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Detection of *Toxoplasma gondii*-specific antibodies in pigs using an oral fluid-based commercial ELISA: Advantages and limitations

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ABSTRACT

Toxoplasma gondii is a major food-borne parasite and undercooked meat of infected pigs represents an important source of infection for humans. Since infections in pigs are mostly subclinical, adequate diagnostic tests for use at the farm level are pursued. Oral fluid (OF) was shown to be a promising matrix for direct and indirect detection of infections with various pathogens in pigs. The objective of this study was to assess whether *T. gondii* infections in pigs could be diagnosed using an indirect ELISA kit adapted for OF samples (OF-ELISA). Routine serology and OF-immunoblot (IB) were used as standards for the comparison. For this, serial OF samples from sows (n = 8) and fatteners (n = 3) experimentally inoculated with *T. gondii* oocysts, individual field samples from potentially exposed sows (n = 9) and pooled OF samples from potentially exposed group-housed fatteners (n = 195 pig groups, including 2,248 animals) were analysed for antibodies against *T. gondii* by ELISA. For individual animals, OF-ELISA exhibited a relative diagnostic specificity of 97.3% and a relative diagnostic sensitivity of 78.8%. In experimentally infected animals, positive OF-ELISA results were observed from 1.5 weeks post inoculation (pi) until the end of the experimental setup (8 to 30 weeks pi); however, values below the estimated cut-off were occasionally observed in some animals despite constant seropositivity. In potentially exposed individual animals, OF- and serum-ELISA results showed 100% agreement. In group-housed fatteners, antibodies against *T. gondii* could be reliably detected by OF-ELISA in groups in which at least 25% of the animals were seropositive. This OF-ELISA, based on a commercially available serum-ELISA, may represent an interesting non-invasive screening tool for detecting pig groups with a high exposure to *T. gondii* at the farm level. The OF-ELISA may need further adjustments to consistently detect individual infected pigs, probably due to variations in OF antibody concentration over time.

1. Introduction

Toxoplasma gondii is a worldwide distributed zoonotic protozoan parasite belonging to the family Sarcocystidae. This parasite has a facultative indirect life cycle with cats and other felids as the only definitive hosts, in which the parasite undergoes a sexual multiplication leading to production of oocysts, which are shed through their faeces. All warm-blooded species (mammals and birds) may act as intermediate hosts, with development of tissue cysts in several organs (e.g., CNS, heart and skeletal musculature) after asexual multiplication of the parasite (Deplazes et al., 2021). Natural infections in pigs may occur by ingestion of infected intermediate hosts such as rodents or birds carrying tissue cysts but more often through oral uptake of fodder or water contaminated with oocysts from cat faeces (Stelzer et al., 2019). Undercooked meat containing tissue cysts represents an important infection source for humans (Dubey, 2021) and pork is considered to be one of the major meat sources associated with human *T. gondii* infections (Guo et al., 2015). Previous studies in Switzerland, based on meat juice analyses by *T. gondii* P30 (*Tg*SAG1) ELISA, showed seroprevalences of 14%, 13% and 36% in finishing pigs (~6 months old), free-ranging pigs (~6 months old) and adult animals (~3–4 years old), respectively (Berger-Schoch et al., 2011). A very recent study investigating the seroprevalence of anti-*T. gondii*

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antibodies from healthy pigs at slaughter in Switzerland showed a decrease in the seroprevalence to only 1.3% with higher seropositivity in free-range pigs (2.9%) than in indoor pigs (0.4%) (Kelbert et al., 2021). It is estimated that approximately one-third of the global human population is infected with *T. gondii* (Montoya and Liesenfeld, 2004). The parasite is prioritised as the second most important food-borne parasite in Europe after *Echinococcus multilocularis* (Bouwknegt et al., 2018). Infections often remain asymptomatic in healthy adults, but it is of high clinical relevance in immunocompromised patients (e.g., AIDS patients, patients under immunosuppressive treatments) and in cases of primary infection during pregnancy, as *T. gondii* may cause severe foetal damage (e.g., hydrocephalus, microcephalus, intracerebral calcifications and chorioretinitis) leading to abortion, stillbirth, or to the birth of asymptomatic children, which may develop learning and visual disabilities or severe life-threatening infections later in life (Koutsoumanis et al., 2018; Deplazes et al., 2021). In addition, ocular toxoplasmosis was described both in immunocompetent and immunocompromised patients after congenital and post-natal infections, and is considered the most common form of infectious posterior uveitis, which can lead to blindness (Maenz et al., 2014). In pigs, *T. gondii* infections are mostly asymptomatic (Stelzer et al., 2019; Dubey et al., 2020). Nevertheless, *T. gondii* has been reported to be a cause of reproductive failure in sows (Basso et al., 2015) and occasionally of severe illness with respiratory, digestive and/or neurological signs and even death in different age categories (Dubey, 2009; Klein et al., 2010; Li et al., 2010; Olinda et al., 2016; Stelzer et al., 2019; Dubey et al., 2020).

