initial endogenous stages of C. suis and invade epithelial cells after they are released from ingested oocysts. They do not replicate but develop to advanced stages during merogony and subsequent asexual multiplication results in the generation of merozoites which destroy their host cells and invade further epithelial cells [29, 46]. Merozoites might be more immunogenic because they are more numerous and thus have a higher destructive potential and longer contact time to the host compared to sporozoites, and might thus provoke a stronger immune response. Likewise, the asexual developing stages of avian coccidia are considered to be particularly immunogenic [20, 49].

In avian coccidiosis, the development of immunity is known to be directly correlated to the primary infection dose [22]. In this study, however, only slight differences in the immune response were observed between the infection models, suggesting that the mode of infection does not noticeably affect the immunological outcome. Similarly, Woidratzek et al. [47] described that the infection dose does not have a significant impact on the clinical signs or the outcome of disease in piglets, but immune responses were not looked at. Herein, titers against merozoites were significantly higher in trickle infected animals on SD 8; however, by that time all animals had just received one infection dose and differences thus have to be attributed to individual variations in the immune response. A higher IgG titer on SD 15 on the other hand might be the result of the booster infection trickle groups received on SD 8. Nonetheless, titers did not significantly differ at later time points. IgA titers against merozoites were significantly higher in group SH compared to the other infected groups at the end of the study, and a similar trend, although not significant, was observed in other titers measured. Moreover, group SH exhibited the strongest increase of all evaluated titers from SD 1 to 29 and comprised most shedders with most excretion days. This is in line with our findings that antibody titers correlate positively with oocyst excretion. Likewise,
studies performed on *Ensetria* in calves, lambs and goat kids showed that IgA and IgG levels were positively correlated with oocyst excretion in most cases [28, 29]. In suckling piglets infected with *C. suis*, titers and oocyst per gram of feces (OpG) were largely unrelated while titers were mostly negatively correlated with fecal consistency [28, 29]. The higher antibody production might be the result of a higher parasite reproduction rate and the completion of the life-cycle and consequently a longer contact time between antigen and immune system in the gut. The presence of intestinal IgA against merozoites supports this hypothesis since they were also positively (although not significantly) correlated with oocyst excretion and were highest in group S4. The detection of IgG in jejunal mucosa of infected animals also indicates a local humoral immune response to *C. suis*; however, levels of IgA were either very low or undetectable which is in contrast to what Schwarz et al. [38] found in 28 day-old infected suckling piglets. A possible explanation could be that IgA, the primary antibody in sow milk and most relevant immunoglobulin of the local, mucosal defense [60], is primarily consumed with the milk and acts locally in the gut after the intestinal barrier becomes impermeable for antibodies rather than endogenously produced IgA, acting at the site of infection.

It has to be concluded that although antibodies seem to reflect the exposure to *C. suis* and possibly the severity of infection, they might not be correlated with anticoccidial immunity and might not contribute to protection. This is in line with the opinion of many authors [20, 22, 30, 31, 33, 51, 52] although some do suggest a protective role of (passively obtained or endogenously produced) antibodies against infection with several coccidia [23, 32, 39].

The mechanisms to successfully control coccidial infections and to develop a protective immunity are considered to be predominantly cell-mediated, and particularly characterized by the production of T helper 1 (Th1) and pro-inflammatory cytokines which are crucial in eliminating intracellular pathogens [20, 28, 51]. For *C. suis* however, only few data are available on the cytokine response of the infected host, particularly on a systemic level. For this, we herein investigated the systemic cytokine profiles of unstimulated white blood cells, MLN and spleens at mRNA level and of stimulated lymphocytes at protein level. Cytokine expression levels were in part rather low, indicating that the systemic cellular immune system might not play a major role in the immune response to *C. suis*. Also, cytokine levels were subject to a high inter-individual variation which is consistent with findings of other studies on cytokine profiles in pigs [45, 53, 54] and similar to the antibody response, differences between infected groups were mostly insignificant. Nevertheless, certain differences between infected and control animals as well as significant changes over time could be observed, particularly in white blood cells.

Expression levels of the Th1 cytokine IFN-γ and the inflammatory cytokine IL-2, an inducer of IFN-γ synthesis, decreased in white blood cells post (first) infection. Thγ−γδ+ T cells are, amongst others, important producers of these cytokines [55–57] and were not only shown to be a highly abundant T cell subset in pigs but also to be significantly increased in the jejunum of *C. suis* infected pigs with numbers being reduced in blood and MLN [26, 27]. This suggests a migration of this subset of T cells to the site of infection and a predominantly local relevance of IFN-γ and IL-2. Local production of these cytokines, particularly of IFN-γ, and their essential role in the immune response against avian and various mammalian coccidia has been extensively described [21, 32, 58–63]. Alternatively, their production might have been inhibited by IL-4 and IL-10 which were both increasingly expressed after infection. Both IL-4, belonging to the Th2 family, and the regulatory cytokine IL-10 suppress the generation of Th1-associated and inflammatory cytokines and, on the contrary, promote Th2 response, B cell proliferation and antibody production [24, 45, 54, 64, 65]. This matches the increase of serum antibody levels observed post-infection and the significant positive relations between IL-4 (IL-10) and antibody titers shown in this study, indicating a Th2-biased immune response. Increased peripheral levels of IL-4 and IL-10 have also been described in *Necrophorus autumnalis* infected cattle, but are thought to be associated with a decreased ability of the host to control infections with *Necrophorus* [52, 61, 66]. IL-10 mRNA expression was also significantly elevated in MLN of infected animals which matches findings of Workneh et al. [26] where the number of B cells was significantly increased in MLN of infected piglets. The simultaneously increased expression of TNF-α mRNA is a commonly observed phenomenon because an elevated production of TNF-α is usually counterbalanced by IL-10 production to avoid excessive inflammatory responses [67]. However, levels of both cytokines were not drastically increased, thus these results have to be interpreted with caution. In contrast to IFN-γ and IL-2, mRNA expression of IL-12p35, a subunit of the Th1-associated cytokine IL-12, was significantly increased on SD 8 after infection. This is surprising since IL-12 is an important enhancer of IFN-γ production [64], thus one would expect a simultaneous increase of both cytokines, and furthermore IL-10 usually downregulates IL-12 [65]. However, inter- and intra-individual variation of IL-12p35 mRNA expression was high and also observed in group C, and differences were only slightly significant (W = 281, P = 0.032). Moreover, IL-12 is known to be regularly produced by a broad range of cells in humans and animals [64, 68], and the
changes observed might be attributed to physiological fluctuation or induced by other stimulators than C. suis. Similarly, mRNA expression of inflammatory TNF-α was subject to some variation in white blood cells of both infected and uninfected animals; group differences rather resulted from varying expression levels in group C, although levels also slightly increased over time in infected animals TNF-α is, in part, produced by TcR-β T cells and its expression was shown to increased in the jejunum of infected piglets [27], thus it might be more relevant and consequently TNF-α producing cells might be more abundant at the site of infection. Expression of TGF-β was significantly downregulated in infected animals in the first two weeks post-infection. Similarly, local TGF-β expression was found to be downregulated in N. caninum infected cattle and sheep [52, 63] and uninfected in the jejunum of C. suis infected piglets [27]. TGF-β is a regulatory cytokine which suppresses Th1 and inflammatory cytokine production in order to avoid excessive inflammatory responses with tissue-damaging consequences [60, 63]. Since IL-10 mRNA expression was increased in this study, one might speculate that C. suis primarily activates the production of IL-10 by regulatory T cells rather than that of TGF-β. Alternatively, TGF-β expression could be suppressed in favor of a local Th1-biased immune response which would explain the severe damage of the intestinal mucosa in the course of C. suis infections in suckling piglets since TGF-β is involved in its protection [60]. However, more data on cytokine expression in the jejunum are needed to test this theory. The increased mRNA expression of IL-6 on SD 8 and 15 indicates an acute-phase response to infection with C. suis. Increased serum and local levels of IL-6 were reported in human mammals and birds after infections with coccidia [52, 62, 63, 69, 73]. In addition, IL-6, secreted in vitro by bone fibroblasts infected with N. caninum, induced the production of IL-17 by T cells which in turn significantly reduced parasite burden [71]. Since IL-6 can also promote a humoral immune response [52], together with IL-4 and IL-10 it may be responsible for the antibody increase observed in this study.

In vitro stimulation of MLN-derived lymphocytes of infected animals with a recombinant merozoite protein resulted in increased levels of IL-12, supporting other studies on coccidia [51, 66, 70] and the assumed key role of a Th1-biased immune response in the successful elimination of protozoa. However, protein levels were low with very high variations, and differences between infected and control animals did not reach significant levels. Also, other cytokines frequently reported to be produced upon stimulation with coccidial antigens were not increased. Potentially, the in vivo application of the protein alone does not sufficiently stimulate the immune cells due to a weak immunogenicity which is also a common issue in vaccines based on recombinant proteins unless combined with immunostimulants [72]. Of the interferon family two members were measured: the type II interferon IFN-γ is widely associated with controlling coccidial infections [24, 73], but an antigen-induced production could not be observed in this study. Production of IFN-γ, a representative of type I interferons, was (although not significantly) reduced in cells of infected animals IFN-γ is primarily recognized for its antiviral activity [73] and has only been studied to a limited extent in the context of coccidial infections; however, it was shown to limit parasite growth in cells infected with N. caninum and other coccidia [73, 74]. Toxoplasma gondii was shown to be able to inhibit IFN-γ production by host cells [73] and C. suis might possess the same ability in order to escape parasite-limiting immune responses. As mentioned above, the changes observed are mostly rather small, since protein levels appeared very low or were not measurable at all in many cases, and warrant cautious interpretation. Further attempts should be made to improve this assay.

Conclusions

Overall, our findings indicate that the infection of immune-competent animals with C. suis does induce a systemic immune response, irrespective of infection dose or frequency, without clinical signs and with negligible oocyst excretion. Immune responses of sow and growers can therefore be considered as comparable, thus they can be used as models to mimic infection or immunization of sows with this parasite. Moreover, the induction of an immune response without clinical signs or oocyst excretion is an ideal situation for a passive vaccination strategy. However, the different modes of oral infection tested here did not significantly differ in their immunological outcome, thus application routes other than the oral one, the use of adjuvants, or the use of recombinant or DNA vaccine should be taken into consideration to enhance immune responses and subsequently improve passive protection for piglets. Systemic immune responses were associated with upregulated Th2 and regulatory cytokines and increased serum levels of C. suis-specific antibodies whereas Th1-associated cytokines typically involved in coccidial infections were downregulated or unaffected by infection. Generally, systemic cytokine responses were weak and showed considerable variation within groups. However, unlike other Eimeria species, C. suis reproduces solely within the superficial layers of the intestinal mucosa, thus the strongest cellular immune reactions, particularly those of the Th1 and proinflammatory type, might take place at the site of infection where the parasite has direct contact to local immune cells. It is conceivable that certain cytokine profiles observed in the blood are simply a spillover effect of the local immune response. Finally, none of the measured parameters could be clearly identified as a marker related to protection. Antibody titers were positively
correlated with oocyst excretion, with IgG titers against mannose-resistant exhibiting the strongest increase, resulting in higher levels. It might thus serve as a correlate for parasite replication or the severity of infection. It cannot, however, be ruled out that an enhanced immune response in sow 2 would confer sufficient passive protection to piglets, either through colostral antibodies or cellular components or both. Since pre-experimental antibody titers indicated previous infections of the growing pigs with C. suis, the reactions to the experimental infections must be seen as immune memory response (which would mimic the situation in sows), although from the generally low responses in most pigs this could not be determined unequivocally.

Additional file

Additional file 1: Table S1. Hematological parameters and white blood cell counts determined on study day 1 and 2: Abbreviations: MCV: mean cell volume; MCH: mean cellular hemoglobin; MCHC: mean cellular hemoglobin concentration. Differences in hematological parameters and white blood cell counts between groups and over time. For Table S1, the mean ± standard deviation is given. Differences in antibody titers between groups and over time. For Table S2, the mean ± standard deviation is given. Differences in antibody titers between groups and over time. For Table S3, the mean ± standard deviation is given. Differences in antibody titers between groups and over time. For Table S4, the mean ± standard deviation is given. Differences in antibody titers between groups and over time. For Table S5, the mean ± standard deviation is given. Differences in antibody titers between groups and over time. For Table S6, the mean ± standard deviation is given.

Abbreviations

BN: Body weight; FC1: Fetal calf serum; PMA: Phorbol 12-myristate 13-acetate; PT: Purified antigen; TRAP: Transmitting growth factor

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Availability of data and materials

All data and materials of the experiments described here are included in the manuscript and its additional file.

