Experimental *Toxoplasma gondii* infections in pigs: Humoral immune response, estimation of specific IgG avidity and the challenges of reproducing vertical transmission in sows

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Ten pregnant sows were experimentally inoculated per os with *T. gondii* in order to investigate vertical and galactogenic transmission of the parasite and the evolution and maturation of the specific IgG humoral response in the sows and piglets. Five seronegative sows received 10⁶ *T. gondii* (CZ isolate clone H3) sporulated oocysts during late-pregnancy (Exp. 1), three sows received 10⁵ oocysts during mid-pregnancy (Exp. 2) and three sows from Exp. 1 (and two seronegative sows) were re-inoculated with 10⁵ oocysts during a further pregnancy (late-pregnancy) (Exp. 3). Besides, six 4.5 week-old piglets inoculated per os with 5 × 10³ oocysts were also included in the serological investigations. All animals seroconverted (PrioCHECK Toxoplasma Ab porcine ELISA, Prionics, Switzerland) by 2–3 weeks post inoculation (vpi) and remained seropositive for at least 38 weeks or until euthanasia. Four chronically infected sows from Exp. 1 and 2 were serologically monitored during a further pregnancy and no reactivation, but a decrease of the antibody levels was observed at farrowing (Exp. 4). In all experiments, the specific IgG-avidity was initially low, increased during the course of infection and after re-inoculations. An avidity index (AI) ≥40% could be used to rule out recent infections (<8 weeks) in most (15 of 16) animals. In some piglets (18.6% of 70) delivered by inoculated sows (Exp. 1 and 2), maternal antibodies were still detectable at 2 months (but not by 3 months) of age, with constant high avidity values, comparable to those of the dams at farrowing.

In all experiments, the sows remained asymptomatic and delivered non-infected offspring at term. A total of 208 normal and 5 stillborn piglets delivered by the inoculated sows (Exp. 1–4) tested serologically negative before colostrum uptake. Placentas (n = 88) from all sows and tissues (brain, liver, lung, heart, and masseter muscle) from 56 delivered piglets were analysed histopathologically and by real-time PCR for *T. gondii* with negative results. Colostrum and milk samples from all sows were negative by real-time PCR for *T. gondii* DNA. In addition, no seroconversion was observed in 16 piglets from seronegative dams that were transferred to infected dams one day after birth to detect a possible infection through colostrum or milk during the suckling period. Although vertical transmission of *T. gondii* was demonstrated in naturally infected pigs, many factors involved in the outcome of vertical transmission and congenital toxoplasmosis in pigs are still unknown.

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1. Introduction

*Toxoplasma gondii* infections in pigs are commonly asymptomatic; however, several cases of clinical disease have been reported mainly in neonatal and weaned piglets. The main clinical signs observed were anorexia, apathy, fever, cyanosis, ocular
and nasal discharge, dyspnoea, diarrhoea, hind limb weakness and even death. Besides, *T. gondii* may cause reproductive failure in sows characterized by abortion, foetal mummification, stillbirth and neonatal mortality (Dubey, 2009a; Dubey and Beattie, 1988). Numerous cases were registered in Asia (i.e. Japan, Taiwan, China, Korea, Thailand), but there are also some reports from Europe and America (reviewed by Dubey, 2009a; Dubey and Beattie, 1988). It is not known if specific genotypes of the parasite played a role in the clinical presentations. Clinical signs, if present, are supposed to occur during the acute phase of infection. Chronically infected animals do not show any clinical signs, however infected pork represents a major source of *T. gondii* infection for humans (Dubey, 2009a). In humans, *T. gondii* infection can cause severe symptoms in individuals with immature or impaired immune systems such as the developing foetus, or medically immunosuppressed or immunocompromised patients (Kayser et al., 2010; Smith, 1997).

In recent years, a study from Handke et al. (2012) showed that the aetiology of over 50% of the cases of reproductive failure in pigs in Switzerland remained unclear, but there is evidence that *T. gondii* could be involved in part of the undiagnosed reproductive losses. Previous studies showed transmission of *T. gondii* and/or placental infection in 3.5% of 113 sows that aborted or delivered a high number of stillborn or weak piglets in Switzerland (Basso et al., 2015).

Contrary to the vast knowledge about toxoplasmosis in small ruminants, little is known about *T. gondii* infection as cause of reproductive disorder in sows, the epidemiological significance of intrauterine infection in asymptomatic piglets, the importance of Colostral and milk transmission, and the immune mechanisms involved in the control of vertical transmission in chronically infected and in naïve sows.

Therefore, in order to better understand the vertical transmission of *T. gondii* in sows and the humoral immune response of sows and piglets, experimental infections of pregnant gilts were performed, together with a clinical and serological monitoring and further histopathological and molecular examination of the offspring and placentas.