In order to reduce human exposure (Djurković-Djaković et al., 2019) and secondly to improve pig health, control of *T. gondii* is necessary. Therefore, adequate diagnostic tests are needed. Currently, serology is the most commonly used method to detect *T. gondii* infections in pigs (Basso et al., 2013) and commercial ELISA kits are confirmed to be a useful tool in the detection of antibodies to *Toxoplasma* in serum or meat juice in pigs (Basso et al., 2013; Macaluso et al., 2019; Liyanage, et al., 2021). Detection of *T. gondii* antibodies in oral fluid (OF) might serve as an alternative to standard serology, aiding in the identification of farms with high exposure to *T. gondii* (Campero et al., 2020). Compared with blood sampling, collection of OF represents a non-invasive, animal welfare friendly method with less discomfort or stress for the animals (Henao-Diaz et al., 2020). OF is not equivalent to saliva. It can be defined as the fluid obtained during the placement of absorbent tissue in the oral cavity (Henao-Diaz et al., 2020). It consists of a combination of saliva and serum transudates from capillaries in the oral mucosa and gingival tissues. In particular, the gingival crevice (space between teeth and gingiva) allows access to systemic immune factors from blood into the oral cavity via crevicular fluid (Challacombe et al., 2015). Therefore, OF contains antibodies deriving from the systemic immune system (from the passage of serum antibodies) but also locally produced antibodies from the secretory immune system in the salivary glands. There are reports of the diagnostic use of OF in swine dating back to 1976 (Prickett and Zimmerman, 2010). So far, OF has been used as matrix for direct and indirect detection of numerous viral and bacterial agents causing disease in pigs (Campero et al., 2020). Recently, Henao-Diaz et al. (2020) published in their "Guidelines for oral fluid-based surveillance of viral pathogens in swine" a list with more than 23 swine viral pathogens that have been successfully detected with OF. OF has also been used in pigs as a matrix for the detection of hormones (Colson et al., 2012) and recently, for the detection of antibodies against *T. gondii* (Campero et al., 2020). As Campero et al. (2020) showed in their study, antibodies to *T. gondii* can be detected in OF from infected pigs by immunoblot (IB). Their results suggested that IgA-antibodies present in OF would represent a better target than IgG, as a higher correlation with the serological status from individual animals was observed. In pooled samples from groups of pigs, positive IB results were obtained only in herds with a high proportion of seropositive pigs. No false positive results were observed in the study, which is particularly important when used as a surveillance strategy (Henao-Diaz et al., 2020). As Campero et al. (2020) suggested, their preliminary results may serve as a basis to develop further serological assays. Indirect ELISA kits designed to detect antibodies in serum samples have been adapted in several studies to detect antibodies against different swine pathogens in OF (Prickett and Zimmerman, 2010; Rotolo et al., 2017; Henao-Diaz et al., 2020). Changes in incubation times or temperature, as well as in the concentration of OF or conjugate, lead to suitable results. Besides, the use of commercial ELISA kits has significant advantages over IB techniques. ELISA kits are easily available to practitioners, are generally cost-effective, time-saving due to a higher throughput, and easy to use. The results are also less biased (i. e., measurable as O,D,) and hence easier to standardize compared with results from IB.

The objective of this study was therefore to determine if a commercial ELISA kit can represent a suitable tool for the detection of antibodies against *T. gondii* in OF samples from experimentally inoculated and naturally infected pigs, to compare the results with standard serology as well as with IB, and to assess its potential as a screening method at the farm level.

2. Material and methods

2.1. Animals (OF and serum samples)

OF and serum samples were simultaneously collected from pigs experimentally inoculated with *T. gondii* oocysts (at several time points after inoculation), as well as from potentially exposed pigs (at one time point) as previously described (Campero et al., 2020).

2.1.1. Experimentally inoculated pigs

This sample set included serial OF and serum samples from eight sows experimentally inoculated with 10^4 *T. gondii* oocysts (CZ isolate, clone H3, type II) in Switzerland, and from three negative control sows (collected at 1, 1.5, 2, 3, 4, 8, 12, 13 and 30 weeks post inoculation (pi)), which derived from previous studies (Basso et al., 2017; Campero et al., 2020) and had been earlier tested for antibodies against *T. gondii* in serum and OF by ELISA and IB, respectively. In addition, new serial OF and serum samples from three Large White fattening pigs (weight 47–58 kg) inoculated with 1.4×10^3 *T. gondii* oocysts (ME49 strain, type II) in France, (collected at 0, 1, 4, 5, 6, 7 and 8 weeks pi), and from three further non-inoculated fatteners from the same experimental setup were included in the study.

2.1.2. Field samples from potentially exposed pigs

Pooled OF samples were obtained from potentially exposed group-housed fattening pigs in Switzerland by hanging one or two (in cases of groups with up to 20 or >20 animals, respectively) cotton ropes in the pens for 45–60 min for the animals to chew on, as previously described by Campero et al. (2020). When two ropes were used, both OF samples were pooled after collection. Pooled OF samples from a total of 195 pig groups (2–40 pigs/group; mean 12 pigs/group; 2,248 pigs in total) deriving from 22 Swiss pig farms were included in the study. Thirty-nine of these OF samples derived from a previous study and had been earlier tested for *T. gondii* antibodies by IB techniques (Campero et al., 2020). Also, blood samples were taken in parallel from each of the group-housed fatteners.