Authors’ contributions

All BF and MP designed the study. AS provided the recombinant mannose resistant protein. AN and BF designed and tested the P57 primers. BR provided acaflusenog and mercoxyls. Al BF and MP carried out the tests. BF and BR processed and analyzed the samples and BF conducted the statistical analysis and drafted the manuscript. All authors read and approved the final version of the manuscript.

Ethics approval

All procedures involving animals were approved by the Animal Ethics Committee of the University of Veterinary Medicine Vienna and the Austrian Federal Ministry of Science and Research according to the Austrian Animal Protection Law (BMV 66.050/188/III/01-4; BMV 66.050/304-WE/9/16).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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5.2. Publication II

Comparison of an injectable toltrazuril-gleptoferron (Forceris®) and an oral toltrazuril (Baycox®) + injectable iron dextran for the control of experimentally induced piglet cystoisosporosis

Anja Joachim1*, Anura Shrestha1, Barbara Preudenschuss1, Nicola Palmieri1, Barbara Hinney1, Hamadi Karembe2 and Daniel Sperzi2

Abstract

Background: Cystoisospora suis causes diarrhoeal disease and reduced weight gain in suckling piglets, and a toltrazuril-based oral suspension is available for treatment. Recently, a combinational product with toltrazuril plus iron has been developed for parental application. In this study we compared the efficacy of the injectable product with the oral suspension against experimentally induced piglet cystoisosporosis.

Methods: In a randomised controlled study, three groups of piglets (n = 10–13) were treated either with a fixed dose of 40 mg toltrazuril + 200 mg gleptoferron im. per piglet (Forceris®) on the second day of life (study day 2; SD 2) or with 20 mg toltrazuril/kg body weight as an oral suspension (Baycox® 3%) on SD 4 or left untreated (Control group). The Baycox® and the Control group received 200 mg of iron dextran/piglet on SD 2. All piglets were infected with 1000 sporulated C. suis oocysts on SD 3. Faecal samples were taken daily from SD 7 to SD 20 to determine faecal consistency, oocyst shedding and other diarrhoeal pathogens. Body weight was recorded on SD 1 and then weekly until SD 29. Animals were observed daily for general health and after treatment for possible adverse events.

Results: In the Control group, all animals shed oocysts for 21 days on average and all animals showed diarrhoea for an average of five days. Faecal score peaked on SD 9 (max. 4, 6 of 8 oocysts per gram of faeces). Treatment with Forceris® completely suppressed oocyst excision. In the Baycox® group, low levels of excitation could be detected. Diarrhoea was reduced to single piglets in the treated groups. Body weight development was reduced in the Control group compared to the treated groups. Enteropathogenic bacteria (Escherichia coli, Clostridium perfringens) could be detected. All parameters related to oocyst excision, faecal consistency and weight gain were significantly improved in the treated groups compared to the Control group without significant differences between the treated groups. Both products were safe to use.

Conclusions: Treatment with both the injectable (Forceris®) and the oral (Baycox®) formulation of toltrazuril in the prepatent period were safe and highly effective against experimental infection with C. suis in newborn piglets.

Keywords: Pig, Swine, Coccidiosis, Cystoisospora suis, Isospora suis, Diarrhoea, Experimental Infection, Toltrazuril, Efficacy

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Background
Porcine neonatal coccidiosis caused by *Cystoisospora suis* (syn. *Isospora suis*) is a major cause of diarrhoea and unthriftiness in piglets worldwide [7–10] and induces substantial economic losses in the pig breeding industry [7–10]. Currently, the only effective chemotherapeutic drug available for treatment is the trithion tolxtrazin [11]. It is available as a 5% suspension to be applied as a single oral treatment during the third to fifth day of life. Oral treatment with toxtrazin is highly effective in controlling oocyst excretion and diarrhoea both in experimental and natural infections of suckling piglets [12–17].

Recently, an injectable combination product, toltazarin + iron (30 mg toltazarin/ml; 133.4 mg iron/ml as gleptoferron) has been developed for the prevention of piglet coccidiosis together with a prevention of iron deficiency (Fercerin®, Ceva, Libourne, France). Treatment is scheduled from the first to the third day of life (i.e. between 24 and 96 h after birth) as a single intramuscular injection of 1.5 ml/piglet corresponding to 45 mg of toltazarin and 200 mg of iron.

Fercerin® is the first toxtrazin product to be marketed in an injectable formulation for the control of *C. suis* in piglets from the first day of life, and this study aimed to evaluate its efficacy against porcine coccidiosis in an experimental infection model [18] in comparison to an established reference product, an oral toxtrazin suspension (Baycox® 5%, Bayer Animal Health, Monheim, Germany).

Methods

Animals and husbandry
A total of 35 piglets from four litters were enrolled in the trial on study day (SD) 1 and finished it on SD 29. Sows (Landrace × Large White) were moved to the large animal facilities of the Institute of Parasitology of the Vetmeduni Vienna two weeks before farrowing to acclimatise to the housing conditions. Animals were kept on straw with a heat lamp for the piglets and fed with conventional feed free of coccidostats. Water was provided ad libitum. On the first day of the study (SD 1; within 24 h after birth of the piglets) animals were marked individually and enrolled in the study if they were clinically healthy and weighed at least 900 g. They were randomly allocated to one of three groups according to body weight on SD 1 in the order of the birth of the litters. The general health of sows and their piglets was observed and recorded daily from SD 1 to SD 29.

Treatment
Animals were treated either with toltazarin + gleptoferron (investigational product: Fercerin®, Ceva, France) at a fixed dose of 1.5 ml/piglet corresponding to 45 mg of toltazarin and 200 mg of iron, i.e. on SD 2 (Fercerin® group, n = 13 piglets) or with a toxtrazin suspension (reference product: Baycox® 5%, Bayer Animal Health, Monheim, Germany) at a dose of 20 mg/kg orally on SD 4 (Baycox® group, n = 12 piglets). A third group (Control group n = 10 piglets) remained untreated. The Baycox® and the Control groups received iron dextran (Unifer® 200, Virbac, Kolding, Denmark) at a fixed dose of 200 mg/piglet, i.e. on SD 2. Allocation to treatment groups and treatment were carried out under blinded conditions, i.e. only the dispenser was aware of the allocation of piglets to a treatment group during the entire study period. Post-treatment observations for any adverse events (swelling/bleeding of the injection site, inability to stand, walk, suckle or other abnormal behaviour, including dyspnoea, vomiting, limping, lateral recumbency, signs of pain, distress or neurological alterations, [19]) were conducted under blinded conditions by a veterinarian at 2, 6 and 24 h after treatment, and after that daily until SD 8.

Experimental infection
Each piglet was orally infected with a single dose of approximately 1000 sporulated oocysts of *C. suis*, strain Wien-1 [13] on SD 3.

Determination of concomitant bacterial and viral infections
On the first day of faecal sampling (SD 7) pooled faecal samples from each litter (n = 4) were submitted for bacteriological and virological examination to the Institute of Microbiology and Institute of Virology, respectively, of the Vetmeduni Vienna. A general qualitative/semiquantitative bacteriological examination, followed by specific differentiation and virulence factor/toxin typing in positive cases, was conducted. Porcine rotavirus A antigen and nucleic acids of porcine coronaviruses TGEV and PRCV were determined from the faecal material. In case of diarrhoea after treatment with toxtrazin, individual samples were taken on the first day of diarrhoea and examined for bacterial pathogens as well.

Efficacy parameters
Individual faecal samples were taken daily from SD 7 to SD 20. Efficacy of toxtrazin was evaluated by qualitative (autofluorescence [20]) and quantitative (McMaster counting of oocysts per gram of faeces, OopG) determination of oocysts and faecal consistency as previously described [18]. Faecal consistency of each sample was scored immediately with faecal score (FS) 1 describing firm, FS 2 pasty, FS 3 semi-liquid and FS 4 liquid faeces, with FS 3 and FS 4 considered as diarrhoea. Individual body weight was recorded for each enrolled piglet on SD 1, 8, 15, 22 and 29.

To evaluate the efficacy of treatment, oocyst excretion, faecal consistency and body weight development were analysed statistically. This included the area under the curve (AUC) for quantitative oocyst excretion and FS, the number of days with oocyst excretion/diarrhoea, and
Table 1: McMaster countable oocyst excretion, autofluorescence detectable oocyst excretion and diarrhoea values per group

<table>
<thead>
<tr>
<th></th>
<th>Forecex*</th>
<th>Baycox*</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
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<td>10</td>
<td>15</td>
<td>10</td>
</tr>
<tr>
<td>No. of samples over all sampling days</td>
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<td>152</td>
<td>140</td>
</tr>
<tr>
<td>McMaster countable oocyst excretion</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>No. (%) positive piglets</td>
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<td>2 (13%)</td>
<td>9 (90%)</td>
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<tr>
<td>No. (%) excretion days</td>
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<td>3 (20%)</td>
<td>9 (90%)</td>
</tr>
<tr>
<td>Autofluorescence detectable oocyst excretion</td>
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<td></td>
<td></td>
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<td>10 (100%)</td>
</tr>
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<td>No. (%) excretion days</td>
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<tr>
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<td>No. (%) FS 4 piglets</td>
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<td>7 (70%)</td>
</tr>
<tr>
<td>No. (%) FS 4 days</td>
<td>1 (50%)</td>
<td>2 (90%)</td>
<td>20 (150%)</td>
</tr>
</tbody>
</table>

The number of animals that showed excretion/diarrhoea in each group, as well as the body weight on each weighing day and body weight gain. Data were evaluated for homogeneity of the groups and analysed by means of the Kruskal-Wallis H-test (for continuous parameters) or by means of the Chi-square test (for discrete parameters). Pairwise comparisons were performed either using Fisher’s exact test (excretion/diarrhoea present or not) or Wilcoxon-Mann-Whitney U-test (other parameters). For all tests, P-value correction with Bonferroni was applied.

Results

Safety

No animal showed treatment-related adverse events that required veterinary intervention. Two animals from the Forecex* group showed a slight temporary swelling at the injection site within the first day of observation after treatment. Two animals from the Control group had to be treated with Ringer’s lactate due to dehydration.

Qualitative oocyst excretion determined by autofluorescence

All piglets of the Control group and 25% of the piglets in the Baycox* group excreted oocysts detectable by autofluorescence (AF), while none of the animals in the Forecex* group shed oocysts. The mean duration of oocyst excretion was 1.3 days in the Baycox* group and 3.1 days in the Control group (Table 1). The number of days with AF detectable oocyst excretion and the number of piglets that excreted AF detectable oocysts were significantly reduced in the Forecex* and Baycox* groups compared to the Control group without statistical difference between the treatment groups (Table 2).

Quantitative oocyst excretion determined by McMaster counting

McMaster countable oocyst excretion was observed in nine out of ten piglets of the Control group, in two animals of the Baycox* group and in none of the piglets of the Forecex* group. When excretion days were evaluated, positive animals in the Control group excreted McMaster countable oocysts between one and four days (mean 2.3 days), whereas in the Baycox* group two positive animals excreted for one and two days, respectively (Table 1).

The maximum OPG value in the Control group was 40.18 OpG on SD 9 (Fig. 1) on that day, the prevalence of McMaster countable oocyst excretion also reached its peak at 50% (Fig. 2), while in the Baycox* group the OPGs did not exceed 333.

The area under the curve for OPG, the number of days with McMaster countable oocyst excretion and the number of piglets that excreted countable oocyst numbers were significantly reduced in the Forecex* and Baycox* groups compared to the Control group without statistical difference between the treatment groups (Table 2).

