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Detection of antibodies to *Toxoplasma gondii* in oral fluid from pigs

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ABSTRACT

Toxoplasma gondii-infected pigs play a major role as a source of infection for humans and detection of high-risk herds is essential to implement control measures at the farm level. The aim of this study was to determine whether oral fluid (OF) could be used as a matrix to detect antibodies against *T. gondii* in infected pigs by immunoblot (IB). For this, OF from experimentally inoculated sows (n = 8) (serial samples) and naturally exposed group-housed fatteners (n = 42 groups, one sample/group) were analysed for IgG and IgA against *T. gondii*-SAG1 antigen by IB. Simultaneously, each animal was serologically tested for anti-*T. gondii* IgG by ELISA. Specific IgG was detected in the sera of all inoculated sows from 2 to 3 weeks post inoculation (pi) and in 3.4 to 92% of the pigs in 13 out of 42 groups. Experimentally inoculated sows showed positive OF-IB results for IgA (100%) and IgG (87.5%) at 1.5 weeks pi and continued yielding positive results for IgA (87.5–75%) and IgG (50%) until 4 weeks pi; however, from 8 weeks pi the frequency of detection of both isotypes was lower, despite constantly positive IgG values in serum-ELISA. Interestingly, consecutive daily samplings for 4 days at 13 and 30 weeks pi showed inconsistent results for some sows, showing that the antibody concentration in OF is prone to timely variations. Pooled OF from groups with 91 and 92% of seropositive pigs yielded positive IB results for IgG and IgA. Fattener groups with ≤13% of seropositive pigs gave negative IB results to both isotypes. Our results showed that antibodies to *T. gondii* can be detected in OF from infected pigs, and that IgA seems to be a more adequate target than IgG. Although OF does not seem to be a robust matrix to assess the serological status for *T. gondii* in individual animals, this diagnostic approach represents an interesting non-invasive, low-cost and animal welfare friendly option as a screening method at the farm level to determine high exposure to *T. gondii* in the herd.

1. Introduction

Toxoplasma gondii (Apicomplexa, Sarcocystidae) is one of the most widespread zoonotic parasites on earth and may potentially affect all warm-blooded species. It is responsible for abortion and disease in many domestic and wild animals (Dubey, 2009; Stelzer et al., 2019) and can be the cause of serious illness and death in humans with underdeveloped or impaired immune systems. If the primary infection occurs during pregnancy, it may be vertically transmitted, producing foetal lesions (e.g. hydrocephalus, microcephalus, intracerebral calcifications and chorioretinitis), stillbirth and abortion. Besides, *T. gondii* can produce life-threatening illness and encephalitis in immunocompromised patients, and ocular disease and blindness both in immunocompromised and immunocompetent humans after congenital and post-natally acquired infections (Maenz et al., 2014; EFSA, 2018; Pardini et al., 2018). In pigs, the infection is mainly acquired by the ingestion of food/water contaminated with oocysts, eliminated by infected felids with their faeces during patency. Alternatively, pigs can become infected by ingestion of intermediate hosts harboring tissue cysts (e.g. rodents, birds) or, less frequently, congenitally (Stelzer et al., 2019). Although *T. gondii* infection in pigs has been associated with clinical disease in young animals and reproductive failure in sows, in most cases the infection is subclinical (Dubey, 2009; Basso et al., 2015; Stelzer et al., 2019). In infected hosts, the parasite persists quiescently within tissue cysts mainly

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located in neural and muscular tissues, probably for life. Therefore, chronically infected pigs (and other meat producing animals) represent an important source of *T. gondii* infection for humans through consumption of undercooked meat (Broglia and Basso, 2014). The European Food Safety Authority (EFSA) recognized toxoplasmosis as the parasitic zoonosis with the highest human incidence (EFSA, 2007) and as one of the major causes of food-borne disease worldwide (EFSA, 2018).