### 2. Materials and methods

#### 2.1. Parasites

All experimental inoculations were performed with the *T. gondii* CZ isolate-clone H3. These parasites derived from the CZ *T. gondii* isolate (type II) (Juráneková et al., 2013) that was obtained in Czech Republic from the faeces of a Siberian tiger. In Switzerland, the parasites were transferred to cell cultures by inoculation of brain tissues from one sheep experimentally infected with oocysts from Czech Republic (Juráneková et al., 2015). The line was maintained in vitro by passing in human foreskin fibroblasts (HFF) for about 3 months and was then cryopreserved. Later, a clonal line (H3) from this isolate was obtained by limiting dilution cloning in HFF. This line was then passaged once in NMRI mice and cats and subsequently once in sheep and cats. Oocysts obtained from faeces of these cats were used for this study. The viability of the *T. gondii* oocysts used as inoculum in the Experiments (Exp.) 1 and 3 (see point 2.2) was confirmed by bioassay in NMRI mice –8 weeks before the inoculation of the sows. Briefly, in each viability test, three groups of 3 mice each were orally inoculated by gavage with dilutions containing 10^4, 10^5 and 10^6 oocysts in 0.3 ml water, respectively. All mice in both assays developed clinical signs (mainly shaggy hair) between 5 and 11 days after inoculation and were subsequently euthanized with CO2. Necropsies were performed and samples from brain, liver, lung and heart tissues were collected and analysed by real-time PCR for *T. gondii* (Cassaing et al., 2006).

For each mouse, 50 mg from each tissue were homogenized in a pool (0.2 g) and DNA was extracted with a commercial kit (QiAmp DNA Mini Kit, QIAGEN GmbH, Hilden, Germany) as described by Glor et al. (2013). Samples from all analysed mice gave positive PCR results (Ct values between 22.6 and 28.5; mean 25.6). Moreover, in some of the mice, *T. gondii* tachyzoites were microscopically visualized in touch imprint slides from lung tissues stained with Diff-Quik®, confirming the viability of the parasites.

#### 2.2. Experimental inoculation of T. gondii

Three experimental inoculations of pregnant sows were performed (Exp. 1–3). In Exp. 1, five gilts (Nr. 1798, 1806, 1818, 1827, 1829), seronegative for *T. gondii* by ELISA (PrioCHECK Toxoplasma Ab porcine, Prionics, Schlieren, Switzerland), were orally inoculated with 10^4 *T. gondii* oocysts diluted in 0.5 ml water during late-pregnancy (79–85 days of pregnancy). Two further seronegative gilts (Nr. 1805 and 1819) in the same stage of pregnancy received only 0.5 ml of water and served as negative controls. In Exp. 2, three gilts (Nr. 1890, 1888, 1874) were inoculated with 10^5 *T. gondii* oocysts during mid-gestation (54–57 days of pregnancy) and one further seronegative gilt (Nr. 1878) in the same stage of pregnancy served as negative control. In Experiment 3, the three remaining of the previously inoculated sows (Nr. 1798, 1818, 1829) and the two negative control sows (Nr. 1805 and 1819) from Exp. 1 were re-inoculated or primo-inoculated, respectively, during a further pregnancy at 75–78 days of gestation (late-pregnancy) with 10^5 oocysts/sow.

After finishing Exp. 2 and 3, one sow from Exp. 2 (Nr. 1890) and three sows from Exp. 3 (Nr. 1798, 1818, 1829) were inseminated for a second or third time, respectively, and were serologically monitored for *T. gondii* infection during pregnancy (without further re-inoculations). In addition, piglets delivered by these sows were serologically tested at birth (beforecolostrum ingestion) in order to detect transplacental infection (Exp. 4).

In all experiments, an ultrasonographical control during pregnancy and a clinical monitoring of the sows and delivered piglets were performed. All animals were held at the experimental facilities of the University of Zurich, in indoor stalls with an outdoor yard and concrete floor and were fed commercial pig feed and hay and water *ad libitum*. During the first week after inoculation, special clothes and boots were used to handle the inoculated sows. One week after inoculation, all bedding material was removed and the boxes were thoroughly cleaned and disinfected with 3% Neopredisan 135–14 in order to eliminate any oocysts from the inoculum that might have passed unexcysted with the faeces.

All animal experiments in swine and the viability assays in mice were authorized by the Cantonal Veterinary Office of Zurich, Switzerland (permission no. 106/2010 and 216/2013) and complied with the current laws of the country.

#### 2.3. Blood sampling

In each experiment, blood samples from the sows were collected from the right *vena cava cranialis* at weeks –1, 1, 2, 3, 4, 8 and 12 after inoculation (Exp. 1–3), or at –1, 9, 14 and 16 weeks after insemination (Exp. 4). Blood from the piglets was taken at birth previous tocolostrum uptake from the umbilical cord, and at weeks 1, 2, 3, 4, 8 and 12 of age from the right *vena cava cranialis* (Exp. 1 and 2) or only at birth (Exp. 3 and 4).

