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Seroprevalence of *Toxoplasma gondii* infection in sows and finishers from conventional and organic herds in Denmark: Implications for potential future serological surveillance

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ABSTRACT

Pigs are one of several host species for *Toxoplasma gondii* parasites, and consumption of infected pork may lead to toxoplasmosis in humans. We estimated seroprevalence in sows and finishers from conventional and organic herds in Denmark and discussed the strategies for reducing the risk from pork. We collected 447 blood samples from 59 herds, and additional meat-juice samples from 212 of the same pigs. Using a *T. gondii* IgG commercial ELISA test, we found 2% (95% CI = 0.4%–5%) apparent seroprevalence of *T. gondii* in conventional finishers, 11% (95% CI = 6%–17%) in organic finishers, 19% (95% CI = 11%–30%) in conventional sows and 60% (95% CI = 47%–72%) in organic sows. The odds of an animal testing positive for *T. gondii* was 16 times higher (95% CI = 4.6–74.3) in organic compared to conventional herds. The odds were 22 times higher (95% CI = 6.5–88.3) if the animal was a sow compared to a finisher. Meat-juice ELISA values were significantly correlated with plasma results ($P < 0.001$), but on average 64% of the blood-plasma ELISA values. Lowering the recommended cut-off from 20 to 13 percent positive values of the positive control for meat-juice ELISA, resulted in the meat-juice ELISA identifying 93% of the plasma positives as positive and 99% of the plasma negatives as negative. The time taken to detect one or more infected pigs from a *T. gondii* positive herd at slaughter was estimated using abattoir data on pigs (17,195,996) and batches (165,569) delivered to Danish abattoirs in 2018. The time to detection was affected by the seroprevalence, frequency at which the pigs were delivered, the number of samples tested per batch delivery and the batch sizes. Time to detection was long in conventional finisher herds due to low prevalence, and in sow herds because of intermittent delivery of a low number of sows. In organic finisher herds, time to detection was short due to medium prevalence and frequent delivery of a high number of finishers. Conventional finisher herds may be classified as low-risk, organic finisher herds as medium-risk, and conventional and organic sow herds as high-risk herds. Risk-mitigation strategies at processing plants (freezing or curing) or at the consumer level (heat treatment) for meat originating from high-risk herds, surveillance of medium-risk herds, and auditing for controlled housing (high biosecurity) in low-risk herds may be cost-effective alternatives to serological surveillance of all Danish pig herds.

1. Introduction

Toxoplasma gondii is the most prevalent coccidian parasite in the world, known to infect a wide range of host species (Tenter et al., 2000; Webster, 2010). Infection with *T. gondii* parasites in humans may occur through accidental ingestion of oocysts from cat feces or the environment or via the consumption of meat from infected animals (Webster, 2010). In the early 1960s, undercooked meat was found to be an

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important route of transmission for *T. gondii* parasites to humans (Frenkel, 1970). In most healthy adults, *T. gondii* infections remains mild or asymptomatic. However, an exposure to the parasite in the early stages of pregnancy, in previously non-infected women, may lead to congenital toxoplasmosis, with long-lasting effects on the child (Weiss and Dubey, 2009). Furthermore, in immunocompromised individuals, reactivation of latent *T. gondii* infections can cause fatal encephalitis (Montoya and Liesenfeld, 2004).

Domestic pigs like any other farm animal, may become infected with *T. gondii* parasites. The seroprevalence in pigs worldwide is reported to range from 0% to 93% (Dubey, 2009). Intensive management of pigs has decreased the prevalence of *T. gondii* on farms due to improved biosecurity measures (Kijlstra et al., 2004). However, in alternative systems such as free-range and organic farms, the prevalence of *T. gondii* is higher (van der Giessen et al., 2007; Swanenburg et al., 2019). And since viable cysts may be present in the commercial pork cuts, and pork is a commonly consumed commodity, pigs are considered a potential source for toxoplasmosis in humans (Dubey et al., 1986).

The parasite is inactivated if the meat is heated to above 67° C or frozen to below −12° C or fermented with NaCl concentration of 1.3% or above for 6 h during the curing process (Dubey et al., 1990; Kotula et al., 1991; Hill et al., 2018). Due to the small size of the tissue cysts, it is impossible to detect the parasite by visual meat inspection. In the European Union (EU), there is no official requirement for surveillance or control for *T. gondii* in pigs or pork. However, to improve food safety, the European Food Safety Authority (EFSA) recommends auditing of biosecurity in conventional finisher herds and serology in other herd types for the identification of high-risk herds and to use this as food chain information (EFSA, 2011a). Testing of pigs for *T. gondii* antibodies using meat-juice is seen as a potential tool in surveillance because collection of blood at the time of exsanguination is complicated compared to the collection of meat samples, and this latter type of sampling is routinely in place for the detection of *Salmonella* in finishers in Denmark and Germany (Alban et al., 2012; Meemken et al., 2014). Therefore, using an existing set up could make surveillance for *T. gondii* feasible and cost-effective.

As described above, the within-herd prevalence of *T. gondii* might be low in certain production systems, and high in others (Kijlstra et al., 2004; van der Giessen et al., 2007; Kofoed et al., 2017). A routine surveillance based on testing the same number of pigs in all herds may therefore not be a cost-effective strategy for identifying the disease (Alban et al., 2020). In conventional production systems with finisher herds, farmers send many pigs for slaughter in frequent batches, while e.g. in organic and free-range production systems, and sow farms, farmers send a lower number of pigs and in fewer batches. In a surveillance system, both the within-herd prevalence and the number of pigs tested, determine the time it takes to detect one or more positive animals. Moreover, if the number of pigs sent for slaughter is very low, or if batches are only sent intermittently, then this will also have an impact on time to detection. If risk mitigation for *Toxoplasma* is deemed necessary in the future, it may consist of different elements involving surveillance, auditing and control, depending on the risk represented by the different subpopulations of pigs (Häsler et al., 2011; EFSA, 2011b).

The objective of this study was to quantify the seroprevalence of *T. gondii* in sows and finishers from Danish conventional and organic production systems and to identify and quantify risk factors that may be used in a potential future risk-based surveillance system. Additionally, we wanted to quantify the number of pigs that were available for testing, and the frequency at which batches of pigs were delivered to the abattoirs from each of these four production categories. Furthermore, we wanted to explore how the frequency of these batch deliveries to the abattoirs together with the observed prevalences, affected the time it would take to detect one or more test-positive pigs. The study also aimed to compare the results from meat-juice ELISA with the blood-plasma ELISA, to explore if existing meat-juice samples from e.g. the national *Salmonella* surveillance program could be used for *T. gondii* screening in pigs in future.