Table 2: P-value (given as -log10) are given for the parameters of oocyst excretion and faecal score. Differences at P < 0.05 are indicated in bold

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Forecex vs Control</th>
<th>Baycox vs Control</th>
<th>Forecex vs Baycox</th>
<th>( \chi^2 )</th>
<th>df</th>
</tr>
</thead>
<tbody>
<tr>
<td>Area under the Curve for OPG</td>
<td>3.09</td>
<td>3.19</td>
<td>0.23</td>
<td>24.07,2</td>
<td></td>
</tr>
<tr>
<td>No. of days with McMaster</td>
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<td>2.70</td>
<td>0.33</td>
<td>23.17,2</td>
<td></td>
</tr>
<tr>
<td>Countable oocyst excretion present or not</td>
<td>4.13</td>
<td>2.24</td>
<td>0.18</td>
<td>23.06,2</td>
<td></td>
</tr>
<tr>
<td>No. of days with AF detectable</td>
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<td>3.80</td>
<td>0.70</td>
<td>28.29,2</td>
<td></td>
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<tr>
<td>Oocyst excretion present or not</td>
<td>5.52</td>
<td>2.65</td>
<td>0.54</td>
<td>25.36,2</td>
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<tr>
<td>Area under the Curve for FS</td>
<td>3.72</td>
<td>4.72</td>
<td>0.00</td>
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<tr>
<td>No. of days with diarrhoea</td>
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<td>3.60</td>
<td>0.00</td>
<td>24.46,2</td>
<td></td>
</tr>
<tr>
<td>Diarrhoea present or not</td>
<td>3.70</td>
<td>2.29</td>
<td>0.00</td>
<td>17.44,2</td>
<td></td>
</tr>
<tr>
<td>Daily body weight gain SD 1-29</td>
<td>1.26</td>
<td>2.59</td>
<td>0.88</td>
<td>14.59,2</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: OPG oocysts per gram of faeces, AF autofluorescence, FS faecal score, \( \chi^2 \) and df of statistics and degrees of freedom according to Kruskal-Wallis rank sum test or Chi-square test.
Fecal consistency and diarrhoea

The average faecal score increased above 2 in the Control group from SD 8 to SD 14, while in the treated groups the mean FS never exceeded 2. The maximum prevalence of diarrhoea was 100% in the Control group (SD 11 and SD 12) with an average duration of 5.0 days, while in the Baycox® group 33.3% of the animals showed diarrhoea for an average of 1.5 days, and in the Foreris® group two animals (12.2%) showed diarrhoea for one and two days, respectively. FS 4 (watery diarrhoea) was observed in 70% of the Control animals (average duration: 3.1 days) while in the Baycox® group one animal had watery diarrhoea for three days and in the Foreris® group one animal for one day (Table 1).

The peak of diarrhoea was observed on SD 11 to SD 12 when all animals in the Control group showed diarrhoea with a mean FS of 3.6 (Figs. 3 and 4). The area under the curve for the FS, the number of days with diarrhoea and the number of piglets that had diarrhoea were significantly reduced in the Foreris® and Baycox® groups compared to the Control group without statistical difference between the treatment groups (Table 2).

Body weight development

Body weights were not significantly different between the groups on SD 1, the day of randomisation (Kruskal-Wallis H-test, $\chi^2 = 95145$, $df = 2$, $P = 0.62$). Daily body weight gain and total weight gain from SD 1 to SD 29 were lowest in the Control group due to a severe depression of weight gain in the acute phase of infection (from SD 8 to SD 15) during which the Control group only gained 975.8 g on average compared to 2088.3 g in the Baycox® group and 1976.9 g in the Foreris® group (Fig 5).

The daily body weight gain from SD 1 to SD 29 was significantly higher in the Foreris® and Baycox® groups compared to the Control group without statistical difference between the treatment groups (Fig 5).

Concomitant infections

All litters showed infections with a similar pattern of bacteria. Haemolytic Escherichia coli which were positive for virulence factors fimH, papC, ivc, D, and cfa and β-2 toxin positive Clostridium perfringens Type A were detected in high amounts at the beginning of sampling and...
were also diagnosed in those animals that showed diarrhoea after treatment. No viral infections were detected.

Discussion
Piglets experimentally infected with *C. suis* were treated with toltrazuril either parenterally or orally before the onset of oocyst excretion and diarrhoea. Both, Forcetis® and Baycox® had a comparable effect on oocyst shedding, faecal consistency and body weight development.

Oocyst shedding was significantly suppressed in both treated groups compared to the Control group. Despite the intra-litter randomisation which entailed a high environmental contamination in all litters due to the presence of untreated control animals, no piglets of the Forcetis® group excreted oocysts during the trial. This indicates that the treatment was highly effective and the level of false-positive samples due to coprophagy [21] in such an experimental setting is likely to be low. In the Baycox® group, three samples were positive with one oocyst in McMaster counting each, resulting in OPG values of 333. It is difficult to unequivocally conclude that these findings are derived from true infections for the reasons stated above; however, one of the two animals that excreted countable oocysts did so on two consecutive days indicating that at least this individual shed oocysts due to infection and not due to coprophagy, but data on the extent of false positive samples due to this in experimentally infected mixed litters are not available. Low levels of oocyst shedding can occur despite treatment with Baycox® [14] while in other experimentally induced infections suppression of oocyst development was complete [15]. Despite low levels of oocyst shedding the reduction of environmental re-contamination by disruption of the parasite’s life-cycle was highly effective.

Diarrhoea is a hallmark of porcine coccidiosis [22, 23, 14, 22]; it can also be induced by parasite infection in the absence of other enteropathogens [23, 24] but exacerbated by rotavirus [24] or *Clostridium perfringens* [25, 26], and probably by other enteropathogens as well. Early studies on the use of toltrazuril in pigs indicated that *C. suis* has a forerunner role for bacterial infections as anticoccidial treatment reduced diarrhoea and simultaneously the amount of antibiotics required to control bacterial infections on affected farms, but a low level of diarrhoea was still seen after toltrazuril application [12]. In the light of these findings, it is conceivable that the *E. coli*
and clostridia that were circulating in the litters (and also detected in the animals with diarrhoea after treatment) contributed to the clinical outcome in some individuals despite successful treatment of coccidiosis.

Body weight development and diarrhoea are inversely related [18] and consequently body weight gain was increased in treated animals (which showed less diarrhoea), with the result of higher weights around weaning on SD 29 compared to the Control group. Improved weight gain has also been shown upon treatment in C. suis-positive herds with no apparent clinical signs [27], indicating that the presence of the parasite may influence performance of piglets even at low infection levels.

Parasite shedding, diarrhoea and body weight development showed no significant differences between the two toltrazuril formulations; both were highly efficacious. Forc fx® can be used to simultaneously treat metaphylactically against C. suis infection and supply the iron required to support the rapid growth and increase in blood volume during the first days of life of the piglets, thus reducing the required handling of the piglets. Time needed for oral treatment of one piglet was estimated at 10 seconds with a labour cost estimation of € 1.8/hour [27], significantly contributing to the costs of coccidiosis control. Forc fx® can be applied from the first day of life (Baycox®: third to fifth day of life) and may thus also be more useful in cases where C. suis infections occur very early after birth and are often seriously exacerbated by bacterial infections, such as with Cl. perfringens [17]. Accurate dosing of piglets by injection may also be of advantage when vomiting occurs for a variety of reasons, including infection with C. suis [26].

Conclusions

Oocyst shedding was reduced to a minimum in the Baycox® group and suppressed completely in the Forc fx® group. Diarrhoea was also reduced significantly in both treatment groups, resulting in significantly better weight gain. The latter was depressed during the acute stage of infection (in the second week of life) in animals that were clinically affected by pasty to watery diarrhoea. Both applications were safe to use and effective in a single application. Early (metaphylactic) treatment of piglet coccidiosis during the prepatent phase of infection can control infection and significantly improve piglet health when C. suis is present on a farm. Treatment with toltrazuril together with iron by injection was safe and effective. Handling of animals for medication was reduced by the combination product without interfering with the efficacy of toltrazuril treatment.

Abbreviations

AF: Airflow measurement, ALV: Area under the curve; TS: Fecal score; Ogg: Oocysts per gram of faeces; SD: Study day

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Availability of data and materials

Raw data will be shared, as study documentation is protected by confidentiality agreements.

Authors' contributions

AJ, HR and CO designed the study. AS and BF sampled and supervised the animal part. NP carried out the statistical analysis and BI carried out the dispensing, blinding and debinding of the staff involved and the sponsor. All authors reviewed and approved the final manuscript.

Ethics approval

The procedures involving piglets for collection of oocysts were approved by the institutional ethics committees and the national authority according to 354 of Animal Experiments Act. Terre de loisirs (2012-REG-2012-286). The experiment number: BWIF 602/00203-7AF/3/6/2016, Austrian Federal Ministry of Science, Health and Economy.

Conflict of publication

Not applicable.

Competing interests

The authors declare that they have no competing interests. DS and HR are employees of Covas. No member of the staff of the Vetmeduni Vienna involved in the trial received any other personal benefits from the Sponsor.

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5.3. Publication III

Experimentally confirmed toltrazuril resistance in a field isolate of *Cystoisospora suis*

Aruna Shrestha1,2, Barbara Freudenthal1,2, Rutger Jansen3, Barbara Hinney1,2, Bärbel Rudikowski1 and Anja Joachim1,2

RESEARCH Open Access

Parasites & Vectors

**Abstract**

**Background:** Constant treatment regimens with toltrazuril against *Cystoisospora suis* infection in piglets are being applied in the intensive production systems for the last two decades, but the possibility of resistance development has not been addressed so far despite limited availability of treatment alternatives. Recently, a pig producer in The Netherlands who routinely used toltrazuril complained about diarrhea in suckling piglets in the absence of bacterial and viral pathogens and oocysts of *C. suis* could be isolated from feces of affected litters.

**Methods:** Piglets from two litters were infected with a field isolate of *C. suis*; Holland 1 and 2, and treated with 0 (Holli-Ctr), 20 (Holli-20) or 30 (Holli-30) mg/kg of body weight (BW) of toltrazuril (Baycos®). The efficacy of toltrazuril was measured by assessment of oocyst excretion, fecal consistency and BW gain. A separate litter was infected with a toltrazuril-resistant strain of *C. suis*, Wien-1, and treated with 0 (Wien-Ctr) or 20 (Wien-20) mg/kg BW of toltrazuril for comparison.

**Results:** Treatment with the recommended (20 mg/kg) dose of toltrazuril completely suppressed oocyst shedding and diarrhea in group Wien-20. The prevalence of oocyst excretion was 100% in the groups infected with Holland 1 and 80% in the group Wien-Ctr. Most days, diarrhea were observed in group Holli-20 with an average of 6.4%, followed by 5.7% in Wien-Ctr, while in Holli-Ctr and Holli-30 diarrhea was only seen in 1.7% of the samples. (n = 12 piglets). Oocyst excretion, fecal consistency and BW gain did not differ significantly among groups infected with Holland 1, indicating loss of efficacy to toltrazuril.

**Conclusion:** Experimental infections and treatment confirmed toltrazuril resistance against the field isolate even at increased dosage. Such isolates are a potential threat to pig production as no other effective and economically sustainable alternative treatment is currently available. In the absence of a standardized protocol for resistance testing in *C. suis*, regular pathobiological examination and, if possible, experimental confirmation should be considered to evaluate the extent and consequences of toltrazuril resistance.

**Keywords:** Efficacy, Toltrazuril, Field isolate, Oocyst excretion, Pig

**Background**

*Cystoisospora suis* (syn. *Isospora suis*) is the most pathogenic species of *Isospora* and most severely affects suckling piglets [1–4]. Clinical signs include pasty to watery non-hemorrhagic diarrhea, weight loss and ill thrift [5–11]. At present, *cystoisosporosis* is considered as one of the leading causes of diarrhea in neonatal piglets with high prevalences worldwide [4, 7, 11–14]. Oocysts are highly resistant to desiccation and antimicrobial compounds [14, 15] making elimination virtually impossible once they have been introduced into the farm. The disease shows a very high morbidity with low mortality, and not all the piglets in a litter are equally affected, resulting in reduced, uneven weaning weights and thus, ultimately in other significant economic losses [5, 11, 16, 17].
In the European Union (EU), control of coccidiosis is commonly achieved with a single oral administration of toltrazuril in the prepotent period (day 3–5 of life). Baycox® is the trade name of a broad spectrum anticoelicial drug containing the triazine trione toltrazuril [18]. Use of Baycox® in piglet coccidiosis was first authorized in Australia in 1998 to be administered once in the first week of life at a dose of 20 mg/kg body weight (BW) [19]. A single oral treatment with toltrazuril administered during the prepotent period provided effective and sustained suppression of coccidial shedding and diarrhea in piglets experimentally infected with C. suis [17, 20–22] and under field conditions [23–28]. However, emerging resistance in poultry coccidia against anticoelicials including toltrazuril is of growing concern [29–31]. Concurrent treatment regimens with toltrazuril have been applied for controlling porcine coccidiosis in the EU for almost two decades now, but the possibility of resistance development in C. suis isolates has not been assessed so far, despite limited availability of treatment alternatives. In 2014, a pig farmer in The Netherlands, with a farrow-to-finish herd of 300 sows, complained about pasty feces in 60% of the piglets from 10 days of age until weaning, despite treatment with the recommended dose of toltrazuril. The involvement of bacterial and viral pathogens was excluded as the possible pathogens associated with piglets' diarrhea could neither be re-isolated nor be detected by polymerase chain reaction (PCR). Presence of vibronulture and furon in the histological sections of jejunum indicated coccidiosis [32, 33], which was later confirmed by the detection and isolation of C. suis oocysts in the fecal samples. Evaluation of the administered amount of toltrazuril on the farm level revealed no under-dosing. Moreover, application of twice the recommended dose (40 mg/kg) of toltrazuril also did not have any effect on the clinical picture and thus loss of efficacy was suspected. In the present study, the efficacy of toltrazuril against C. suis infections in suckling piglets was evaluated in experimental infections with the mentioned field isolate, Holländ-1, and a toltrazuril-sensitive strain, Wien-l. In the past, experimental studies have indicated development of resistance under field conditions in Exneria of poultry [34–36]. To our knowledge, this is the first report of experimentally confirmed toltrazuril resistance in a field isolate of C. suis.