#### 2.4. Serological analyses for IgG against T. gondii

Serum samples collected from the sows and their offspring (stillborn and normally delivered piglets) and colostrum samples were tested for IgG against *T. gondii* using a commercial ELISA kit (Pri-
2.5. Assessment of galactogenic transmission of T. gondii

In order to detect galactogenic transmission of T. gondii, colostrum and milk samples were taken from the sows at farrowing and after 3 days (10 min after oxytocin (Pitocin-10 °, Dr. E. Graeub AG, 1 ml im) administration) for PCR analysis. DNA was extracted from 200 µl of colostrum or milk respectively using a commercial kit (QiAamp DNA Mini Kit, QIAGEN GmbH, Hilden, Germany) following the protocol for blood. Every DNA sample was tested in duplicate. In addition, PCR inhibition was ruled out by a parallel plasmid specific PCR in spiked samples (internal control).

Additionally, with the purpose of detecting seroconversion during the lactation period due to a possible T. gondii galactogenic transmission, 16 piglets born to two seronegative dams in Experiments 1 and 2 (9 piglets from sow Nr. 1819 in Experiment 1 and 7 piglets from sow Nr. 1878 in Experiment 2) were transferred one day after birth (after having ingested colostrum from their own mothers) to five of the experimentally inoculated, seropositive dams that had farrowed simultaneously with the negative sows (Exp. 1: three piglets to each of sows Nr. 1806, 1798 and 1827; Exp. 2: four and three piglets to sows Nr. 1874 and 1890, respectively). The piglets were serologically tested simultaneously with the non-transferred piglets (see point 2.3).

2.6. Histopathological examination and real-time PCR for T. gondii of placentas and tissues of piglets from inoculated sows

A total of 56 piglets derived from inoculated sows in all three experiments were stillborn (n=48), random chosen and euthanized after birth (n=48) or crushed by the dam (n=4) during the first days of life (suppl. Table 1). In order to check transplacental infection by histopathological and molecular methods, necropsies were performed and samples from brain, heart, liver, lung and masinger muscle were collected. Part of these tissues were fixed in 10% buffered-formalin, embedded in paraffin, cut at 2–3 µm sections, stained with H&E and examined histopathologically. Parts from the same tissues were conserved at −20 °C and analysed by real-time PCR for T. gondii DNA (Casaiaga et al., 2006), performing DNA extraction from 0.5 g of each tissue with a commercial kit (QiAamp DNA Mini Kit, QIAGEN GmbH, Hilden, Germany) as described previously (Glor et al., 2013). Additionally, 3 mummies delivered during Exp. 3 were analysed by real-time PCR.

Additionally, a total of 88 placentas (4–14 placenta/sow, mean 6.8; Exp. 1 n = 39, Exp. 2 n = 20; Exp. 3 n = 29) delivered by the inoculated sows in all three experiments and 4 placentas from the control sows in Exp. 1 and 2 were also collected and analysed by histopathology and real-time PCR as indicated above for tissues of piglets.

2.7. Real-time PCR and bioassay in mice of tissues from sows experimentally inoculated with T. gondii

Six of the inoculated sows (Exp. 2: 1874, 1888 and 1890; Exp. 3: 1798, 1819, 1805) were euthanized with overdose of pentobarbital i.v. (Esconaron® Streuli Pharma AG) after anaesthesia with 1.5 mg/kg azaperone i.m. (Stresnil® Provet AG) and 15 mg/kg ketamine i.m. (Narketan® Vetoquinol AG) at five months post inoculation (mpi) (sows Nr. 1874 and 1888), eight mpi (sows Nr. 1805 and 1819), 13 mpi (sow Nr. 1890) or 17 mpi (sow Nr. 1798) (sow Nr. 1798 had been re-inoculated with 107 T. gondii oocysts eight months before euthanasia). Necropsies were performed and heart and brain tissues were collected. Brain tissues from all sows were analysed by real-time PCR for T. gondii DNA (Cassaing et al., 2006), performing DNA extraction from 0.5 g of tissue as described in point 2.6. Subsequently, brains were homogenized in physiological saline with antibiotics while hearts were digested artifically in trypsin. Homogenized brains and digested heart samples were inoculated intraperitoneally (ip) in Swiss Webster mice (two mice/tissue/sow, 1 ml/mouse). Six weeks after inoculation, the mice were euthanized with CO2 blood from the heart was collected for serology and brain tissues were analysed by real–time PCR for T. gondii. Mouse sera were analysed by the modified agglutination test (MAT) at two-fold dilutions beginning from a 1/6 dilution (Djokic et al., 2016). Detailed protocol of the mouse bioassay can be found in the Appendix A of the EFSA report “Experimental studies of T. gondii in the main livestock species” (Opsteegh et al., 2016). The bioassays of swine tissues in mice were authorized by the Ethical committee C2EA-16 (ComEth ANSES/UPEC/ENVA) of France, under the permission no. 21/01/13-2 and complied with the current laws of the country.