Pigs are not routinely tested for *T. gondii* infection at slaughter because current meat inspection practices do not allow the detection of tissue cysts due to their small size (generally < 100 μm) (EFSA, 2018) and no rapid diagnostic test at slaughter has yet been established. Currently, serological tests represent the most commonly used diagnostic tools to detect *T. gondii* infections in pigs (Basso et al., 2013), and a good correlation between seropositivity and the presence of cysts in the tissues was observed; however, tissue cysts were also found in approximately 5% of seronegative pigs tested by the modified agglutination test and Western blot (Dubey et al., 2002), showing that testing of individual animals may have limitations. Therefore, screening at the farm level was proposed as a valid option to identify high risk herds in order to implement control measures to reduce the prevalence of infection (EFSA, 2018).

In recent years, sampling of oral fluid (OF) for diagnostic purposes in pigs using cotton ropes has gained interest and OF already has been used as a matrix for screening of several viral and bacterial swine infections, either by direct detection of pathogens or by indirect detection of specific antibodies (IgA and IgG) (Prickett and Zimmerman, 2010; Rotolo et al., 2017). Oral fluid is a combination of saliva and serum transudates from capillaries in the oral mucosa (oral mucosal transudate) and gingival tissues (gingival crevicular fluid) (Delima and Van Dyke, 2003; Prickett and Zimmerman, 2010; Kittawornrat et al., 2014). The junction between the teeth and the mucosa (gingival crevice) allows blood components (e.g. immunoglobulins and blood cells) greater access to the mucosal surface than in any other part of the mucosal system (Challacombe and Shirlaw, 1994). Immunglobulins present in OF cannot only derive from the passage of serum antibodies (mainly IgG, but also IgA, IgM) of the systemic immune system, but they also can be locally produced (mainly IgA but also IgG and IgM at low levels) by blood-derived plasma cells of the secretory immune system in the major salivary glands (parotid, mandibular and sublingual glands) and duct-associated lymphoid tissue (DALT) of minor salivary glands distributed around the oral cavity (Kittawornrat et al., 2010, 2014; Prickett and Zimmerman, 2010).

Antibodies against *T. gondii* in saliva were already detected in seropositive humans, showing a good correlation with the serological status (Stroehle et al., 2005), but to our knowledge, no similar studies in pigs have been reported to date.

The aim of this work was to determine the presence of antibodies against *T. gondii* antigen in oral fluid samples from experimentally inoculated and naturally exposed pigs from Switzerland by immunoblot (IB) and to determine if this method could represent an alternative to standard serology.

2. Materials and methods

2.1. Animals (oral fluid and serum samples)

Serial OF samples were collected from eight sows experimentally inoculated with 10^4 sporulated *T. gondii* oocysts (CZ isolate-clone H3) during pregnancy, and from three negative control sows (Basso et al., 2017) at different time points after inoculation (i.e. at 1, 1.5, 2, 3, 4, 8, 12 and 13 or 30 weeks post inoculation (pi)) (Fig. 1) (Table 1).

Additionally, pooled OF samples were obtained from group-housed fattening pigs aged three to six months from 10 farms in Switzerland (n = 42 groups with three to 29 pigs, average 13 animals/group; 552 pigs in total).

OF samples from each sow were collected using cotton gauze swabs (three layers of 10 × 10 cm gauze, IVF Hartmann AG, Switzerland). For this, the swabs were held inside the mouths of the sows for several minutes for them to chew, using 30 cm long stainless steel forceps. Afterwards, the swabs were placed on a