In addition, individual OF and serum samples were taken from nine potentially exposed sows aged 8–34 months from one further farm.

All animal experiments were authorized either by the Cantonal Veterinary Offices of Zurich, (permission no. ZH 216/2013) and Luzern (permission no. LU 03/2014) in Switzerland or by the APAFIS N° 14363-2018032908554996v3 in France, and complied with Swiss and French Animal Welfare guidelines, respectively.

2.2. Toxoplasma gondii serum-ELISA

To assess the serostatus of all pigs included in the study, individual serum samples of all animals were tested for anti-*T. gondii* antibodies with commercial ELISA kits. Either the ELISA PrioCHECK™ Porcine Toxoplasma Ab Kit, Thermo Fisher Scientific, Schlieren, Switzerland (for testing of the experimentally infected sows and group-housed pigs), which was validated for detecting antibodies in sera and meat juice from swine (Basso et al., 2013), or the ID Screen® Toxoplasmosis Indirect Multi-Species Test, ID.vet, Grabels, France (for testing of the experimentally infected fatteners and individual sows) were used. The analyses were performed as described by the manufacturers. Results of the ELISA assays were measured as O.D. at 450 nm and the values were normalised by calculating a sample/positive control ratio (SP; $SP\% = \frac{OD\ sample - OD\ negative\ control}{OD\ positive\ control - OD\ negative\ control} * 100$). According to the manufacturer, animals with $S/P\% \leq 40\%$ were considered negative, inconclusive if $40\% < S/P\% > 50\%$ and positive if $S/P\% \geq 50\%$.

2.3. TgSAG1 (P30) immunoblot (IB)

As it was previously shown that antibodies against *T. gondii* could be detected in OF from infected pigs by IB techniques, OF samples were tested in parallel with a *T. gondii* tachyzoite surface antigen *Tg*SAG1 (P30)-based IB to detect specific antibodies (IgG and IgA) against *T. gondii* as a standard of comparison. In order to achieve the highest possible comparability, all samples were treated in exactly the same way as described by Campero et al. (2020). The reaction between antibody and the immunodominant antigen *Tg*SAG1, visible as a sole band of a relative molecular mass of 30 kDa, was recorded in the following way: strong band = high positive (++); clear band = positive (+); weak band = weak positive (+/-); no band = negative (-) (Campero et al., 2020). For evaluation in this study, weak positive, positive, and high positive reactions of IgA or IgG were considered as positive IB reactions.

2.4. Toxoplasma gondii OF-ELISA

OF samples were tested for antibodies against *T. gondii* with a commercial indirect ELISA kit (ID Screen® Toxoplasmosis Indirect Multi-Species Test, IDvet, Grabels, France), which was validated for its use in serum and meat juice of pigs. To assess the most adequate conditions for testing OF, a small sample set including known positive and negative OF samples was first tested under different incubation conditions (temperature and time of incubation) and using different sample and conjugate concentrations (Supplementary Table S1). Based on these preliminary results, OF samples were tested under the following conditions: sample dilution 1:2 in sample buffer (serum samples were tested at a 1:10 concentration), sample incubation with the antigen at 4 °C overnight (16 h +/- 1 h) and conjugate dilution 1:2 in conjugate buffer (double concentration as used with serum samples). Except for these modifications, the ELISA was carried out as described by the manufacturer (TOXO-MS ver 1014 DE, Stand 08.2018). ELISA values were measured as O.D. at 450 nm and normalised by calculating a sample:-positive control ratio

$$\left(SP\% = \frac{OD\ sample - OD\ negative\ control}{OD\ positive\ control - OD\ negative\ control} * 100\right).$$

Two separate cut-offs were calculated: one cut-off for the group of pooled OF samples, and another for samples from individual animals.

For the calculation of the cut-off for pooled samples, ELISA results from OF samples from pens in which all individual animals in the group tested serologically negative in serum-ELISA were considered (n = 167 samples). The S.D. as well as mean value (MV) were calculated, and the cut-off was defined as follows: cut-off = MV + 3*SD (Classen et al., 1987; Lardeux et al., 2016).

For the calculation of the cut-off-value for individual animals, only individual negative animals from an experimental setting were included: serial samples from negative control sows (28 samples, three animals), and single samples before inoculation (five samples, five animals) (n total = 33 samples). Calculations were performed the same way as described above for pooled samples (cut-off = MV + 3*SD).

The sensitivity, specificity as well as predictive values for the OF-ELISA were calculated in relation to a reference standard of comparison. An animal was regarded as reference standard-positive if it tested serum ELISA- or OF-IB IgG- and/or IgA-positive. All remaining animals were regarded as reference standard-negative.

3. Results

3.1. Analyses of samples from individual animals

3.1.1. Serum-ELISA

Serum-ELISA results from the eight experimentally inoculated sows included in this study were previously described (Basso et al., 2017). Briefly, all sows seroconverted between two and 3 weeks pi and remained seropositive until the end of OF sampling (30 weeks pi) or until euthanasia (between eight and 22 weeks pi).