2.8. Assessment of specific IgG-avidity to characterize recent and chronic T. gondii infections

In order to diagnose recent or long-term T. gondii infections in sows and piglets, we adapted the commercial ELISA kit (PriCHECK Toxoplasma Ab porcine ELISA, Prionics, Switzerland) to measure specific IgG avidity (functional affinity). The avidity assay is based on the fact that antibodies produced early after an infection have lower binding affinities to the antigen than those produced later on and can be eluted by including an additional incubation step with urea (Björkman et al., 1999). For this, serial serum samples collected from all sows in Exp. 1–3 and from 14 piglets delivered by the sows in Exp. 2 were tested. Additionally, in order to test if there were differences in the immune response between sows and piglets, also serum samples from six 4.5 week-old piglets experimentally infected with 5 × 103 T. gondii oocysts (CZ isolate), collected at weeks 0, 1, 2, 3, 4, 8 and 11 post inoculation (Basso et al., 2013) were included in the study. All absorbance values were normalized by calculating a percentage of positivity (PP) value relative to the O.D. of the positive control. For the assessment of IgG avidity, positive sera were titrated in duplicate (four-fold dilutions starting at 1:12.5) with and without an additional incubation step with 6 M urea in washing buffer (10 min at room temperature) after the incubation of the serum dilutions with the antigen. For each serum, two endpoint titres were determined using PP 15 as cut-off. The avidity index (AI) was calculated as the ratio between endpoint titre with urea/endpoint titre without urea x 100. The end-point titres were calculated by following formula, adapted from Jenum et al. (1997): Endpoint titre = dilution x−1 + 10°, where dilution x is the highest dilution giving a PP ≥ 15 and a is equal to log4x (PPa – 15)/(PPa – PPb), where 4 is the dilution factor, PPa is PP at dilution x, 15 is the cut-off PP, and PPb is the PP at the next higher dilution (+y) from dilution x.
3. Results

3.1. Clinical outcome of the experimental infections

*T. gondii* inoculations did not produce any clinical signs in the sows in any of the experiments. Rectal temperature measured during the first three weeks post inoculation remained within the normal parameters in all cases. All sows delivered at term between 4 and 17 piglets/sow (Suppl. Table 1). Considering experiments 1–3 together, a total of 174 normal piglets, four stillborn and three mummies were delivered by the inoculated sows. The non-inoculated control sows delivered 43 piglets at term (Suppl. Table 1). In Exp. 4, all four chronically infected sows monitored during a further pregnancy without performing *T. gondii* re-inoculations delivered between 13 and 17 piglets/sow at term (Total = 62 normal piglets, 1 stillborn) and did not show any clinical signs (Suppl. Table 1).

3.2. Serological analyses

In Exp. 1, ELISA values (IgG) above the cut-off were first detectable in three of the gilts at two weeks post inoculation (wpi) and in the remaining two gilts at three wpi. Positive values were detected until at least 12 wpi in two sows that had to be euthanized due to claw disease (Nr. 1806 and 1827) or until 38 wpi in the remaining three sows (Nr. 1798, 1818 and 1829), when they were re-inoculated for Exp. 3 (Fig. 1A). In Exp. 2, one of the sows seroconverted at 2 wpi (Nr. 1874) and two sows (Nr. 1888 and 1890) at 3 wpi, and all remained seropositive at least until 22 wpi. Interestingly, a transient decrease of the antibody levels was noticed in all three sows around farrowing (Fig. 2A). Antibody levels in colostrum were generally higher than those in serum at farrowing in the sows in Exp 1 and 2, however, the ratios between ELISA value in serum/ELISA value in colostrum showed a great variation among them (suppl. Fig. 1 and 2). Sows re-inoculated in Exp. 3 showed a notably increase in ELISA values at 1–2 wpi. (Fig. 1A).

All piglets delivered from inoculated and non-inoculated control sows in Exp. 1–3 that could be analysed before colostrum ingestion (150/181 and 41/43, respectively) tested negative for *T. gondii* antibodies by ELISA. After ingestion of colostrum they seroconverted. In 70 piglets from Exp. 1 (n = 49) and 2 (n = 21), which were serologically monitored until 3 months of age, maternal antibodies accounted for positive ELISA values since colostrum ingestion (95.7% seropositive piglets at 7 days of age) until 2 months of age (18.6% of seropositive piglets), however, by 3 months of age maternal antibodies were undetectable in all piglets (Fig. 2C; Suppl. Fig. 1A and Suppl. Table 1).

In Exp. 4, the specific IgG levels of the chronic infected sows seemed to decrease from insemination to farrowing date but remaining above the cut-off (Fig. 4) and no sign of reactivation was noted. All 63 piglets delivered by the four sows and tested were serologically negative at birth (Suppl. Fig. 1).

![Fig. 1. Development of anti- *T. gondii* IgG ELISA values (PP) (A) and specific IgG avidity (AI) (B) in sows (n = 7) experimentally inoculated with 10⁴ and 10⁵ *T. gondii* oocysts during two consecutive pregnancies, respectively (inoculations at late-pregnancy) (Experiments 1 and 3).](image-url)
3.3. Assessment of galactogenic transmission of T. gondii

No seroconversion was observed in the 16 piglets from seronegative dams that were transferred to infected dams one day after birth to detect a possible infection through colostrum or milk in Exp. 1 and 2. Moreover, all colostrum and milk samples analysed by PCR yielded negative results for T. gondii DNA.

