From the Department of Pathobiology

University of Veterinary Medicine, Vienna

Institute of Parasitology

(Head: Univ.-Prof. Dr.med.vet. Anja Joachim)

Efficacy of bumped kinase inhibitor BKI 1369 against experimental infections of suckling piglets with *Cystoisospora suis* - Follow-up study on reduced treatment frequencies

Diploma Thesis

University of Veterinary Medicine, Vienna

Submitted by

Patricia Greber

Vienna, January 2020

Supervisors: Univ.-Prof. Dr.med.vet. Anja Joachim

and

Aruna Shrestha, PhD

Institute of Parasitology

Department of Pathobiology

University of Veterinary Medicine, Vienna

Reviewer: Univ.-Prof. Dr.med.vet. Andrea Ladinig

Contents

Abbreviations

AF	Autofluorescence
AUC	Area under the curve
BKI	Bumped Kinase Inhibitor
BW	Body weight
CDPK	Calcium-dependent protein kinase
Cl. perfringens	*Clostridium perfringens*
C. suis	*Cystoisospora suis*
DBWG	Daily body weight gain
E. coli	*Escherichia coli*
OpG	Oocysts per gram of feces
sd	Standard deviation
SD	Study day

1. Introduction

In intensive pig production, neonatal porcine cystoisosporosis is a major enteric disease in suckling piglets caused by an apicomplexan parasite *Cystoisospora suis*. Infected piglets suffer from diarrhea and dehydration, while high morbidity and poor weight gain lead to severe economic losses. *C. suis* is an obligatory intracellular parasite which develops in the host's intestinal epithelium after oral ingestion of fully sporulated oocysts. Afterwards, excreted oocysts sporulate in the environment to achieve the infectious stage and reinitiate a new developmental cycle.

So far, toltrazuril is the only commercially available drug to treat infections with *C. suis* in suckling piglets. Recently, a toltrazuril resistant field isolate of *C. suis* was reported from the Netherlands (Shrestha et al. 2017). This discovery made it necessary to search for new efficient pharmaceuticals to treat suckling piglet coccidiosis. A new group of compounds called bumped kinase inhibitors (BKIs), which selectively bind to and inhibit calcium-dependent protein kinases (CDPKs) of apicomplexan parasites but not to the mammalian kinases, has already been studied for efficacy against several apicomplexan parasites, including *C. suis*. In a previous study, treatment with BKI 1369 (10 mg/kg BW twice a day for five days) suppressed oocyst excretion and diarrhea and increased body weight gain in treated piglets without noticeable side effects in both a toltrazuril-sensitive and a resistant strain of *C. suis* (Shrestha et al. 2019).

However, because of the repeated and long treatment period and the subsequent amount of workload, farmers might not be able to use this treatment on their farms. Therefore, a follow-up study was planned to evaluate the efficacy of reduced treatment frequencies i.e., administration of a single or double dose of BKI 1369 at 20 mg/kg of body weight, and the most appropriate time point of treatment against porcine cystoisosporosis. The hypothesis was that because of the limited window of time in which *C. suis* can cause disease in piglets, treatment efficacy can be maintained despite reduced treatment frequencies.

2. Literature overview

2.1. *Cystoisospora suis*

Cystoisospora suis (syn. *Isospora suis*) is the pathogenic agent of cystoisosporosis in suckling piglets. It is an obligatory intracellular parasite and belongs to the class Coccidia (Shrestha et al. 2015).

The oocysts are smooth- and thin-walled, colorless and measure about 21 x 18 µm in diameter (Joachim and Schwarz 2016). After sporulation, each oocyst contains two sporocysts each with four sporozoites (Harleman and Meyer 1983). The predilection site of *C. suis* is the small intestine, especially the jejunum, and the prepatent period is of about five days (Lindsay et al. 1980).

2.1.1. Life cycle of *Cystoisospora suis*

The oral ingestion of sporulated and infectious oocysts marks the beginning of the life cycle of *C. suis*. After excystation and invasion of intestinal epithelial cells, it passes through three main phases: merogony, gamogony and sporogony. Merogony and sporogony are forms of asexual reproduction and gamogony is the sexual form (Karamon et al. 2008). Caused by the low pH value and enzymes in the stomach, sporozoites are released which then invade villous epithelial cells of the small intestine. In the enterocytes of the jejunum and ileum, different generations of meronts develop. After maturation, merozoites leave the epithelial cell and subsequently penetrate adjacent cells where they undergo gamogony. After gamogony oocysts are set free into the gut lumen by destroying the host cells and excreted together with the feces (Karamon et al. 2008). The sporogony takes place outside of the host in the environment. Under favorable environmental conditions, temperature (between 20 °C and 37 °C) and moisture, sporulation takes place within twelve to 56 hours (Chae et al. 1998, Lindsay et al. 1982). Infected piglets excrete oocysts in a cyclical pattern with two peaks at five to nine and eleven to 14 days post infection (Harleman and Meyer 1983, Worliczek et al. 2009).

2.1.2. Occurrence and epidemiology

C. suis has a worldwide distribution, regardless of the farrowing facilities and the management (Taylor et al. 2007). Oocysts of *C. suis* can withstand harsh environmental conditions and are resistant to most of the commonly used disinfectants. Contaminated farrowing crates and bedding materials are the most common source of infection. Once introduced, the spreading of oocysts takes place very quickly due to the short prepatency and fast sporulation (Eckert et al. 2008, Shrestha et al. 2015).

The age-related prevalence of oocyst shedding manifests itself in increased shedding of oocysts by piglets aged two to three weeks, which decreases to very low levels in the following weeks. Older pigs show low frequencies of oocyst shedding which normally cannot be detected in the feces of sows from infected herds (Lindsay and Blagburn 1994, Shrestha et al. 2015). In the United States studies showed that nursing piglets are not infected by the sows as the primary source of *C. suis* but contaminated farrowing crates are supposed to be the highest infection risk, once a farm is contaminated with *C. suis* (Lindsay and Blagburn 1994).

2.1.3. Immunology

C. suis mainly affects piglets in the first three weeks of life, when their immune system is functionally immature and unable to control the infection (Lindsay and Blagburn 1994, Koudela and Kučerová 2000). Therefore, antibodies and other immune components might be of greater importance to control cystoisosporosis in piglets. Unlike human beings, immunity does not pass across the placenta to the piglets. Antibodies and cellular components found in colostrum and later in milk, provide passive immunity to piglets against infections. However, although piglets receive naturally acquired *C. suis*-specific antibodies from the colostrum, these only persist for a short time and therefore do not provide passive protection against infection (Schwarz et al. 2013).

Older piglets are less susceptible and excrete no or very low oocysts. In *C. suis* infections a strong age resistance is observed and piglets also develop a strong immunity against homologous infections (Bowman et al. 2014, Stuart et al. 1982). The actual mechanism for this age resistance is not clear. It might be due to the maturation of the immune system with the growing age of the piglets or the changing physiology of the gut (Koudela and Kučerová 2000).

2.1.4. Pathogenesis and clinical signs

Suckling piglets can contract the infection soon after birth until their third week of life. Morbidity tends to be very high, but mortality is normally moderate (Lindsay and Blagburn 1994). The major clinical signs associated with cystoisosporosis include watery-to-pasty yellowish diarrhea for four to six days, dehydration, unthriftiness, and body weight loss (Karamon et al. 2008, Worliczek et al. 2009, Shrestha et al. 2015). Microscopically, villous atrophy and fusion, crypt hyperplasia and catarrhal to necrotic enteritis can be seen (Chae et al. 1998). Clinical signs and mortality depend mostly on concomitant infections with further viral (e.g. rotavirus) or bacterial (e.g. *Clostridium perfringens)* pathogens and the age of the infected piglets and tend to be inapparent in older pigs (Stuart and Lindsay 1986, Taylor et al. 2007, Bowman et al. 2014, Shrestha et al. 2015).

2.1.5. Chemotherapeutic control of infections with *Cystoisospora suis*

2.1.5.1. Toltrazuril

In Europe, the only available treatment against cystoisosporosis is a toltrazuril 5% oral solution (Baycox® Multi 50 mg/ml, Bayer Vital GmbH, Leverkusen, Germany). It is a triazinone-based antiprotozoal drug with a broad spectrum anti-coccidial activity. This coccidiostat is licensed for the treatment and prophylaxis against coccidiosis in calves, suckling piglets, lambs, and rabbits. After oral admission, the active agent is resorbed by at least 70% in the form of the main metabolite toltrazuril-sulphone. Highest concentrations of the agent are found in the liver, kidney and in fat tissue, the plasma half-life in pigs takes six days (Institut für Veterinärpharmakologie und -toxikologie, Zürich, Switzerland, 2019).

The oral application of a single dose of toltrazuril at 20 mg/kg of body weight on the third or fourth day of life of the piglets is recommended for treatment of an infection with *C. suis*. Toltrazuril therapy reduces oocyst shedding and diarrhea, and improves body weight gains (Scala et al. 2009, Skampardonis et al. 2010, Joachim and Mundt 2011). Toltrazuril can preferably be used as a metaphylactic treatment in three to five days old piglets in herds where the problem of infections with *C. suis* has been diagnosed (Joachim and Mundt 2011, Noack et al. 2019).

Besides Baycox®, some other generic toltrazuril products against cystoisosporosis are available in Austria. To name only a few, these are Cevazuril® 50 mg/ml (Ceva Santé Animale, Libourne, France), Chanox® Multi 50 mg/ml (Chanelle Pharmaceuticals Manufacturing Ltd, Dublin, Ireland), Dozuril® 50 mg/ml (Dopharma Research B.V., Raamsdonksveer, the Netherlands) and Espacox® 50 mg/ml (Industrial Veterinaria SA, Barcelona, Spain), which are all applied orally (Bundesamt für Sicherheit im Gesundheitswesen, Austria, 2019). Recently, two new injectable toltrazuril plus iron combination products, Forceris® (Ceva Santé Animale, Libourne, France) and Baycox® Iron (Bayer Animal Health Care, Leverkusen, Germany), have been launched for parenteral application for simultaneous prevention of cystoisosporosis and for prophylaxis of an iron deficiency anemia (Bundesamt für Sicherheit im Gesundheitswesen, Austria, 2019).

In the absence of effective treatment alternatives, farmers have continuously been using toltrazuril for more than two decades. Over-use (and occasional under-dosing) of toltrazuril for so long has led to the appearance of toltrazuril resistance, which was reported recently in a field isolate of *C. suis,* obtained from a pork producer from the Netherlands (Shrestha et al. 2017).

2.1.5.2. Sulphonamides

Sulphonamides and its derivates were the first synthetic anticoccidials used for prevention of coccidiosis in farm animals (Kaufmann 1996).

Several studies have been published, comparing the efficacy of toltrazuril and sulphonamides against cystoisosporosis in piglets. A study conducted by Scala et al. in 2009 reported less efficacy of injectable sulfamethazine and trimethoprim combination in terms of oocyst excretion and occurrence of diarrhea compared to the ones which were treated with toltrazuril. Therefore, they concluded that treatment with toltrazuril is the best way to handle infections with *C. suis* in piglets and that the application of sulphonamides is not recommended (Scala et al. 2009). In another study by Mundt et al. oral formulations of sulphadimidine administered two to four days after infection had no effect on oocyst excretion and diarrhea (Mundt et al. 2007).

Injectable sulphonamides applied continuously for six to seven days after infection on the other hand have been shown to effectively reduce clinical disease, while reduced treatment frequencies with the same drug had no effects on disease outcomes (Joachim and Mundt 2011). Since injectable sulphonamide treatment requires an extensive workload and time, another trial was performed and showed that the application of sulphamethoxypyrimidine (SMP) can have satisfying effects on diarrhea and oocyst shedding only if it is applied at the right time during the acute infection phase. Treatment just a few days earlier or later did not show a reduction of the above-mentioned parameters. Therefore, its use was not recommended under field conditions (Joachim and Mundt 2011).

2.1.6. Prevention

Because of limited compounds for prophylactic treatment, hygiene factors and sanitation such as cleaning of the farrowing crates with a steam cleaner, chemical disinfection, and prevention of oocyst spreading within the farm are very important to break the cycle of reinfection (Harleman and Meyer 1983, Lindsay and Blagburn 1994). Also, staff hygiene and change of clothes should be considered, because boots and clothing have an enormous impact on crate-to-crate contamination. After removal of all animals, the whole facility should be sanitized properly with effective cresol-based disinfectants (Ruzicka and Andrews 1983, Straberg and Daugschies 2007, Zimmerman and Dunne 2012).