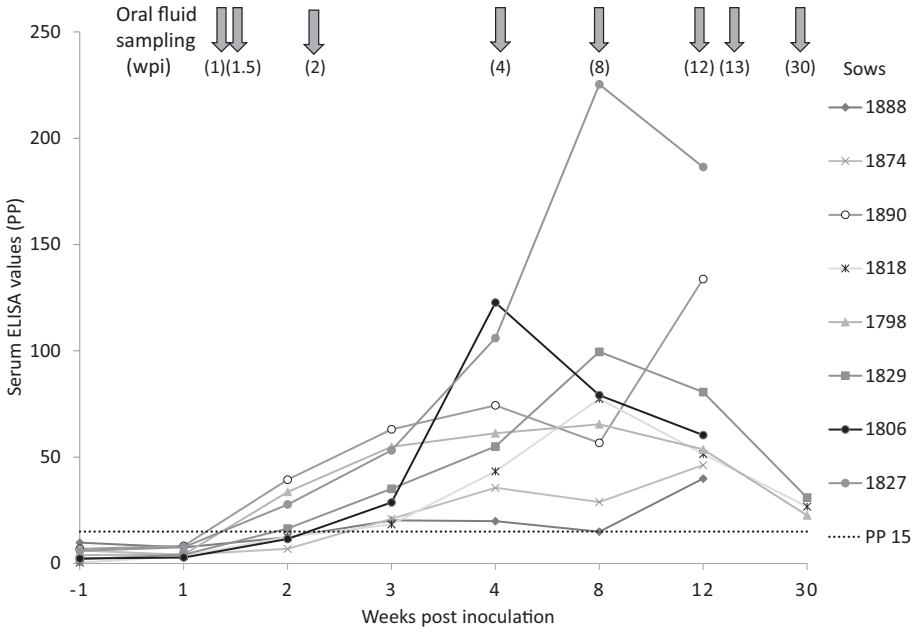

Fig. 1. Anti-*Toxoplasma gondii* IgG response in sows (n = 8) experimentally inoculated with 10^4 *T. gondii* oocysts in ELISA (PrioCHECK™ Porcine Toxoplasma Ab Kit) over time, and time points of oral fluid sampling. wpi, weeks after inoculation; PP, percentage of positivity relative to the reaction of the positive control (PP sample = O.D. 450 nm sample/O.D. 450 nm positive control × 100). Three parallelly tested naïve sows (Sows Nos. 1878, 1805 and 1819) yielded ELISA values below the cut-off during the whole study and were not included in the figure for a better visualization of the antibody response over time in the inoculated animals.

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Table 1
Immunoblot results for IgA and IgG against TgSAG1 antigen in oral fluid samples from sows experimentally inoculated with *Toxoplasma gondii*.

wpi (dpi)	0 (0)	1 (7)	1.5 (10)	2 (14)	3 (21)	4 (28)	8 (56)	12 (84)	13 (88)	13 (89)	13 (90)	13 (91)	30 (208)	30 (209)	30 (210)	30 (211)	Ig isotype
Sow 1888	–	–	+	++	+	+	+		+/–	+/–	++						IgA
	–	–	+	++	+	–	–		–	–	++						IgG
Sow 1874	–	–	+	+	+	+/–	–		+/–	+/–	+	–					IgA
	–	–	+	+	+	+/–	–		–	–	+/–	–					IgG
Sow 1890		–	+/–	+	+	–	+/–				+	+/–					IgA
		–	–	+	+/–	–	–				–	–					IgG
Sow 1818			+	+	+	+	+/–	+						+	+/–	–	IgA
			+	+	–	+	–	–						–	–	–	IgG
Sow 1798			+/–	+/–	–	+/–	–	–					–	–	–	–	IgA
			+/–	–	–	+/–	–	–					–	–	–	–	IgG
Sow 1829			++	–	–		–	+/–					–	–	–	–	IgA
			++	–	–		–	+/–					–	–	–	–	IgG
Sow 1806			+	+	+	+	–	–									IgA
			+/–	–	–	–	–	–									IgG
Sow 1827			+	+/–	+	+											IgA
			+	–	+	+											IgG

wpi, weeks post inoculation; dpi, days post inoculation; blank, sample not available; +/–, weak positive sample; +, positive sample; ++, strong positive sample; –, negative sample.

new piece of gauze fixed to the upper part of a 50 ml plastic tube, transported to the laboratory on ice in a polystyrene foam container and centrifugated at 500 *g* for 5 min at 4 °C. The OF was subsequently collected from the tube bottom and frozen at −20 °C until analysis. The time between collection and freezing was 1–3 h.

Pooled OF samples from the fattening pigs were obtained by a three-strand-twisted cotton rope of 30–40 cm length with a diameter of 12 mm (Seilerei Kislig, Winterthur, Switzerland), hanging at shoulder height into the pens for 45–60 min (Fig. 2). For each pen, one rope (groups $\leq$ 20 animals) or two (groups with 21–29 animals) were used (when two ropes were used, both OF samples were pooled after collection). After that time, the ropes were cut, individually placed into single-use plastic bags, transported to the laboratory and refrigerated using ice packs within 1–3 h. Oral fluid was extracted manually by squeezing the rope inside the plastic bag or with a press roller, and collected into a 10 ml plastic tube by cutting a bottom corner of the bag, refrigerated at 4 °C overnight and afterwards aliquoted and conserved at −20 °C until testing for antibodies against *T. gondii*.