2. Materials and methods

2.1. Sample size and animal selection

Due to financial constraints, the total number of samples to be collected in the study was limited to 450 pigs (five sows per herd and 10 finishers per herd) from organic and conventional herds for blood samples, and from 210 out of these 450 pigs, meat-juice samples were collected. The aim was to sample equal number of animals from conventional and organic herds. To get an idea of the within-herd prevalence, we would have liked to sample ten animals per herd. However, this number was limited to five in sow herds as they were known to send few animals per batch to the abattoirs. To ensure a reasonable balance between the number of pigs and the number of herds, the sampling was distributed among 15 herds in each of the four production categories (conventional finisher herds, conventional sows herds, organic finishers herds, organic sows herds) resulting in a target of 150 finishers from 15 conventional herds, 75 sows from 15 conventional herds, 150 finishers from 15 organic herds, and 75 sows from 15 organic herds. Furthermore, three of the nine large abattoir plants in Denmark were selected to ensure a substantial geographical coverage of the herds.

At the abattoirs, finishing pigs are received from the producers in batches, and a batch usually consists of a truckload with 200–240 animals, whereas for sows, the number of animals delivered is much smaller. A day before the sampling, a list containing information on all batch deliveries was obtained from the abattoir. From this list, different herds to be sampled were selected based on the number of pigs registered for slaughter on the scheduled day of sampling (a minimum of ten finishers or five sows per batch) and the time of day of the delivery of the batches to the abattoirs (with early deliveries being preferred to later deliveries). The finishers and the sows were convenience samples from a batch and were marked with a spray in the lairage area as they exited the transport truck at the abattoir. From the animals that moved into the lairage area, those that were easiest to catch (excluding any injured animals) were selected for sampling. Before exsanguination at the slaughter line, each selected pig was marked with a rubber stamp consisting of a unique number in the shank area. This was done to identify the carcass in the cold-storage room for the meat sample collection.

2.2. Sample collection and laboratory analysis

Blood samples were collected in a 5 mL tube (3.2% sodium citrate anticoagulant) and were taken from pigs at exsanguination. The meat tissue samples (15–20 *g*) were taken from *M. sternomastoideus* muscle of the pig carcasses and stored in a container (in EZ-DripLoss). The samples were collected by trained technicians from Danish Meat Research Institute (DMRI). Both blood and meat tissue samples were transported to the DMRI laboratory for analysis. At the laboratory, the blood samples were centrifuged, and while awaiting further analysis the separated plasma samples were stored at −20° C. The meat tissue samples were also frozen at −20° C, and the meat-juice samples for meat-juice serology were obtained after thawing.

Blood-plasma and meat-juice serology analyses were done using a commercial ELISA kit (PrioCHECK® Toxoplasma Ab SR, Prionics, Schlieren-Zurich, Switzerland) following the manufacturer's instructions for the detection of IgG antibodies against *T. gondii* (Thermo Fisher Scientific, 2020a). According to the manufacturer, the blood-plasma sensitivity and specificity values are 98% and 100%, respectively, and the meat-juice sensitivity and specificity values are 97% and 100%, respectively (Thermo Fisher Scientific, 2015b). The ELISA results were expressed as percent of positive control (PP), where PP = ((optical density of sample - optical density negative of control) / (optical density of positive control - optical density of negative control)) *100. The manufacturer's recommended cut-off value of 20 PP for blood-plasma and meat-juice serology was chosen for classifying a sample positive ($\geq$ 20) or negative ($<$ 20) for *T. gondii*.

2.3. Serology data

The data from blood-plasma and meat-juice serology of all animals, along with their respective batch delivery numbers were stored in an Excel spreadsheet. For all batch delivery numbers, herd identification numbers (ID) were traced, and this information was further used to retrieve the herd size and to locate the geographical coordinates of the herds from the Central Husbandry Register database. Hence, the serology dataset had one row (observation) for each animal with its respective batch delivery number, herd ID, PP value, blood-plasma and meat-juice serological status (positive or negative), herd size, and geographical coordinates (UTM coordinate system) across the columns (variables). The farm geographical coordinates were used to identify the region (North-Jutland, Mid-Jutland, Southern-Jutland, Zealand) of the farms.

From the serology dataset, the overall apparent seroprevalence was estimated separately for blood-plasma and meat-juice serology from total positives / total samples. For estimating the true seroprevalence, a Bayesian model for a single test was applied using uniform priors for sensitivity (range = 96.4% and 100%) and specificity (range = 83.9% and 100%), where these values were taken from studies that had tested meat-juice samples from pigs using PrioCheck ELISA (Felin et al., 2017; Macaluso et al., 2019). The model was implemented using the "prevalence" package in R for estimating the true seroprevalence in each of the four production categories (Devleesschauwer et al., 2014).

2.4. Statistical analysis for the identification of risk factors

To identify risk factors for *T. gondii* seroprevalence, the blood-plasma serology data were used. A hierarchical mixed-effects logistic regression model was fitted using Gauss-Hermite approximation of the likelihood estimation method (using 10 quadrature points), implemented using the "lme4" package in R (Bates et al., 2015; R Core Team, 2018). A two-level hierarchical modelling approach was chosen to account for clustering of animals (level 1) within herds (level 2). The dependent variable was the serological status of the animal (seropositive = 1, seronegative = 0) from blood-plasma ELISA, whereas the production system (organic and conventional), herd size, animal-type (sow and finisher) and region (North-Jutland, Mid-Jutland, Southern-Jutland, Zealand) were the independent variables with herd ID as a random effect (u_{herdID}) in the model. u_{herdID} was assumed to be normally and independently distributed with a standard deviation, σ_u.

$$\text{logit}\,(p_i) = \beta_{0j} + \beta_1 * x1_{ij} + \ldots. + \beta_n * xn_{ij} + u_{\text{herdID}(j)}$$

where,

p_i is the probability that pig (i) is seropositive for *T. gondii*
$u_{\text{herdID}(j)}$ is the random effect of herd *j*
(x1,…, xn) are the independent variables

The independent variables in the model were included, if they were associated (P-value $\leq$ 0.25) with *T. gondii* blood-serology status in the univariable mixed effects logistic regression model, with herd ID as a random effect. In the univariable analysis, the herd size (continuous variable) was explored separately for finisher and sow herds because of substantial variation in the herd sizes between the two. The final model was built using backward elimination, where all the variables with a P-value $\leq$ 0.05 in the likelihood ratio test were retained. Two-way interactions were then added to the model and retained if found to be significant (P $\leq$ 0.05). To identify potential confounding variables, we removed the variables one-by-one from the final model to assess the relative change in the β estimates of the remaining variables, where a relative change of $\geq$ 25% was defined as a confounding effect (Noordhuizen et al., 2001). The intraclass correlation coefficient (ICC) for the model was estimated by $\sigma^2_{\text{herd-level}} / (\sigma^2_{\text{herd-level}} + \pi^2/3)$, where $\pi^2/3$ is the assumed variance at level 1 i.e., $\approx$ 3.29 (Dohoo et al., 2012). Using the fitted model, we calculated the predictive probability of a pig testing seropositive for each of the four production categories (Noordhuizen et al., 2001).