A study of Langkjær et al. investigated the development and survival of oocysts of *C. suis* under different temperatures (20 °C, 25 °C and 30 °C) and different relative humidities (RH) (53-100%) (Langkjaer and Roepstorff 2008). The outcome of the study showed that the number of viable oocysts rapidly decreased when subjecting them to high temperatures (25 °C and 30 °C) and a low relative humidity (53% and 62% RH). The oocysts died within 24 hours under these conditions. The authors concluded that the spreading of *C. suis* oocysts can be controlled by establishing a warm and dry microclimate in the stable during the time from one litter to the next, although it cannot be eliminated completely (Langkjaer and Roepstorff 2008).

2.1.7. *Cystoisospora* in other mammals

Besides pigs, *Cystoisospora* can also infect humans, other domestic animals, and non-human primates (Lindsay et al. 1997). Several species of *Cystoisospora*, such as *C. canis, C. ohioensis, C. neorivolta,* and *C. burrowsi* are known to infect dogs. Cats can be infected by *C. felis* and *C. rivolta* (Dubey 2009, Eckert et al. 2008, Lindsay and Blagburn 1994). The *Cystoisospora* species of dogs and cats can have either a direct or an indirect cycle. While animals can become directly infected by oral ingestion of sporulated oocysts, the indirect cycle includes paratenic hosts. These animals, such as rodents, ruminants, and pigs, accommodate *Cystoisospora* in its dormancy period and the dormozoites continue their development once they are ingested by their definite host (Frenkel and Dubey 1972, Eckert et al. 2008, Lindsay et al. 2014). This indirect way of infection is also one of the reasons why, unlike pigs, stray dogs and cats are exposed to the parasites via feeding (Lindsay and Blagburn 1994). The clinical symptoms of coccidiosis in dogs are diarrhea (sometimes hemorrhagic), dehydration, abdominal distress, and vomiting. When animals acquire a severe infection, they can also show anorexia, mental depression, and even death (Mitchell et al. 2007, Dubey and Lindsay 2019). Even though the clinical significance is not ascertained, coccidial oocysts can be found in many young dogs suffering from diarrhea (Lindsay and Blagburn 1994). A study conducted in Vienna, Austria showed that oocyst shedding dogs had a greater risk for hemorrhagic and non-hemorrhagic diarrhea than dogs with no detectable oocysts of *Cystoisospora* in their feces (Buehl et al. 2006).

Stray cats are also more vulnerable to being infected by *Cystoisospora* because of the ingestion of paratenic hosts (Becker et al. 2012). Feline coccidiosis has the same clinical signs as the infection in dogs. It is mostly seen in naturally infected kittens which may also suffer from other enteral diseases (Lindsay and Blagburn 1994). The best way to control oocyst excretion in dogs and cats is a single dose of 10 to 20 mg/kg of body weight of toltrazuril (Daugschies et al. 2000, Eckert et al. 2008).

Coccidiosis in humans (cystoisosporosis) was first described by Woodcock in 1915. There are several case reports about people infected by *Cystoisospora belli* or *Cystoisospora natalensis*. Immuno-competent individuals are usually asymptomatic. Only mild clinical signs such as transient diarrhea and abdominal pain can occur. However, immune-compromised individuals can be severely affected by chronic recurrent diarrhea and weight loss. Human infection with *Cystoisospora* is usually treated with trimethoprim/sulfamethoxazole for ten days (Lindsay et al. 1997, Ros Die and Nogueira Coito 2018, Skinner 1972).

2.2. Bumped kinase inhibitors

2.2.1. Efficacy of bumped kinase inhibitors

Bumped kinase inhibitors (BKIs) are a group of synthetic competitive inhibitors of ATP-binding (van Voorhies et al. 2017). They were first described in 1998 by Shokat et al. as specific protein kinase inhibitors, which compete for the kinase's active localization against ATP (Bishop et al. 1998). The basis for the efficacy of BKIs is that calcium-dependent protein kinases (CDPKs) are crucial for several physiological functions of apicomplexan parasites. Therefore, a new way to treat parasitic diseases is to interrupt the function of indispensable protein kinases to block the parasites life cycle without affecting the mammalian kinases of the host cells.

The impact of BKIs on apicomplexan parasites depends on a small gatekeeper residue, which is located in the CDPK1 of these parasites. The oscillation in calcium iron concentrations regulates gliding movement and microneme secretion in apicomplexan parasites, which makes it easier for the parasite to invade the host cell. CDPKs have a fundamental physiological role but only occur in Apicomplexa, plants, and ciliates, not in vertebrates (Larson et al. 2012, Shrestha et al. 2019).

Different classes of BKIs have been examined for their efficacy against several apicomplexan parasites. Due to its potency, stability, metabolism, pharmacokinetics and lesser potential cardiotoxicity than other BKIs, BKI 1369 was identified as a promising therapeutic agent against apicomplexan parasites (Hulverson, Choi et al. 2017, Lee et al. 2018). *In vivo*, two metabolites of BKI 1369 could be identified, the first one is BKI 1318 and the second one is BKI 1817 (Hulverson, Choi et al. 2017). The structure of BKI 1369 and its metabolites is presented in Figure 1.

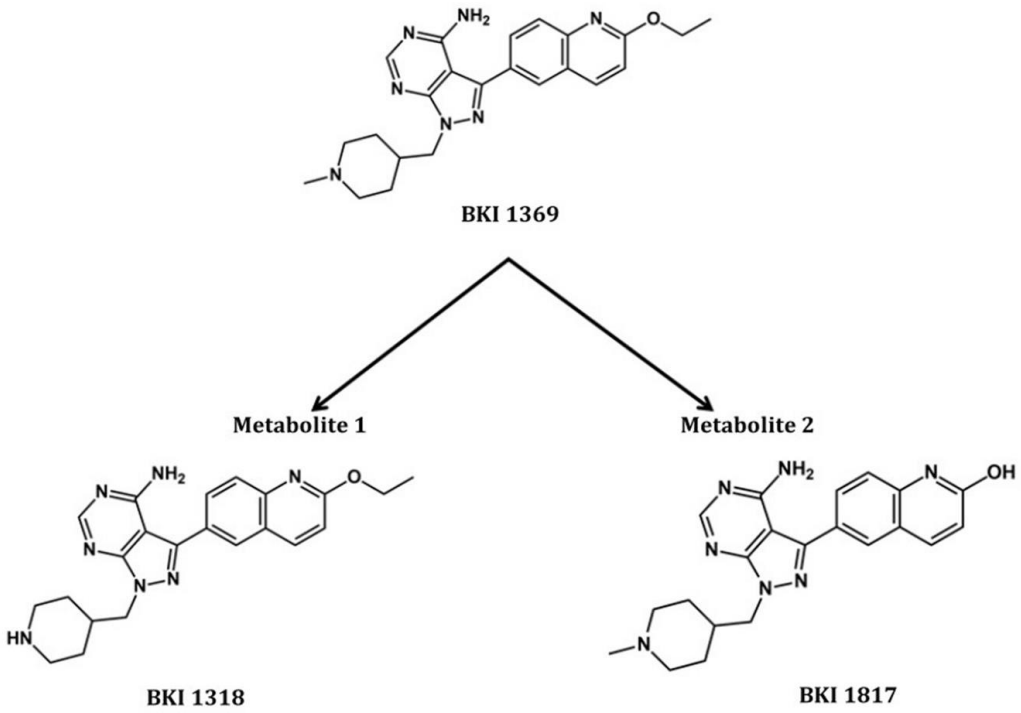

Figure 1: Chemical structure of BKI 1369 and its corresponding metabolites (see Shrestha et al. 2019).

2.2.2. Structure of calcium-dependent protein kinases (CDPKs)

Bumped kinase inhibitors target the calcium-dependent protein kinases (CDPKs) domain which is similar in structure and sequence to other members of threonine kinases. The conformation of these CDPKs can be either in an active or inactive state, which determines their ability to bind to and act on their protein substrates. In the active state, the calcium-binding domain is organized in such a way that substrate proteins can readily reach to the active site of CDPKs, in the inactive state the access is blocked (van Voorhis et al. 2017).

One reason for BKIs being effective against apicomplexan parasites but not against mammalian kinases is the presence of a small gatekeeper residue, such as glycine in apicomplexan CDPK1, which makes it possible for selective inhibitors such as BKIs to access the small hydrophobic pocket lying next to the ATP binding site. In contrast, mammalian protein kinases have large gatekeeper residues, such as methionine, that block BKIs to reach the hydrophobic pocket (Murphy et al. 2010, van Voorhis et al. 2017, Shrestha et al. 2019).

2.2.3. Efficacy of BKI in other apicomplexan parasites

2.2.3.1. *Toxoplasma*

Toxoplasma gondii is an obligate intracellular protozoan parasite and the only definite host where *T. gondii* forms microgametes and macrogametes are cats and other members of the family Felidae (Blader et al. 2015). Any mammal can serve as an intermediate host because the sporulated oocysts are infective to nearly all warm-blooded animals after ingestion. The clinical signs vary from enteritis, enlarged mesenteric lymph nodes and pneumonia to degenerative transformations in the central nervous system and encephalitis (Bowman et al. 2014, Kochanowsky and Koshy 2018). In addition, toxoplasmosis is an important zoonotic disease especially for pregnant women and immunodeficient persons. Primary infections during pregnancy can lead to severe affections of the fetus and even to abortion (Gao et al. 2012). In immunodeficient patients *T. gondii* can cause a generalized or cerebral infection, in persons with an intact immune system, the symptoms of an infection with *T. gondii* normally remain mild (Eckert et al. 2008, Kochanowsky and Koshy 2018).

Doggett et al. demonstrated that BKI 1294 is effective against acute infections with *T. gondii* in a mouse model. Oral application of BKI 1294 at 100 and 30 mg/kg of body weight daily for five days effectively reduced parasite numbers at both doses (Doggett et al. 2014).

2.2.3.2. *Neospora*

Neospora caninum is a coccidian which is known to cause abortion in cattle (Dubey 2005). In dogs, *N. caninum* causes progressive ascending paralysis of the hindlimbs, ataxia and even sudden death may occur (Barber and Trees 1996). *N. caninum* has also been reported in other livestock species such as sheep, goats, horses, and deer (Dubey 2003).

The efficacy of BKIs against *N. caninum* was evaluated by Sánchez-Sánchez et al. Pregnant ewes were treated with a subcutaneous injection of BKI 1553 at different dose combinations. The results of the study showed that treatment resulted in a reduced fetal mortality up to 37–50%, a decreased fever in pregnant ewes and a modulation of immune responses was also detected. Although *N. caninum* could still be detected in placental tissues in abundance, parasite counts in fetal brain tissue were reduced from 94% in untreated animals to 69–71% in the medicated groups (Sánchez-Sánchez et al. 2018).

In another study by Winzer et al., the effects of BKI 1294 in pregnant mice infected with *N. caninum* were evaluated. After oral treatment with 50 mg/kg of body weight BKI 1294 per day for eight days, parasite burden in brains of treated dams and their litters were significantly lower than the ones of the control groups. Also, a highly decreased mortality and transfer of *N. caninum* to the pups was recorded (Winzer et al. 2015).

2.2.3.3. *Cryptosporidium*

Member of the genus *Cryptosporidium* are causative agents of a sometimes-zoonotic disease called cryptosporidiosis, a significant diarrheal disease in humans and animals. It can affect mammals, birds, reptiles, and fish. In calves, the leading clinical sign of cryptosporidiosis are profuse diarrhea with liquid and light-yellow feces. Also, anorexia, fever, and poor growth rates can be observed. Most symptoms disappear after a few weeks, but in severe cases, cryptosporidiosis can also be fatal (Davies and Chalmers 2009, Shahiduzzaman and Daugschies 2012).

Several classes of BKIs have been tested for their efficacy against cryptosporidiosis *in vitro*, in mouse and in calf models. A study by Hulverson et al. showed that BKIs are effective against infections with *C. parvum* in mice. In one part of the study, oral applications of twelve different BKIs (BKI 1294, 1318, 1369, 1412, 1534, 1546, 1550, 1553, 1556, 1557, 1649 and 1664) at 25 mg/kg of body weight resulted in a decreased infection level by 14–76%. In another part of the study, the efficacy of BKIs in adult interferon-γ knockout mice was tested. Three days after infection, the animals were treated with BKIs 1294, 1318, 1369, 1534, 1550 and 1649 orally for five days. The results showed that the parasite shedding was decreased significantly by BKIs 1294, 1369 and 1534, whereas infection was only slightly limited by BKIs 1550 and 1318 (Hulverson, Vinayak et al. 2017). However, the further consideration of BKIs 1534 and 1649 was declined due to their failure in a mouse pregnancy safety test. Considering the safety, lack of toxicity and the low systemic exposure, BKI 1369 was concluded as a potential candidate to treat cryptosporidiosis (Hulverson, Choi et al. 2017).