Methods

Study animals

A total of 34 conventionally raised healthy piglets from three crossbred sows (Landrace x Large White) were allotted to five treatment groups (Table 1). Sows were housed on straw in individual farrowing crates in the animal husbandry facility of the Institute of Parasitology, University of Veterinary Medicine Vienna, Austria. All rooms were equipped with daylight and ventilation, and room temperature of 18–20 °C was maintained throughout the trial. Fresh drinking water was provided ad libitum to the sows and piglets. The sows were fed once daily with a commercial feed without coccidiosis according to the manufacturer’s recommendation and the piglets received milk from the sow followed by starter feed from the second week of life. The day of birth of piglets was considered as study day 1 (SD 1). All piglets were ear-marked and received 100 mg iron dextran on SD 2 to prevent iron deficiency. The sows arrived two weeks prior to the expected parturition date for adaptation to the new environment.

Study design

The clinical trial followed a blinded and incompletely randomized block design consisting of two blocks (one for each C. suis strain), each containing the control and the treatment group(s). The experimental unit was the individual animal. Randomization was carried out in each block assigning piglets to the respective treatment group (n = 5 to 8 piglets/group), ranking animals based on decreasing birth weight. The animals were distributed among the litter as described in Table 1.

Parasite material and experimental infection

Oocysts of the Holländ-1 field isolate of C. suis were obtained from fecal samples originating from the mentioned commercial farm in The Netherlands with suspected reduced sensitivity to toltrazuril. Before performing resistance studies, the field isolate was passaged once through piglets for collection of fresh viable oocysts. A toltrazuril-sensitive strain of C. suis, Wien-1 [20], was used for comparison between the strains, which was maintained and passaged regularly in suckling piglets for the production of infectious oocysts at the Institute of Parasitology, University of Veterinary Medicine Vienna, Austria. The strain is passaged every 3–6 months and infectivity in vivo and susceptibility to toltrazuril are assessed regularly. Each piglet was inoculated orally with 1000 C. suis oocysts, suspended in 1 ml of tap water, of the respective C. suis strain on SD 4 using a flexible plastic Pasteur pipette. The groups were denominated on the basis of treatment (Table 1) on SD 6 and received either sham-treatment or a commercial formulation of toltrazuril (Baycox® 5% oral suspension; Batch no: K90008X9; Expiry date: 06/2023, Levetrolsen, Germany). Animals infected with Wien-1 received the recommended dose of 20 mg/kg of body weight (BW) of toltrazuril (Wien-20). Piglets infected with Holländ-1 were treated with 20 mg/kg BW (Holl-20) or an elevated dose of 30 mg/kg BW (Holl-30) of toltrazuril. Piglets in the sham-treated control groups
(Wien-Crl and Holl-Crl) received 1 ml of tap water orally. The efficacy of toltrazuril was evaluated by assessment of body weight development, fecal consistency and oocyst excretion.

### Evaluation of fecal samples

Individual fecal samples were collected daily from SD 8 to 21 for the evaluation of fecal consistency and oocyst excretion. Fecal consistency was scored immediately after sampling according to the following key: FC 1 = normal, FC 2 = pasty, FC 3 = semi-liquid, and FC 4 = liquid, with FC 3 and FC 4 considered as diarrhea [7]. Fecal samples were first screened for oocysts by autofluorescence (AF) detection under UV light [37] with a sensitivity of ca. 10 OpgI in positive samples oocyst excretion was determined quantitatively using a modified McMaster technique [3].

### Body weight and general health observation

The piglets were weighed on SD 1, 8, 15 and 22. Additionally, the body weight of each piglet was recorded on the day of treatment for calculation of the treatment dose. All piglets were observed daily during the course of the studies to ensure good general health and any condition that required veterinary care was recorded and addressed.

### Differential diagnosis

Pooled fecal samples of each litter were screened on SD 8 for the presence of any other pathogens causing diarrhea in neonatal piglets including rotavirus, coronavirus, *E. coli* and *C. perfringens*.

### Statistical analysis

Statistical calculations were performed with RStudio version 0.99.896 (RStudio Team, 2016), descriptive statistics with Microsoft Excel 2010 and GraphPad Prism version 5.04 for Windows (GraphPad Software, San Diego, California USA). Differences in clinical and pathological parameters between groups were analyzed applying an ANOVA in case of normal distribution and variance homogeneity of the data, or a Kruskal-Wallis rank sum test if this was not the case. In the event of significance for the omnibus tests parametric or non-parametric post-hoc tests for multiple comparisons were performed (according to Tukey and Conover, respectively), using P-value adjustment after Bonferroni. Statistical calculations were restricted to groups Holl-Crl, Holl-20 and Holl-30 (n = 8 animals/group) due to the small size of groups Wien-Crl and Wien-20 (n = 5 animals/group). Spearman’s rank correlation coefficient was calculated to describe the relationship between selected parameters. \( r \) values ≤ 0.05 were considered significant.

### Results

#### Oocyst excretion

Excretion of *C. suis* oocysts was completely suppressed by the treatment in group Wien-20 while all other groups excreted oocysts detectable in AF (Fig. 1) and McMaster (Table 2) techniques. Oocyst shedding was first observed in these groups on SD 9, and by SD 12 all animals except one had been positive at least once (Fig. 2). In groups Holl-Crl, Holl-20 and Holl-30 every piglet excreted oocysts at least once, whereas in group Wien-Crl all piglets except one shed oocysts (Table 2). The prevalence in piglets infected with Holl-1 peaked on SD 11 with 87.5% positive piglets in group Holl-Crl and 100% positive piglets in groups Holl-20 and Holl-30. In group Wien-Crl the prevalence reached its maximum (80%) on SD 12 (Fig. 1). Prepatency tended to be shorter in piglets infected with Holl-1 (Table 2). The number of excretion days (AF) was similar in all groups with excretion, ranging from 301 to 364% of all sampling days in the different groups (Table 2).

The highest individual oocyst shedding (max OpgI) was seen in group Holl-Crl on SD 14. Generally, max OpgIs were higher in groups infected with the Holl-1 isolate (Table 2). Oocyst excretion reached its peak on SD 11–12 (Fig. 3), and was most pronounced in group

<table>
<thead>
<tr>
<th>Group</th>
<th>C. ruiz strain</th>
<th>litter no.</th>
<th>Treatment/Dose</th>
<th>No. of piglets</th>
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<td>Wien-Crl</td>
<td>Wien-1</td>
<td>1</td>
<td>Tap water; 1 ml</td>
<td>5</td>
</tr>
<tr>
<td>Wien-20</td>
<td>Wien-1</td>
<td>1</td>
<td>Toltrazuril; 20 mg/kg body weight</td>
<td>5</td>
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<tr>
<td>Holl-Crl</td>
<td>Holl-1</td>
<td>2</td>
<td>Tap water; 1 ml</td>
<td>4</td>
</tr>
<tr>
<td>Holl-20</td>
<td>Holl-1</td>
<td>3</td>
<td>Toltrazuril; 20 mg/kg body weight</td>
<td>4</td>
</tr>
<tr>
<td>Holl-30</td>
<td>Holl-1</td>
<td>2</td>
<td>Toltrazuril; 30 mg/kg body weight</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td></td>
<td>4</td>
</tr>
</tbody>
</table>
Holl-30 with a mean OpG value of 196.603. The amount of shed oocysts exhibited high variabilities between individuals, ranging from 333 to 365.301 within the same group, without significant differences in daily mean OpG values, the area under the curve (AUC) for OpG SD 9 to 19 as well as the mean OpG SD 9 to 19 between groups infected with Holland-I.

Fecal consistency and diarrhea

None of the study animals had diarrhea on the day of infection. Moreover, the mean fecal score of Wien-20 did not exceed 1.20 throughout the study. The mean FS reached its peak on SD 13 in groups Wien-Ctrl, Holl-Ctrl and Holl-30 with 2.80, 2.13 and 2.00, respectively, and with 2.25 on SD 12 in group Holl-20 (Fig. 4). Neither the overall mean FS from SD 9 to 18 (Table 3) nor the mean FS on single study days differed significantly between groups infected with Holland-I.

Diarrhea was not observed at all in group Wien-20. The prevalence of diarrhea peaked on SD 13 in groups Wien-Ctrl and Holl-Ctrl with 60 and 26% of the piglets having FS > 2, respectively. In group Holl-30, the prevalence reached its maximum already on SD 9 (28.37%) and on SD 12 and 13 in group Holl-30 with 12.5% each. Most days with diarrhea were observed in group Holl-20 with an average of 64.0%, followed by 57.1% in group Wien-Ctrl, while in groups Holl-Ctrl and Holl-30 diarrhea was only seen in 1.79% of the samples. Percentage of piglets with FS > 2 at least once ranged from 12.5% (Holl-30) to 62.5% (Holl-20) (Table 3). However, the number of diarrhea days and piglets that had diarrhea at least once did not differ significantly between groups infected with Holland-I. FS 4 (watery) was only observed in two piglets of group Wien-Ctrl on SD 13.

Body weight

Since the birth weight of two individual piglets was below 0.9 kg, they were excluded from the body weight calculation. Mean body weights and mean body weight gains (baseline: SD1) did not differ significantly (P > 0.05) between groups Holl-Ctrl, Holl-20 and Holl-30 throughout the study (Additional file 1: Table S1) although a

| Table 2: Overview of the parasitological parameters (AF and McMaster countable excetration) for all experimental groups |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Group | % piglets with target excetration | Mean ± SD | % excetration on days ± SD | % excetration on days ± SD | Mean ± SD | Mean ± SD | Mean ± SD | Mean ± SD | Mean ± SD | Mean ± SD |
| | | | | | | | | | | | | |
| Wien-Ctrl | 29 | 4.2 ± 3.0 | 38.1 ± 31 | 37.1 ± 23.0 | 7.0 ± 1.4 | 0.06 ± 0.2 | 1.06 ± 0.2 | 2.07 ± 0.2 | 12.30 ± 2.5 | 3.20 ± 0.2 |
| Wien-20 | 29 | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 |
| Holl-Ctrl | 100 | 4.2 ± 1.0 | 31.5 ± 1.0 | 25.8 ± 14.8 | 6.3 ± 1.0 | 16.4 ± 1.0 | 23.4 ± 14.8 | 36.0 ± 14.8 | 46.0 ± 14.8 | 13.2 ± 14.8 |
| Holl-20 | 100 | 5.0 ± 1.0 | 35.3 ± 12.3 | 25.4 ± 15.0 | 6.0 ± 1.0 | 19.4 ± 1.0 | 21.2 ± 15.0 | 31.2 ± 15.0 | 41.2 ± 15.0 | 11.2 ± 15.0 |
| Holl-30 | 100 | 5.4 ± 1.0 | 38.4 ± 13.2 | 30.4 ± 15.3 | 6.4 ± 1.0 | 21.9 ± 1.0 | 26.9 ± 1.0 | 35.9 ± 13.2 | 45.9 ± 13.2 | 12.9 ± 13.2 |

For parameters related to quantitative excetration, only the period with observed McMaster counts was included.

Auction: All values were calculated using mean values of all piglets per group. SD ± standard deviation.
significant negative correlation between the mean fecal score and the individual weight gain from SD 1 to 22 was observed for all included animals ($p = -0.486$, $P = 0.006$).

**Differential diagnosis**
Fecal samples pooled by litter on SD 8 tested negative for rotavirus and coronavirus while E. coli as well as C. perfringens could be isolated from all litters.

**General health**
Piglets showed softened feces (FS 2) or diarrhea (FS 3 or 4) as described. No condition related to the experimental infection requiring veterinary treatment was observed.