3.4. Histopathological examination and real-time PCR for T. gondii of placentas and tissues of piglets from inoculated sows

No histopathological alterations as inflammation or T. gondii parasites were detected by histological examination of 88 placentas from inoculated sows and tissues from 53 normal and 4 stillborn piglets. No positive PCR results were obtained.

3.5. Real-time PCR and bioassay in mice of tissues from sows experimentally inoculated with T. gondii

Real-time PCR analysis of 0.5 g of brain from all sows yielded negative results. However, the success of the experimental infection could be additionally confirmed by bioassay in two sows (Nr. 1874 and 1888) from Exp. 2, euthanized 5 months after inoculation. Four mice (2 mice/sow) inoculated with heart tissues from these sows seroconverted after 4 weeks with MAT titres between 1/200 and 1/6400. Additionally, T. gondii DNA could be detected in mice brains by real-time PCR (Ct values 17–27). Bioassays performed with brain and heart tissues from the further four euthanized sows yielded negative results.

3.6. Assessment of specific IgG-avidity to characterize recent and chronic T. gondii infections

All experimentally inoculated sows and piglets seroconverted by 2–3 wpi (Figs. 1A, 2A and 3A). The specific IgG avidity was low during the first weeks after inoculation and increased during the course of infection in all animals (Figs. 1B, 2B and 3B).

At 4 weeks after primo-infection, all animals showed AI ≥ 7 in all experiments; the sows had mean AIs of 28.7 ± 10.3% and the piglets of 8.9 ± 1.7% (Figs. 1B, 2B and 3B). From 8 wpi, until at least 51 wpi (Exp 1 and 3, Fig. 1B) or 22 wpi (Exp 2, Fig. 2B) the AIs were always ≥40% in all sows. Notably, the AI values increased markedly after re-inoculation at 38 wpi in sows Nr. 1818, 1829 and 1798 (Exp 3, Fig. 1B), they decreased in two of the sows (Nr. 1818 and 1829) during the following 12 weeks, but remaining above 40% in all three animals. The experimentally infected piglets showed AI values ≥40% only from 8 wpi (in three piglets) or later (in two further piglets) (Fig. 3B). Interestingly, one of the piglets had lower (≤28%) AI values during the whole observation period of 11 weeks (Fig. 3B).

All piglets from sows inoculated at mid-pregnancy (Exp. 2) were seronegative at birth and most showed positive ELISA values after colostrum ingestion when first re-tested at 1 week of age (Suppl. Table 2, Fig. 2C). Although the ELISA values decreased over time and were undetectable at 13 weeks of age, the AI remained constantly high (mean 58.3 ± 10.2%) in seropositive piglets when tested at 1, 4 and 8 weeks of age and were comparable to those of the dams at the time of farrowing (Fig. 2D).

4. Discussion

The effect of T. gondii during gestation varies tremendously among the different animal species, and even among very related species such as ruminants. For example, T. gondii causes abortion in sheep only if the primo-infection occurs during pregnancy, and abortion normally occurs only once. In goats, it can cause repeated abortion without reinfection, and in cattle T. gondii is not even considered a cause of abortion (Dubey, 2009b). Therefore, the observations made in other animal species cannot be directly extrapolated to pigs.
In sows, natural cases of congenital toxoplasmosis have been reported worldwide. The main clinical signs of transplacentally acquired toxoplasmosis are abortion, stillbirth, gait abnormalities, dyspnoea, diarrhoea and neonatal death, with presence of *T. gondii* parasites in several tissues (e.g. brain, lung, liver, heart, spleen) (Dubey, 2009a; Dubey and Beattie, 1988). Rarely, clinical signs such as fever, anorexia, neurological signs and even death were observed in sows that aborted and transmitted the infection to the foetuses (Kim et al., 2009). The association of *T. gondii* infection with reproductive disorders in sows was pointed out in a large serosurvey performed in 94 pig farms in Germany, in which it was observed that the within-herd prevalence of antibodies against *T. gondii* was significantly higher in farms with reproductive disorders than in those without such problems (Damriyasa et al., 2004). Recently, *T. gondii* infection was detected in placenta and/or foetuses from 3.5% of 113 sows on Swiss pig farms that aborted or delivered a high number of stillborn or weak piglets (Basso et al., 2015). However, a review of the literature suggested that it is difficult to consistently reproduce congenital toxoplasmosis in pigs experimentally (Dubey, 1986, 2009a). So far, a number of experimental infections in pregnant sows have been performed with variable results (Boch et al., 1965; Dubey et al., 1990; Dubey and Urban, 1990; Moller et al., 1970; Sanger and Cole, 1955; Work et al., 1970). The stage of *T. gondii*, route of inoculation, stage of gestation and breed of the sow have been suggested as possible factors that might influence the outcome of the infection (Dubey, 1986, 2009a) but also the infection dose and the virulence and genetic background of the *T. gondii* strains used in the experiments might account for the differences observed.