As a standard of comparison, blood samples from the sows and from every fattening pig in each group were collected on the same day from the vena cava cranialis for serological analysis.

All animal experiments were authorized by the Cantonal Veterinary Offices of Zurich, (permission no. ZH 216/2013) and Luzern, Switzerland (permission no. LU 03/2014) and complied with Swiss Animal Welfare guidelines.

Fig. 2. Group of fattening pigs chewing at the cotton rope in the pen.

2.2. ELISA PrioCHECK™ porcine Toxoplasma Ab Kit

Serum samples from all animals were individually tested for anti-*T. gondii* antibodies with a commercial ELISA (ELISA PrioCHECK™ Porcine Toxoplasma Ab Kit, Thermo Fisher Scientific, Schlieren, Switzerland) previously evaluated for its use in pigs (Basso et al., 2013) as reported.

2.3. TgSAG1 (P30)-immunoblot

The presence of specific antibodies (IgG and IgA) against *T. gondii* in OF was assessed using a *T. gondii* tachyzoite surface antigen TgSAG1 (P30)-based immunoblot. The immunoblot was performed as previously described for detection of antibodies against *Besnoitia besnoiti* in cattle (Garrido-Castañe et al., 2019), with a few modifications. Briefly, ~3 μg of native *T. gondii* TgSAG1 antigen obtained by immunoaffinity chromatography (TOXO P30 B-10 Antigen, SR2B: Société de Recherche et de Réalisations Biotechnologiques, Avrillé, France) were electrophoresed in a precast polyacrylamide gel (Criterion™ TGX Stain-Free™ 4–20% precast gels for PAGE, Bio-Rad, USA) under non-reducing conditions. Subsequently, the antigen was electrophoretically transferred to a nitrocellulose membrane (Trans-Blot Turbo™, Bio-Rad, USA), cut into strips and blocked for 30 min with blocking solution (PBS with 0.05% (V/V) Tween 20 (Sigma, Germany) and 2% (V/V) fish gelatin (Serva Electrophoresis GmbH, Heidelberg, Germany)). OF samples were tested in duplicate at a 1:2 dilution in blocking solution and incubated at room temperature for 60 min. Afterwards, the strips were washed five times for 5 min with 0.05% Tween 20-PBS solution. For detection of specific IgG and IgA against Tg-SAG1 antigen, the strips were incubated with either rabbit anti-pig IgG (whole molecule) peroxidase conjugate (Sigma-Aldrich, St. Louis, USA) at 1:150 dilution in 0.05% Tween 20-PBS or goat anti-pig IgA peroxidase conjugate (Bio-Rad, Hercules, CA, USA) at 1:600 dilution in 0.05% Tween 20-PBS, respectively, at room temperature for 60 min. Washing was performed as indicated above and followed by a 15–20 min incubation step with substrate solution (40 μl of H_2O_2 (30% (V/V)) and 30 mg of 4-chloro-1-naphthol (Sigma-Aldrich, St. Louis, USA) in 40 ml of PBS, 20% (V/V) methanol). Finally, the reaction was stopped by addition of distilled water. Reaction against the immunodominant antigen TgSAG1, visible as a sole band of a rela-

tive molecular mass of 30 kDa, was recorded (strong band: high positive (++); clear band: positive (+); weak band: weak positive (+/-); no band: negative (-)). Sera from experimentally infected and naïve sows (Basso et al., 2017) were used as positive and negative controls, respectively, at a 1:100 dilution in blocking solution.

Additionally, pooled OF samples from groups containing > 91% seropositive fattening pigs were serially diluted (80%, 60%, 40% and 20%) in OF from seronegative pigs and tested by IB as indicated above, in order to make a preliminary assessment of the potential limit of detection of this test.

3. Results

3.1. Toxoplasma gondii ELISA in serum

The experimentally inoculated sows showed seroconversion in the commercial serum-ELISA at 2–3 weeks pi, and remained seropositive until the end of the experiment. The negative control animals showed ELISA values under the cut-off value at all time points (Basso et al., 2017) (Fig. 1)

Sixty out of 552 (10.9%) individually tested fattening pigs yielded positive results in the serum-ELISA. The seropositive animals belonged to 13 of the 42 groups. Eleven groups had 3.4 to 13% seropositive pigs and two groups had 91 and 92% seropositive pigs. The remaining 29 groups contained 100% seronegative animals (Table 2).