Furthermore, in an experimental calf model, the efficacy of three different BKIs (BKI 1294, 1517 and 1553) was tested. At first, treatment was applied every other day for eight days after infection at a dose of 10 mg/kg of body weight. Due to a lack of significant reduction in diarrhea, another trial was performed where BKIs were given every twelve hours for five days, starting two days after infection. This treatment regimen significantly improved clinical health, diarrhea,

and parasitological outcomes. The calves had firmer feces and a decreased fecal volume and parasite shedding was significantly inhibited. Summing up the outcomes of these studies, BKI 1294 seemed to be the most effective agent, followed by BKI 1517 (Schaefer et al. 2016).

2.2.3.4. *Babesia*

Over 100 *Babesia*-species parasitize in the erythrocytes mainly of cattle, sheep, horses and dogs and can also affect humans. *Babesia* is a tick-borne zoonotic hemoparasite and sporozoites are inoculated with the saliva into the host during the tick's blood meal. After infection of the erythrocytes, *Babesia* multiplicates by merogony and generates merozoites. While feeding on blood from the host, ticks take up merozoites which then undergo gamogony and sporogony and at later blood meals release sporozoites via their salivary gland (Taylor et al. 2007, Jalovecka et al. 2019).

Babesia bovis is the most important species of *Babesia* in cattle, due to its wide distribution and high pathogenicity. It is often associated with another pathogenic species of this genus, *Babesia bigemina*. In Europe, in some regions *Babesia divergens* occurs, while *Babesia major* is rare (Uilenberg 2006, Eckert et al. 2008). Clinical signs of babesiosis in cattle include a decreased milk yield, fever, anemia, hemoglobinuria, icterus and tachycardia and can even result in death (Bowman et al. 2014, Kaufmann 1996, Rojas-Martínez et al. 2018).

In an *in vitro* study, BKI RM-1-152 completely inhibited *B. bovis* within 48 hours together with noticeable phenotypic changes to the parasite due to its *Babesia*-static activity (Pedroni et al. 2016).

2.2.3.5. *Theileria*

There are several species of *Theileria* in horses and ruminants. The hosts become infected by sporozoites injected with the saliva of hard ticks. Reproduction first takes place in lymphocytes, later in the erythrocytes of the host by merogony. After ingestion of infected erythrocytes, gamogony occurs in the tick gut and the parasites are transmitted to the host via tick-bite. After an incubation period up to three weeks, clinical signs like an increase in body temperature, depression, icterus, anemia, colic, and hemoglobinuria appear and mortality can reach up to 50% (Eckert et al. 2008, Onyiche et al. 2019).

The efficacy of several classes of BKIs against *T. equi* was tested in an *in vitro* assay. After 72 hours of incubation with BKIs RM-1-132, RM-1-152, 1735, 1369 and 1318, parasite growth was effectively suppressed (Gimenez et al. 2018).

2.2.3.6. *Besnoitia*

The exact transmission path of *Besnoitia besnoiti* remains unknown, but cattle, goats and wild ruminants are intermediate hosts. The parasite is transmitted by direct contact between animals or mechanically by biting insects. Moreover, transmission via infected bulls during mating has also been suggested. Affected animals may have high fever, skin thickening, swollen lymph nodes, alopecia, and tissue cysts. Morbidity is usually high, whereas mortality is only ≤10% (Alvarez-García et al. 2013, Alvarez-García et al. 2014).

The therapeutic potential of BKIs against *B. besnoiti* was evaluated *in vitro*. The study concluded that BKIs 1294, 1517, 1553 and 1571 successfully inhibited tachyzoite invasion and proliferation, indicating that BKIs may be effective drug candidates to control infections with *B. besnoit*i (Jiménez-Meléndez et al. 2017).

3. Materials and Methods

3.1. Study design

The clinical study was performed according to a parallel, blinded experimental block design comparing four different treated groups. The day after the piglets' birth was referred to as the first day of the study (SD 1), the last day of the study was four weeks later (SD 29). The experimental unit was the individual piglet. The study was undertaken in accordance with the guidelines of Good Scientific Practice rules of the University of Veterinary Medicine Vienna and the national animal welfare requirements. The study was approved by the Animal Ethics Committee of the University of Veterinary Medicine Vienna and the national authority according to § 26ff of Animal Experiments Act, Tierversuchsgesetz 2012-TVG 2012 (license number: BMWF-68.205/0034-WF/V/3b/2016; Austrian Federal Ministry of Science, Health and Economy).

3.1.1. Study animals

At least two weeks before farrowing, gravid crossbred sows from a conventional pig farm were moved to the experimental site to acclimatize to the housing conditions.

All animals were held under conditions conducive to the maintenance of good health. The sows were fed with a standard non-medicated feed twice daily and water was provided *ad libitum*. The piglets received milk from the sow and piglet starter *ad libitum* from the second week of life.

Individual identification of piglets was ensured by ear tattooing and parenteral iron injection (Ferriphor®, OGRIS Pharma, Austria; 2 ml per piglet) was administered to all piglets on SD 2 for the prevention of iron deficiency anemia.

3.1.2. Treatment groups

The piglets were assigned to the four treated groups based on ascending body weight at birth within each litter. Fine powder of BKI 1369 was dissolved in the solvent (70% Ethanol + 30% Tween 80) with heating (56 °C for 2 hours) to get a slurry of 20% stock concentration. On the day of treatment, the stock was diluted 1:4 in 0.9% NaCl to receive the final concentration of 5% BKI 1369.

Group BKI-A consisted of ten piglets which received a single dose of BKI 1369 at 20 mg/kg of body weight (5% BKI 1369 = 0.4 ml/kg of body weight) on their third day of life (SD 3). Group BKI-B consisted of eleven piglets which were treated with a single dose of BKI 1369 at 20 mg/kg body weight (5% BKI 1369 = 0.4 ml/kg of body weight) on their fifth day of life (SD 5). The eleven piglets of group BKI-C were given two doses of BKI 1369 at 20 mg/kg body weight (5% BKI 1369 = 0.4 ml/kg of body weight) on their fifth and seventh day of life (SD 5 and SD 7). The negative control group D consisted of ten piglets which were sham-treated with a solvent in the same dosage as group BKI-A (0.4 ml/kg of body weight) on their third day of life (SD 3). The treatment groups used in the study are represented in Table 1.

Table 1: Treatment groups used in the study. BW: body weight. SD: Study day.

Group	No. of piglets	Treatment dosage	Time points of treatment
BKI-A	10	20 mg/kg BW of BKI 1369 (5%) = 0.4 ml/kg BW once daily	SD 3
BKI-B	11	20 mg/kg BW of BKI 1369 (5%) = 0.4 ml/kg BW once daily	SD 5
BKI-C	11	20 mg/kg BW of BKI 1369 (5%) = 0.4 ml/kg BW once daily	SD 5 and SD 7
Group D (Control)	10	0.4 ml/kg BW of solvent	SD 3

3.1.3. Inclusion and exclusion criteria

Piglets with a birth weight of at least 0.9 kg and a normal health status were included in the study. All piglets with a birth weight less than 0.9 kg, piglets which were unable to suckle and/or unable to stand and walk normally and piglets with malformations and other conditions that precluded the animals from developing normally were excluded from the study.

3.2. Experimental infection and treatment

All piglets were infected with a single dose of approximately 1000 sporulated oocysts of a laboratory strain of *C. suis* (Wien-I) on SD 3. The infection dose was prepared in 1.0–1.5 ml of

tap water per dose and applied with a single use flexible pasteur pipette over the tongue of the piglets.

The doses of treatment were calculated referring to the body weight of the piglets on the day of treatment and were rounded to 0.05 ml. The drug was applied orally with a 1 ml syringe individually over the piglets' tongue.

During treatment and two hours after treatment a veterinarian monitored the piglets' health (in addition to the daily general health observation) to evaluate potential adverse events after the application.

3.3. Evaluation of efficacy and safety

3.3.1. Oocyst count

Fecal samples were collected rectally from each included piglet daily from SD 7 to SD 27. Fecal samples were first screened for the presence or absence of oocysts qualitatively by autofluorescence (AF) detection under ultraviolet light (Daugschies et al., 2001). For positive samples, quantitative assessment of oocyst excretion were determined using a modified McMaster counting method (Joachim et al. 2018).

First, fecal samples were diluted in tap water, spread evenly on a slide using a spatula and covered with a glass cover slip. Each sample was examined under light microscope with an ultraviolet filter and a wavelength between 340 and 380 nm. When the presence of oocysts could be observed by the emission of faint blue light, samples were considered as AF-positive.

In addition to autofluorescence, positive samples were examined by a modified McMaster counting method. For this purpose, 0.5 g of feces were weighed into a plastic bowl and suspended in 4.5 g of saturated sugar-salt solution. In case there was not enough sample material, the amount of sugar-salt solution was reduced according to the amount of available feces. The fecal sample was mixed thoroughly and then transferred to a cylindrical tube; a double-layered gauze (2x2 cm) was gently pushed to the bottom of the tube using a wire loop, to filter larger particles. Then 200 µl of this suspension were pipetted and mixed into 1800 µl of the sugar-salt solution in a 2 ml Eppendorf tube. The tube was closed and mixed by inverting several times. At the end, around 1 ml of the suspension was pipetted out and transferred to McMaster counting chambers without formation of air bubbles. After two minutes of resting, filled McMaster chambers were examined under the microscope at 100x magnification.

The total number of oocysts counted in these two chambers corresponds to the amount in 300 µl of the diluted suspension. The initial dilution factor was 1:10 (0.5 g of feces plus 4.5 g of saturated sugar-salt solution) and dilution was repeated (1:10; 200 µl of the first suspension plus 1800 µl of saturated sugar-salt solution), so that the final dilution factor was 100.

Resulting from that, the counted number of oocysts was multiplied with 3.33 (1000 µl:300 µl) and 100 (dilution factor) to determine the number of oocysts per gram of feces (OpG):

$$\mathrm{OpG} = \mathrm{x} * 100 * 3.33 \text{ or } \mathrm{OpG} = \mathrm{x} * 333$$

with x = number of oocysts counted in two chambers.

Whenever there were countable oocysts present in the sample, it was determined to be positive. Furthermore, an excretion day was defined as a day with AF and/or McMaster countable excretion of oocysts by a piglet. The mean OpG per group was calculated by averaging over all counted oocyst numbers, also including negative samples with no McMaster-countable oocyst excretion.

3.3.2. Fecal score

The fecal score (FS) of each fecal sample was recorded daily from SD 7 to SD 27. Fecal consistency was scored as follows: firm (FS 1), pasty (FS 2), semi-liquid (FS 3) and liquid (FS 4). FS 3 and 4 were considered as diarrhea.

3.3.3. Body weight

For inclusion or exclusion, all piglets were weighed on their day after birth (SD 1). For calculation of the individual treatment doses, all included piglets were weighed on the day/s of treatment/s and also on SD 8, SD 15, SD 22 and SD 29. Weighing was performed with a Bosche Industrielle Paketwaage PS 60 SST with an accuracy of 20 g according to the manufacturer's instructions. The scale was calibrated before every weighing.

The daily body weight gain was calculated by subtracting the first measured body weight (e.g. on SD 8) from the second measured body weight on the following week (e.g. SD 15) and dividing this result by seven days.

3.3.4. Concomitant infections

On the first day of sampling (SD 7), a pooled sample from each of the four litters was sent to the Institute of Virology and the Institute of Microbiology, Department of Pathobiology, University of Veterinary Medicine Vienna, to evaluate the presence of viral and/or bacterial entero-pathogens causing diarrhea such as rotavirus and coronavirus, *Escherichia coli* and *Cl. perfringens*.

3.3.5. Adverse events and safety

Any observation of an unfavorable and unintended reaction that occurred after the use of a veterinary product or an investigational veterinary product, whether product-related or not, was defined as an adverse event. In case the treated animals suffered from a fatal or life-threatening situation or showed permanent or prolonged clinical signs, the adverse event was considered serious. As diarrhea was expected after infection, it was not considered as an adverse event.

3.4. Statistical analysis

Statistical calculations were performed with Microsoft Excel 2010. For continuous parameters, a Kruskal-Wallis-One-Way Analysis of Variance test and for discrete parameters, a Chi-square test was used. *P*-values ≤0.05 were considered significant.

4. Results

4.1. Oocyst excretion

4.1.1. Autofluorescence

All piglets in the control group D (*n*=10) were positive at least once by autofluorescence, whereas 70.00% of the piglets in group BKI-A (*n*=11), 18.18% in groups BKI-B (*n*=11) and BKI-C (*n*=11) showed an autofluorescence-detectable excretion of oocysts (Table 2).

Compared to the control group D, the results of Chi-square test for the number of piglets with a positive result in AF were statistically significant for groups BKI-B and BKI-C ($P = 0.00015$) with a level of significance $\alpha = 0.05$.

Similarly, the percentage of AF excretion days was highest in the control group D (28.10%), followed by BKI-A with 8.10% group BKI-B with 2.60% and the group BKI-C with 0.87%. These results were significantly different for groups BKI-A, BKI-B and BKI-C compared to the control group D (Table 2).