**Discussion**
Resistance to all classes of anticoccidials is well described in avian coccidia [30, 38, 89]. Despite the long-term use of toltrazuril in piglet production and the lack of satisfying treatment alternatives, the possibility of resistance development has not been addressed so far. In the present study we evaluated the efficacy of toltrazuril against a C. suis isolate from a Dutch farm complaining about symptoms typical of coccidiosis despite metaphylactic treatment with the recommended dose of toltrazuril. In an experimental setup, two litters were infected with the isolate in question and treated with 0, 20 or 30 mg/kg BW of toltrazuril. Additionally, a third litter was infected with a toltrazuril-sensitive strain (Wien-I) and treated with 20 mg/kg BW of toltrazuril or sham.

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**Fig. 2** Percentage of piglets with oocyst excretion at least once post-infection as detected by fluorescence microscopy.

**Fig. 3** Daily oocyst excretion ($\ln(OgC+1)$) in piglets from study days 8-21 as determined by McMaster technique.
treated to confirm effectiveness of the Baycox® batch used. Due to animal welfare reasons only a minimum number of animals were infected with this strain; therefore, statistics could not be employed for these groups. However, the positive effect of toltrazuril on oocyst excretion, fecal score and body weight gain was sufficiently shown in several earlier field and experimental studies [7, 17, 20, 21, 24, 25, 40, 41]. It completely inhibits the development of all parasitic stages of C. suis and, given during the prepatent period of infection, prevents tissue damage and consequently the emergence of diarrhea [42].

Resistance is described by the World Health Organization [43] as the "ability of a parasite strain to survive and/or multiply despite the administration and absorption of a drug given in doses equal to or higher than those usually recommended but within tolerance of the subject". This definition can also be employed for coccidia. The reliability of toltrazuril to significantly reduce the excretion of C. suis oocysts was already shown in a number of experimental studies [20, 21, 41, 42]. Mundt et al. [7] described a complete suppression of oocyst shedding in experimentally infected and toltrazuril-treated animals while every untreated animal shed parasites at least once. In another study conducted by Mundt et al. [17], the treatment with toltrazuril resulted in significantly fewer mean excretion days (0.6 vs 4.0), fewer piglets with oocyst excretion (30 vs 91%) and a significantly lower mean OpG (146 vs 17.79) 5 to 11 days post-infection (dpi). This is in accordance to a field study conducted by Kreiner et al. [24] where the number of C. suis positive samples was significantly lower in toltrazuril-treated compared to untreated piglets in different herds. The same effect was observed in this study for group Wien-20, where oocyst shedding was completely suppressed by the treatment. By contrast, treatment with toltrazuril did not impair oocyst development and excretion in the groups infected with the Dutch isolate, Holland-I, in avian coccidiosis complete drug resistance is defined by ineffectiveness despite higher doses [44]. Every single piglet infected with the Holland-I isolate shed oocysts regardless of the toltrazuril dose. Moreover, the number of AFI countable excretion days, the AUC for OpG, the maximum individual OpG as well as the mean OpG from SD 9 to 19 was comparable between the control and treatment groups of this isolate, indicating complete drug resistance. The above mentioned parameters

<p>| Table 3 Clinical parameters in the forms of fecal consistency and intensity of diarrhea in all experimental groups over the study period |
|-----------------|-----------------|---------------------|-----------------|</p>
<table>
<thead>
<tr>
<th>Group</th>
<th>% piglets with diarrhea at least once</th>
<th>% diarrhea days ± SD (14 sampling days/dpiglet)</th>
<th>Mean ± SD fecal score (study days 6–18)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wien-Ctr</td>
<td>100</td>
<td>5.7 ± 6.0</td>
<td>1.6 ± 0.4</td>
</tr>
<tr>
<td>Wien-20</td>
<td>0</td>
<td>0</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td>HolCtrl</td>
<td>75.0</td>
<td>1.8 ± 3.3</td>
<td>1.7 ± 0.4</td>
</tr>
<tr>
<td>Hol20</td>
<td>62.5</td>
<td>6.4 ± 6.0</td>
<td>1.9 ± 0.3</td>
</tr>
<tr>
<td>Hol50</td>
<td>12.5</td>
<td>1.8 ± 3.1</td>
<td>1.5 ± 0.3</td>
</tr>
</tbody>
</table>

*For quantification evaluation of the fecal score only the period of mean values >1.00 was evaluated for study days 6–18.*
tended to be higher in groups Holl-Crl, Holl-20 and Holl-30 compared to group Wien-Crl, and the prepatent period for excretion detected by AF technique was shorter in Holland-4 compared to Wien-1. Variations in prepatent periods can be attributed not only to factors such as infection dose or age and health of piglets (which were comparable between all groups) but also to the virulence of *C. suis* isolates [7, 45, 46]. However, as oocyst excretion underlies large individual variations [7, 47–49] reliable conclusions about possible differences between strains regarding this particular trait cannot be drawn at this point in time.

While mean fecal consistencies remained below 2.2 in group Wien-20, in all other groups developed increased mean fecal scores after infection, indicating enteritis as a consequence of parasite replication [50]. Diarrhea was observed in all groups except group Wien-20, but prevalences and numbers of diarrhea days were generally low. It has been previously shown that toltrazuril successfully reduces fecal scores and suppresses diarrhea in piglets infected with *C. suis* [17, 41, 42, 51]. In a field study, Sala et al. [28] found the overall diarrhea prevalence to be significantly lower in toltrazuril-treated animals compared to untreated animals. Similarly, Kreiner et al. [24] found treated animals to have a significantly lower mean FS and significantly fewer diarrheic fecal samples in the field. In two trials conducted by Jouchein & Mundt [20] not a single piglet with diarrhea was observed after treatment with toltrazuril while almost all untreated animals had a FS of 3 or 4 (85.7 and 100%, respectively) at least once. This was also described by Mundt et al. [7] who observed an average FS of 2 or more in experimentally infected piglets throughout the study, with daily diarrhea prevalences between 25 and 75%. On the other hand, the mean FS of animals treated with toltrazuril remained between 1 and 2 during the entire sampling period. In this study, treatment had obviously no effect on fecal consistency of the piglets infected with the Dutch isolate. The group Holl-20 showed the highest mean fecal score, the highest prevalence of diarrhea and most diarrhea days among the three groups. These parameters were also similar in groups Holl-Crl and Holl-30, indicating complete lack of clinical efficacy of toltrazuril. Despite the limited data set available so far for Holland-4, this strain might be of low virulence since overall prevalences and days of diarrhea were low and a FS of 4 was not observed even once. However, inter-and intra-litter deviations cannot be excluded in this setting. Just as with oocyst excretion, the development and severity of diarrhea varies between litters and individuals [48, 52, 53] and is also influenced by other factors [7].

Chapman [54] considered an *Eimeria* strain to be resistant if the weight gain of treated infected chicken did not differ significantly from that of untreated infected controls. In fact, treatment with the recommended or the elevated dose did not have a significant effect on body weight gains in this experiment, indicating resistance to toltrazuril. This finding is not surprising as the mean fecal score, which was negatively correlated with the individual weight gain, did not differ significantly between the control and treatment groups of isolate Holland-4. It has previously been shown that diarrhea is negatively correlated with the body weight gain in *C. suis* infections and that the application of toltrazuril significantly increases the weight gain of piglets compared to untreated infected controls [7, 17, 26, 40], although for the groups infected with Wien-1 this could not be shown due to the small group size.

On grounds of the clinical picture observed during the study, it can be assumed that the bacterial agents identified by microbiological examination are facultative pathogenic subtypes regularly found in porcine intestinal flora [55, 56]. This is underpinned by the fact that no sign of disease occurred in group Wien-20, despite the presence of the same bacteria as in all other groups. Lonach et al. [57] did not find statistical differences in the intestinal abundance of *E. coli* or *C. perfringens* when comparing piglets with and without diarrhea. This was confirmed by Bali et al. [58] who found similar prevalences in piglets with diarrhea and control piglets while frequencies of *C. suis* were significantly higher in diarrheic piglets.

To our knowledge, this study is the first to experimentally confirm toltrazuril resistance in a *C. suis* isolate. There are currently no satisfying treatment alternatives available [29, 33], underlining the need of new intervention strategies against porcine neonatal colicosis. Sulphonamides have been suggested against porcine diarrheal colicosis but a short-term oral administration was shown to have an unsatisfying effect [17, 26]. Only an injectable sulphonamide, repeatedly administered for 6 to 7 days, had an effect on parasite development and clinical outcome comparable to that of toltrazuril under experimental conditions [20]. Such a treatment may be considered as an alternative in cases of toltrazuril resistance, but it is labor-intensive and unsuited for routine treatment. Therefore, alternative control strategies to chemotherapy are desirable [59].

Luckily, it seems that resistance in *C. suis* develops far slower than in avian coccidia. Vrontommen et al. [60] described the development of resistance to Baycox against *Eimeria* on a broiler farm within four fattening periods. Nevertheless, the rise of further resistant *C. suis* isolates may just be a matter of time as the extensive use of a drug over a longer period of time will inevitably lead
to decreased efficacy [29, 44]. Currently, the sensitivity of C. suis isolates to toltrazuril can only be evaluated in vivo. A less laborious and faster in vitro assay, possibly employing the already established cell culture system [41], would be advantageous.

Conclusions
Toltrazuril resistant C. suis isolates are a potential threat to pig farming as no other effective and economically sustainable alternative treatment is available. All pigs infected with the field isolate of C. suis Holland-1 showed appreciable levels of diarrhea and ocular excretion unresponsive to treatment. Therefore, veterinarians and farmers should be aware of the possibility of resistance development with long-term application of toltrazuril in intensive piglet production systems. In the absence of vaccines and effective anticoagulants agents other than toltrazuril routine fecal screening and periodical assessment of efficacy of toltrazuril must be considered essential for the sustainable control of cystoisosporosis. In cases of reduced efficacy optimized hygiene measures employing regular chemical disinfection with a cresol-based product must be enforced.

Additional file

Additional file 1: Table S1. Mean body weights and body weight gains in grams with standard deviations in brackets. SD: Study day. (DOCX 15.6 kb)

Abbreviations
AP, Antiparasitascience; AUC, Area under the curve; BW, Body weight; AP Day, post-infection day; E3, European 3rd European; FS, Faecal score; OPG, Opgypters per gram feces; SIT, Study day.

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Availability of data and materials
All the datasets analyzed during the study are included in the manuscript.

Authors’ contributions
AF, FA, AS, BR and BF conceived and designed the study. BF analyzed the samples. AS and BF drafted the manuscript. All authors read and approved the final manuscript.

Ethics approval
All procedures involving animals were approved by the Animal Ethics Committee of the University of Veterinary Medicine Vienna and the Austrian Federal Ministry of Science and Research according to the Austrian Animal Protection law EMAV-19205/10/644/2015.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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References


6. DISCUSSION AND CONCLUSIONS

With the introduction of intensive pig production the coccidium *C. suis* became one of the most relevant causes for diarrhoea in neonatal and suckling piglets (Harleman and Meyer 1984, Lindsay et al. 1992, Mundt et al. 2006), with a high prevalence on farms worldwide (Roepstorff et al. 1998, Meyer et al. 1999, Mundt et al. 2005, Hamadejová and Vítovec 2005) and a poor chance to eradicate the disease once introduced into a facility, unless toltrazuril is continuously applied to all newborn litters (Niestrath et al. 2002, Mundt et al. 2005, Kreiner et al. 2011). Oral administration of toltrazuril is effective against all endogenous *C. suis* stages and has successfully been in use for 20 years as the only compound licensed for the treatment of porcine neonatal coccidiosis (Bach et al. 2003, Mundt et al. 2007, Rypula et al. 2012, Mehlhorn and Greif 2016). It almost completely prevents the development and excretion of oocysts and subsequent diarrhoea in experimental infections as well as in the field, thus increasing weaning weights when compared to untreated animals (Mundt et al. 2006, 2007, Maes et al. 2007, Scala et al. 2009, Joachim and Mundt 2011, Kreiner et al. 2011).