The serology data were further used to explore the performance of meat-juice ELISA in comparison with blood-plasma ELISA, by selecting all samples that were tested with both blood-plasma and meat-juice ELISA. The sensitivity and the specificity of the meat-juice ELISA (relative to the test results from the blood-plasma) were calculated with blood-plasma ELISA used as a reference test, with a cut-off value of 20 PP. Subsequently, an appropriate cut-off for meat-juice ELISA was explored by calculating the sensitivity and the specificity at various cut-off values. The sensitivity and the false positive fraction (1-specificity) values were plotted using a receiver operating characteristic (ROC) curve. This was done to determine an optimal cut-off for the meat-juice ELISA, where most of the test positive and test negative samples in the blood-plasma ELISA also tested positive and negative, respectively, in the meat-juice ELISA. Additionally, Spearman's rank correlation was calculated to test the association between the blood-plasma and the meat-juice ELISA PP values. The mean difference in the PP values from blood-plasma and meat-juice ELISA was also explored using a Bland-Altman plot in R (Bernhard, 2015). Finally, to test whether the seropositive pigs from organic herds had higher level of antibodies (PP) compared to seropositive pigs from conventional herds, the within-herd average of seropositive PP values (to account for clustering of animals within a herd) in organic and conventional production system were calculated. These within-herd average seropositive PP values of the two groups were then tested with a two-sample *t*-test while checking for equal variances (F-test) in the two populations. Similarly, to test whether the seronegative PP values were higher in sows than in finishers, the within-herd level average of seronegative PP values from both sows and finishers were calculated and the means of the two groups were compared using two-sample *t*-test as described above.

2.5. Time to detection at varying seroprevalence

To explore the potential and limitations for developing a future serological surveillance system, we estimated the time (in days) taken to detect one or more *T. gondii* seropositive pigs from an infected herd belonging to one of the four production categories. First, we identified the following relevant variables for the surveillance system in the four production categories: expected within-herd *T. gondii* seroprevalence in four production categories, frequency of batches delivered to the abattoirs, number of animals sent to the abattoirs, number of animals [one, two or six samples per batch, based on the sample sizes used in the recent Dutch study by Swanenburg et al. (2019)] tested per batch delivery at the abattoir, desired probability of detection (95%), and the number of samples needed for the detection of one or more seropositive pigs from a herd (accumulated sample size). Thereafter, we estimated the time to detection in each production category. The observed seroprevalences (apparent prevalence from blood-plasma serology) found in this study for conventional finishers, organic finishers, conventional sows, and organic sows were used to determine the sample size required to have a $\geq$ 95% probability of detecting a minimum of one pig with *T. gondii* for each of the four apparent prevalence values, based on the quantile function of the negative binomial distribution (i.e. number of failures before one success). For the number of pigs sent to slaughter from each of the four production categories, we used the abattoir delivery data from all Danish pig herds from January 2018 to December 2018. From the raw abattoir data, four datasets were created, with each dataset containing information on animals belonging to either one of the four production categories previously defined (for additional information on the raw abattoir data and its handling, please see the Supplementary material I). To ensure that the dataset comprised of active and commercial herds, herds delivering less than 200 finishers per year were removed in line with the approach in the Danish *Salmonella* program for finishers (Alban et al., 2012). Similarly, we chose to remove herds from which fewer than 20 sows per year were delivered to slaughter. Each of

the reduced datasets was used to create a final herd level abattoir dataset which included the variables shown in Table 1. From these variables, the time to detection in days was calculated using the following formula:

Time to detection = average number of days between the batch deliveries (DD) * average number of deliveries needed to meet the required sample size for each herd (NoD)

Time to detection was calculated for each production category using each of the four identified seroprevalences. For each herd in the production category dataset, and for each of the four seroprevalences, the time to detection was classified into thirteen intervals (30 days) for up to 1 year (0–30 days, 31–60 …. 331–365 days and > 365 days), and the number of farms with a given time to detection was estimated. The time to detection at four seroprevalences for each of the four production categories in Denmark were presented as cumulative graphs, showing the increasing proportion of herds, that on an average would deliver one or more test positive animals (with 95% probability) as a function of time (days) for up to 1 year.

3. Results

Between June 2017 and September 2018, 447 pigs from 59 herds (conventional finisher herds, n = 15; organic finisher herds, n = 15; conventional sow herds, n = 16; organic sow herds, n = 13) were sampled for blood-plasma. Additionally, 212 of the same 447 animals from 32 herds (conventional finisher herds, n = 7; organic finisher herds, n = 8; conventional sow herds, n = 8; organic sow herds, n = 9) were also sampled for meat-juice. The distribution of number of animals tested for blood-plasma and meat-juice serology from each production category is shown in Table 2. From the 59 herds sampled in this study, 30 herds were from the Mid-Jutland, 15 from South-Jutland, eight from Zealand and six from the North-Jutland region.

3.1. Seroprevalence of T. Gondii

The apparent seroprevalence and the true seroprevalence estimates of *T. gondii* were similar in sows and finishers originating from conventional and organic herds (Table 2). The observed seroprevalence was lowest (meat-juice = 0.0%, 95% CI = 0.0%–4.8% ; blood-plasma = 1.9%, 95% CI = 0.4%–5.4%) in conventional finisher pigs and highest in organic sows (meat-juice = 48.6%, 95% CI = 31.9%–65.6% ; blood-plasma = 59.7%, 95% CI = 47.0%–71.5%).

Table 1
Description of the variables in the final abattoir dataset used for the estimation of time to detection of one or more *Toxoplasma gondii* seropositive pigs in a herd.