Table 2: Summary of results for AF. N = total number; sd = standard deviation. Level of significance $\alpha = 0.05$. For group descriptions see Section 3.1.2 Treatment groups, Table 1.

Group [N]	N sampling days	N positive piglets [%]	*P*-value no. positive piglets	% of excretion days [sd]	*P*-value % of excretion days
BKI-A [10]	21	7 [70.00]	0.06029	8.10 [8.41]	0.0000
BKI-B [11]	21	2 [18.18]	0.00015	2.60 [5.78]	0.0000
BKI-C [11]	21	2 [18.18]	0.00015	0.87 [1.93]	0.0000
D (Control) [10]	21	10 [100.00]		28.10 [14.63]	

The control group D showed two peaks of autofluorescence-positive piglets over the entire sampling period (SD 7 to SD 27); the first one on SD 10-13 (max. 90.00%), the second one on SD 17-18 (max. 30.00%). Group BKI-A showed one peak on SD 14-16. One small peak was observed for group BKI-B from SD 17-18 (18.18%). In group BKI-C, single positive samples were observed on SD 15 and SD 21 (Figure 2).

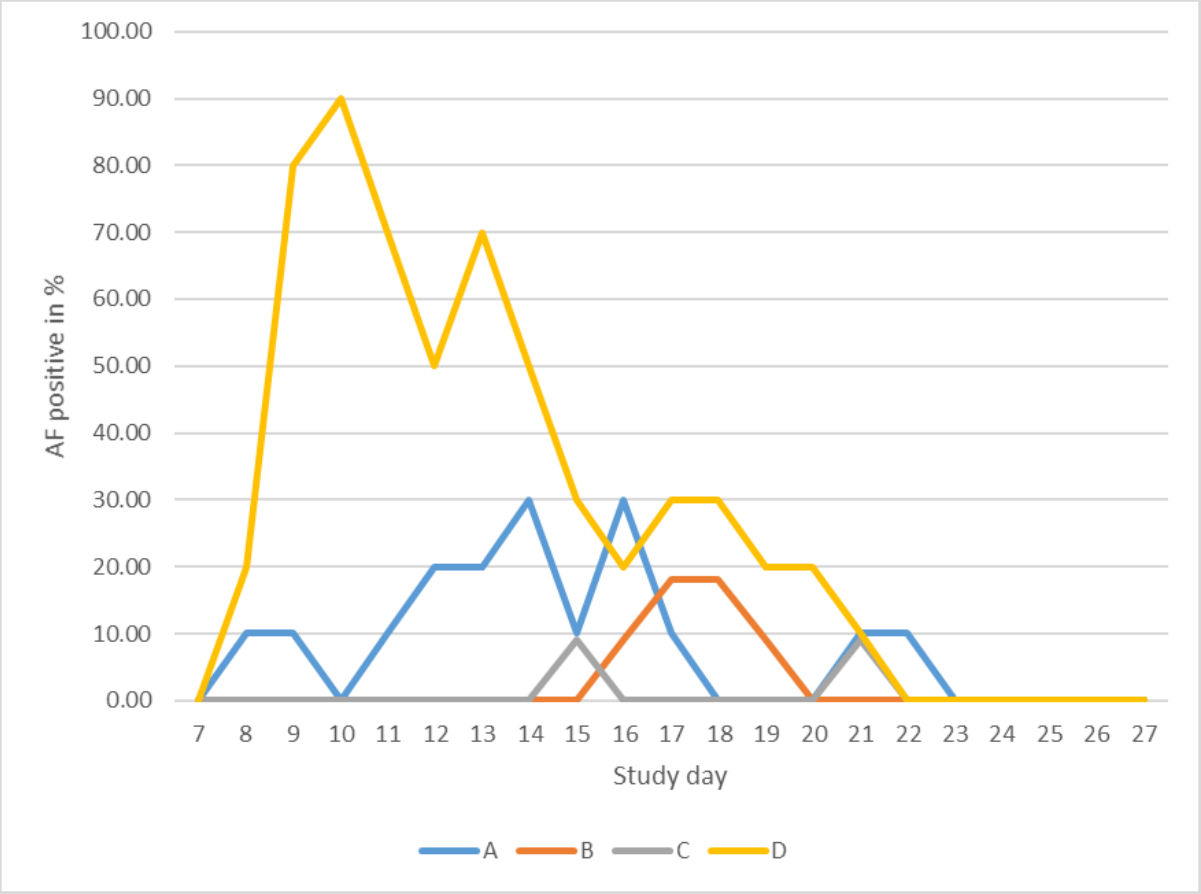

Figure 2: Percentage of AF positive piglets from SD 7 to SD 27. For group descriptions see Section 3.1.2 Treatment groups, Table 1.

4.1.2. McMaster oocyst counting

McMaster-countable excretion of oocysts was detected in all piglets of the control group D, while 50.00% of the piglets in group BKI-A and 18.18% of group BKI-B were positive in McMaster counting. In contrast, none of the piglets in group BKI-C excreted McMaster countable oocysts (Table 3).

The Chi-square test for the number of positive piglets and the percentage of excretion days showed a significant difference for all treated groups compared to the control group D (Table 3).

Table 3: Summary of results for McMaster counting. N = total number of piglets; sd = standard deviation. Level of significance α = 0.05. For group descriptions see Section 3.1.2 Treatment groups, Table 1.

Group [N]	Total sampling days	N positive piglets [%]	*P*-value no. positive piglets	% of excretion days [sd]	*P*-value % of excretion days
BKI-A [10]	21	5 [50.00]	0.00982	4.76 [7.45]	0.0000
BKI-B [11]	21	2 [18.18]	0.00015	2.16 [4.93]	0.0000
BKI-C [11]	21	0 [0.00]	0.00001	0.00 [0.00]	0.0000
Control D [10]	21	10 [100.00]		20.95 [13.87]	

The highest individual oocyst excretion (max OpG) was observed in the control group D on SD 14, additionally this group also showed the highest mean OpG (Table 4).

Table 4: Results of oocyst counting (OpG) per group. N = total number of piglets; AUC: Area under the curve; sd = standard deviation. For group descriptions see Section 3.1.2 Treatment groups, Table 1.

Group	Mean OpG [sd]	Median OpG	Max. OpG (study day)	Mean AUC [sd]
BKI-A [10]	231.51 [591.15]	0.00	24,975 (11)	4862 [10,0040]
BKI-B [11]	15.86 [35.33]	0.00	1332 (19)	333 [745]
BKI-C [11]	0.00	0.00	0.00	0.00
Control D [10]	4852.29 [8471.73]	333.00	269,730 (14)	101,898 [192,569]

The control group D and group BKI-A showed two noticeable peaks of the mean OpG during the entire sampling days. The mean OpG in the control group peaked on SD 9 and SD 14 and in group BKI-A on SD 11 and from SD 16-17 (Figure 3).

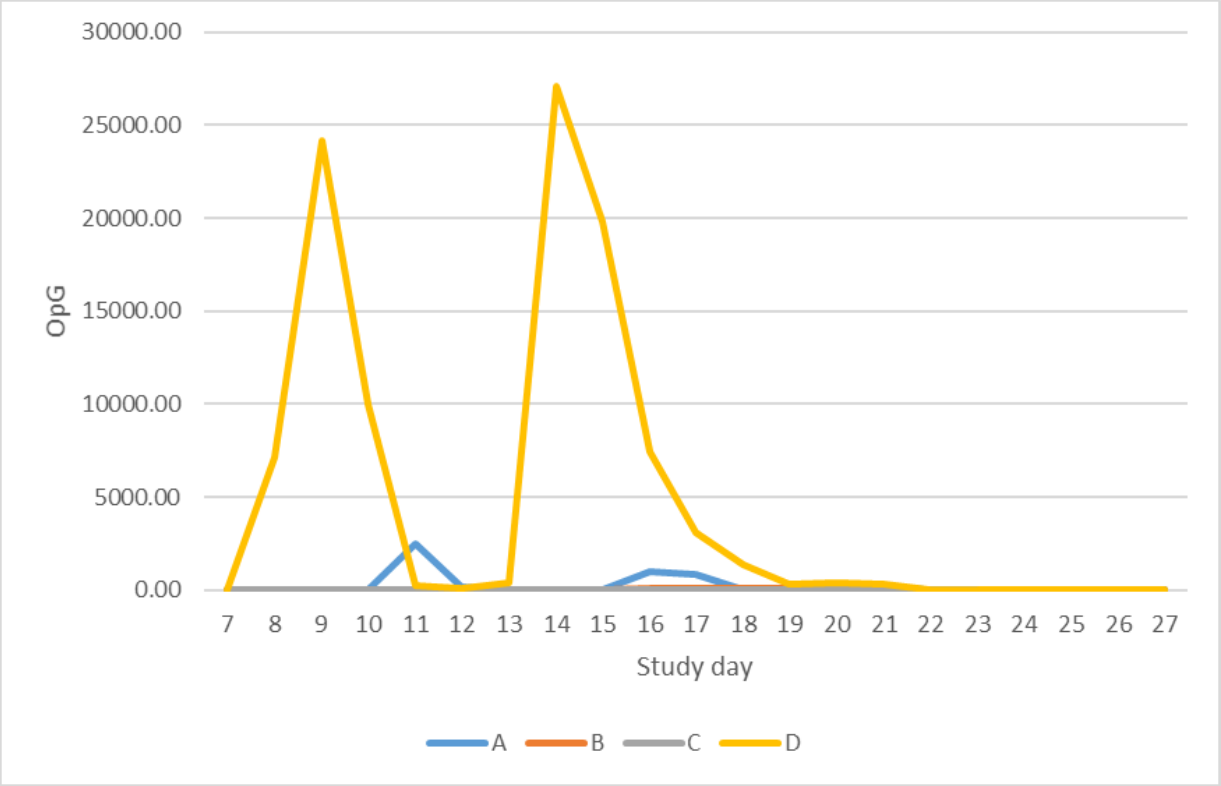

Figure 3: Mean OpG per group from SD 7 to SD 27. For group descriptions see Section 3.1.2 Treatment groups, Table 1.

4.2. Fecal score

Nine out of ten piglets (90.00%) in the control group D showed diarrhea at least once. In contrast, seven out of ten piglets (70.00%) of group BKI-A, two out of eleven piglets (22.00%) of group BKI-B and three out of eleven piglets (33.00%) of group BKI-C had a FS of 3 or 4 at least once. The Chi-square test showed a statistically significant difference compared to the control group D for group BKI-B (P = 0.001) and for group BKI-C (P = 0.004) (Table 5).

Table 5: Prevalence of diarrhea per group. [N] = total number of piglets. AUC: area under the curve. Level of significance α = 0.05. For group descriptions see Section 3.1.2 Treatment groups, Table 1.

Group [N]	N piglets with diarrhea at least once [%]	*P*-value N piglets with diarrhea at least once	Mean days with diarrhea [%]	*P*-value of % days with diarrhea	Mean AUC for fecal score
BKI-A [10]	7 [70.00]	0.26	1.40 [6.67]	0.073	27.15
BKI-B [11]	2 [22.00]	0.001	0.27 [1.30]	0.003	22.14
BKI-C [11]	3 [33.00]	0.004	0.45 [2.16]	0.005	23.32
Control D [10]	9 [90.00]		3.10 [14.76]		30.65

The control group D showed a prominent peak on SD 12 with a mean FS of 3.10. Groups BKI-A and BKI-C had their highest values on SD 14 with an average of 2.30 and 1.73 for group BKI-A and BKI-C, respectively. On SD 15, group BKI-B showed the highest mean FS with a value of 1.36 (Figure 4).