In this study, although different time points and inoculation doses were evaluated, inoculations of pregnant sows with *T. gondii* oocysts resulted always in seroconversion of the dams but not in clinical signs or foetal infection. In the first Experiment, the inoculation was performed late in pregnancy as it was previously suggested that porcine placenta would be more permeable to *T. gondii* during the later stages of pregnancy (De Meuter et al., 1975; Dubey and Urban, 1990; Work et al., 1970) but no transplacental transmission could be reproduced (Exp. 1 and 3). Therefore, in Exp. 2 the sows were inoculated in mid-pregnancy as reported by Jungersen et al. (2001) but also in this case the inoculation did not lead to infection of the offspring. Besides, a 10 times enhancement of the inoculation dose (from $10^4$ oocysts in Exp. 1 and 2–$10^5$
oocysts in Exp. 3) did not influence the clinical outcome of infection. Although due to technical reasons only descending doses up to 10 oocysts but not doses containing only one oocyst were tested for vitality (and thus potentially not all 100% inoculated oocysts might have been viable), the vitality of at least $10^3$ or $10^4$ oocysts/inoculation dose (higher than in many other studies) can be certainly assumed.

All new-born piglets from inoculated sows that were analysed in Exp. 1–4 ($n = 213$) tested seronegative before colostrum ingestion and no *T. gondii* parasites (or DNA) could be detected by histopathological observation and real-time PCR in the tissues of the tested piglets ($n = 59$). It is possible that different *T. gondii* strains could be responsible for different clinical outcomes in pigs.

Similarly, in the study by Durfee et al. (1974), the inoculation of 10 sows in early pregnancy (between 28–53 days) with 10–150 oocysts of the P–800 *T. gondii* strain (isolated from a sow) lead to infection of the sows without causing any clinical signs or abortion. However, the dose used in the study from Durfee et al. (1974) was much lower than that used in the present study. Nonetheless, they showed that the inoculation of a higher oocysts dose (1000–2000) of this same strain produced severe clinical toxoplasmosis with fever over 41 °C after 3 dpi in a further gilt. In the present study, also much higher doses of a different *T. gondii* strain did not cause clinical signs in the sows. In the study from Dubey and Urban (1990) a lower inoculation dose than in this study ($10^3$ oocysts) but different *T. gondii* strains (PT-1 or GT-1), were used. Oral inoculations with the GT-1 strain lead to transplacental infection in 5/11 pregnant sows, while *T. gondii* was found in only one placenta from 1/6 sows inoculated with the PT-1 strain and none of the piglets was infected. GT-1 is a highly virulent type I strain, which consistently induced transplacental transmission in sheep and goats and was pathogenic for mice (Dubey, 1980; Su et al., 2002), whereas the strain used in the present study is a typical European type II strain, and appears to be low virulent (Basso et al., 2013; Juráňková et al., 2013, 2015). While Dubey and Urban (1990) reported lethargy, anorexia and respiratory distress in the inoculated sows between 5 and 15 dpi, the sows in this study did not develop any clinical signs. Also high inoculation doses ($5 \times 10^2$ oocysts) of the CZ isolate in very young piglets (4.5 week-old) caused only fever for a few days and only 1 out of 6 piglets showed apathy, anorexia and soft faeces for 2 days as only clinical signs, suggesting a low virulence of this isolate for pigs (Basso et al., 2013). Differences in pathogenicity among *T. gondii* strains isolated from different hosts were also observed in experimental infections of 7-week-old pigs and pregnant minipig gilts by Jungersen et al. (1999, 2001) in Denmark. Pigs inoculated i.v. with $10^4$ tachyzoites of four *T. gondii* strains (i.e. NED: type III and SSI 119, SVS P14 and SVS Fox2: Type II) developed fever for some days, while pigs inoculated with a low pathogenic strain (SVS O14 strain: Type II) showed normal body temperature during the whole observation period, as it occurred in the sows in the present study (Jungersen et al., 2001; Jungersen et al., 1999). Interestingly, the same strains inoculated i.v. (at a 3 times higher dose) into pregnant minipig gilts evidenced a different pathogenicity: two of the strains (SVS P14 and Fox2) lead to severe illness of the sows with abortion of non-infected foetuses; one strain (SSI 119) produced mild or no illness of the sows causing transplacental infection and sometimes foetal mummification, and two strains (NED and SVS O14) caused no clinical signs of disease in the sows but lead to transplacental infection of the foetuses (Jungersen et al., 2001). The authors hypothesised that *T. gondii* strains causing a slowly progressing infection in the dam may have a better chance of establishing placental infections followed by transplacental transmission and congenital toxoplasmosis in the piglets before development of a limiting immune response in the sow (Jungersen et al., 2001) and considered low pathogenic *T. gondii* strains as good candidates to reproduce vertical transmissions in sows. However, in contrast to the present study, in which a natural route of infection was chosen, the experimental infections in Denmark were performed by the i.v. route, what may have contributed to the severity of the clinical signs observed, enhancing the potential of low virulent strains to propagate and leading to transplacental infection of the foetuses. Also Work et al. (1970) could reproduce severe toxoplasmosis in four sows (with fever, anorexia, dyspnoea and even death) and transplacental infection (with foetal death, stillbirth and perinatal mortality) after i.v. inoculation of high numbers ($5.5 \times 10^6$ to $2 \times 10^8$) of tachyzoites of the virulent *T. gondii* RH strain (Type I).