Variable	Abbreviation	Value
Unique herd identification number	Herd ID	n.a
Total number of batches delivered per herd per year	TNBD	Table 4
Total number of animals delivered per herd per year	TNAD	Table 4
Number of samples tested per batch delivered to the abattoirs	NoS	One, two or six samples as in the *T. gondii* screening of pigs in the Netherlands (Swanenburg et al., 2019)
Average number of days between the batch deliveries	DD	Calculation, 365 days divided by TNBD
Required sample size to detect a minimum of one seropositive pig from a herd with 95% probability, at a seroprevalence of 2%, 11%, 19% and 60% was 148, 25, 14 and 3 animals, respectively.	RSS	Calculated using the quantile function of the negative binomial distribution
Average number of deliveries needed to meet the required sample size, at a seroprevalence of 2%, 11%, 19% and 60%	NoD	Calculation, RSS divided by NoS

3.2. Risk factor analysis

Herd size was not significant (finisher herds: P-value = 0.37; sow herds: P-value = 0.26) in the univariable analysis and was therefore not added to the full model. The categorial variables, animal-type (finisher or sow), production system (conventional or organic) and region (Southern-Jutland or North-Jutland or Zealand or Mid-Jutland) were significant (Table 3). There was no interaction between the variables animal-type and production system (P-value = 0.78) and no confounding between the variables in the model. The random effect of herd was substantial, accounting for 45% of the variation in the model. The results of the analysis showed that the odds of testing positive for *T. gondii* was 16 times higher for an animal in an organic herd (95% CI = 4.6–74.3) compared to a conventional herd. Moreover, the odds were 22 times higher, if the animal was a sow (95% CI = 6.5–88.3) compared to a finisher. The predicted probability for a pig being seropositive for *T. gondii* (averaged over regions) from conventional-finisher herds, conventional-sow herds, organic-finisher herds and organic-sow herds was 1%, 22%, 15% and 79%, respectively.

3.3. Comparison of meat-juice ELISA with blood-plasma ELISA

Ignoring animal-type (finishers or sows), the average blood-plasma PP values in seropositive animals were significantly higher (Welch Two-sample *t*-test, P = 0.005) in the organic herds (overall average = 117 PP from 19 herds) compared to the average PP values in seropositive animals from the conventional herds (overall average = 62 PP from 10 herds). Ignoring production system (conventional or organic), the average blood-plasma PP values were significantly higher (Two-sample *t*-test, P = 0.04) in seronegative animals from sow herds (overall average = 3.9 PP from 10 herds) compared to the average PP values in seronegative animals from finisher herds (overall average = 2.6 PP from 20 herds).

Meat-juice ELISA PP values were significantly correlated with blood-plasma ELISA PP values (Spearman's rank correlation coefficient, $\rho = 0.76$, $P < 0.001$) (Fig. 1). When using the cut-off of 20 PP recommended by the manufacturer for the meat-juice ELISA, 83% (33/40) of the positive values in the blood-plasma ELISA also tested positive in meat-juice ELISA, and 100% (172/172) of the negative values in the blood-plasma ELISA also tested negative in meat-juice ELISA. However, a comparison of meat-juice PP values with blood-plasma PP values, showed PP values for meat-juice ELISA were 64% of the blood-plasma PP values (Fig. 2). The Bland-Altman plot of differences showed that the difference in PP values between the two tests increased as the average PP values increased, and that blood-plasma ELISA were consistently higher than meat-juice ELISA (Supplementary material II, plot A). It can therefore be concluded that the two tests were not directly equivalent. Exploration of various cut-off's using a ROC curve (Fig. 3) revealed that the highest combined sensitivity and specificity for meat-juice ELISA (relative to blood-plasma ELISA) was obtained with cut-off values ranging from 11 to 13 PP, each resulting in a sensitivity of 93% and specificity of 99%.

3.4. Time to detect one or more positive pig in each of the four production categories

The raw abattoir data included 17,239,339 slaughtered animals, and after removal of herds that sent less than 200 finishers or 20 sows, 17,195,996 (99.8%) remained (Table 4). The distributions of the annual number of animals delivered, and the number of batches sent to the abattoir in 2018 is shown as percentiles in Table 4. The conventional finisher herds delivered the highest median number of pigs per herd followed by the organic finisher herds, the conventional sow herds, and the organic sow herds. The organic finisher herds delivered the highest median number of annual batches followed by the conventional finisher herds, the conventional sow herds, and the organic sow herds (Table 4).

Table 2
Total number of blood-plasma and meat-juice samples tested, and the number of sows and finishers found positive for *Toxoplasma gondii* antibodies, divided into production categories, Denmark, 2017-18.

Animal-type (Production system)	Blood-plasma ELISA				Meat-juice ELISA			
	N[a]	Pos[b]	Apparent prevalence (95% CI)[c]	Mean true prevalence (95% CI)[d]	N[a]	Pos[b]	Apparent prevalence (95% CI)[c]	Mean True prevalence (95% CI)[d]
Finishers (Conventional)	159	3	1.9 (0.4–5.4)	1.6 (0.1–4.6)	75	0	0.0 (0.0–4.8)	1.3 (0.0–3.9)
Finishers (Organic)	148	16	10.8 (6.3–16.9)	6.3 (0.3–14.2)	64	10	15.6 (7.8–26.9)	10.3 (0.7–22.6)
Sows (Conventional)	73	14	19.2 (10.9–30.1)	13.4 (1.7–25.9)	36	5	13.9 (4.7–29.5)	10.4 (0.6–25.0)
Sows (Organic)	67	40	59.7 (47.0–71.5)	56.7 (42.7–69.6)	37	18	48.6 (31.9–65.6)	44.7 (25.9–62.6)
Total	447	73			212	33		

[a] Total number of animals tested.
[b] Total number of seropositive animals when using 20 percent positive (PP) as a cut-off value.
[c] Exact 95% confidence interval calculated using the Clopper-Pearson method.
[d] Mean and 95% Confidence Intervals (2.5% and 97.5% Percentiles) for the true prevalence estimated from a Bayesian model using uniform priors for the sensitivity (range = 96.4% and 100%) and the specificity (83.9% and 100%).

Table 3
Predictor variables found to be significantly associated with *Toxoplasma gondii* (yes, no) in blood-plasma ELISA, estimated using a two-level hierarchical logistic regression model.

Variables	Categories	Beta coefficient estimate	SE[b]	Odds ratio (95% CI)[c]	N[d]	% Pos[e]
Fixed part						
Intercept		−4.5	0.7			
Farm-type	Organic	2.8	0.7	15.7 (4.6–74.3)	215	26.0
	Conventional[a]	0.0		1	232	7.3
Animal-type	Sow	3.1	0.6	21.6 (6.5–88.3)	140	38.6
	Finisher	0.0		1	307	6.2
Region	Southern-Jutland	−3.0	1.2	0.1 (<0.0–0.4)	108	19.4
	North-Jutland	−2.4	1.5	0.1 (<0.0–1.1)	37	8.1
	Zealand	−1.4	0.7	0.3 (0.5–1.0)	69	1.4
	Mid-Jutland[a]	0		1	233	20.6
Random part	Variance			SD[f]	ICC[g]	
Herd	2.7			1.5	0.45	

[a] Reference group.
[b] Standard error of the beta coefficient estimate.
[c] Confidence interval of the odds ratios.
[d] Total number of animals tested.
[e] Proportion of animals tested seropositive at 20 percent positive (PP) cut-off value.
[f] Standard deviation.
[g] Intraclass correlation coefficient for the model.