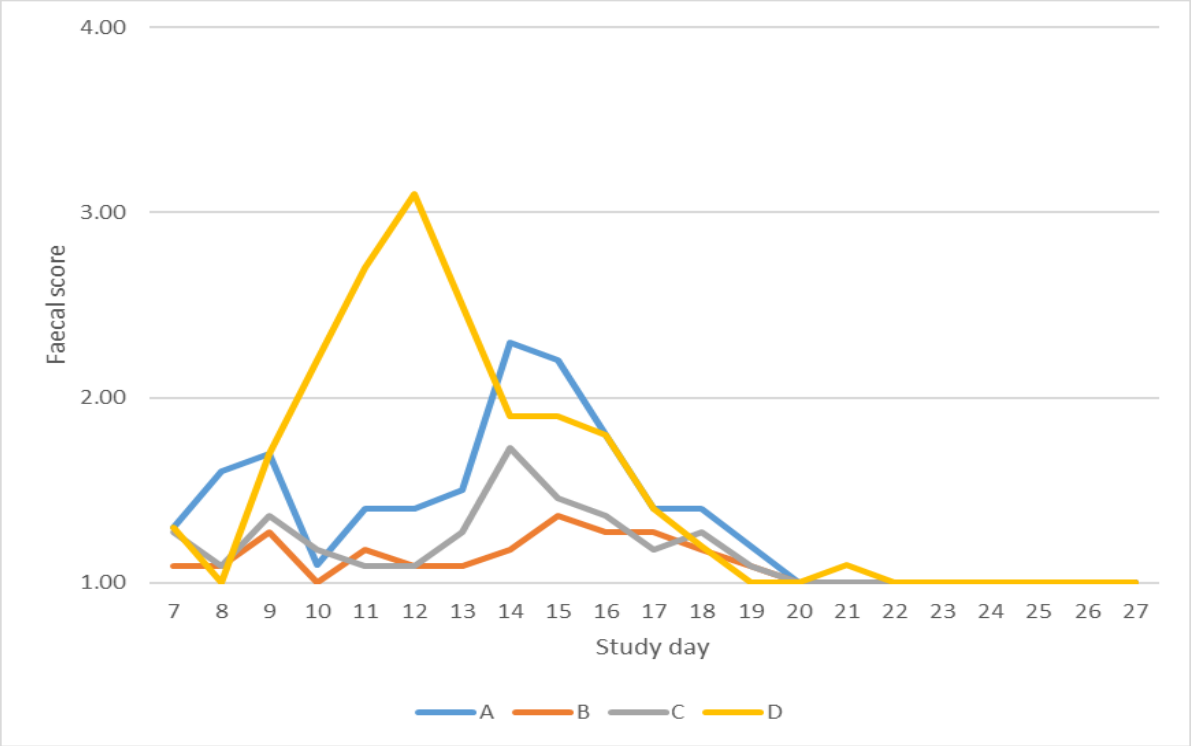

Figure 4: Mean fecal score per group from SD 7 to SD 27. For group descriptions see Section 3.1.2 Treatment groups, Table 1.

Considering every piglet with an FS >2 on a sampling day as an individual day with diarrhea, group BKI-A had a total of 14 diarrhea days, group BKI-B three days, group BKI-C five days and the control group D 31 days. Here also only groups BKI-B (*P* = 0.003) and BKI-C (*P* = 0.005) showed a statistically significant difference compared to the control group D.

Diarrhea in group BKI-C already started on SD 7, in group BKI-A on SD 8 and in the other groups BKI-B and the control group D from SD 9. All in all, the control group D had the longest period where piglets showed diarrhea (nine days), group BKI-A had seven, group BKI-B three and the piglets in group BKI-C four days of diarrhea (Figure 5).

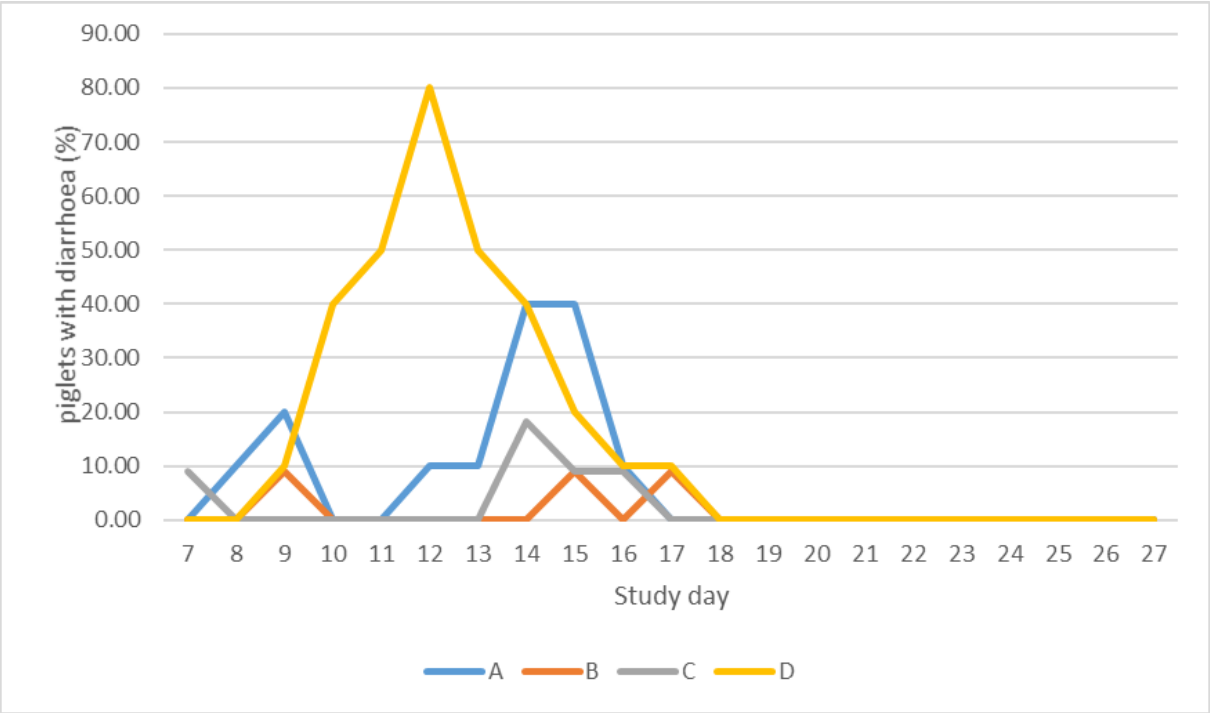

Figure 5: Distribution of piglets with diarrhea. For group descriptions see Section 3.1.2 Treatment groups, Table 1.

4.3. Body weight

The body weight of the piglets per group did not differ on SD 1 and SD 8. Differences in the body weight between groups BKI-A, BKI-B and BKI-C and the control group D were evident on SD 15 onwards, when the control group D gained less weight than the other groups (Figure 6). Body weights of all treated groups were significantly higher compared to the control group D on SD 15 with a *P*-value of 0.016. On the other weighing days (SD1, SD 8, SD 22 and

SD 29) the Kruskal-Wallis-One-Way Analysis of Variance test did not show a significant difference in the body weight development (Table 6, Figure 6).

Table 6: Results of Analysis of variance (ANOVA) of the body weight of piglets per group on the different study days.

Study day	*P*-value (α=0.05)
1	0.55
8	0.87
15	0.016
22	0.07
29	0.21

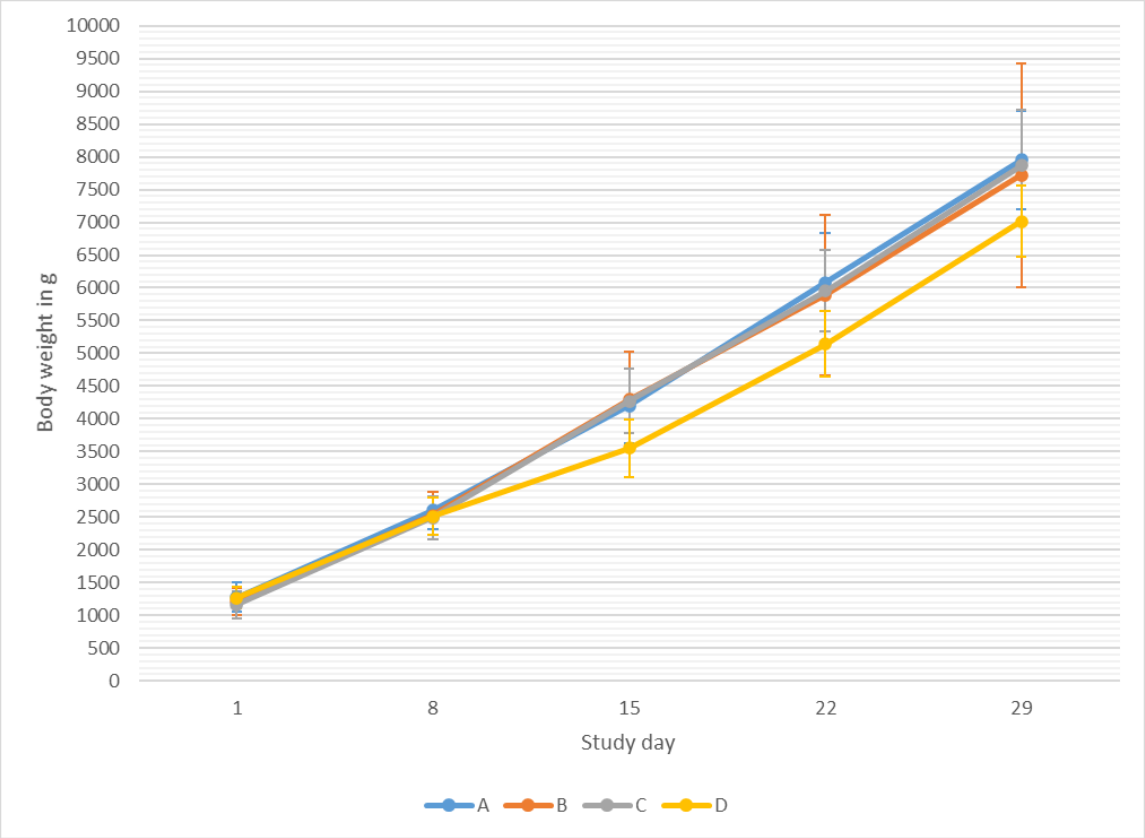

Figure 6: Mean body weight per group and standard deviations on weekly weighing days. For group descriptions see Section 3.1.2 Treatment groups, Table 1.

On average, group BKI-A gained 6680.00 g body weight between SD 1 and SD 29, group BKI-B gained 6507.27 g, group BKI-C 6703.64 g and the control group gained 5744.00 g. During this period, the mean daily weight gain of group BKI-A was 238.57 g and for group BKI-B 232.40 g. During the same time, group BKI-C gained on average 239.42 g per day and the control group D gained 205.14 g daily. Daily weight gains were depressed in the control group in the second week of life and differences in the mean daily body weight gain were only statistically significant between SD 9 and SD 15 (Table 7).

Table 7: Mean daily body weight gain (DBWG) per group in gram during different time periods. sd = standard deviation. SD: study day. For group descriptions see Section 3.1.2 Treatment groups, Table 1.

Group	Mean DBWG SD 1-8	Mean DBWG SD 9-15	Mean DBWG SD 16-22	Mean DBWG SD 23-29	Mean DBWG SD 1-29 [sd]
BKI-A	189.71	227.77	269.09	267.71	238.57 [37.79]
BKI-B	188.05	251.69	228.57	232.40	232.40 [32.60]
BKI-C	190.13	253.25	273.77	239.42	239.42 [35.60]
Control D	177.14	148.86	267.14	267.14	205.14 [52.58]
***P*-value**	0.67	0.0002	0.25	0.97	0.11

4.4. Concomitant infections

Fecal samples pooled by litter on SD 7 were negative for rotavirus and coronavirus. However, a high-grade infection with *E. coli* and *Cl. perfringens* could be detected in all four litters.

4.5. Safety

Since all piglets born had a normal health status and a birth weight of at least 0.9 kg, they were included in the study. All included piglets completed the study except for one piglet of the control group D, which was found dead in the stable on SD 11, due to other reasons not related to the treatment. Furthermore, none of the piglets showed treatment-related adverse effects that required veterinary interventions.

5. Discussion

This study aimed to evaluate whether reduced treatment frequencies with BKI 1369 are effective against experimental infections of suckling piglets with *C. suis*. The fundamental study design was adopted from a previous study (Shrestha et al. 2019). In that study, piglets were treated repeatedly for five days with 10 mg/kg of body weight twice daily, i.e. a total of 20 mg/kg of body weight per day (Shrestha et al. 2019). The same treatment dose was adapted in the present study with slight modifications; instead of 10 mg/kg of body weight of BKI 1369 twice daily for five days, piglets received 20 mg/kg of body weight of BKI 1369 either only once on SD 3 or SD 5 or twice on SD 5 and SD 7.

BKI 1369 has already been tested for efficacy against various other apicomplexan parasites *in vitro* and *in vivo*. Efficacy of BKI 1369 against *Cryptosporidium hominis* was tested in a gnotobiotic pig model, where piglets received BKI 1369 twice daily for five days (Lee et al. 2018). Another study used both, the mouse and new-born calf infection model, to test efficacy of BKI 1369 against *C. parvum* (Hulverson, Choi et al. 2017). In above mentioned studies, repeated applications of BKI 1369 have been shown to inhibit clinical outcomes of disease in the host. In the present study, suckling piglets were used as experimental animals because they belong to the most susceptible age group for infection with *C. suis* and clinical signs of cystoisosporosis can be observed primarily in neonatal piglets (Shrestha et al. 2015). Weaned and older piglets rarely show clinical signs and do not excrete oocysts (Stuart et al. 1982), which are the most important parameters for evaluation of treatment efficacy (Mundt et al. 2006, Joachim et al. 2018a). Therefore, older piglets are not useful in an experimental animal infection model for cystoisosporosis.