Recently an injectable toltrazuril formulation, combined with iron, was developed (30 mg toltrazuril/ml and 133.4 mg gleptoferron/ml, 1.5 ml/piglet i.m., Forceris®, Ceva, Libourne, France) for the metaphylactic treatment of porcine neonatal coccidiosis and the simultaneous prophylaxis of iron deficiency, and its efficacy was proven in an animal experiment (Joachim et al. 2018). The intramuscular injection of Forceris® completely suppressed oocyst excretion in piglets experimentally infected with *C. suis* and significantly less diarrhoea was observed when compared to an untreated control group, resulting in significantly increased body weight gains. Its efficacy in terms of the abovementioned parameters was moreover comparable to that of an established oral toltrazuril product (20 mg/kg bodyweight, Baycox® 5 % oral suspension, Bayer Animal Health GmbH, Leverkusen, Germany), and its application was safe with none of the animals needing treatment-related veterinary care. The use of Forceris® reduces the handling of animals since both the metaphylaxis of coccidiosis and the supply with iron can be performed at once, saving time and subsequently labour costs. It is additionally licensed to be administered
from the first day of life already, possibly being of advantage for farms with early \textit{C. suis} infections shortly after birth.

However, it was only a matter of time until the emergence of resistant isolates of \textit{C. suis}, a phenomenon that is already known from avian \textit{Eimeria} where toltrazuril resistance seemed to develop rather quickly (Chapman 1984, Vertommen et al. 1990, Stephan et al. 1997).

Indeed, the first resistance of a \textit{C. suis} field isolate against toltrazuril was recently reported from a farm in The Netherlands (Shrestha et al. 2017a). Toltrazuril had regularly been in use on this farm for many years, but lately piglets excreted oocysts and showed diarrhoea typically associated with cystoisosporosis despite treatment. Under-dosing could be ruled out as the cause of poor toltrazuril efficacy, and subsequently the resistance of this isolate (Holland-I) against toltrazuril, even at increased doses [which is an indication of complete resistance according to Peek and Landman (2011)], was shown in an animal experiment. Despite treatment, animals infected with Holland-I excreted considerable amounts of oocysts and suffered from diarrhoea to the same extent as untreated piglets did, whereas animals infected with a toltrazuril-sensitive strain (Wien-I) were completely asymptomatic and showed no excretion after treatment. The resistant strain seems to be of relatively low virulence since diarrhoea was less severe than with the strain Wien-I, nevertheless the farm is now without meaningful treatment options. We conclude that this isolate shows a complete drug resistance since even increased doses of toltrazuril remained ineffective.

Suspected resistance can currently only be confirmed \textit{in vivo} which is labour-intensive and impracticable for routine and large-scale resistance evaluations. Therefore the establishment of an \textit{in vitro} assay for anticoccidial efficacy testing, possibly employing the cell culture model (Worliczek et al. 2013), or the identification of the genes responsible for conferring resistance as molecular markers would be a major improvement for future research and routine diagnostics since the demand for toltrazuril sensitivity testing might soon increase.

The mode of toltrazuril activity against coccidia is not fully elucidated, thus the mechanisms responsible for the development of resistance cannot easily be determined. Most probably, toltrazuril inhibits the activity of the parasite’s apicoplast (the eponymous plastid-like organelle
of the Apicomplexa) or interferes with certain enzymes of the mitochondrial respiratory chain (Harder and Haberkorn 1989, Chapman 1997). Just like in avian coccidia, the long-term, intense application of toltrazuril will unavoidably favour the emergence of more widespread resistance through gene mutations and subsequent selection and spread of resistant phenotypes (Chapman 1997). If not managed adequately, this could soon lead to an alarming shortfall of effective control options, underpinning the necessity of medical alternatives to keep porcine neonatal coccidiosis under control, and immunoprophylaxis could be a sustainable, consumer-friendly option.

In the early phase of life, when the infection is most relevant, the newborn’s immature immune system and the presence of maternal antibodies would hamper its active immunization (Becker and Misfeldt 1993, Schwager and Schulze 1997, Morein et al. 2002, Stepanova et al. 2007, Chase et al. 2008). Therefore, maternal immunization and subsequent passive transfer of immune components via colostrum (Salmon et al. 2009) might be an attractive alternative.

A good understanding of the immune response and of the immunological control mechanisms against C. suis is a prerequisite for the development of a passive vaccine. Although previous studies indicate that the infection of immune-competent pigs does induce an immune response (Worliczek et al. 2010b, Schwarz et al. 2014), data on this age group are limited, particularly on cytokine responses. The successful control of coccidial infections and the development of a protective immunity are largely regarded to be cell-mediated, and particularly characterized by the secretion of Th1 and pro-inflammatory cytokines (Rose 1987, Bliss et al. 1999, Hermosilla et al. 1999, Daugschies and Najdrowski 2005, Frölich et al. 2012, Verhelst et al. 2015). However, humoral immune activity was also documented after C. suis infections (Schwarz et al. 2013, Schwarz et al. 2014), but its contribution to the elimination of or the protection against an infection is not elucidated yet.

Therefore, the aim of the present project was to further study the antibody and cytokine response of immunocompetent animals undergoing an infection with C. suis.

The high-dosed infection of sows shortly before birth was shown to lead to a milder disease in their offspring after experimental infections with C. suis, but an adequate protection could not be
achieved (Schwarz et al. 2014). Additionally, the routine administration of oocysts to sows in such high doses is not feasible. Although the cell culture model of C. suis mimics its complete life cycle, oocyst output and sporulation rates are low (Worliczek et al. 2013). Thus, a continuous, large-scale in vivo passage of oocysts would be necessary to provide these stages in large enough numbers for the immunization of animals. Therefore, other sow immunization protocols must be validated for their practicability and for their potential to increase the intensity of the sow’s immune response to higher levels and subsequently achieve a better passive protection of piglets. In various avian Eimeria species, the continuous inoculation of chicks with low doses of oocysts over several days, a so called ‘trickle’ infection, induces a stronger immune response and subsequently a more potent protective immunity than a single exposure to a higher dose (Reid 1990, Chapman et al. 2005, Price 2012). In calves, on the other hand, the level of immunity seems to correlate directly with the amount of E. bovis oocysts exposed to during primary infection (Daugschies and Najdrowski 2005).

Thus, this project additionally aimed to evaluate the character and intensity of immune responses induced by different oral immunization protocols in order to identify a potentially more successful immunization protocol for sows.

For this, 50 growers, ten to eleven weeks of age, were split up into groups of ten animals each and were either single or trickle infected with either a low or a high dose of C. suis oocysts. A sham-treated group served as control. Although ultimately sows are supposed to be immunized, growers were used because they are easier to handle and to accommodate. The growers used in this study were regarded as immunologically mature because at that age pigs are considered to have a fully developed immune system (Becker and Misfeldt 1993, Schwager and Schulze 1997, Bailey et al. 2001, Stepanova et al. 2007) and immune responses were assumed to be comparable to those of sows. It was also assumed that results from growers can be conferred to sows since specific antibody titres in sow blood were shown to be positively correlated with those in colostrum and milk (Schwarz et al. 2013, 2014). This indicates that the antibody level in the blood reflects the situation in the mammary secretions and ultimately the antibody transfer to the
piglets, although large amounts of antibodies, particularly IgA and IgM, are also produced locally in the mammary tissue (Salmon et al. 2009, Hurley and Theil 2011).

Infection, irrespective of the protocol applied, successfully induced a systemic immune response as demonstrated by the significant increase of all evaluated serum antibody titres, namely immunoglobulin (Ig) A and IgG against *C. suis* sporozoite and merozoite antigens. This is in line with the antibody response of pregnant sows described by Schwarz et al. (2014), indicating similar immune responses of sows and growers and thus supporting our assumption that growers can be used to mimic immunizations of sows. However, highly similar immunological outcomes in all infection models suggest that the mode of infection does not noticeably influence the character of the humoral immune response and its intensity. This is in strong contrast to bovine and avian coccidia where immune response and development of protective immunity are directly dependent on infection dose and scheme, respectively (Reid 1990, Chapman et al. 2005, Daugschies and Najdrowski 2005, Price 2012). Conversely, in piglets infected with *C. suis*, the severity of clinical disease is not significantly influenced by the infection dose (Worliczek et al. 2009a); however, differences in the immune response after different doses were not evaluated. Generally, although *C. suis* and *Eimeria* belong to the same family of parasites and are similar in many aspects, they still differ in certain biological aspects. For example, unlike *Eimeria*, *C. suis* reproduces only within the superficial layers of the intestinal mucosa, thus the parasite might above all trigger the local immune system (Worliczek et al. 2009b). Moreover, in cattle and rabbits, infections with *Eimeria* are sometimes more severe at some weeks of age than straight after birth (Daugschies and Najdrowski 2005, Pakandl et al. 2008), which is opposite to what is observed in *C. suis* (Worliczek and Joachim 2011). Thus the host’s immune system might just react differently upon infection, with the activation of other pathways or a predominantly local activity.

While the control group stayed negative throughout the trial, oocyst excretion occurred in each infected group. The low excretion levels compared to those typically observed in experimentally infected piglets (Shrestha et al. 2017a) are regularly seen in older pigs and are likely the result of a maturation of the immune system, an altered gut physiology (for example, a faster epithelial
turnover than in piglets) or of a developed immunity after an earlier infection (Lindsay et al. 1985, Koudela and Kučerová 1999, Worliczek et al. 2009a). Consequently, *C. suis* might only rarely reproduce and finish its life cycle, and infection is most probably rapidly cleared from the intestines of immune-competent pigs. This, together with the successfully induced immune response in the absence of clinical signs, is a good starting point for the development of a vaccine; although sows are not regarded important infection sources for piglets (Lindsay et al. 1997), a contamination of the surroundings with oocysts from sows after infection or immunization should be avoided.

Before infection, low antibody levels could be detected in every group, supporting the assumption of an earlier contact to *C. suis* since it is almost impossible to conventionally breed pigs in a *C. suis*-free environment. Alternatively, these titres could be explained by the presence of extraintestinal stages (Harleman and Meyer 1984) constantly triggering the immune system, although we could not detect any parasite-specific DNA in mesenteric lymph nodes (MLN) or spleens.

In studies on *Eimeria* infections in goat kids, calves and lambs, levels of IgA and IgG were shown to be either positively correlated or unrelated with oocyst excretion. Thus, the authors concluded that antibodies might simply reflect the infection and possibly the replication rate of the parasite, but are not involved in its elimination or the development of a protective immunity (Faber et al. 2002, Reeg et al. 2005, Matos et al. 2017), an opinion supported by many authors (Rose 1987, Daugschies and Najdrowski 2005, Worliczek et al. 2007, Frölich et al. 2012). Likewise, in this study, the number of days with excretion of *C. suis* oocysts was significantly positively correlated with antibody titres of single study days and with the titre development during the course of the study. Positive correlations were particularly frequent with titres against merozoites which overall exhibited the most pronounced increases after infection, thus they may be used as markers for infection or parasite load. However, a possible application as marker for protection is questionable. In a study conducted by Schwarz et al. (2014), blood serum titres and oocyst excretion of infected suckling piglets did mostly not correlate, and although a passive
transfer of antibodies from sows to piglets was shown, a protective effect of antibodies themselves could thus not be unequivocally proven.

Additionally, IgG titres against merozoites were increased in the jejunal mucus after infection and exhibited a positive (although insignificant) correlation with oocyst excretion. This supports the hypothesis of an increased antibody production as result of a longer contact time between parasite and immune system due to a higher replication rate and the successful completion of the developmental cycle. However, respective mucus IgG titres were low and levels of other antibody classes were even lower or not present at all. In piglets infected with *C. suis*, the acute phase, characterized by severe clinical symptoms, oocyst excretion and tissue damage, is typically observed in the first two weeks post infection (Harleman and Meyer 1984, Vitovec and Koudela 1990, Mundt et al. 2006, Worliczek et al. 2009a). One would expect the highest intestinal antibody concentrations to be found during that time span, thus the time point chosen for sampling in this study, i.e. four weeks post (first) infection, was presumably too late. This assumption is underpinned by the fact that neither *C. suis*-specific DNA could be detected in jejunum samples of SD 29, nor were parasites or tissue lesions typically related to *C. suis* infections detected in histological sections. It seems that the infection is already cleared by that time, consequently followed by a rapid decline of antibody titres.

To summarize, it has to be debated that even though immunoglobulins seem to mirror the exposure to *C. suis* and possibly reflect the severity of an infection, they may not correlate with anticoccidial immunity and may not play a crucial role in protection.