In each production category, time to detection was affected by the apparent seroprevalence (the four different seroprevalences identified), the frequency and the size of batch deliveries to the abattoirs, and the number of samples tested per batch (one, two or six samples). Time to detection in the four production categories, based on two and six samples tested per batch are shown in Fig. 4-I and 4-II, respectively; whereas, the results for one sample tested per batch is shown in Fig. S-I in the Supplementary material III.

The estimated four different seroprevalences had a large effect on time to detection within each given production category (Fig. 4). For example, in the conventional sow herds, the proportion of herds with one or more seropositive pig detected at 90 days with two samples tested per batch delivery were 0%, 5%, 32%, and 85% at 2%, 11%, 19% and 60% seroprevalence, respectively (Fig. 4-I). The large variation in the number of pigs, and the frequency of batches sent to slaughter between the four different production categories, also had a large effect on the time to detection. For example, 0% of the organic sow herds were detected at 120 days; whereas, 15% of the conventional sow herds, 38% of the conventional finisher herds and 47% of the organic finisher herds were detected at 120 days when assuming a seroprevalence of 11% in all four production categories (Fig. 4-I). In conventional finishers, the seroprevalence was the lowest (2%), which will increase time to detection. However, the conventional finishers sent more batches to the abattoirs, and this reduced the time to detection. On the other hand, in the organic sows, the seroprevalence was highest (60%), which in theory should shorten time to detection. However, the frequency of batch delivered by the organic sows was the lowest. Hence, the effect of seroprevalence and frequency of batches delivered tended to counteract each other such that the difference in the time to detection in the two production categories was reduced despite the observed large variation in both the seroprevalence and the delivery patterns.

4. Discussion

The results of this study showed that the seroprevalence of *T. gondii* differed substantially among animals sampled from the four different pig production categories. The apparent seroprevalence in sows from conventional herds (19%) was slightly over half (Fisher's exact test P = 0.05) of the previous estimate reported from Denmark of 34% (Kofoed et al., 2017). The apparent seroprevalence for conventional finishers of 2% was comparable with the estimates from other studies in Denmark, Finland and the Netherlands (0.4%–3%) (Kijlstra et al., 2004; van der Giessen et al., 2007; Kofoed et al., 2017; Felin et al., 2019).

For the organic finishers, the apparent prevalence of 11% matched the estimate reported previously from Denmark by Kofoed et al. (2017). However, the *T. gondii* seroprevalence in organic finishers in the present study and the Kofoed et al. study was higher compared to the estimates reported from the Netherlands (1% and 3%) (Kijlstra et al., 2004; van der Giessen et al., 2007).

The highest apparent seroprevalence (60%) in the present study was reported in sows from the organic herds. We were unable to find seroprevalence estimates in sows from organic farms from European countries. Therefore, it was impossible to assess if the seroprevalence was higher in Denmark than elsewhere. However, the risk of exposure to *T. gondii* is known to be higher on free-range and organic farms than on conventional farms, as the animals have an increased risk of being exposed and infected with *T. gondii* with environmental oocysts or from ingesting infected rodents (Kijlstra et al., 2004; van der Giessen et al., 2007). In the organic herds, the *T. gondii* apparent between-herd prevalence was approximately twice the prevalence (68% out of 28 farms) on the conventional farms (32% out of 31 farms). This finding is comparable with the results from a recent Dutch survey for *T. gondii* in finishers, where they found the risk of infection to be 2.6 times higher in

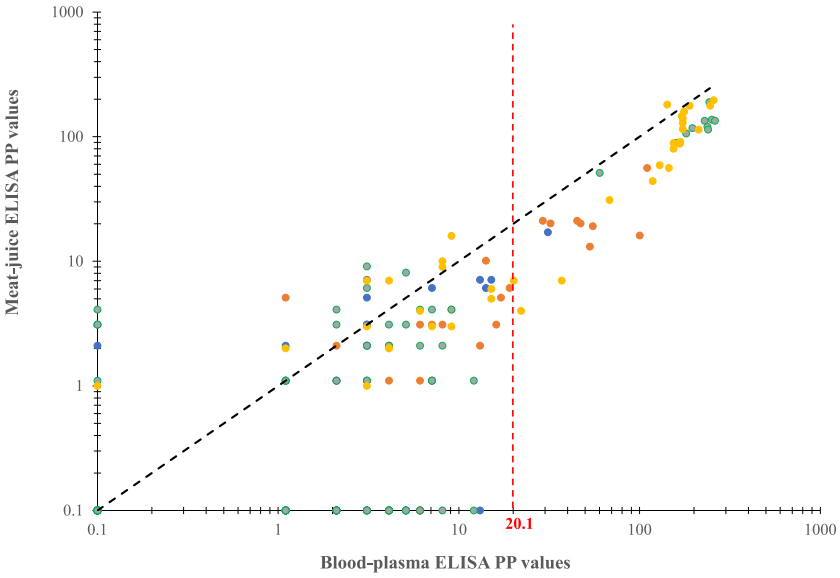

Fig. 1. Comparison of percent positive values of the positive control (PP) for meat-juice ELISA (y) with blood-plasma ELISA (x) PP values in conventional finishers (blue dots), conventional sows (brown dots), organic finishers (green dots) and organic sows (black dots). The PP values are presented on a base-10 log scale of x and y axes. Due to the presence of zeros in the raw data, a constant of 0.1 was added to all data points, with the black dotted line indicating the theoretically desirable line of equality.

pigs raised on organic farms compared to conventional farms (Swanenburg et al., 2019).

It is plausible that housing and management factors unaccounted for in the statistical model may have a role to play in the observed differences in the between-herd prevalence in the four production categories. A large amount of unexplained variation (45%) in seroprevalence was attributable to herds, indicating further exploration of herd level factors could be beneficial.