The primary parameter used to evaluate efficacy of the investigated product was the oocyst excretion. In order to determine oocyst excretion quantitatively per gram feces, fecal samples were analyzed by a modified McMaster technique (Joachim et al. 2018b). Secondary parameters were the reduction of diarrhea and improvement in the body weight gain. The main hypothesis of the present study was that reduced treatment frequency with BKI 1369 (single or double treatment) is effective in preventing cystoisosporosis in piglets as compared to the sham-treated piglets. This proposition could be verified, since all treated groups showed a statistically significant reduction in McMaster countable oocyst excretion, improved body weight gain during the acute phase of infection as well as reduced occurrence of diarrhea in treated groups BKI-B (single treatment on SD 5) and BKI-C (double treatments on SD 5 and

SD 7). BKI 1369 therefore overcomes the practicability issue associated with multiple dosing and can be considered as a promising new candidate for treatment of neonatal porcine cystoisosporosis.

An anticoccidial is considered to be efficient *in vivo* when oocyst shedding is noticeably reduced (reduced environmental contamination) and animal health (reduced diarrhea) and weight gain are improved compared to the sham-treated control group (Joachim et al. 2018a). In the present study, compared to the control group, the piglets in the treated groups BKI-B and BKI-C which received treatment/s three days post infection showed a significant reduction in oocyst shedding. In the control group, all piglets were positive in AF as well as in McMaster counting at least once. While 18.18% of the piglets in group BKI-B were positive by both AF and McMaster technique, none of the piglets from group BKI-C showed a McMaster countable oocyst shedding, despite of two AF positive samples throughout the screening period. Only McMaster positive samples are considered truly positive since randomization within the litter and the coprophagic nature of the piglets might lead to false positive results in AF (Joachim et al. 2018a). Although treated with the same dose of BKI 1369, more than half of the piglets in group BKI-A were both AF and McMaster positive. In this experiment, BKI 1369 in group BKI-A was applied on the day of infection (SD 3) with the intention to block host cell invasion by sporozoites as in other apicomplexan parasites (Doggett et al. 2014, Ojo et al. 2014). However, the role of BKI 1369 does not seem to be limited only to inhibition of host cell invasion in case of *C. suis,* since piglets in group BKI-A started to excrete oocysts as early as SD 11. Rather it seems that BKI 1369 has inhibitory effects on the development of endogenous stages of *C. suis* and probably as well on cell egress, since there was a significant reduction in oocyst excretion when piglets were treated two days post infection instead of on the day of infection. Oocyst excretion was completely suppressed in group BKI-C which received two applications of BKI 1369 (on SD 5 and SD 7). The suppression of oocyst shedding by BKI 1369 in groups BKI-B and BKI-C is comparable to the effect of toltrazuril (Joachim and Mundt 2011, Skampardonis et al. 2010). Therefore, BKI 1369 could be a potential alternative to toltrazuril, even under field conditions.

Diarrhea is one of the clinical hallmarks of cystoisosporosis (Lindsay and Blagburn 1994, Shrestha et al. 2015). Apart from limiting the environmental contamination with oocysts (Ruzicka and Andrews 1983), effective control of coccidial infections should also ensure limiting epithelial tissue damage, thereby preventing the occurrence of diarrhea. In the present study, the prevalence of diarrhea in group BKI-B and BKI-C was significantly lower compared

to the control group, whereas in the control group and group BKI-A diarrhea started two days after the beginning of oocyst excretion. In cystoisosporosis, more or less simultaneous excretion of oocysts and the occurrence of diarrhea, caused by the destruction of intestinal epithelium during parasite replication followed by malabsorption, is common (Chae et al. 1998, Ruzicka and Andrews 1983, Shrestha et al. 2015). In the control group, the mean FS peaked on SD 12 with 80.00% of the piglets showing a FS >2, while in group BKI-A the mean FS peaked on SD 14 with 40.00% of the piglets showing diarrhea. Although morbidity of cystoisosporosis is very high, mortality is usually low unless it is complicated with secondary infections (Lindsay and Blagburn 1994). Since none of the piglets died during the experiment and diarrhea subsided within a few days of appearance, it can be assumed that diarrhea was caused by *C. suis*. After SD 18, none of the piglets showed diarrhea which might be due to gradual maturation of intestinal epithelium and age-related immunity against *C. suis* (Stuart et al. 1982).

The body weight of the piglets did not differ among the control group and the treated groups on SD 1 and SD 8. On SD 15, treated piglets had a significantly higher body weight and body weight gain (SD 9 to SD 15) compared to the control group. This is in accordance with the occurrence of clinical signs, as piglets in the control group had maximum oocyst excretion and maximum diarrhea days compared to the treated ones during this acute phase of infection. Moreover, the higher the mean FS, the lower the body weight gain in experimentally infected piglets (Mundt et al. 2006, Shrestha et al. 2019). Once the intensity of oocyst excretion decreased, the occurrence of diarrhea and the differences in the mean body weight also decreased. This indicates that the control group gradually recovered from the infection to such an extent that there was no difference in the mean body weight gain in the last week of the experiment. However, at the end of the study the mean body weight of the control group was still lower than that of the BKI-treated groups, although not significant, indicating that the piglets cannot compensate for the weight loss induced by cystoisosporosis at least during the suckling period. A study conducted by Shrestha et al. reported significantly higher daily weight gain and total weight gain in BKI-treated piglets compared to the control group on all days, except for SD 1 and SD 8 (Shrestha et al. 2019), which is in line with the finding of the present study that infections with *C. suis* cause weight gain depression that can be attenuated by BKI application.

Regarding all evaluated parameters, group BKI-B with a single treatment on SD 5 and group BKI-C which received treatment on SD 5 and SD 7 showed statistically significant differences in comparison to the control group. One of the reasons for the unsatisfying results of group

BKI-A could be the early application of the investigated product immediately after infection on SD 3 such that the bioavailability was too low during the acute phase of infection, and hence was not protective. This underlines the importance of the right time of treatment. The outcomes of this current study added to the findings of the previous study by Shrestha et al. (2019) and confirmed that reduced treatment frequencies with BKI 1369 are also effective against experimental infections with *C. suis* making the treatment applicable also in the field.

During the whole animal experiment, only one piglet from the control group was found dead on SD 11, indicating that this incident was not caused by the treatment. Moreover, none of the treated piglets showed signs of treatment-related abnormalities. Therefore, it is confirmed that BKI 1369 does not have any treatment-related adverse effects on the health of piglets. The results are in accordance with the previous study in which none of the piglets treated with BKI 1369 showed treatment-related indications of toxicity nor signs of undesirable reactions with clinical relevance (Shrestha et al. 2019). Furthermore, Lee et al. also could not find significant microscopic lesions in the extra-intestinal tissues of piglets treated with BKI 1369 (Lee et al. 2018).

Considering the efficacy parameters, treatment regimens BKI-B and BKI-C, in which piglets were treated either only once on SD 5 or treated twice on SD 5 and SD 7, were considered best. However, keeping in mind the applicability in the field, treatment regimen BKI-C would mean increased workload for the farmers, as they have to treat all piglets twice, compared to single dosing in treatment regimen BKI-B. Although two piglets in group BKI-B excreted oocysts for three days, oocyst excretion was observed only from SD 16 onwards and also the number of oocysts excreted was far lower compared to the control group. In cystoisosporosis, it takes at least six days from the day of infection until sporulated oocysts are available again for re-infection or infection of other healthy piglets (Mundt et al. 2006, Ruzicka and Andrews 1983). If it is assumed that oocysts excreted by piglets in group BKI-B could have served as a source of environmental contamination, then another phase of oocyst excretion would have been noticeable from SD 22 onwards, which was not the case in the present study and is probably clinically irrelevant due to immunity and age resistance in piglets older than three weeks (Koudela and Kučerová 2000, Worliczek et al. 2009). Therefore, it can be concluded that the treatment scheme of group BKI-B, with a single dose of BKI 1369 on SD 5, is effective enough to inhibit outcome of cystoisosporosis.

The confirmation of toltrazuril resistance in a field isolate of *C. suis* demands identification of alternative control options against cystoisosporosis (Shrestha et al. 2017). The efficacy of BKI 1369 against experimental infections with *C. suis* in suckling piglets has already been validated in the previous study (Shrestha et al. 2019). However, repeated applications of BKI 1369 therapy against cystoisosporosis are not practical in the field. Therefore, as a follow-up of the previous study, the present study has confirmed that if applied at the correct timepoint, even the single dose of BKI 1369 is effective to suppress clinical outcomes of cystoisosporosis.

Nevertheless, in order to develop a practical drug application scheme, it is recommended to perform dose titration experiments in the future, because the currently used dose of 20 mg/kg of body weight of BKI 1369 is quite high, especially for food animals. Moreover, it is indispensable to carryout drug-residue testing experiments to ensure that carcasses from BKI 1369-treated piglets are safe for the consumers as well as to determine drug withdrawal period.

6. Summary

Coccidiosis in neonatal piglets caused by *Cystoisospora suis* is one of the most frequent health problems in intensive pig production. Cystoisosporosis is characterized by diarrhea, wasting and low weaning weight in infected piglets. Morbidity tends to be high, while mortality is moderate. The most commonly used drug to treat cystoisosporosis is toltrazuril, but after confirmation of a toltrazuril-resistance in a field isolate of *C. suis,* there is an urgent need for the identification of therapeutic alternatives. Bumped kinase inhibitors, synthetic competitive inhibitors of ATP-binding, have already been examined for their efficacy against numerous apicomplexan parasites like *Toxoplasma gondii, Neospora caninum* and *Cryptosporidium*. Among different classes of tested BKIs, BKI 1369 so far has been shown to be effective against apicomplexan diseases, including *C. suis*, as well as is considered safe for the hosts. In a previous study, multiple applications of BKI 1369 were effective in controlling cystoisosporosis but repeated dosing seems impracticable in the field, owing to increasing labor costs and stress to the animals. Therefore, in order to test the most appropriate time point of treatment as well as to test efficacy of reduced treatment frequencies with BKI 1369 against *C. suis*, the present study was performed. A total of 42 piglets were allocated to three different treated groups and a control group. All piglets were orally inoculated with 1,000 sporulated oocysts of *C. suis* (strain Wien-I) on study day (SD) 3, three days after birth. Piglets in group BKI-A were treated with 20 mg/kg of body weight of BKI 1369 once on SD 3, group BKI-B received the same dose of treatment but on SD 5, group BKI-C was treated with the same dose twice on SD 5 and SD 7 and the piglets in the control group D were sham-treated. Efficacy of the investigated product was assessed considering oocyst shedding as a primary parameter and the occurrence of diarrhea and the body weight gain as secondary parameters. Fecal samples were collected daily from SD 7-27 to determine oocyst excretion and fecal consistency and the body weight was determined at birth and weekly thereafter. Treatment of piglets twice on SD 5 and SD 7 with 20 mg/kg of body weight of BKI 1369 completely suppressed oocyst excretion. A single treatment on SD 5 suppressed oocyst excretion in 82.00% of the piglets and reduced the quantitative excretion in those that shed oocysts to 98.40% oocysts per gram feces. Moreover, significantly increased body weight gains during the acute phase of infection (SD 8-15) and reduced numbers of diarrhea days were observed in all BKI 1369-treated piglets, compared to the sham-treated control piglets, irrespective of time points and frequencies of treatment. Since the efficacy of reduced treatment frequencies with BKI 1369 are comparable

to repeated applications in the previous study this could be considered as a practical therapeutic alternative against porcine cystoisosporosis, also under field conditions.