Nonetheless, some authors do support the theory of a contribution of (passively obtained or endogenously produced) antibodies to protection against infections with various coccidia (Wallach 2010, Frölich et al. 2012), and a maternal vaccine conferring passive protection to broiler chicks is available against *E. maxima* (Sharman et al. 2010). Casadevall (2003) stated that specific antibodies are indeed able to confer immunity to intracellular pathogens in general and to protozoa in particular. On the one hand most intracellular pathogens can be found outside their host cells at least briefly during their life cycle where they are then exposed to neutralizing antibodies. On the other hand he claimed that protective antibodies can exist even if respective
experiments turned out negative, since many factors, including host and pathogen strains, may influence their actual potential to mediate protection. In passive antibody experiments, the transfer of too few or even too many antibodies can hamper the successful control of an infection; but not only quantitative parameters, also qualitative variables such as immunoglobulin specificity and isotype can influence the efficacy of antibody-mediated immunity. Moreover, where naturally occurring antibodies do not confer consistent protection, \textit{in vitro} produced monoclonal antibodies might do so and could thus be an attractive option for the establishment of an effective, antibody-mediated immunity.

Moreover, even though antibodies might not have a direct protective effect, they possibly contribute to the elimination of a parasite by stimulation and modulation of the cellular immunity. A successful immune response often requires a tight interplay of antibody- and cell-mediated immunity where the presence of antibodies could lead to a more effective cellular response, for example by promoting phagocytosis and antigen presentation, secretion of cytokines and chemokines or attraction of effector cells (Casadevall and Pirofski 2003, Wallach 2010). Likewise, the \textit{in vitro} phagocytosis of \textit{E. bovis} sporozoites by macrophages and polymorphonuclear neutrophils (PMN) was significantly enhanced after addition of immune serum (from 35.6 \% reduction of sporozoites to 73.6 \% in macrophages and from 30 \% to 66 \% in PMN), suggesting an antibody-dependent efficacy of cytotoxicity (Behrendt et al. 2008, Taubert et al. 2009). The elucidation of a similar role of \textit{C. suis}-specific immunoglobulins would need further investigation.

In this study the immunofluorescence antibody test (IFAT) was used for the detection and quantification of \textit{C. suis}-specific antibodies, a semi-quantitative serological tool which is, however, not standardized and a rather subjective method. To overcome this limitation, all samples were examined by the same person. Nevertheless, the IFAT is also a time consuming and labour-intensive test, thus the development of a more sensitive, more quantitative and high-throughput method would certainly represent an improvement for future immunological, prevalence or vaccination studies.
In addition to the antibody response, we herein investigated the systemic cytokine response of immune-competent animals in unstimulated white blood cells, MLN and spleens at mRNA level and in stimulated lymphocytes isolated from MLN at protein level. The systemic cytokine response to *C. suis* was only weak: cytokine expression and protein levels were rather low and showed considerable variation between individual animals, also within the same groups, thus the results have to be interpreted with caution. The variation is in line with results of other studies on porcine cytokine profiles (De Groot et al. 2005, Käser et al. 2012, Ladinig et al. 2014) and, similar to the humoral response, the various infection protocols hardly differed in the cytokine response they induced. Yet, certain differences between the whole of infected animals and the control group and significant changes over time were detected.

Splenic mRNA expression of the evaluated cytokines did not alter upon infection, indicating that this organ might not noticeably respond to *C. suis* infections. This is supported by the fact that no *C. suis*-specific DNA was detected in the spleen samples. However, our findings are in disagreement with a study performed by Schwarz et al. (2014) where splenocytes of piglets previously infected with *C. suis* responded to antigen with production of IFN-γ; however these results were obtained after stimulation of cells whereas in this study spleen cells were not stimulated before measuring mRNA levels. Moreover, since the study of Schwarz et al. (2014) did not include control animals, it cannot be determined whether the infection led to an actual increase of antigen-specific IFN-γ production. Preliminary experiments on the stimulation of splenocytes from a small set of animals with another antigen (Shrestha et al. 2017b) did not produce promising results (data not shown), thus this path was not followed.

Instead, lymphocytes isolated from MLN were stimulated with the above mentioned antigen, a recombinant merozoite protein previously characterized by Shrestha et al. (2017b). This protein provides a defined, pure antigen compared to parasite stages grown in cell culture and can be produced in large amounts, enabling the stimulation of a larger set of samples. In addition, the protein used is considered to be immunogenic *in vivo* (Shrestha et al. 2017b). Indeed, stimulation of lymphocytes and subsequent analysis of supernatants revealed an increased production of IL-12 by cells from infected animals, supporting the general recognition of a Th1-biased immune
response and of IL-12 as important player in the control of coccidial infections (Bliss et al. 1999, Yap and Sher 1999, Behrendt et al. 2008, Almería et al. 2012). Moreover, the increase was most prominent in cells from single infected animals, indicating that repeated infections do not alter or improve the immune response. The reduced production of the type I interferon IFN-α by cells of infected animals might be seen as an attempt of *C. suis* to escape a growth-inhibiting immune response, a mechanism already observed *in vitro* in *T. gondii* infections (Beiting et al. 2014). However, only few studies are available on the role of IFN-α in protozoan infections; further investigations on its ability to limit the intracellular growth of coccidia might be of interest.

However, the stimulatory potential of the recombinant protein remained below expectations since the measured cytokine concentrations were overall low - some cytokines were not detectable at all - and differences between groups did not reach levels of significance. Also an antigen-induced secretion of IFN-γ, a type II interferon regarded as effector molecule in infections with intracellular pathogens in general and with coccidia in particular (Lundén et al. 1998, Worliczek et al. 2010b, Beiting et al. 2014), was not observed. As the stimulation of immune cells with coccidial antigens is a commonly used, attractive way to analyse the host’s cellular immune response, further attempts should be made to improve this assay. Possibly, a single recombinant protein is of too weak immunogenicity and may have to be combined with an immunostimulans. Alternatively, the improvement of parasite stage purification methods and subsequent protein extraction and concentration techniques could make it possible to use complete native proteins for stimulation assays.

In unstimulated MLN samples, in contrast, the expression of IL-10 mRNA was significantly increased after infection. IL-10 inhibits inflammatory and at the same time supports Th2-biased immune responses, the proliferation of B cells and the subsequent production of immunoglobulins (D'Andrea et al. 1993, Xu et al. 2004, Sand et al. 2016) which is in line with our findings of increased blood serum antibody titres post infection. Additionally, it matches the increase of B cells found in MLN of *C. suis* infected piglets in a study conducted by Worliczek et al. (2010a).

Together with increased expression levels of IL-10 and the Th2-associated cytokine IL-4 in white blood cells of infected animals, these findings point towards a Th2-dominated immune response,
underpinned by positive correlations observed between IgA and IgG titres and abovementioned cytokines. Increased peripheral levels of IL-4 and IL-10 have also been described in *N. caninum* infected cattle, but are thought to be associated with a decreased ability of the host to control infections (Rosbottom et al. 2007, Almería et al. 2011, Almería et al. 2012, Bartley et al. 2013). Local IL-10 expression was shown to be upregulated in avian *Eimeria* infections (Hong et al. 2006), and the oral application of an anti-IL-10 antibody markedly improved body weight gains of *Eimeria* infected broiler chicks (Sand et al. 2016), indicating that the immunosuppressive properties of this cytokine aid a successful infection with coccidia. Likewise, IL-10 deficient mice infected with *Cr. parvum* shed significantly less oocysts and cleared the infection much faster than control animals (Campbell et al. 2002). The severity of *E. vermiformis* infection in terms of oocyst output did not differ between IL-4 knockout and wild type mice, thus the authors concluded that a Th2 response is not important for the successful elimination of primary or challenge infections (Ovington et al. 1995). On the other hand, the *in ovo* application of an IL-4 plasmid together with an *in ovo* coccidia vaccine stimulated the antibody- and cell-mediated immunity and consequently improved body weight gain and reduced oocyst excretion (Annamalai and Selvaraj 2012).

The simultaneous increase of the mRNA expression of IL-6 in the first two weeks post infection further strengthens the impression of a Th2-biased immune response since IL-6 can promote Th2 differentiation, B cell proliferation and antibody production (Dienz et al. 2009, Scheller et al. 2011) and might, together with IL-10 and IL-4, be responsible for the increase of serum titres observed in this study. However, in contrast to the other cytokines, significant correlations between IL-6 and antibody titres were not found. Elevated systemic and local IL-6 levels were also found in infections with other avian, mammalian and human protozoa (Titus et al. 1991, Behrendt et al. 2008, Almería et al. 2011, Ozmen et al. 2012, Arranz-Solís et al. 2016), and according to Lynagh et al. (2000) increased serum and local values of IL-6 in mice infected with *E. vermiformis* were related to increased resistance to infection.

IL-6 also plays a pivotal role in inflammatory immune responses, particularly through the production of acute phase proteins (Stadnyk and Gauldie 1991, Titus et al. 1991), thus the observed increased IL-6 expression might as well simply reflect inflammation. The serum
concentration of acute phase proteins could be measured to test this theory (Stadnyk and Gauldie 1991, Tanaka et al. 2014). In addition, local expression of IL-6 in the jejunum should be evaluated to elucidate its role in the local mucosal acute phase response to the infection with *C. suis*.

The significant decrease of the mRNA expression of both IFN-γ and IL-2 in white blood cells after infection might be the result of the dominating Th2-associated and regulatory cytokines since IL-4, IL-6 and IL-10 inhibit the Th1 response and the accompanying production and activity of pro-inflammatory and Th1-related cytokines (D’Andrea et al. 1993, De Groot et al. 2005, Rosbottom et al. 2007, Dienz et al. 2009, Sand et al. 2016). Nonetheless, the involvement and the essential role of both the Th1-associated cytokine IFN-γ and the inflammatory cytokine IL-2 in the control of coccidia, particularly on a local level, have been widely described (Lillehoj and Trout 1996, Lundén et al. 1998, Inagaki-Ohara et al. 2006, Rosbottom et al. 2007, Taubert et al. 2009, Almeria et al. 2011, Ozmen et al. 2012, Arranz-Solís et al. 2016). IFN-γ was shown to effectively downregulate intracellular parasite multiplication in *N. caninum* infected bovine cells (Nishikawa et al. 2001), and mice deficient of IFN-γ are highly susceptible to infection with this parasite and other coccidia (Beiting et al. 2014). Its local expression was significantly increased in the caecal intraepithelial lymphocytes of *E. tenella* infected chickens (Zhang et al. 2012). Likewise, local production of IFN-γ was shown to be crucial for the elimination of *Cr. muris* infections in mice (Culshaw et al. 1997). Calves infected with *E. bovis* exhibited significantly increased local IL-2 mRNA levels, highlighting once again the dominance of Th1 immunity at the site of infection (Hermosilla et al. 1999). In pigs, TcR-γδ+ T cells are a highly abundant subset of T cells and were identified as important source of the abovementioned cytokines (Follows et al. 1992, Takamatsu et al. 2006, Sedlak et al. 2014). Moreover, in the intestinal mucosa of piglets infected with *C. suis*, these T cells were present in significantly higher numbers than in control animals while their abundance was decreased in MLN and blood (Worliczek et al. 2010a, Gabner et al. 2014). This could indicate a migration of TcR-γδ+ T cells to the site of infection, namely the jejunum, and subsequently a primarily local secretion of IFN-γ and IL-2. The local expression of these cytokines in the small intestines of *C. suis* infected animals should
be evaluated to test this hypothesis. Until now, only few data are available on the local cytokine expression in the course of *C. suis* infections. Gabner et al. (2014) found the mRNA levels of TNF-α to be increased, however, expression patterns of IFN-γ and IL-2 were not measured. Since TNF-α is also produced, among others, by porcine TcR-γδ+ T cells (Sedlak et al. 2014), the abovementioned theory of a predominantly local production might as well apply for this pro-inflammatory cytokine, particularly because of the fairly low and also inconsistent systemic expression witnessed in white blood cells during this study.

Another cytokine found to be mainly expressed by porcine TcR-γδ+ T cells is IL-17 (Stepanova et al. 2012, Sedlak et al. 2014). It is produced in response to *N. caninum* and effectively reduces the parasite burden in infected bovine fibroblasts *in vitro* (Peckham et al. 2014). Additionally, it seems to be involved in several other protozoan infections, although its contribution to either immunoprotection or immunopathology is somewhat contradictory (Kelly et al. 2005, Ribot et al. 2010, del Cacho et al. 2014). The immune function of IL-17 during *C. suis* infections is unknown, however, we were not able to design reliable, specific primers to evaluate its expression. Several attempts were made to produce primers from a partial sequence of porcine IL-17A mRNA but their efficiency or specificity were always below the set limits and could not be improved. Nonetheless, further attempts should be made in the future to investigate the systemic and local involvement of this cytokine in the immune response to *C. suis* since its role in protozoan infections is of increasing interest (Kelly et al. 2005, del Cacho et al. 2014).