We found that the risk of infection with *T. gondii* was higher in sows (OR = 22) than in finishers. And though these findings correspond with the results from other studies; our estimates were higher than the estimates reported from Estonia and France (OR = 6, OR = 3, respectively) (Djokic et al., 2016; Santoro et al., 2017). A higher seroprevalence in sows than in finishers from the conventional production category may be attributed at least partially to the longer exposure time in sows (Kofoed et al., 2017). However, it could also be related to a higher probability of exposure to infected cats or rodents, as many Danish sow farms keep cats for rodent control (Nielsen et al., 2019). Moreover, there

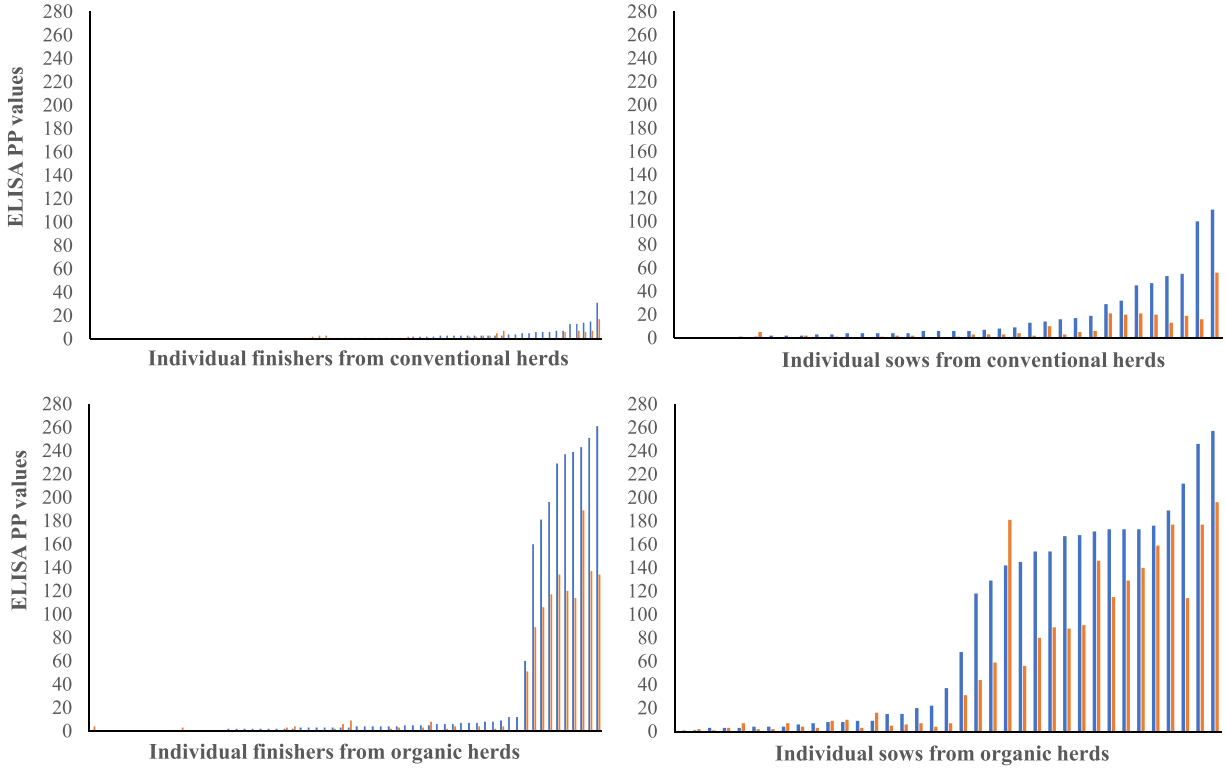

Fig. 2. The blood-plasma ELISA (blue vertical bars) percent positive values of the positive control (PP) and meat-juice ELISA (brown vertical bars) PP values, with the blood-plasma values sorted in ascending order shown for each of the four production categories (conventional finishers, conventional sows, organic finishers and organic sows). The selected cut-off for the identification of seropositive animals in the study was 20 PP.

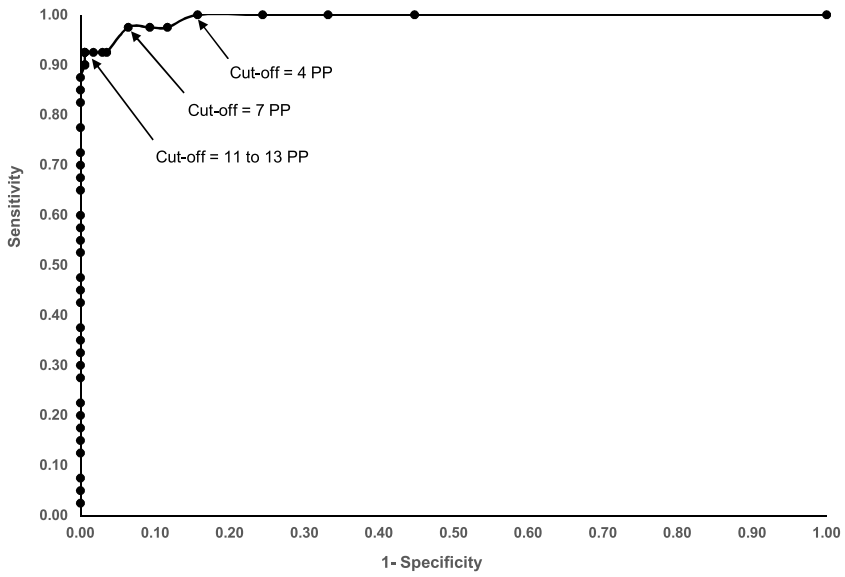

Fig. 3. Exploration of optimal cut-off for percent positive values of the positive control (PP) for meat-juice ELISA illustrated in a Receiver Operating Characteristic (ROC) with true positive fraction (sensitivity) on the y-axis, and false-positive fraction (1-specificity) on the x-axis.

Table 4
The 5%, 50% and the 95% percentiles for the number of animals and the total number of batches delivered per herd (based on herds delivering ≥ 200 finishers and ≥ 20 sows annually) to the abattoirs in 2018 from each of the four production categories in Denmark.

		Percentiles for animals delivered				Percentiles for the batches delivered			
Production categories	Total no. of farms[a]	Total no. of animals delivered[b]	5%	50%	95%	Total no. of batches delivered[c]	5%	50%	95%
Conventional finisher herds	3,561	16,615,520	429	3,701	11,914	125,202	9	34	64
Conventional sow herds	1,567	343,826	23	137	654	36,667	5	20	52
Organic finisher herds	96	234,423	295	1,581	7,637	3,410	7	38	52
Organic sow herds	34	2,227	21	42	188	290	4	8	17

[a] 37% of the farms were removed from the full dataset.
[b] 0.3% of the animals were removed from the full dataset.
[c] 8% of the batches removed from the full dataset.

is a high focus on hygiene when sows are farrowing and when piglets are weaned and moved to the grower and finisher section. This may be reducing the exposure in piglets, weaners, growers, and finisher pigs to the parasite, as suggested by Kofoed et al. (2017).