3.2. TgSAG1 (P30) immunoblot

The immunoblot results are displayed in Table 1. Sows experimentally inoculated with *T. gondii* showed positive IB results for IgA (8/8 sows) and IgG (7/8 sows) against TgSAG1 antigen in OF at 1.5 weeks pi, but the frequency of detection of both Ig isotypes decreased over time, with intermittent positive IB results (Table 1). Only two sows (Sows 1888 and 1818) had positive IgA results at all sampling times between 1.5 and 12 or 13 weeks pi. However, detection of specific IgG during the same period was intermittent (Table 1). Consecutive daily samplings for 4 days within weeks 13 (Sows 1888, 1874 and 1890) and 30 (Sows 1829, 1818 and 1798) pi showed inconsistent results day after day for some sows (Table 1). During week 13 pi, all three sows yielded positive IgA results with variations in the intensity of reaction from day to day (- to ++), and only two of the sows (Sows 1888, 1874) showed positive results for IgG on only 1 day. Only one of the sows (Sow 1818) sampled during week 30 pi yielded positive results for IgA, and all three sows gave negative results for IgG. Three negative control sows (Sows 1805, 1878 and 1819) sampled in parallel to the inoculated animals did not react with Tg-SAG1 antigen at any sampling time point (data not shown).

Analysis of pooled OF from fattening pigs yielded positive results for both IgA and IgG (with a stronger signal for IgA) only in the two groups with the highest rates of seropositive pigs (91 and 92%). All 11 groups with up to 13% seropositive pigs and the 29 groups with 100% seronegative pigs yielded negative OF-IB results for both Ig isotypes.

Due to the lack of groups with intermediate percentages of seropositive pigs, serial dilutions (80%, 60%, 40% and 20%) of OF from the two groups with 91–92% seropositive pigs were performed and evaluated by IB to make a preliminarily assessment of the potential limit of detection. Positive results to both Ig isotypes (IgG and IgA) were observed with OF dilutions of 80%, 60% and 40% from the two groups. Regarding OF dilution of 20%, IgA was detected only in the group with 92% seropositive animals, while IgG was not detected in either group.

4. Discussion

Pigs are considered one of the most important meat sources associated with *T. gondii* infection in humans (Guo et al., 2015); however, despite their importance in the epidemiology of human toxoplasmosis, pigs are not routinely tested for *T. gondii* infection at slaughterhouses. Serological monitoring at the primary production level has been suggested as a more feasable alternative to assess the exposure of pigs to the parasite in order to implement control measures (EFSA, 2007,2018). Traditional serology involves blood sampling, which requires trained personnel, is time consuming, may be stressful for the animals and risky for the personnel, and it may be expensive for producers if large numbers of animals need to be sampled for a solid evaluation of the herd status (Pol et al., 2017). Therefore, alternative sampling methods and diagnostic tools are particularly being looked for. In recent years, OF sampling in pigs has gained awareness, and it has been successfully used for detection of antibodies against several swine viral and bacterial pathogens in pig populations (Prickett et al., 2008,

Table 2
Percentage of seropositive (serum–ELISA: ELISA PrioCHECK™ Porcine Toxoplasma Ab Kit) fattening pigs (n = 552) in each of 42 sampled pens from 10 Swiss pig farms and oral fluid-TgSAG1 (P30) immunoblot results.