The mRNA expression of IL-27, a cytokine only recently identified and associated with both pro- and anti-inflammatory properties (Yoshida and Miyazaki 2008, Meurens et al. 2009), was also measured in this study. However, its expression was low and showed considerably high variation in both infected and uninfected pigs, and differences between groups mostly resulted from varying expression levels in the control group. Thus, no influence of *C. suis* on the expression of IL-27 could be determined, indicating that it does not play a pivotal role in porcine coccidiosis, however its local production was not investigated and also suboptimal primers or PCR conditions might have negatively influenced the results.
Transforming Growth Factor (TGF)-β is, besides IL-10, an important regulatory cytokine that modulates and delimits Th1 and inflammatory responses in order to avoid damage by immunopathology due to excessive inflammation. It plays a critical role in maintaining the integrity of the intestinal lining and in protecting it from inflammation (Omer et al. 2000, Inagaki-Ohara et al. 2006, Nguyen et al. 2015). In this study, its systemic mRNA expression was significantly downregulated in the first two weeks post infection. Likewise, the local expression of TGF-β was shown to be decreased in the course of *N. caninum* and *E. vermiformis* infections (Inagaki-Ohara et al. 2006, Almería et al. 2011, Arranz-Solís et al. 2016). Its production might be suppressed in favour of a Th1-associated and inflammatory, parasite-limiting immune response. This could also explain the severe tissue damage and clinical signs observed during coccidiosis in piglets - provided that the situation in neonates is a similar one. Accordingly, low levels of TGF-β were found to be associated with increased mortality and parasite growth in mice infected with *Plasmodium* (Omer et al. 2000).

Overall, this study shows that the infection with *C. suis* does induce a systemic immune response in immunocompetent animals, regardless of the oocyst dose or infection frequency, whereas clinical signs were not observed and oocyst excretion was negligible. It can be concluded that growers can be used as models to mimic the immunization or infection of sows with *C. suis* due to comparable immune responses.

The tested oral infection models did not produce profound differences regarding the immune response; only the group which received a single high dose of oocysts (SH) stood out in a few parameters evaluated, however differences were not striking enough to undoubtedly promote this protocol for subsequent sow immunizations. Therefore, other protocols should be considered to improve the immunological outcome in sows and consequently the passive protection of piglets. The effectivity of other routes of application should be investigated; for *Cr. parvum* it was shown that antibody responses in serum and colostrum of sheep markedly differ depending on the vaccination route applied (Frölich et al. 2012). Also, the immune response might as well differ when other stages of *C. suis* are used for immunization. Moreover, utilizing stages that can be produced *in vitro* in large amounts, like merozoites, would drastically reduce the number of
animals needed for oocyst and therefore vaccine production. Furthermore, adjuvants could be employed to enhance the immune response to protective levels. IL-6, for example, was suggested as a potent coadjuvant through stimulation of the humoral immunity (Dienz et al. 2009), and since its expression is also involved in the response against *C. suis* infections, administration of IL-6 might increase specific antibody production and thus further enhance the immune response. Additionally, the development of a recombinant vaccine could be an attractive alternative, potentially applying the recombinant merozoite protein described by Shrestha et al. (2017b).

The systemic immune response was characterized by an increased expression of Th2-associated and regulatory cytokines, along with elevated serum levels of *C. suis*-specific antibodies. Inflammatory cytokines and those associated with a Th1 response were mostly downregulated or unaltered upon infection. They might be suppressed in favour of the dominating Th2 and anti-inflammatory immune response. Since a Th1-biased response is, however, regarded as more relevant and much more effective in controlling coccidial infections, the dominant Th2 response in pigs might be the reason why piglets cannot adequately control the disease and why the parasite causes so much damage of the intestinal lining. Likewise, mice that react with a Th1-biased immune response to infection with *Leishmania major* overcome the infection, whereas mice with a Th2 response die (Titus et al. 2001). However, the low and varying expression and protein levels observed in this study might indicate an only subordinate role of the systemic cellular immune system in the response to *C. suis*. Immune reactions of the Th1 and inflammatory type might thus primarily take place locally, namely in the small intestines, where the parasite is in direct contact with the local immune system and causes the greatest harm.

Lastly, none of the parameters evaluated in this study could be clearly identified as a marker that is related to immunoprotection. Oocyst excretion and titres of both IgA and IgG were positively correlated. The IgG titre against merozoites exhibited the most prominent increase, thus it might serve as a marker for the rate of parasite replication or for the severity of infection. A contribution of immunoglobulins to protection against *C. suis* can, however, not be completely ruled out. They might still play a role in either conferring protection or promoting cellular immunity, particularly in conjunction with an enhanced immune response due to optimized immunization protocols.
7. SUMMARY

The immune response to *Cystoisospora suis* infections was until now mostly investigated in suckling piglets since they are particularly susceptible to disease. While in *Eimeria* the immunization of chicks is well established, piglets cannot be actively immunized, additionally effective treatment options against porcine coccidiosis are limited to a single licensed drug, toltrazuril. While usually administered orally, it was recently shown to be also efficacious as an injectable formulation combined with iron. The simultaneous intramuscular injection of toltrazuril and iron reduces the required handling of animals, thus saving time and labour costs. However, complete resistance of a *C. suis* field isolate against toltrazuril was recently described for the first time, and the emergence of further resistant isolates has to be expected as the consequence of the long-term blanket use of a single drug. The aim of this study was thus to investigate the immune response of immunocompetent pigs and the impact of the infection strategy on it in order to evaluate the possibility of a passive immunization of piglets. To examine their systemic antibody and cytokine response to different oral immunization protocols, growers were single or trickle infected with *C. suis* oocysts in low and high doses (600 and 6,000 or 200 and 2,000, respectively). Feces were investigated for oocysts and consistency, IgA and IgG titers in blood and jejunal mucus were evaluated by IFAT. Cytokines were measured in different immune compartments at mRNA level and, after stimulation of mesenteric lymph node-derived lymphocytes with a recombinant protein, at protein level. Irrespective of the protocol, infection significantly increased IgA and IgG titers against *C. suis* merozoites and sporozoites in blood serum. Clinical or hematological deviations were not observed; low oocyst excretion was detected in all infected groups, and antibody titers were positively correlated with excretion days. IL-4, IL-6 and IL-10 mRNA expression significantly increased in white blood cells after infection, while IL-2, IFN-γ and TGF-β levels decreased. In mesenteric lymph nodes, TNF-α and IL-10 expression was increased upon infection. Supernatants of stimulated lymphocytes from infected animals contained elevated concentrations of IL-12 whereas IFN-α production was reduced. We conclude that all evaluated infection protocols induce a similar systemic immune response in immunocompetent pigs, and that growers can serve as models for sow immunization.
studies. The immune response to *C. suis* was mainly represented by Th2, regulatory cytokine and specific antibody production. However, cytokine profiles underlay high variation and levels were relatively low. Th1-associated and inflammatory activities might be more relevant at the site of infection. A correlation between any evaluated parameter and immunoprotection could not be established; antibody titers could potentially serve as markers for the severity of an infection.
8. ZUSAMMENFASSUNG

Porzine neonatale Cystoisosporose: alternative Strategien zur Kontrolle

*Cryptosporidium suis* ist ein häufiger Durchfallerreger bei Saugferkeln, was Gewichtsverluste und wirtschaftliche Einbußen zur Folge hat. Aufgrund der frühen Infektion und ihres unausgereiften Immunsystems ist eine aktive Immunisierung von Ferkeln nicht möglich, und zur effektiven Behandlung steht nur ein Wirkstoff zur Verfügung, Toltrazuril. Kürzlich konnte gezeigt werden dass auch die intramuskuläre Injektion von Toltrazuril, welches normalerweise oral verabreicht wird, wirksam gegen die porzine Kokzidiose ist. Das Produkt wurde kombiniert mit Eisen, sodass nun zwei Arbeitsschritte zu einem vereint werden können, was Zeit und wiederum Geld spart. Nichtsdestotrotz besteht durch die langjährige Applikation eines einzigen Wirkstoffs die Gefahr der Resistenzbildung, und so wurde erst kürzlich das erste vollständig Toltrazuril-resistente Feldisolat von *C. suis* identifiziert. Das Ziel dieser Studie war daher die Untersuchung und Charakterisierung der Immunantwort immunkompetenter Tiere auf *C. suis* sowie die Auswirkung verschiedener oraler Immunisierungsprotokolle darauf um die Möglichkeit einer passiven Immunisierung von Ferkeln über die Muttersau zu evaluieren. Um die Antikörper- und Zytokinantwort auf verschiedene Infektionsprotokolle zu untersuchen, wurden zehn bis elf Wochen alte Schweine einmalig oder dreimalig mit jeweils einer hohen und niedrigen Dosis *C. suis*-Oozysten oral infiziert. Individuelle Kotproben wurden auf Oozysten und Konsistenz untersucht, IgA- und IgG-Titer gegen zwei Stadien von *C. suis* in Blutserum und Darm-Mukus mittels IFAT quantifiziert. Das Zytokinprofil dreier Immunkompartimente wurde auf mRNA-Ebene mittels real-time PCR bestimmt, außerdem wurde die Zytokinproduktion von aus mesenterialen Lymphknoten isolierten Lymphozyten nach Stimulation mit einem rekombinantigen Protein mittels Multiplex-Fluorescent-Immunoassay gemessen. Die Infektionen führten unabhängig vom Protokoll zu einem signifikanten Anstieg sämtlicher im Blutserum gemessener Titer, wobei jene gegen Merozoiten am stärksten zunahmen. Klinische Symptome oder hämatologische Abweichungen traten nicht auf. Geringe Oozystenausscheidung wurde in allen infizierten Gruppen beobachtet, welche eine positive Korrelation mit der Höhe der
Antikörpertiter aufwies. Die Zytokinexpressions war sehr variabel, dennoch zeigte sich nach der Infektion eine signifikant erhöhte mRNA-Expression von IL-4, IL-6 und IL-10 in weißen Blutkörperchen, während die Expressionslevels von IL-2, IFN-γ und TGF-β abnahmen. In mesenterialen Lymphknoten wurden nach Infektion erhöhte mRNA-Werte von IL-10 und TNF-α gemessen, und ihre Lymphozyten produzierten nach Stimulation vermehrt IL-12, während die IFN-α-Sekretion im Vergleich zur Kontrollgruppe reduziert war.

9. ABBREVIATIONS

* C. suis: *Cystoisospora suis*

* CTL: Cytotoxic T lymphocytes*

* IFAT: Immunofluorescence antibody test*

* IFN: Interferon*

* Ig: Immunoglobulin*

* IL: Interleukin*

* MLN: Mesenteric lymph nodes*

* NK cells: Natural killer cells*

* PMN: Polymorphonuclear neutrophils*

* SD: Study day*

* SL: Single low*

* SH: Single high*

* TcR: T cell receptor*

* TGF: Transforming growth factor*

* Th: T helper*

* TL: Trickle low*

* TNF: Tumor necrosis factor*

* TH: Trickle high*
10. REFERENCES


Peckham RK, Brill R, Foster DS, Bowen AL, Leigh JA, Coffey TJ, Flynn RJ. Two distinct populations of bovine IL-17⁺ T-cells can be induced and WC1⁺IL-17⁺γδ T-cells are effective killers of protozoan parasites. Sci Rep. 2014:4:5431.


11. APPENDIX

11.1. Further Publications


**Impact factor (2017):** 2.422

**Authors’ contributions:** Shrestha A established the ELISA, performed the sample analyses and drafted the manuscript. Schwarz L and Freudenschuss B provided the serum samples, and Freudenschuss B performed the statistical analyses. Joachim A revised and edited the manuscript. All authors read and approved the final version of the manuscript.


**Impact factor:** /

**Authors’ contributions:** Shrestha A drafted the introduction and compiled the manuscript. Abd-Elfattah A compiled the chapter on immunology and age resistance, Freudenschuss B drafted the chapter about the life cycle and created the respective figure. Palmieri N drafted the chapter about the genomics analyses; Joachim A, Ruttkowski B and Hinney B devised the outline, added data, and revised and edited the manuscript together with the other authors. All authors read and approved the final version of the manuscript.
11.2. Conference contributions


**Freudenschuss B.** Ruttkowski B, Abd-Elfattah A, Pagès M, Joachim A: Cytokine responses to *Cystoisospora suis* infections in immune competent pigs. 4th International Meeting on Apicomplexa in Farm Animals (APICOWPLEXA). October 11-14 2017, Madrid, Spain. [poster]
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