![Fig. 4. Evolution of anti-*T. gondii* IgG ELISA values (PP) during pregnancy in sows chronically infected with *T. gondii* (*n* = 4) without experimental re-inoculation during the observation period (Experiment 4).](image-url)
during the third month of pregnancy (Work et al., 1970). It seems that transplacental transmission of \textit{T. gondii} in pregnant sows can be more frequently reproduced after i.v. inoculation of tachyzoites than after oral administration of tissue cysts or oocysts, and also the development of clinical manifestations in the sows was more commonly seen after i.v. inoculations of high infection doses. However, the use of an unnatural route may not exactly reproduce the pathogenesis of natural infection cases, what could be a limitation, depending of the specific objectives followed with the experimental model. Therefore, the variations in the intrinsic proliferation potential of different \textit{T. gondii} strains, as it has been observed in vitro (Jungersen et al., 1999), but also the infection dose and route of infection could influence the clinical outcome of experimental infections.

The success of the infection of the sows in this study was confirmed by serology in all sows and by bioassay in mice in two of six tested sows. Demonstration of \textit{T. gondii} in the tissues of pigs is considered difficult (Dubey, 2009a); however, the possibilities of parasite detection could have been probably enhanced by performing bioassay in cats, which can be fed higher amounts of tissues, by inoculation of a higher amount of mice/sample or by using immunosuppressed or gamma interferon knockout mice that showed a higher susceptibility to the infection with cyst-forming coccidia (Basso et al., 2009).

Transmission of \textit{T. gondii} through colostrum in pigs was described by Sanger and Cole (1955) in the U.S., who reported the isolation of \textit{T. gondii} in mice inoculated (i.p.) with colostrum from two sows, presumably chronically infected (toxoplasmin-skin test positive) before breeding, and from one sow experimentally infected by three routes (i.e. orally, s.c. and i.m.), at the end of gestation. However, experiments performed before the life cycle of \textit{T. gondii} was completely understood and reliable serological tests were available, should be regarded with care, because the infection-free status of the experimental animals employed as well as the prevention of infection from external sources during the experiments might have not been always guaranteed. Boch et al. (1965) could not isolate \textit{T. gondii} from colostrum of experimentally infected sows (i.e: eight sows inoculated at different times before pregnancy, seven chronically infected sows re-inoculated during pregnancy and four sows primo-inoculated during pregnancy) by bioassay in mice, although high inoculation doses were used (feeding of infected mice and/or i.p. inoculation of brain and/or peritoneal exudate from infected mice). Neither could the authors reproduce transplacental transmission in any of the sows (Boch et al., 1965). In this study, all colostrum samples investigated by PCR yielded negative results. Moreover, no seroconversion was observed in piglets from seronegative dams that were transferred to infected dams one day after birth and monitored serologically during the suckling and weaning periods, excluding the possibility of a postnatal infection through ingestion of colostrum and milk. Thus the occurrence of galactogenic transmission of \textit{T. gondii} from the sow to the piglets might be an uncommon event.

According to the study by Garcia-Bocanegra et al. (2010) in \textit{T. gondii} naturally infected pigs in Spain, the persistence of maternal derived antibodies in piglets was directly correlated with the antibody levels of the dam, being highest (up to 6 weeks) in piglets whose dam had high \textit{T. gondii} antibody level (MAT $\geq 1:500$), while in piglets born from sows with low antibody titres ($\leq 1:50$) the persistence lasted only up to 3 weeks of age (Garcia-Bocanegra et al., 2010). In the present study (using ELISA instead of MAT), we could observe that maternal antibodies lasted longer in some of the piglets. At 8 weeks of age up to 12.2% and 33.3% of the piglets in Exp. 1 and 2, respectively still had detectable maternal antibodies (ELISA values $\geq 15$ PP), what could confound a serological diagnosis at that age (Fig. 2C; suppl. Fig. 1A; suppl. Table 2). Maternal antibodies disappeared by 3 months of age, in agreement with observations from Dubey and Urban (1990). Therefore, this fact should be considered when performing serological monitoring for \textit{T. gondii} infection in young pigs. Also in this study, the persistence and levels of maternal antibodies in the piglets correlated with the serum antibody levels of the dams at farrowing; however, the correlation appeared to be better when the antibody levels in colostrum were considered (Fig. 2C; suppl. Fig. 1A and B; suppl. Fig. 2). However, other factors related to the sow (e.g. maternal instinct, excitement, aggressiveness towards the piglets, colostrum production), the new born piglets (e.g. maturity, size, weakness) or management factors that would prevent the early contact between piglets and their dams (and subsequently the colostrum uptake during the first hours of life), could influence the levels of maternal antibodies in the piglets. Interestingly, a transient decrease of serum antibody levels around farrowing was noticed in the sows in Exp. 2, which had been infected around 8 weeks before farrowing (Fig. 2A). This fact might be related with colostrum production. Also the chronically infected sows, monitored during a further pregnancy without re-infections, showed a decrease in serum antibody levels at farrowing, however, they were not controlled afterwards.