Farm No.	Pen No.	Serum-ELISA *n*Pos/ *n*Total in the pen	Serum-ELISA % Pos (95% CI)	OF-IB IgA	OF-IB IgG
1	1, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12	0/12, 0/12, 0/13, 0/11, 0/10, 0/12, 0/12, 0/11, 0/13, 0/11, 0/11	0	–	–
	2	1/12	8.3 (0–23.9)	–	–
2	1, 2, 3, 4	0/14, 0/5, 0/11, 0/11	0	–	–
3	1, 2, 3	0/14, 0/5, 0/9	0	–	–
	4	1/11	9 (0–6.9)	–	–
	5	1/11	9 (0–6.9)	–	–
4	1, 2, 3, 4, 5, 6, 8	0/10, 0/10, 0/10, 0/10, 0/10, 0/18, 0/18	0	–	–
	7	1/18	5.5 (0–16)	–	–
5	1	3/23	13 (0–26.7)	–	–
	2, 3, 4	0/9, 0/3, 0/6	0	–	–
6	1	0/10	0	–	–
7	1	3/24	12.5 (0–25.7)	–	–
	2	3/23	13 (0–26.7)	–	–
	3	22/24	92 (81.1–100)	++	+
	4	21/23	91 (79.3–100)	++	+
8	1	1/29	3.4 (0–10)	–	–
	2	1/13	7.7 (0–22.2)	–	–
9	1	1/10	10 (0–28.6)	–	–
10	1	1/20	5 (0–14.6)		

Pos, seropositive; OF-IB, oral fluid *Toxoplasma gondii*-TgSAG1 (P30) immunoblot; +, positive sample; ++, strong positive sample; –, negative sample; CI: confidence interval.

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2011; Kittawornrat et al., 2010, 2012; Prickett and Zimmerman, 2010; Ramirez et al., 2012; Pol et al., 2017; Almeida et al., 2018; Barrera-Zarate et al., 2019) as well as for direct detection of pathogens by PCR (Prickett and Zimmerman, 2010; Ramirez et al., 2012; Vosloo et al., 2015; Cheong et al., 2017; Senthilkumaran et al., 2017; Almeida et al., 2018; Panyasing et al., 2018).

Oral fluid can be easily collected by hanging a cotton rope over the box, with the end of the rope hanging at the height of the pigs' shoulders. The ropes provide a novel stimulus for oral exploration and the pigs deposit OF while chewing on those. Oral fluid sampling can be quickly carried out by a single person even in several pens at the same time, it can be frequently performed if needed, without stress for animals and practitioners, and it is inexpensive and animal welfare friendly (Olsen et al., 2013; Rotolo et al., 2017).

To determine if OF sampling could represent an alternative to traditional serum screening for *T. gondii* in pigs, we analyzed serial OF from experimentally inoculated sows (Basso et al., 2017), pooled OF from group-housed fattening pigs, as well as OF from naïve seronegative animals by a TgSAG1-based immunoblot, and compared these results with serological ELISA results (IgG) from all individual pigs. Additionally, to assess the robustness of the test, individual sampling of experimentally infected sows was repeated for four consecutive days (either at 13 or 30 weeks pi) in order to detect possible sample-to-sample variations.

In a study in which human saliva was analyzed at a 1:4 dilution for specific *T. gondii* IgG antibodies by ELISA using a streptavidin–biotin detection system with a recombinant SAG1 antigen, a good agreement with matched serum samples was observed (Chahed Bel-Ochi et al., 2013). The commercial ELISA used in this study was previously shown to be adequate for the detection of antibodies against *T. gondii* in porcine serum and in meat juice samples when lower dilutions (1:10) than serum were used (Basso et al., 2013). However, when OF samples from the sows in this study were preliminarily tested at a low dilution (1:4) to enhace specific IgG detection (Chahed Bel-Ochi et al., 2013), no clear differences in O.D. values between OF samples from seropositive and seronegative animals were consistently observed (data not shown). Nevertheless, when the same OF samples were tested by IB, positive results for both IgG and IgA isotypes were found in individual samples from seropositive animals and in pooled samples from group-housed pigs containing high proportions of seropositive animals. Several studies have pointed out the importance of modifing not only the concentration and volume of sample to improve antibody detection in OF, but also the incubation time and temperature (Kittawornrat et al., 2012; Panyasing et al., 2014; Gonzalez et al., 2017). The commercial ELISA kit that was preliminarily evaluated in our study does not seem to be a suitable test to be used with OF under the applied conditions. Several studies adapted serum antibody detection kits for different swine diseases to OF samples, showing variable results. However, a common kinetics pattern of IgG and IgA in pigs' OF should not be assumed for all pathogens because it depends on the microorganism, pathogens and infection kinetics (Panyasing et al., 2018). Also, diagnostic characteristics of the tests employed, volume of sample tested, stress factors and lacerations of the oral mucosa (Brandtzaeg, 2013) may influence the concentration of immunoglobulins in the OF sample. Some tests targeting IgG in OF provided better diagnostic performance than those targeting IgA, such as indirect ELISAs for porcine reproductive and respiratory syndrome virus (Kittawornrat et al., 2012), porcine circovirus type 2 (Prickett et al., 2011), influenza A virus (Panyasing et al., 2014) and classic swine fever virus (Panyasing et al., 2018) or immunoperoxidase in a monolayer assay for *Lawsonia intracellularis* infections (Barrera-Zarate et al., 2019). However, in the case of porcine epidemic diarrhea virus, the diagnostic performance of OF IgA indirect ELISA was better than that of OF IgG ELISA (Bjustrom-Kraft et al., 2016), in agreement with the results of the present study.