The three experimentally inoculated fatteners (Pigs 836, 838, 848) started yielding positive results between 1.5 and 2.5 weeks pi and remained seropositive until the end of the experiment (8 weeks pi). The highest serum antibody levels were reached between 5.5 and 8 weeks pi (Fig. 1). All non-inoculated pigs used as negative controls (n = 6) tested negative. The sample set of nine potentially exposed sows included one seropositive (serum-ELISA S/P% 144.98), and eight seronegative sows.

3.1.2. OF-IB

OF-IB results from the eight experimentally inoculated sows were previously described by Campero et al. (2020), and were included in Table 1 of this paper. Briefly, positive OF-IB results for IgA and IgG were first detected at 1.5 weeks pi in eight and seven of the inoculated sows, respectively, and the frequency of detection of both antibody isotypes decreased over time. At 30 weeks pi only one out of three sampled sows yielded positive results in IgA-IB and none of them in IgG-IB. In a total of 62 samplings performed between detection of the first positive results and the end of the experiment at 30 weeks pi, 42 (67.7%) and 22 (35.5%) positive IB reactions for IgA and IgG, respectively, were recorded. Some of the sows showed inconsistent IB results when consecutive daily samplings during weeks 13 and 30 pi were performed (Table 1).

Immunoblot results from the three experimentally inoculated fatteners showed the same trend as in the sows: first positive IB results were detected between 1.5 and 2 weeks pi, with a decreasing frequency of detection of both Ig isotypes over time. None of the three pigs remained positive at all sampling times. In contrast to the sows, the frequency of IB detection of IgA and IgG was com-

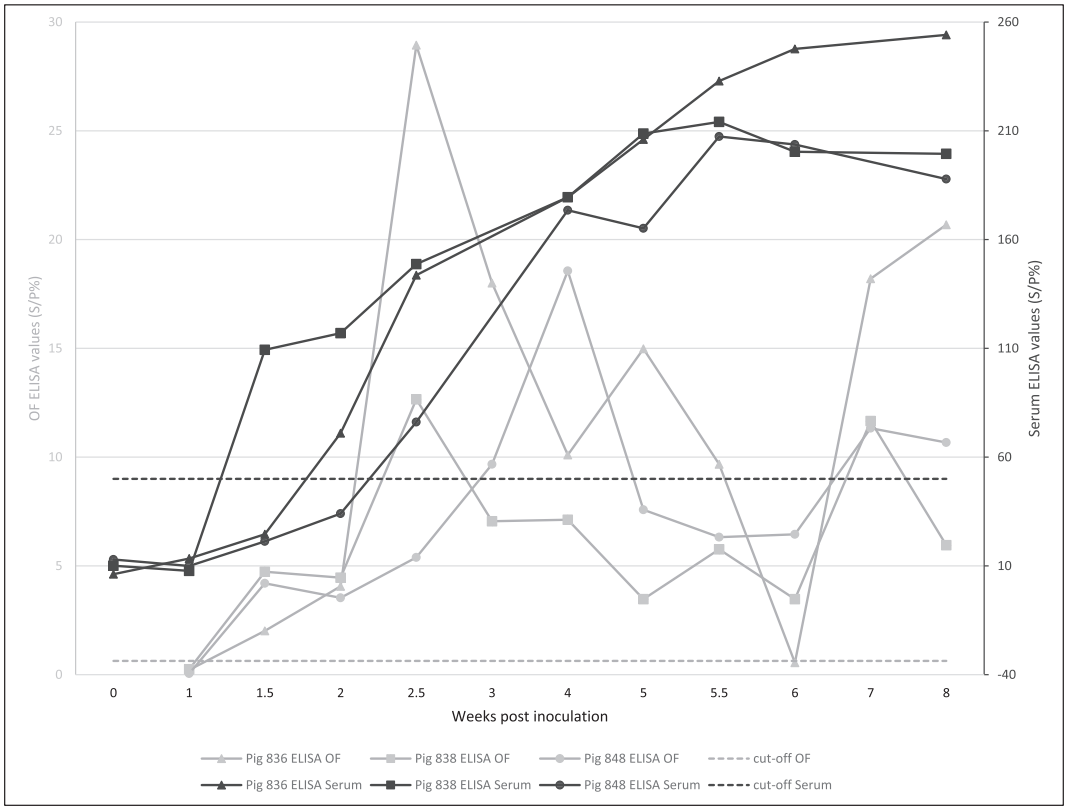

Fig. 1. Anti-*Toxoplasma gondii* response in fattening pigs (n = 3) experimentally inoculated with 1,400 *T. gondii* oocysts (ME49 strain, clonal type II) in oral fluid- and serum-ELISA (ID Screen® Toxoplasmosis Indirect Multi-Species Test) over time. OF, oral fluid; S/P%, sample:positive control ratio.

parable (i.e., 15 IB reactions positive for IgA versus 16 reactions positive for IgG antibodies from a total of 25 samplings were registered after the first positive results) (Table 1). All non-inoculated pigs used as negative controls (n = 6) tested negative by IB.

The only seropositive sow within the group of potentially exposed sows yielded also positive results by OF-IB for IgA. All seronegative sows yielded negative IB results for both IgA and IgG.