7. Zusammenfassung

Behandlung von experimentell mit *Cystoisospora suis* infizierten Saugferkeln mit dem Bumped Kinase Inhibitor BKI 1369 – Folgestudie über reduzierte Behandlungshäufigkeiten

Die Kokzidiose bei neugeborenen Ferkeln, die durch *Cystoisospora suis* verursacht wird, ist eines der häufigsten Gesundheitsprobleme in der intensiven Schweineproduktion. Die Cystoisosporose ist gekennzeichnet durch Durchfall, Kümmern und ein geringes Absetzgewicht infizierter Ferkel. Die Morbidität ist hoch, die Mortalität jedoch mäßig. Das am häufigsten verwendete Medikament zur Behandlung von Cystoisosporose ist Toltrazuril, doch nachdem eine Toltrazuril-Resistenz bei einem Feldisolat von *C. suis* nachgewiesen wurde, besteht ein dringender Bedarf an therapeutischen Alternativen. Bumped Kinase Inhibitoren, synthetische kompetitive Inhibitoren der ATP-Bindung, wurden in Bezug auf ihre Wirksamkeit gegen verschiedene zu den Apicomplexa zählende Parasiten wie *Toxoplasma gondii*, *Neospora caninum* und *Cryptosporidium* bereits untersucht. Unter den verschiedenen untersuchten Klassen von Bumped Kinase Inhibitoren hat sich BKI 1369 als wirksam gegen Krankheiten, die von Apicomplexa ausgelöst werden, bewiesen und ist zusätzlich sicher für den behandelten Wirt. In einer vorangegangenen Studie war die mehrmalige Gabe von BKI 1369 wirksam in der Bekämpfung der Cystoisosporose. Jedoch scheinen die wiederholten Applikationen unter Feldbedingungen wenig praktikabel zu sein, da diese die Arbeitskosten und den Stress, dem die Tiere ausgesetzt werden, erhöhen. Um den bestmöglichen Behandlungszeitpunkt und die Wirksamkeit der reduzierten Behandlungshäufigkeiten mit BKI 1369 gegen *C. suis* zu untersuchen, wurde die hier beschriebene Folgestudie durchgeführt. Insgesamt 42 Ferkel wurden in drei verschiedene Behandlungsgruppen und eine Kontrollgruppe aufgeteilt. Sie alle wurden am dritten Studientag (SD 3), entsprechend dem dritten Lebenstag, oral mit 1.000 sporulierten *C. suis*-Oozysten (Stamm Wien-I) infiziert. Die Ferkel der Gruppe BKI-A wurden mit 20 mg/kg Körpergewicht BKI 1369 einmalig am SD 3 behandelt, die Gruppe BKI-B erhielt die gleiche Behandlungsdosis aber am SD 5, die Gruppe BKI-C wurde in gleicher Dosierung zweimal, nämlich am SD 5 und SD 7, behandelt und die Ferkel der Kontrollgruppe erhielten eine Scheinbehandlung. Die Wirksamkeit des untersuchen Produktes wurde anhand der Oozystenausscheidung als primärer Parameter und dem Auftreten von Durchfall und der Körpergewichtszunahme als sekundäre Parameter bemessen. Kotproben wurden täglich vom 7. bis zum 27. Studientag (SD 7-SD 27) gesammelt, um die Oozystenausscheidung und die Kotkonsistenz zu bestimmen. Zusätzlich wurde das

Körpergewicht am Tag der Geburt und anschließend wöchentlich ermittelt. Die zweimalige Behandlung der Ferkel am fünften und siebten Studientag (SD 5 und SD 7) mit 20 mg/kg Körpergewicht BKI 1369, unterdrückte die Oozystenausscheidung vollständig. Eine einmalige Behandlung am Tag der Infektion (SD 3) oder am fünften Studientag (SD 5) supprimierte die Oozystenausscheidung bei 82,00% der Ferkel und reduzierte die quantitative Ausscheidung der Ferkel, die Oozysten ausschieden auf 98,40% Oozysten pro Gramm Kot. Darüber hinaus konnten einerseits eine signifikant gestiegene Körpergewichtszunahme während der akuten Infektionsphase (SD 8 bis SD 15) und andererseits weniger Tage, an denen die Ferkel Durchfall zeigten, bei allen mit BKI 1369 behandelten Ferkeln im Vergleich zur Kontrollgruppe beobachtet werden, unabhängig von Behandlungszeitpunkt und -frequenzen. Da die Wirksamkeit von reduzierten Behandlungshäufigkeiten mit BKI 1369 vergleichbar mit den wiederholten Behandlungen in der vorangegangenen Studie ist, könnte dies als eine praktikable therapeutische Alternative gegen die Saugferkel-Cystoisosporose, auch unter Feldbedingungen, in Betracht gezogen werden.

8. References

Alvarez-García G, Frey CF, Mora LMO, Schares G. 2013. A century of bovine besnoitiosis: an unknown disease re-emerging in Europe. Trends in Parasitology, 29 (8): 407–415. DOI 10.1016/j.pt.2013.06.002.

Alvarez-García G, García-Lunar P, Gutiérrez-Expósito D, Shkap V, Ortega-Mora LM. 2014. Dynamics of *Besnoitia besnoiti* infection in cattle. Parasitology, 141 (11): 1419–1435. DOI 10.1017/S0031182014000729.

Barber JS, Trees AJ. 1996. Clinical aspects of 27 cases of neosporosis in dogs. The Veterinary Record, 139 (18): 439–443. DOI 10.1136/vr.139.18.439.

Becker A-C, Rohen M, Epe C, Schnieder T. 2012. Prevalence of endoparasites in stray and fostered dogs and cats in Northern Germany. Parasitology Research, 111 (2): 849–857. DOI 10.1007/s00436-012-2909-7.

Bishop AC, Shah K, Liu Y, Witucki L, Kung C-y, Shokat KM. 1998. Design of allele-specific inhibitors to probe protein kinase signaling. Current Biology, 8 (5): 257–266.

Blader IJ, Coleman BI, Chen C-T, Gubbels M-J. 2015. Lytic cycle of *Toxoplasma gondii*: 15 years later. Annual Review of Microbiology, 69: 463–485. DOI 10.1146/annurev-micro-091014-104100.

Bowman DD, Georgi JR, Georgi ME. 2014. Georgis' Parasitology for Veterinarians. 10. Ausg., Elsevier Saunders, St. Louis.

Buehl IE, Prosl H, Mundt AG, Tichy AG, Joachim A. 2006. Canine isosporosis – epidemiology of field and experimental infections. Journal of Veterinary Medicine, 53 (10): 482–487.

Bundesamt für Sicherheit im Gesundheitswesen Österreich. https://aspregister.basg.gv.at/aspregister/faces/aspregister.jspx?_afrLoop=762627112772279&_afrWindowMode=0&_adf.ctrl-state=uzfunjx6f_4 (accessed Aug 15, 2019).

Chae C, Kwon D, Kim O, Min K, Cheon DS, Choi C, Kim B, Suh J. 1998. Diarrhoea in nursing piglets associated with coccidiosis: prevalence, microscopic lesions and coexisting microorganisms. The Veterinary Record, 143 (15): 417–420. DOI 10.1136/vr.143.15.417.

Committee for Medicinal Products for Veterinary Use. https://www.ema.europa.eu/en/documents/product-information/baycox-iron-epar-product-information_de.pdf (accessed Aug 15, 2019).

Daugschies A, Mundt H.-C., Letkova V. 2000. Toltrazuril treatment of cystoisosporosis in dogs under experimental and field conditions. Parasitology Research, 86 (10): 797–799.

Daugschies, A, Bialek, R, Joachim A, Mundt, HC. 2001. Autofluorescence microscopy for the detection of nematode eggs and protozoa, in particular *Isospora suis*, in swine faeces. Parasitology Research 87(5): 409-412

Davies AP, Chalmers RM. 2009. Cryptosporidiosis. British Medical Journal, 339 (7727): 963–967.

Doggett JS, Ojo KK, Fan E, Maly DJ, van Voorhis WC. 2014. Bumped kinase inhibitor 1294 treats established *Toxoplasma gondii* infection. Antimicrobial Agents and Chemotherapy, 58 (6): 3547–3549. DOI 10.1128/AAC.01823-13.

Dubey JP. 2003. Review of *Neospora caninum* and neosporosis in animals. The Korean Journal of Parasitology, 41 (1): 1–16.

Dubey JP. 2005. Neosporosis in cattle. The Veterinary Clinics of North America. Food Animal Practice, 21 (2): 473–483. DOI 10.1016/j.cvfa.2005.03.004.

Dubey JP. 2009. The evolution of the knowledge of cat and dog coccidia. Parasitology, 136 (12): 1469–1475. DOI 10.1017/S003118200900585X.

Dubey JP, Lindsay DS. 2019. Coccidiosis in dogs-100 years of progress. Veterinary Parasitology, 266: 34–55. DOI 10.1016/j.vetpar.2018.12.004.

Eckert J, Friedhoff KT, Zahner H, Deplazes P. 2008. Lehrbuch der Parasitologie für die Tiermedizin. 2. Aufl., Enke, Stuttgart.

Frenkel JK, Dubey JP. 1972. Rodents as vectors for feline coccidia, *Isospora felis* and *Isospora rivolta*. The Journal of Infectious Diseases, 125 (1): 69–72.

Gao X-J, Zhao Z-J, He Z-H, Wang T, Yang T-B, Chen X-G, Shen J-L, Wang Y, Lv F-L, Hide G, Lun Z-R. 2012. *Toxoplasma gondii* infection in pregnant women in China. Parasitology, 139 (2): 139–147. DOI 10.1017/S0031182011001880.

Gimenez F, Hines SA, Evanoff R, Ojo KK, van Voorhis WC, Maly DJ, Vidadala RSR, Mealey RH. 2018. In vitro growth inhibition of *Theileria equi* by bumped kinase inhibitors. Veterinary Parasitology, 251: 90–94. DOI 10.1016/j.vetpar.2017.12.024.

Große Beilage E, Wendt M, Hrsg. 2013. Diagnostik und Gesundheitsmanagement im Schweinebestand. Stuttgart: UTB.

Harleman JH, Meyer RC. 1983. *Isospora suis* infection in piglets. A review. The Veterinary Quarterly, 5 (4): 178–185. DOI 10.1080/01652176.1983.9693894.

Hulverson MA, Choi R, Arnold SLM, Schaefer DA, Hemphill A, McCloskey MC, Betzer DP, Müller J, Vidadala RSR, Whitman GR, Rivas KL, Barrett LK, Hackman RC, Love MS, McNamara CW, Shaughnessy TK, Kondratiuk A, Kurnick M, Banfor PN, Lynch JJ, Freiberg GM, Kempf DJ, Maly DJ, Riggs MW, Ojo KK, van Voorhis WC. 2017. Advances in bumped kinase inhibitors for human and animal therapy for cryptosporidiosis. International Journal for Parasitology, 47 (12): 753–763. DOI 10.1016/j.ijpara.2017.08.006.

Hulverson MA, Vinayak S, Choi R, Schaefer DA, Castellanos-Gonzalez A, Vidadala RSR, Brooks CF, Herbert GT, Betzer DP, Whitman GR, Sparks HN, Arnold SLM, Rivas KL, Barrett LK, White AC, Maly DJ, Riggs MW, Striepen B, van Voorhis WC, Ojo KK. 2017. Bumped kinase inhibitors for cryptosporidiosis therapy. The Journal of Infectious Diseases, 215 (8): 1275–1284. DOI 10.1093/infdis/jix120.

Institut für Veterinärpharmakologie und -toxikologie, Zürich, Switzerland. https://www.vetpharm.uzh.ch/TAK/05000000/00056127.01 (accessed Aug 15, 2019).

Jalovecka M, Sojka D, Ascencio M, Schnittger L. 2019. *Babesia* life cycle - when phylogeny meets biology. Trends in Parasitology, 35 (5): 356–368. DOI 10.1016/j.pt.2019.01.007.

Jiménez-Meléndez A, Ojo KK, Wallace AM, Smith TR, Hemphill A, Balmer V, Regidor-Cerrillo J, Ortega-Mora LM, Hehl AB, Fan E, Maly DJ, van Voorhis WC, Álvarez-García G. 2017. In vitro efficacy of bumped kinase inhibitors against *Besnoitia besnoiti* tachyzoites. International Journal for Parasitology, 47 (12): 811–821. DOI 10.1016/j.ijpara.2017.08.005.

Joachim A, Mundt H-C. 2011. Efficacy of sulfonamides and Baycox(®) against *Isospora suis* in experimental infections of suckling piglets. Parasitology Research, 109 (6): 1653–1659. DOI 10.1007/s00436-011-2438-9.

Joachim A, Schwarz L. Coccidia of Swine: *Eimeria* species, *Cystoisospora* (syn. *Isospora*) *suis*. In: Mehlhorn H, (Hrsg.). Encyclopedia of Parasitology. Berlin; Heidelberg: Springer (2015). S. 253. DOI 10.1007/978-3-642-27769-6_3487-1.

Joachim A, Altreuther G, Bangoura B, Charles S, Daugschies Arwid, Hinney B, Lindsay DS, Mundt H-C, Ocak M, Sotiraki S. 2018a. W A A V P guideline for evaluating the efficacy of anticoccidials in mammals (pigs, dogs, cattle, sheep). Veterinary Parasitology, (253): 102–119.

Joachim A, Ruttkowski B, Sperling D. 2018b. Detection of *Cystoisospora suis* in faeces of suckling piglets - when and how? A comparison of methods. Porcine Health Management, 4 (1): 1–11. DOI 10.1186/s40813-018-0097-2.

Karamon J, Ziomko I, Cencek T, Sroka J, Kozaczynski W, Karpinska T. 2008. Course of experimental *Isospora suis* infection in piglets. Bulletin of the Veterinary Institute in Pulawy, 52 (4): 537–540.

Kaufmann J. 1996. Parasitic infections of domestic animals. A diagnostic manual. Basel, Boston: Birkhäuser Verlag.

Kochanowsky JA, Koshy AA. 2018. *Toxoplasma gondii*. Current Biology, 28 (14): R770-R771. DOI 10.1016/j.cub.2018.05.035.

Koudela B, Kučerová Š. 2000. Immunity against *Isospora suis* in nursing piglets. Parasitology Research, 86 (10): 861–863.