Meat-juice serology has been used in screening of German pig herds and the authors recommend use of meat-juice for a future risk-based meat inspection (Meemken et al., 2014). At the abattoir, meat samples are relatively easier to collect than blood samples, and in Denmark these samples are already available from the Danish National *Salmonella* surveillance program. Therefore, screening of herds for *T. gondii* using meat-juice samples is potentially a useful tool for risk-based surveillance in Denmark. Therefore, we explored how well the ELISA test results from meat-juice correlated with the blood-plasma ELISA results. The correlation plot and the Bland-Altman difference plot showed that the majority of values in blood-plasma were higher than the corresponding values in meat-juice, indicating that the two tests cannot be used interchangeably. Based on the ROC curve, we found that cut-off values between 11 and 13 percent positive (PP) for meat-juice gave the highest combined sensitivity and specificity for meat- juice ELISA relative to blood-plasma ELISA. Our inability to distinguish these cut-off values reflects the lack of observations within this range, so a larger dataset is therefore needed to determine a more precise cut-off value. A high degree of agreement (Kappa-value varied between 0.65 to 0.93) between blood-serum and meat-juice ELISA results has previously been reported (Meemken and Blaha, 2011). The lower PP values of meat-juice ELISA in comparison with blood-plasma may be due to a lower and varying concentration of IgG antibody levels in the meat-juice compared to serum (Wallander et al., 2015). Still we demonstrated that a high agreement between the meat-juice and blood-plasma ELISA could be achieved, when the cut-off value for meat-juice was lowered.

In the present study, we also found that seronegative sows i.e., those with PP values below the cut-off, had significantly higher PP values than the seronegative finishers, which could indicate an elevated level of cross-reacting antibodies in sows. Therefore, future studies could further explore adjusting the cut-off for the older animals such as sows to avoid false-positives. Additionally, there was a grey-zone around the current cut-off value of 20 PP, where many animals were sensitive to the selected cut-off (results not shown). Hence, retesting the samples from these animals using an alternative diagnostic test or a confirmatory test such as Western-Blot, may potentially be useful in the classification of the animals into true positives and true negatives. However, such an approach would not be suitable for a surveillance program, because simplicity is needed to ensure feasibility.

The Dutch own-check program run by Vion Food Group, is the only active surveillance program on *T. gondii* in EU. The testing of samples in this program is limited to one to two samples per batch in low-risk herds and six samples in high-risk herds (Swanenburg et al., 2019). Hence, the time-to-detection exploratory analysis was based on testing one, two or six samples per batch delivery at slaughter.

The present study shows that high-risk herds may be predicted by simple risk factors such as animal-age (sow versus finisher) and production system (organic versus conventional). Use of these risk factors for targeting of herds with high average within-herd seroprevalence has been recommended for *T. gondii*, as surveillance of all herds might not be

