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Looking for new approaches for the use of serology in the context of control programmes against pig salmonellosis

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Summary

Most swine *Salmonella* national control programmes in Europe have been based on the categorization of herds according to risk levels based on serological results. However, none of the non-Scandinavian countries have reported of any significant success on *Salmonella* infection reduction in fattening pigs or the number of human cases attributable to pigs or pork. The limited accuracy of the tests used, the small number of animals sampled and the likely lack of herd representativeness of the samples used could be major factors affecting the suitability of these programmes. Focusing on minimizing *Salmonella* shedding at slaughter appears more important to prevent human infections than focusing on detection of seropositive pigs/herds at this stage. This study assessed whether performing on-farm serology may help to predict shedding at slaughter. Between 2010 and 2016, pigs from six cohorts from a *Salmonella*-positive herd were bled at 30, 60 and 90 days on fattening and before slaughter, and faecal samples collected at slaughter. Serology on days 60, 90 and before slaughter predicted somewhat shedding at slaughter with no significant differences among them. Pigs with higher OD% values at these point times would have higher risk of shedding when arriving to slaughter. The probability of shedding for a pig sampled on day 90 and showing an OD% value of 10 was 43%, and the risk increased up to 65% if the OD% was 40. Concluding, on-farm serology may help to determine to some extent the risk of *Salmonella* shedding at slaughter from seropositive fattening units, which would allow for prompt on-farm and slaughter interventions to reduce the likelihood of slaughter contamination with *Salmonella*.

KEYWORDS
control programmes, *Salmonella*, serology, swine

1 | INTRODUCTION

In the mid-1950s, Sweden experienced severe *Salmonella* outbreaks, and contaminated animal products were associated with these outbreaks (Lundbeck, Plazikowski, & Silverstolpe, 1955). This prompted the beginning of the first comprehensive *Salmonella* National Control Program in Europe (Wierup, 2006). The objective of the Swedish *Salmonella* control programme was to deliver animal products for human consumption free from *Salmonella*, and it was based on preventing *Salmonella* contamination of the whole production chain. Pigs were for first time considered as a potential source of human salmonellosis. Recently, the contribution of pigs to human salmonellosis in the EU has been quantified, and it is now considered as its second major source (de Knegt, Pires, & Hald, 2015; Pires, Knegt, & Hald, 2011).

After the Swedish control programme, other Scandinavian countries followed suit with Denmark, one of the major European pig-exporting countries, starting its own programme in 1995 (Mousing et al.,

1997). The success of the Scandinavian programmes along with new European regulations (Regulation -EC- No 2160/2003 on the control of *Salmonella* and other specified food-borne zoonotic agents) triggered the implementation of national control programmes in other European countries (Germany and United Kingdom in 2002, Ireland in 2003, the Netherlands in 2005 and Belgium in 2007; Anonymous, 2008; Blaha, 2003; Hanssen, Swanenburg, & Maassen, 2007; Méroc et al., 2012). In general, these programmes were mostly based on the Danish model, with the use of serology as the cornerstone of all them. In brief, a small number of pigs are sampled annually, usually at slaughter (blood or meat juice), and pig farms are characterized within different risk categories (I, II, and III) based on some sort of estimation of a weighted seroprevalence (levels of infection may vary widely among batches and years), with those farms with higher seroprevalence (i.e. category III) being considered the "highest-risk" herds. Farmers from these farms are encouraged to initiate herd-specific activities aimed at reducing their risk level, that is, at decreasing the exposure to *Salmonella*, so they can reduce seroprevalence and move to lower categories.

Although some positive achievements have been reached after the implementations of these programmes, to the authors′ knowledge, none of the non-Scandinavian countries has reported of any overall significant success on *Salmonella* infection reduction in fattening pigs or on the number of human cases attributable to pigs or pork. On the contrary, United Kingdom suspended its meat juice testing for *Salmonella* antibodies in 2012 and moved towards an "on-farm risk assessment" approach based on a scoring system (Anonymous, 2012). Belgium discontinued its serological programme in 2015 but kept advisory veterinarians on the field (Brossé, 2015). Germany, although still keeps the original programme based on serology and herd categorization, has not detected a significant reduction in category III farms (Blaha, 2017).