The main salivary immunoglobulin is secretory IgA (Challacombe and Shirlaw, 1994). Once B cells are activated in the mucosa-associated lymphoid tissue (MALT), procceses occurring in the germinative center induce IgA-committed plasmablasts (Brandtzaeg, 2003). Homing via peripheral blood to mucosal effector compartments takes place and plasmablasts differentiate to IgA-producing plasma cells. Dimeric IgA is synthesized, associated with a "J chain", and by transcytosis it is exported from the lamina propria to the surface of the epithelium, mediated by the union to the polymeric Ig receptor. Cleavege of this receptor results in a secretory component which binds to the IgA, protecting the immunoglobulin from proteolytic degradation (Brandtzaeg, 2007). Production of antibodies in saliva can be stimulated locally or at remote sites (e.g. intestine), but secretory antibodies are generally short-lived and of low titre; therefore, persistent antigen presentation might be necessary to mount a long-lasting salivary response (Challacombe and Shirlaw, 1994). Accordingly, when OF were tested by IB in this study, most positive results were observed during the first weeks after inoculation (1.5 to 4 weeks pi for both IgA and IgG), the frequency of positive detections at later time points being lower. Moreover, IgA was more frequently detected than IgG, suggesting that locally produced secretory antibodies represented an important proportion of the antibodies detected.

Unlike IgA, IgG concentration in OF is much lower, because although some IgG may be locally produced, it mainly derives from passive leakage of gingival crevicular fluid, mostly having a systemic origin (Challacombe and Shirlaw, 1994; Brandtzaeg, 2013).

While all animals showed seroconversion by 2–3 weeks pi in serum-ELISA (no serum samples were tested at 1.5 weeks pi) and remained seropositive (for IgG) for at least 30 weeks or until euthanasia (Basso et al., 2017), the frequency of specific IgG detection in OF was not constant. Positive results were intermittently obtained until 13 weeks pi. Testing for four consecutive days at 13 weeks pi revealed that some sows (e.g. Sow 1888) showed differences in IB results on OF ranging from negative to high positive from one day to the next. Only three sows (Sows 1818, 1798 and 1829) were tested at a later time point (30 weeks pi), and all three gave negative results for IgG in IB; nevertheless, it should be mentioned that the serum-ELISA values of these sows at that time point were low (ELISA PP values 26.7, 22.6 and 31.0%, respectively), which could have accounted for negative results in OF. IgG in OF derives mainly from passive transudate from gingival crevicular fluid, with inconstant leakeage (Brandtzaeg, 2007), hence, variable concentrations may be expected. It was estimated that IgG antibody concentration in OF from pigs and humans is ~ 800 times lower than that in serum (Parry et al., 1987; Olsen et al., 2013). Therefore, not only the amount of gingival crevicular fluid that reaches the oral cavity but also the IgG concentration in serum may have a direct influence on the observed results for OF, more evident when testing individual animals. Tissue lacerations in the oral cavity may contribute with additional IgG to OF. Overall, although specific IgG against *T. gondii* could be detected at some time points in OF, it seems that this may not be the most adequate target for diagnosing *T. gondii* infections on this type of matrix for individual animals.