Langkjaer M, Roepstorff A. 2008. Survival of *Isospora suis* oocysts under controlled environmental conditions. Veterinary Parasitology, 152 (3-4): 186–193. DOI 10.1016/j.vetpar.2008.01.006.

Larson ET, Ojo KK, Murphy RC, Johnson SM, Zhang Z, Kim JE, Leibly DJ, Fox AMW, Reid MC, Dale EJ, Perera BGK, Kim J, Hewitt SN, Hol WGJ, Verlinde CLMJ, Fan E, van Voorhis WC, Maly DJ, Merritt EA. 2012. Multiple determinants for selective inhibition of apicomplexan calcium-dependent protein kinase CDPK1. Journal of Medicinal Chemistry, 55 (6): 2803–2810. DOI 10.1021/jm201725v.

Lee S, Ginese M, Beamer G, Danz HR, Girouard DJ, Chapman-Bonofiglio SP, Lee M, Hulverson MA, Choi R, Whitman GR, Ojo KK, Arnold SLM, van Voorhis WC, Tzipori S. 2018. Therapeutic efficacy of bumped kinase inhibitor 1369 in a pig model of acute diarrhea caused by *Cryptosporidium hominis*. Antimicrobial Agents and Chemotherapy, 62 (7). DOI 10.1128/AAC.00147-18.

Lindsay DS, Blagburn BL. 1994. Biology of mammalian *Isospora*. Parasitology Today, 10 (6): 214–220.

Lindsay DS, Stuart B. P., Wheat BE, Ernst JV. 1980. Endogenous development of the swine coccidium, *Isospora suis* Biester 1934. The Journal of Parasitology, 66 (5): 771–779.

Lindsay DS, Current WL, Ernst JV. 1982. Sporogony of *Isospora suis* Biester, 1934 of swine. The Journal of Parasitology, 68 (5): 861–865.

Lindsay DS, Dubey JP, Blagburn BL. 1997. Biology of *Isospora spp*. from humans, nonhuman primates, and domestic animals. Clinical Microbiology Reviews, 10 (1): 19–34.

Lindsay DS, Houk AE, Mitchell SM, Dubey JP. 2014. Developmental biology of *Cystoisospora* (Apicomplexa: Sarcocystidae) monozoic tissue cysts. The Journal of Parasitology, 100 (4): 392–398.

Mitchell SM, Zajac AM, Charles S, Duncan RB, Lindsay DS. 2007. *Cystoisospora canis* Nemeséri, 1959 (syn. *Isospora canis*), infections in dogs: clinical signs, pathogenesis, and reproducible clinical disease in beagle dogs fed oocysts. The Journal of Parasitology, 93 (2): 345–352.

Mundt HC, Mundt-Wüstenberg S, Daugschies A, Joachim A. 2007. Efficacy of various anticoccidials against experimental porcine neonatal isosporosis. Parasitology Research, 100 (2): 401–411. DOI 10.1007/s00436-006-0314-9.

Mundt H-C, Joachim A, Becka M, Daugschies A. 2006. *Isospora suis*: an experimental model for mammalian intestinal coccidiosis. Parasitology Research, 98 (2): 167–175. DOI 10.1007/s00436-005-0030-x.

Murphy RC, Ojo KK, Larson ET, Castellanos-Gonzalez A, Perera BGK, Keyloun KR, Kim JE, Bhandari JG, Muller NR, Verlinde CLMJ, White AC, Merritt EA, van Voorhis WC, Maly DJ. 2010. Discovery of potent and selective inhibitors of calcium-dependent protein kinase 1 (CDPK1) from *C. parvum* and *T. gondii*. ACS Medicinal Chemistry Letters, 1 (7): 331–335. DOI 10.1021/ml100096t.

Noack S, Chapman HD, Selzer PM. 2019. Anticoccidial drugs of the livestock industry. Parasitology Research, 118 (7): 2009–2026. DOI 10.1007/s00436-019-06343-5.

Ojo KK, Reid MC, Kallur Siddaramaiah L, Müller J, Winzer P, Zhang Z, Keyloun KR, Vidadala RSR, Merritt EA, Hol WGJ, Maly DJ, Fan E, van Voorhis WC, Hemphill A. 2014. *Neospora caninum* calcium-dependent protein kinase 1 is an effective drug target for neosporosis therapy. PLOS ONE, 9 (3): e92929. DOI 10.1371/journal.pone.0092929.

Onyiche TE, Suganuma K, Igarashi I, Yokoyama N, Xuan X, Thekisoe O. 2019. A review on equine piroplasmosis: epidemiology, vector ecology, risk factors, host immunity, diagnosis and control. International Journal of Environmental Research and Public Health, 16 (10). DOI 10.3390/ijerph16101736.

Pedroni MJ, Vidadala RSR, Choi R, Keyloun KR, Reid MC, Murphy RC, Barrett LK, van Voorhis WC, Maly DJ, Ojo KK, Lau AOT. 2016. Bumped kinase inhibitor prohibits egression in *Babesia bovis*. Veterinary Parasitology, 215: 22–28. DOI 10.1016/j.vetpar.2015.10.023.

Rojas-Martínez C, Rodríguez-Vivas RI, Millán JVF, Bautista-Garfias CR, Castañeda-Arriola RO, Lira-Amaya JJ, Urióstegui PV, Carrasco JJO, Martínez JAÁ. 2018. Bovine babesiosis: Cattle protected in the field with a frozen vaccine containing *Babesia bovis* and *Babesia bigemina* cultured *in vitro* with a serum-free medium. Parasitology International, 67 (2): 190–195. DOI 10.1016/j.parint.2017.11.004.

Ros Die A, Nogueira Coito JM. 2018. *Isospora belli*. Clinical Microbiology and Infection, 24 (1): 43–44. DOI 10.1016/j.cmi.2017.07.009.

Ruzicka CW, Andrews JJ. 1983. Porcine neonatal coccidiosis: A clinical review. Iowa State University Veterinarian, 45 (2): 90–95.

Sánchez-Sánchez R, Ferre I, Re M, Vázquez P, Ferrer LM, Blanco-Murcia J, Regidor-Cerrillo J, Pizarro Díaz M, González-Huecas M, Tabanera E, García-Lunar P, Benavides J, Castaño P, Hemphill A, Hulverson MA, Whitman GR, Rivas KL, Choi R, Ojo KK, Barrett LK, van Voorhis WC, Ortega-Mora LM. 2018. Safety and efficacy of the bumped kinase inhibitor BKI-1553 in pregnant sheep experimentally infected with *Neospora caninum* tachyzoites. International Journal for Parasitology: Drugs and Drug Resistance, 8 (1): 112–124. DOI 10.1016/j.ijpddr.2018.02.003.

Scala A, Demontis F, Varcasia A, Pipia AP, Poglayen G, Ferrari N, Genchi M. 2009. Toltrazuril and sulphonamide treatment against naturally *Isospora suis* infected suckling piglets: is there an actual profit? Veterinary Parasitology, 163 (4): 362–365. DOI 10.1016/j.vetpar.2009.04.028.

Schaefer DA, Betzer DP, Smith KD, Millman ZG, Michalski HC, Menchaca SE, Zambriski JA, Ojo KK, Hulverson MA, Arnold SLM, Rivas KL, Vidadala RSR, Huang W, Barrett LK, Maly DJ, Fan E, van Voorhis WC, Riggs MW. 2016. Novel bumped kinase inhibitors are safe and effective therapeutics in the calf clinical model for cryptosporidiosis. The Journal of Infectious Diseases, 214 (12): 1856–1864. DOI 10.1093/infdis/jiw488.

Schwarz L, Joachim A, Worliczek HL. 2013. Transfer of *Cystoisospora suis*-specific colostral antibodies and their correlation with the course of neonatal porcine cystoisosporosis. Veterinary Parasitology, 197 (3-4): 487–497. DOI 10.1016/j.vetpar.2013.07.007.

Shahiduzzaman M, Daugschies A. 2012. Therapy and prevention of cryptosporidiosis in animals. Veterinary Parasitology, 188 (3-4): 203–214. DOI 10.1016/j.vetpar.2012.03.052.

Shrestha A, Abd-Elfattah A, Freudenschuss B, Hinney B, Palmieri N, Ruttkowski B, Joachim A. 2015. *Cystoisospora suis* - A model of mammalian cystoisosporosis. Frontiers in Veterinary Science, 2: 68. DOI 10.3389/fvets.2015.00068.

Shrestha A, Freudenschuss B, Jansen R, Hinney B, Ruttkowski B, Joachim A. 2017. Experimentally confirmed toltrazuril resistance in a field isolate of *Cystoisospora suis*. Parasites & Vectors, 10 (1): 317. DOI 10.1186/s13071-017-2257-7.

Shrestha A, Ojo KK, Koston F, Ruttkowski B, Vidadala RSR, Dorr CS, Navaluna ED, Whitman GR, Barrett KF, Barrett LK, Hulverson MA, Choi R, Michaels SA, Maly DJ, Hemphill A, van Voorhis WC, Joachim A. 2019. Bumped kinase inhibitor 1369 is effective against *Cystoisospora suis in vivo* and *in vitro*. International Journal for Parasitology: Drugs and Drug Resistance, 10: 9–19. DOI 10.1016/j.ijpddr.2019.03.004.

Skampardonis V, Sotiraki S, Kostoulas P, Leontides L. 2010. Effect of toltrazuril treatment in nursing piglets naturally infected with *Isospora suis*. Veterinary Parasitology, 172 (1): 46–52. DOI 10.1016/j.vetpar.2010.04.020.

Skinner JI. 1972. Human infection with *Isospora belli* in England: A case report. Journal of Medical Microbiology, 5 (2): 271–273.

Straberg E, Daugschies A. 2007. Control of piglet coccidiosis by chemical disinfection with a cresol-based product (Neopredisan 135-1). Parasitology Research,101 (3):599–604.

Stuart BP, Lindsay DS. 1986. Coccidiosis in Swine. Veterinary Clinics of North America: Food Animal Practice, 2 (2): 455–468. DOI 10.1016/S0749-0720(15)31256-1.

Stuart BP, Sisk DB, Bedell DM, Gosser HS. 1982. Demonstration of immunity against *Isospora suis* in swine. Veterinary Parasitology, 9 (3-4): 185–191.

Taylor MA, Coop RL, Wall R. 2007. Veterinary parasitology. 3. Ausg., Blackwell, Oxford, UK.

Uilenberg G. 2006. *Babesia* - a historical overview. Veterinary Parasitology, 138 (1-2): 3–10. DOI 10.1016/j.vetpar.2006.01.035.

van Voorhis WC, Doggett JS, Parsons M, Hulverson MA, Choi R, Arnold SLM, Riggs MW, Hemphill A, Howe DK, Mealey RH, Lau AOT, Merritt EA, Maly DJ, Fan E, Ojo KK. 2017. Extended-spectrum antiprotozoal bumped kinase inhibitors: A review. Experimental Parasitology, 180: 71–83. DOI 10.1016/j.exppara.2017.01.001.

Winzer P, Müller J, Aguado-Martínez A, Rahman M, Balmer V, Manser V, Ortega-Mora LM, Ojo KK, Fan E, Maly DJ, van Voorhis WC, Hemphill A. 2015. *In vitro* and *in vivo e*ffects of the bumped kinase inhibitor 1294 in the related cyst-forming apicomplexans *Toxoplasma gondii* and *Neospora caninum*. Antimicrobial Agents and Chemotherapy, 59 (10): 6361–6374. DOI 10.1128/AAC.01236-15.

Worliczek HL, Mundt H-C, Ruttkowski B, Joachim A. 2009. Age, not infection dose, determines the outcome of *Isospora suis* infections in suckling piglets. Parasitology Research, 105 (Supplement 1): 157-162. DOI 10.1007/s00436-009-1507-9.

Zimmerman JJ, Dunne HW. 2012. Diseases of Swine. 10. Ausg., Wiley-Blackwell, Chichester, West Sussex, UK.

9. List of Figures and Tables

Acknowledgements

First of all, I would like to thank Prof. Anja Joachim for supervising my diploma thesis and for supporting me whenever I needed help.

Also, I really appreciate all help from Aruna Shrestha, PhD. Not only during the trial, but also afterwards while writing this thesis, she was always on the spot for helping and providing advice, regardless of which kind.

I also want to thank Prof. Andrea Ladinig for reviewing my diploma thesis and for her feedback.

Furthermore, I will miss the time in the stable with Martina Lastufka and Sonja Rohrer. Thank you so much for teaching me how to handle sows and piglets, and for the funny time we had together. Of course, I also don’t want to forget to thank Anna Feix, MSc, and Bärbel Ruttkowski for the collaboration in the laboratory.

Last but not least, I want to thank my whole family for their aid, even they are so far away. Thank you for letting my dream come true and for always being there, whenever I need you.