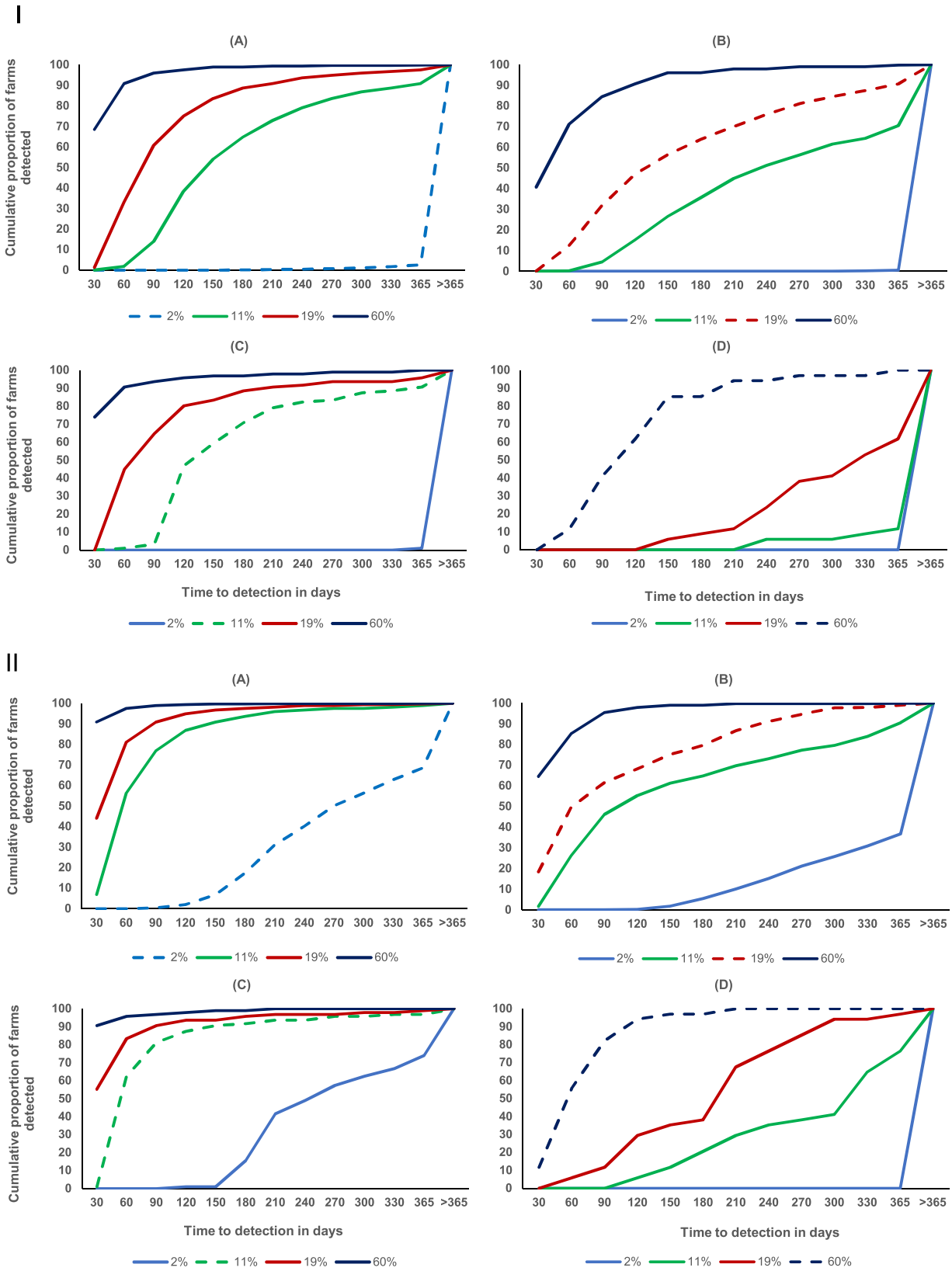

Fig. 4. I & II. Cumulative proportion of herds that meet the sample size required to detect at least one *Toxoplasma gondii* positive pig, from the population of pigs sent to Danish abattoirs in the year 2018, when testing a maximum of 2 (I) or 6 pigs (II) per delivery. In the plot for each production type (A = conventional finisher herds, B = conventional sow herds, C = organic finisher herds, D = organic sow herds), the four lines indicate the estimated average time in days to detect one positive pig based on the frequency by which these pigs are sent to the abattoirs in each production category at sample size determined at 60%, 19%, 11% and 2% seroprevalence. The dotted line indicates the time to detection identified for the production category based on the observed seroprevalence for that specific production category, where the other three non-dotted lines are the observed seroprevalence estimates for the three other production categories.

economically feasible or useful (Alban et al., 2020). If it is judged that mitigation for *T. gondii* in pigs and pork is necessary, then different initiatives can be put in place, such as auditing, surveillance and control. In low-risk herds, auditing of biosecurity may be the most cost-effective approach, in line with EFSA's recommendation (EFSA, 2011a). The vast majority (96.6%) of the animals sent to slaughter in this study originated from conventional finisher herds, and thus contributed to most of the pork produced in Denmark. Additionally, this study showed that the time to detection of one positive pig was long in conventional finisher herds at low prevalence (2%); therefore, relatively many samples must be tested for the detection of an infected animal. The EU controlled housing concept is already in use for the control of *Trichinella* on Danish pig farms (Alban and Petersen, 2016). This could explain the low *T. gondii* seroprevalence among the conventional finishers indicating that the current requirements for controlled housing is already effective in controlling *T. gondii* infections in pigs. Therefore, the conditions for controlled housing for conventional finishers proposed for *Trichinella* could also be used for *T. gondii* (EFSA, 2011a). At present, approximately 95% of the conventional finisher herds in Denmark are audited for controlled housing conditions by an independent third-party company (Asger Kjær Nielsen, SEGES, personal communication). Therefore, for further control of *T. gondii*, adapting the controlled housing requirements could be considered. The remaining 5% of the herds, originating from conventional herds that do not meet the controlled housing conditions, could be tested at the abattoirs or could be classified as high-risk (EFSA, 2011a). Nevertheless, the current measures for controlled housing could also be modified to include control and prevention of cats into the pig barns and restrict cats' access to feed, water and bedding along with use and cleaning of boots and disinfection of pig barns (EFSA, 2011a; Limon et al., 2017).

In medium-risk herds such as organic finisher herds, a surveillance for *T. gondii* may be meaningful, if the information collected can lead to a subsequent lower prevalence in the herds e.g. through targeted improvements of the biosecurity. However, only 1.4% of all the pigs slaughtered in 2018 in Denmark originated from organic finisher herds (which sent $\geq$ 200 animals annually). There has been a growing demand from Danish consumers for organic and free-range pork, and this meat sells at a high price for its brand value. Therefore, it may be of value for the industry to invest in maintaining the brand reputation. Based upon results from surveillance, organic finisher herds could be categorized into high or low-risk herds as part of an own check program (Alban et al., 2020). Our findings show that organic finisher herds with high seroprevalence may be detected in a short time-period as they send animals frequently to slaughter. Therefore, surveillance would enable identification of high-risk herds, and the meat from this risk group could be subjected to post-harvesting methods such as freezing, heat-treatment or curing to destroy any viable parasites present in the product (Kijlstra and Jongert, 2008). Moreover, actions to be taken in high-risk herds would have to be defined, such as a visit to the farm to investigate reasons for the high prevalence. However, before surveillance can be fully recommended, we need to know more regarding whether such follow-up visits can lead to a subsequent lower prevalence in the herds. In all cases, consumers and food service retailers should be made aware of the proper methods to handle, and cook raw meat, and avoid pink pork to ensure that pig meat is safe for consumption (Kijlstra and Jongert, 2009).

For meat originating from high-risk herds, control measures at the processing plants may be considered as an option; e.g. recommendation of freezing of meat intended for use in production of ready-to-eat products. Meat from conventional and organic sows constituted only 2% of the total number of animals slaughtered in 2018. The average seroprevalence in these production categories were 19% and 60%, respectively, and the time to detection was long because sow herds send animals intermittently, and in small batches to the abattoirs. Sows are reared for reproduction, and their meat is mainly used in the production of sausages and other cured meat products, where a high NaCl content will inhibit the viability of the parasite (Kijlstra and Jongert, 2008). Therefore, sows from conventional and organic herds could be categorized into high-risk per se without surveillance, and all the meat from this risk-group could be subjected to post-harvest strategies to destroy *T. gondii parasites* through freezing, salting, or curing (Kijlstra and Jongert, 2008).

5. Conclusion

The apparent *Toxoplasma gondii* seroprevalence varied within the four pig production categories, from 2% positives in conventional finishers to 60% in organic sows. Organic versus conventional production and sows versus finishers were two important risk factors for testing seropositive. The meat-juice and blood-plasma ELISA values were strongly correlated, and based on a cut-off of 13 PP we estimated a sensitivity and specificity of 93% and 99%, respectively, for meat-juice ELISA relative to blood-plasma ELISA. This may suggest that the meat-juice samples collected at slaughter for the ongoing Danish *Salmonella* control program could be used to identify herds with high *T. gondii* seroprevalence; however, an additional study is needed to ensure feasibility. Time to detection of one or more seropositive pigs will be long in conventional finisher herds due to low prevalence and in many sow herds because of intermittent delivery of a low number of sows. In organic finisher herds, the time to detection will be short due to medium prevalence and frequent delivery of a high number of finishers. Auditing of biosecurity in conventional finisher herds, surveillance of organic finishers with meat-juice at slaughter, and freezing, curing or heat-treating sow meat prior to making ready-to-eat products may be cost-effective solutions to reduce the risk for consumers.

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Declaration of Competing Interest

Abbey Olsen, Lis Alban, Marianne Sandberg, and Tina Birk Jensen work for an organization that offers advice to farmers and abattoirs. The sample collection for the project was funded by the "Danish slaughterhouse committee".

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Supplementary data.

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.prevetmed.2020.105149.

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