On the other side, detection of IgA in OF appeared to be more consistent and positive IgA results were more frequently detected over time. While IgA was detected in 67.7 % (42/62) of the OF samplings from seropositive sows, IgG was only detected in 35.5% (22/62) of the cases. In five of the sows, positive IgA results were constantly observed between 1.5 and 4 weeks pi and in some sows positive results were also obtained when tested later at 13 and 30 weeks pi; however, when the same sows were tested for four consecutive days during weeks 13 or 30 pi, variable results were

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also obtained for IgA in six animals. These daily variations may be atributed to several factors. For example, variations in secretion flow rate may affect the salivary IgA level. The 'unstimulated' parotid secretion, as well as whole saliva, contains at least three times more IgA than the stimulated counterpart (Brandtzaeg, 2013). Besides, although the rope material does not seem to influence IgG concentration in collected OF samples, lower IgA and IgM concentrations have been associated with cotton-based collection ropes compared with hemp or nylon devices (Olsen et al., 2013). No reactions were detected for IgA and IgG in naïve sows by TgSAG1 IB, in accordance with their serological status.

In order to evaluate the feasabilitiby of using OF sampling in a surveillance program at the farm level, we analysed pooled OF from naturally exposed group-housed fattening pigs, and in parallel we serologically tested each individual pig in the groups. IB analysis of pooled OF could clearly detect groups in which 91–92% of the animals were seropositive. No positive results were obtained in the groups in which ≤13% of the pigs were seropositive or in the 29 groups containing only seronegative animals. These results suggest that this method would be able to detect herds with a high proportion of seropositive pigs, as it may occur after simultaneous infection of many animals with a common external infection source such as oocysts contaminating fodder or water. However, this method might fail to detect herds in which only one or a few animals had been infected by accidental uptake of a few *T. gondii* oocysts from the environment or after ingestion of an infected intermediate host (e.g. rodent or bird) caught in the premises. Unfortunately, the sampled groups in this study contained only very low (≤13%) or very high (92–91%) numbers of seropositive pigs. Therefore testing of serial dilutions of positive OF samples was performed to have an initial estimation of the possible limits of detection at the herd level. These results suggested that the TgSAG1 IB might detect groups with ≥40% *T. gondii* seropositivity; however, analysis of a higher number of pooled OF samples is needed to support these initial results.

Pooled OF samples should reflect the immunological status of the entire group, but several factors such as dominance status, number of ropes in the pen, group size, sampling duration and ages of the pigs (among others) might influence the likelihood of individual pigs being represented in the sample (Seddon et al., 2012). We performed the samplings over 45–60 min, using one rope for groups of up to 20 pigs and an additional one in the case of larger groups of up to 29 pigs. It was reported that one rope is sufficient for groups of ≤25 pigs (Turner et al., 2000) and that 45–60 min were enough to generate chewing by >80% of the pen group (Seddon et al., 2012). Longer sampling times did not significantly improve the percentage of pigs chewing (Seddon et al., 2012). Young pigs are more curious and motivated to explore objects than older animals (Docking et al., 2008). In a few attempts, we observed that the sows in this study did not show interest in the ropes during the 45–60 min observation period as was generally the case with the young pigs. Therefore, and in order to collect individual samples, we decided to use gauze swabs in this pig category. The sows were housed in an enriched environment with sawdust and long-stemmed straw bedding, and during part of the study they were allocated to groups of two animals. Not only age, but also housing conditions, could have contributed to the lack of interest of the sows in the rope. It was reported that pigs bred in enriched environments (e.g. pens with straw bedding) show less curiosity towards new objects in the pen than do pigs in barren environments (e.g. fully slatted pens) (Stolba and Wood-Gush, 1980; Scott et al., 2006; Seddon et al., 2012).

Our results showed that antibodies to *T. gondii* can be detected in OF from infected pigs by IB, and that specific IgA seems to be a more adequate target than IgG. These results may serve as a basis to develop further serological assays, preferably in ELISA format, with a higher throughput and lower cost. It is of note that OF is not equivalent to serum, and it does not seem to be a very robust matrix to assess the serological status of *T. gondii* in individual animals. Variable sensitivity may depend on days p.i., individual response to infection and timely oscilations in the antibody concentration in OF, and a serological examination should be performed to rule out contact with *T. gondii* in the case of negative IB results on OF. Nevertheless, this diagnostic approach might be an interesting non-invasive, low-cost and animal welfare friendly option as a screening method at the farm level to determine high exposure to *T. gondii*.

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