3.1.3. OF-ELISA

A total of 147 OF samples from 26 individual animals (i.e., eight experimentally inoculated sows, three experimentally inoculated fatteners, three non-inoculated negative sows, three non-inoculated negative fatteners and nine potentially exposed sows) were tested for antibodies against *T. gondii* in OF by ELISA (out of these, 137/147 samples were also tested by IB). For the evaluation of the ELISA results, a cut-off for individual animals was calculated as described above (cut-off = 0.63 S/P%). The OF-ELISA results from the experimentally inoculated animals are shown in Table 1. In the sows, the first positive results were observed between 1.5 and 3 weeks pi. After that, positive OF-ELISA reactions were observed in a total of 37/57 (64.9%) samples. Two of the sows (Sows 1827 and 1890) yielded consistently positive results from the first detection until the end of the study (at 8 and 13 weeks pi, respectively). Five out of six sows sampled daily at 13 or 30 weeks pi for up to four consecutive days showed positive results at one or more of those time points.

The three experimentally inoculated fatteners showed the first positive results by OF-ELISA at 1.5 weeks pi. They continued yielding positive results in all samplings until the end of the experimental period (8 weeks pi), with the exception of Pig 836 which tested negative once, at 6 weeks pi (29/30 samplings; 96.7% positive). The highest antibody levels in OF were observed between 2.5 and 4 weeks pi but these values seemed to show a greater variation over time than the values in serum (Fig. 1).

The three serially tested non-inoculated control sows and the three non-inoculated fatteners yielded negative OF-ELISA and -IB results at all time points (results not displayed in Table 1 for better readability).

The potentially-exposed seropositive sow also presented an ELISA value above the cut-off in OF (S/P% OF-ELISA 1.16). All remaining sows were seronegative and had negative values by OF-ELISA.

Using the reference standard described above (either OF IB IgA-, OF IB IgG- or serum ELISA-positive), a total of 80 OF samples were classified as positive and 46 samples as negative by definition and could therefore be included for further calculations. Hence a relative specificity of 97.83% and a relative sensitivity of 78.75% could be calculated for OF ELISA, when used for testing of samples from individual animals. The negative predictive value (NPV) of this test was 72.58%, and the positive predictive value (PPV) 98.44%.

3.2. Analysis of samples from group-housed animals

3.2.1. Toxoplasma gondii ELISA in serum and OF

A total of 195 pooled OF samples from 195 groups of fattening pigs were tested for antibodies against *T. gondii* by OF-ELISA. The serum of each animal in these groups (n = 2,248) was tested individually for antibodies against *T. gondii*, revealing, that 167 of the groups contained 100% seronegative animals (n = 1,755). In the remaining 28 groups tested, at least one pig per pen was positive for antibodies against *T. gondii* by serum-ELISA. The percentage of seropositive pigs per pen ranged from 2.5% up to 92% (Table 2).

Table 1
Results of immunoblot for IgA (IgA-IB) and IgG (IgG-IB) against *Toxoplasma gondii* (*Tg*)SAG1 antigen and ELISA (ID Screen® Toxoplasmosis Indirect Multi-Species Test, ID.vet, Grabels, France) (cut-off: 0.63 S/P%) in oral fluid samples (ELISA) from animals experimentally inoculated with *Toxoplasma gondii* oocysts.

	Diagnostic method	Weeks after inoculation (days after inoculation)																			
		0	**1**	**1.5**	**2**	**2.5**	**3**	**4**	**5**	**5.5**	**6**	**7**	**8**	**12**	**13 (88)**	**13 (89)**	**13 (90)**	**13 (91)**	**30 (208)**	**30 (209)**	**30 (210)**	**30 (211)**
Sow 1888	**IgA-IB**	-	-	+	++		+	+					+		+/-	+/-	++					
	IgG-IB	-	-	+	++		+	-					-		-	-	++					
	ELISA (S/P%)	0.08	0.02	0.04	0.62		**1.31**	0.62					**1.31**		0.38	0.5	**1.13**					
Sow 1874	**IgA-IB**	-	-	+	+		+	+/-					-		+/-	+/-	+	-				
	IgG-IB	-	-	+	+		+	+/-					-		-	-	+/-	-				
	ELISA (S/P%)	0.29	0.14	**0.74**	**1.37**		**1.1**	**3.44**					0.62		**0.98**	**1.1**	**1.58**	0.41				
Sow 1890	**IgA-IB**		-	+/-	+		+	-					+/-				+	+/-				
	IgG-IB		-	-	+		+/-	-					-				-	-				
	ELISA (S/P%)			0.53	**1.4**		**1.4**	**1.64**					**0.77**				**9.22**					
Sow 1818	**IgA-IB**			+	+		+/-	+					+/-	+						+	+/-	-
	IgG-IB			+	+		-	+					-	-						-	-	-
	ELISA (S/P%)			**1.61**	**1.67**		0.53	**4.44**					**1.07**	**1.4**							**1.22**	0.29
Sow 1798	**IgA-IB**			+/-	+/-		-	+/-					-	-					-	-	-	-
	IgG-IB			+/-	-		-	+/-					-	-					-	-	-	-
	ELISA (S/P%)			**0.98**	**0.98**		0.62	**3.53**					**0.98**	0.59					0.59	**0.98**	**1.01**	0.41
Sow 1829	**IgA-IB**			++	-		-						-	+/-					-	-	-	-
	IgG-IB			++	-		-						-	+/-					-	-	-	-
	ELISA (S/P%)			**2.09**	0.23		0.17						**0.86**						0.35	0.38	0.38	0.41
Sow 1806	**IgA-IB**			+	+		+	+					-	-								
	IgG-IB			+/-	-		-	-					-	-								
	ELISA (S/P%)			**1.64**	0.56		**0.89**	**1.28**					**0.92**	**0.89**								
Sow 1827	**IgA-IB**			+	+/-		+	+														
	IgG-IB			+	-		+	+														
	ELISA (S/P%)			**3.62**	**1.85**		**3.65**	**9.43**					**8.77**									
Pig 836	**IgA-IB**		-					++	++	-	-		++									
	IgG-IB		-					+/-	+	-	-		+									
	ELISA (S/P%)		0.22	**2.01**	**4.06**	**28.94**	**18**	**10.1**	**14.98**	**9.67**	0.55	**18.2**	**20.68**									
Pig 838	**IgA-IB**		-	-	-	+/-	+	+	+/-	-	-	+/-	-									
	IgG-IB		-	-	+/-	+	+	+	+/-	+/-	+/-	+	-									
	ELISA (S/P%)		0.25	**4.73**	**4.46**	**12.66**	**7.05**	**7.12**	**3.47**	**5.76**	**3.47**	**11.66**	**5.95**									
Pig 848	**IgA-IB**		-	+	++	+	+	++	+/-	+/-	-	-	-									
	IgG-IB		-	+	++	+	+	+/-	-	-	-	-	-									
	ELISA (S/P%)		0.05	**4.2**	**3.53**	**5.39**	**9.67**	**18.56**	**7.58**	**6.32**	**6.45**	**11.33**	**10.67**									