Routine serological assays used to detect antibodies (IgG) against \textit{T. gondii} in pigs do not distinguish between recent and chronic subclinical infections. The level of specific IgG alone is not a reliable indicator of infection acuteness. Nonetheless, the estimation of the moment of infection might be desired in some occasions, e.g. in the diagnosis of clinical toxoplasmosis, investigation of disease breaks or in epidemiological studies. The measurement of specific IgG-avidity (functional affinity) can aid in the diagnosis of recent or longstanding \textit{T. gondii} infections. Antibody avidity has been defined to be the overall strength of binding affinities between multivalent antigens and their antibodies (Dimitrov et al., 2011).

Usually, after a primary antigenic challenge, IgG avidity is initially low, and, by antigen-driven B cell selection, it increases during the following weeks and months (Lappalainen and Hedman, 2004); IgG antibodies produced after a secondary antigenic challenge would typically exhibit high avidity. It is known that with repeated exposures to the same antigen, a host will produce antibodies of successively greater affinities for the antigen. This process primarily occurs on surface immunoglobulin of B cells in the centres of the secondary lymphatic organs as a direct result of somatic hypermutation and selection by TH cells. Therefore, in a secondary immune response, antibodies with higher affinity for the antigen may be expected, accounting for higher AIs (Kontio et al., 2012 Steward and Chargeleug, 1997).

Avidity tests have been used in the diagnosis of toxoplasmosis in humans (Jenum et al., 1997; Lappalainen and Hedman, 2004) and sheep (Sager et al., 2003) and also in the investigation of epidemic abortion outbreaks due to Neospora caninum infection, which were associated with the presence of low avidity antibodies in the aborted cows (Basso et al., 2010; Schaeres et al., 2002). However, this diagnostic approach has been only rarely intended for toxoplasmosis in pigs (Suarez-Aranda et al., 2000). In this study, we adapted a commercial ELISA kit to measure specific IgG avidity against \textit{T. gondii} in pigs by adding an extra step (i.e. incubation with 6 M urea in washing solution for 10 min) after serum-antigen incubation, in order to dissociate the antigen-antibody bond of low avidity IgG. Different ways of for the calculation of IgG avidity have been described (Lappalainen and Hedman, 2004). In this study, an antibody titration with and without urea treatment was performed for each serum sample and both end-point titres (with/without urea) were compared, being the IgG avidity expressed as “avidity index” (in percent). This procedure is more time- and reagent-consuming than estimations on single serum dilutions (Suarez-Aranda et al., 2000) but has the advantage that the results are independent of the IgG concentration in the sample and therefore more accurate.
However, care should be taken with the interpretation of individual ALs, thus in some cases even slight variations of the ELISA PP (or OD) values used for the AI calculation could account for certain fluctuation of the ALs. In general, low AI values are suggestive of recent infections (Basso et al., 2010; Sager et al., 2003; Schares et al., 2002) and this was also observed in this study; however, in some individual cases, low avidity values can persist for longer periods, as it was observed in one of the experimentally infected pigs in this study, which had low avidity values for at least 11 weeks (Fig. 3B). On the other side, a high AI (>40%) could be effectively used to rule out a recent (<8 weeks) infection in most animals. In this study, all experimentally infected pigs and all but one sow first showed AI > 40 at 8 wpi or even later (Figs. 1B, 2B and 3B). Therefore, as it is the consensus for diagnosis of toxoplasmosis in humans, the measurement of IgG avidity in individual cases would serve best in ruling out (by high avidity) rather than in ruling in (by low avidity) a recently acquired infection (Lappalainen and Hedman, 2004).

Once avidity has matured, high values would persist over time, and the test would no longer provide consistent information about the time point of infection (Sager et al., 2003). In this study, two sows (Nr. 1818 and 1829) showed a decrease of ALs over the time after initial peaks following the first and second T. gondii inoculations. However, the AI values remained over 40%. This could be related to an aging of pre-existent specific IgG antibodies in absence of new infections or reactivation of tissue cysts. A decrease in specific IgG ALs over the time in absence of new antigen challenge was also observed by Kontio et al. (2012) in humans that were vaccinated twice with a measles, mumps and rubella (MMR) vaccine at 14–18 months and 6 years of age and re-tested 20 years after the second vaccination. The antibody levels for all 3 viruses waned and while the mean avidity index remained unchanged for rubella, it decreased by 8% for measles and 24% for mumps (Kontio et al., 2012).

On the other side, production of high avidity-antibodies will be expected in case of a secondary antigen challenge, as we could observe in the sows in Exp. 3, which showed a notable increase in the AI values after re-inoculation (Fig. 1B) suggesting further avidity maturation. Therefore, the IgG avidity-ELISA might represent a valuable diagnostic tool for epidemiological and immunological studies and for diagnosis of clinical T. gondii infections in pigs, aiding in the estimation of the time point of infection.

Conflict of interest
The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data
Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vetpar.2017.01.026.

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