+/-, weak positive reaction; +, positive reaction; ++, strong positive reaction; -, negative reaction; blank: sample not available/not tested; S/P%: sample/positive control-ratio (SP% = (O.D. sample – O.D. negative control)/ (O.D. positive control – O.D. negative control) * 100); O.D., optical density at 450 nm; ELISA values in bold font, positive values which are above the cut-off.

Table 2
Oral fluid (OF) ELISA (ID Screen® Toxoplasmosis Indirect Multi-Species Test, ID.vet, Grabels, France) and OF-*Tg*SAG1 (P30)-immunoblot (IB) results of pens (n = 28, including 493 animals) containing different percentages of seropositive fattening pigs from Swiss pig farms.

Farm No.	Pen No.	Serum-ELISA n positive pigs /n pigs in the pen	Serum-ELISA% positive pigs in the pen (95% CI)	OF-IB IgA	OF-IB IgG	OF-ELISA (S/P%)
1	2	1/12	8.3 (0–23.9)	-	-	- (0.08)
3	4	1/11	9.0 (0–6.9)	-	-	- (-0.11)
	5	1/11	9.0 (0–6.9)	-	-	- (-0.31)
4	7	1/18	5.5 (0–16)	-	-	- (-0.11)
	9	8/18	44.4 (21.5–67.4)	-	-	+ (2.07)
	10	2/19	10.5 (0–24.3)	-	-	- (0.66)
5	1	3/23	13.0 (0–26.7)	-	-	- (0.02)
6	2	1/19	5.3 (0–15.3)	+/-	-	- (0.50)
7	1	3/24	12.5 (0–25.7)	-	-	- (0.21)
	2	3/23	13.0 (0–26.7)	-	-	- (0.24)
	3	22/24	92.0 (81.1–100)	++	+	+ (23.63)
	4	21/23	91.0 (79.3–100)	++	+	+ (9.75)
8	1	1/29	3.4 (0–10)	-	-	- (-0.02)
	2	1/13	7.7 (0–22.2)	-	-	- (0.47)
9	1	1/10	10.0 (0–28.6)	-	-	- (-0.05)
10	1	1/20	5.0 (0–14.6)	-	-	- (0.08)
11	1	1/40	2.5 (0–7.3)	-	-	- (0.45)
12	1	3/30	10.0 (0–20.7)	-	-	- (0.20)
	4	1/6	16.7 (0–46.5)	-	-	- (0.10)
13	1	8/36	22.2 (8.6–35.8)	-	-	- (0.00)
14	2	1/17	5.9 (0–17.1)	n.a.	n.a.	- (0.15)
	5	4/19	21.1 (2.7–39.4)	n.a.	n.a.	- (0.20)
15	1	1/4	25.0 (0–67.4)	-	-	- (0.67)
	6	1/5	20.0 (0–55.1)	-	-	- (0.39)
	25	1/4	25.0 (0–67.4)	+/-	-	+ (1.56)
	26	1/4	25.0 (0–67.4)	-	-	+ (2.11)
16	1	1/16	6.3 (0–18.1)	+	-	- (0.56)
	2	1/15	6.7 (0–19.3)	+	-	- (0.56)

CI, confidence interval; S/P%: sample/positive control-ratio (SP% = (O.D. sample – O.D. negative control)/ (O.D. positive control – O.D. negative control) * 100); Positive, seropositive; OF-IB, oral fluid *Toxoplasma gondii*- *Tg*SAG1 (P30)-immunoblot; +, positive sample; ++, strong positive sample; -, negative sample; n.a., not analysed. Results from pens containing exclusively seronegative pigs (n = 167 pens, including 1,755 pigs; 1–40 pigs/pen), which yielded negative OF-ELISA results, are not included.

A cut-off value for OF-ELISA from pooled samples was calculated considering the OF-ELISA results of the seronegative animals as explained above (cut off: S/P% = 1.11). All pooled samples from pens with a seropositivity of at least 25% (n = 5) yielded positive results by OF ELISA (Table 2) with the exception of one group of four animals which contained one seropositive pig with a very low value by serum-ELISA (S/P% 24.46), which was not detected by OF-ELISA (S/P% 0.67). Also, when tested by OF ELISA, one out of 167 (0.67%) samples from pens with exclusively seronegative pigs yielded a value above the cut-off (1.17 S/P%), accounting for a false-positive result.

3.2.2. Toxoplasma gondii antibodies in OF-IB

A total of 67 pooled OF samples were additionally tested by IgA and IgG IB (i.e., 26/28 samples from pig groups containing at least one seropositive animal and 41/167 samples from groups containing 100% seronegative pigs).

All 41 OF samples from groups containing exclusively seronegative pigs yielded negative IB results for IgG, but five (12.2%) samples yielded (“false”) positive results for IgA. The seropositive groups with the highest rates of seropositive pigs (91% and 92%) showed positive results for IgG and IgA (Campero et al., 2020). The remaining 26 groups with lower percentages of seropositive pigs yielded negative IgG IB results; however, samples derived from five groups with 5.3%, 6.3%, 6.7%, 7.7% and 25% of seropositive pigs yielded positive results for IgA (Table 2).

4. Discussion

The objective of this study was to test whether a commercial indirect ELISA kit, evaluated for its use in serum and meat juice, could represent a suitable tool for the detection of antibodies against *T. gondii* in OF samples from pigs. Standard serology as well as OF-IB were used as standards for comparison. Adequate monitoring methods at the primary production level are particularly being looked for in order to detect high-risk herds and to implement control measures against this highly important zoonotic food-borne parasite (Campero et al., 2020), and an OF-based antibody detection method would offer many advantages in comparison to standard serology. Collection of OF represents a non-invasive, animal welfare friendly method with less discomfort or stress for the animals (Henao-Diaz et al., 2020). Furthermore, it is easy to perform and requires fewer personnel (Pol et al., 2017). The natural exploratory behaviour of these curious animals makes OF sampling using cotton ropes possible. When cotton ropes are presented to pigs as new environmental enrichment, the typical porcine response consists of chewing on them, with the consequent deposition of OF, which can then be easily collected from the ropes (Kittawornrat and Zimmerman, 2011; Campero et al., 2020).

For a broad evaluation of the adapted OF-ELISA, both serial individual samples from experimentally inoculated animals, as well as a large number of field samples from potentially exposed pigs, were examined. Samples from experimentally infected animals represent valuable reference material because in these cases, the exact time point of infection is known and an evolution of antibody levels over time can be evaluated. Therefore, these samples were included in the study, even though testing of individual animals was not the primary objective for future use. Since the prevalence of *T. gondii* in Swiss conventional pig farms is low (Berger-Schoch et al., 2011; Kelbert et al., 2021) only 28 OF pooled samples from groups containing seropositive pigs were available. However, this sample set had the advantage that the OF samples were very well characterised, as every single pig in the groups had been serologically tested.

To our knowledge, there is not yet any commercial ELISA kit validated for the detection of antibodies against *T. gondii* in OF. Several studies showed that ELISA kits could be effectively adapted to detect antibodies against different swine pathogens in OF by changing the testing conditions (e.g., temperature and time of incubation with the antigen and dilution of the sample), which were optimal for a serum matrix (Kittawornrat et al., 2012; Olsen et al., 2013; Bjustrom-Kraft et al., 2016; Panyasing et al., 2018; Schott et al., 2021). Therefore, different testing conditions were evaluated in this study too (Supplementary Table S1). The most promising results were achieved when the small sample set was tested using the protocol indicated in the Section 2 (protocol 2 in Supplementary Table S1), which was then used for all OF-ELISA runs in the study.

Due to potential differences between the field pen-based samples from potentially exposed pigs and the individual samples from mostly experimentally inoculated pigs, two different OF-ELISA cut-offs were a priori calculated for both sample groups (i.e., 0.63% S/P for individual OF samples and 1.11% S/P for pooled OF samples).

Seroconversion occurred between 1.5 and 2.5 weeks after experimental inoculation with *T. gondii* oocysts, as reported in the present study in sera of fattening pigs. This is in agreement with observations from previous reports (Basso et al., 2013, 2017). Similarly, the first positive results in OF-ELISA were observed at 1.5 weeks pi, in agreement with the results from Campero et al. (2020) using IB. In some animals, positive ELISA results in OF were observed shortly before seroconversion (i.e., Pig 836, Pig 848, Sow 1874, Sow 1818 and Sow 1806). A possible explanation for these findings could be that locally produced antibodies appear earlier because local production is faster than passive diffusion and/or transportation from the circulatory and/or lymphatic system. As the mouth is part of the mucosal immune system, the local immune response with antibody production in the salivary glands can be stimulated not only through pathogen contact in the oral cavity but through pathogen contact in other mucosal linings at distant sites such as the gut (Challacombe et al., 2015). Local antibody production in the oral cavity might be generally faster than systemic antibody production. Therefore, this fact was considered when defining the positive reference standard; i. e., in addition to positive serum- ELISA results, positive OF-IB (IgA or IgG) results were used. This definition led to a reduced number of false-positive results by OF ELISA and in combination with the relatively low cut-offs, to a high relative specificity of 97.8% for OF ELISA in individual animals. A high specificity, or a low rate of false-positive results, is particularly important when used as a surveillance method (Henao-Diaz et al., 2020). However, this high diagnostic specificity goes together with a relatively low diagnostic sensitivity (78.8%). When calculating sensitivity and specificity as well as the predictive values, it must be considered that the experimentally inoculated animals were tested multiple times, so that several samples from the same animal were used for the calculations. Thus, relative diagnostic sensitivity and specificity as reported in this study are probably biased to some extent (Trevethan, 2017). Furthermore, predictive values (NPV: 72.6%; PPV: 98.4%) are influenced by the prevalence of a disease (Trevethan, 2017). In this experimental setting, prevalence is artificially increased, leading to an overestimation of PPV and an underestimation of NPV. To define the statistical safety conclusively, a larger group of naturally exposed, individual animals would be needed.

Comparing ELISA and IB results from 84 OF samples from individual, experimentally inoculated animals collected from 1.5 weeks pi, when positive results may be first expected, OF-ELISA detected a higher number of positive results (62/84; 73.8%) than IgA-IB (54/84; 64.3%) and IgG-IB (37/84; 44.0%) (Table 1). Considering the time and effort in the laboratory, the possibility of standardization and the availability, ELISA has clear practical advantages over IB techniques.

Pens with animals potentially exposed to *T. gondii* generally contained only a very few seropositive animals (i.e., 22 pens with seropositivity between 1–24%, four pens with seropositivity between 25–90%, two pens with seropositivity over 90%). OF-ELISA seemed to reliably detect antibodies against *T. gondii* in pens with a proportion of seropositive pigs of at least 25%; however, the number of pooled samples with a potentially detectable proportion of seropositive animals was small, and further studies to confirm these detection limits would be desirable. In contrast, a high number of negative pooled OF samples from potentially exposed animals (n = 167) was tested, and only one false-positive result was obtained. OF-ELISA offers a number of advantages over traditional serology as a screening method such as cost-effectiveness (given that pooled and not individual samples are tested), ease of implementation, safety for the personnel and improved animal welfare (Ramirez et al., 2012; Olsen et al., 2013; Pol et al., 2017; Rotolo et al., 2017; Henao-Diaz et al., 2020). A further advantage of OF over serum, which is particularly important when used for pathogens with a low prevalence, would be that OF samples are directly pooled in the pens when several animals chew on the same rope, reducing the impact of day-to-day variability in individual animals. However, this method may also introduce variability via the unpredictable chewing frequency of individual animals in the group.

OF ELISA values were generally very low compared with those from sera. Even with the adjustment of testing conditions as discussed above, values were approximately 100 times lower. One possible explanation is that IgA and IgG antibody concentrations in OF from pigs are 10 and 800 times lower than in serum, respectively (Olsen et al., 2013). OF samples from the three experimentally infected fattening pigs clearly yielded higher S/P% values in ELISA than did samples from the eight experimentally infected sows during the same sampling period (mean from reference standard-positive, inoculated fattening pigs: 8.4 S/P%; mean from reference standard-positive sows: 1.6 S/P%). Sample collection and testing of the samples were performed in the same way. Putative factors that might account for the higher ELISA values in OF from fattening pigs are the difference in age, a lower volume of saliva production in the fatteners, which could lead to a higher antibody concentration in OF, the *T. gondii* strain used for the infection (fattening pigs: ME49 strain, type II; sows: CZ isolate-clone H3, type II) and individual factors of each animal.

In conclusion, this study showed that antibodies to *T. gondii* can be detected in OF with an ad hoc modified commercial ELISA, and that the diagnostic sensitivity of the method seems to be higher than that previously observed using either IgG- or IgA-IB techniques (Campero et al., 2020). Nevertheless, we observed that it may fail to consistently detect individual infected pigs over time, probably due to timely variations in the OF antibody concentration, as was previously reported using IB techniques (Campero et al., 2020). In pooled OF samples from group-housed fatteners, antibodies against *T. gondii* could be reliably detected by ELISA in groups in which at least 25% of the animals tested positive in sera. These results suggest that this ad hoc adapted OF-ELISA may represent a promising non-invasive screening tool to detect groups of pigs with a high exposure to *T. gondii* at the farm level; however, it may fail to detect individual seropositive animals in large groups of seronegative pigs.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpara.2022.11.003.

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