There are many country-specific factors that may be responsible for the lack of success of these programmes, but a major and common contributor to all of them may have relied on serological tests of limited diagnostic accuracy. Several studies have shown the lack of correlation between serological and microbiological results for detection of pig salmonellosis at the individual level (Farzan, Friendship, & Dewey, 2007; Funk, Harris, & Davies, 2005; Methner, Rammler, Fehlhaber, & Rösler, 2011; Nollet et al., 2005; Vico, Engel, Buist, & Mainar-Jaime, 2010). The variability associated with the use of different ELISAs or matrices (serum or meat juice) has been also reported (Farzan et al., 2007; Mainar-Jaime, Atashparvar, Chirino-Trejo, & Blasco, 2008; Mejía, Casal, Mateu, & Martin, 2005; Vico & Mainar-Jaime, 2011). Thus, the bias assumed when these ELISAs are used on individuals should be translated to the herd level as well, which probably make them unsuitable for proper herd risk characterization (Gradassi et al., 2015; Sørensen, Alban, Nielsen, & Dahl, 2004; Vico et al., 2010). The small sample size usually considered (between 36 and 100 pigs/year) for an infection whose presentation varies among batches and years, and the lack of representativeness of the on-farm animal distribution, as pigs are usually sampled at slaughter, are factors that also may have contributed to increase that bias. The wrong *Salmonella* characterization of a pig herd certainly leads to a misperception of its actual *Salmonella* risk. In addition, the lack of good, reliable and cost-effective on-farm interventions may also explain in part the lack of success of these programmes, as the efficacy of the different interventions seems to be very variable (FAO-WHO, 2015). Thus, a new approach may be needed to tackle this problem, and, if serology has to be used, then, it seems reasonable to look for a different role for it.

Impacts

- Most swine *Salmonella* national control programmes in Europe have failed to report any significant success on *Salmonella* infection reduction in fattening pigs or the number of human cases attributable to pigs or pork.
- On-farm serology may help to predict to some extent the probability of a pig shedding *Salmonella* at slaughter.
- The use of on-farm serology would allow for prompt on-farm and slaughter interventions to reduce the likelihood of slaughter contamination with *Salmonella*.

The sources of carcass contamination are multiple, and global approaches considering the whole food chain (i.e. from stable to table) are required (Alban & Stärk, 2005), but the presence of *Salmonella* in the pig′s faeces is with no doubt a major source of slaughter and carcass contamination (Argüello, Álvarez-Ordoñez, Carvajal, Rubio, & Prieto, 2013; Swart, Simons, Evers, Snary, & Swanenburg, 2015). Thus, for the pig farms, one of the most important objectives to address should be the reduction in the proportion of pigs shedding *Salmonella* that arrive to the slaughterhouse. Indeed, a reduction in *Salmonella* loads in the guts of slaughtered pigs might help to reduce the proportion of contaminated carcasses in the slaughter (Pesciaroli et al., 2017). The slaughter plants should, in turn, maintain strict hygienic measures (i.e. improving singeing procedures, reducing probability of cross-contamination at degutting and handling), as these measures are likely the best way to reduce the number of contaminated carcasses (Alban & Stärk, 2005). Therefore, focusing on the prevention of *Salmonella* shedding when pigs arrive to the slaughterhouse may be an initial step far more important than focusing on detection of seropositivity at this stage.

It has been observed that pigs shedding *Salmonella* at slaughter seroconverted earlier during the fattening period than non-shedders pigs (Casanova-Higes, Andrés-Barranco, & Mainar-Jaime, 2017a), which may help to predict the risk of shedding at slaughter. Thus, in this study, we assess whether performing on-farm serology may help to predict shedding at slaughter and, if so, when, during the fattening period, serology would predict it better.

2 | MATERIAL AND METHODS

2.1 | Animal selection and sample collection

This study takes advantage of results from six field trials carried out between 2010 and 2016 in a small fattening unit ($N \approx 110$) to

TABLE 1 Proportion of slaughter pigs shedding *Salmonella* and infected with *Salmonella* (presence of the bacterium in mesenteric lymph nodes.-MLN-) for each batch of pigs analysed

	Batch (no. of pigs)					
	A (25)	B (28)	C (49)	D (48)	E (41)	F (42)
% of shedders at slaughter (95%CI)	60 (39.4, 80.6)	21.4 (5.2, 37.6)	69.4 (66, 82.8)	75 (62.3, 87.7)	9.7 (0.3, 19.2)	14.3 (3.2, 25.3)
% of carrier pigs in MLN at slaughter (95%CI)	76 (58, 94)	18.5 (2.8, 34.2)	40.8 (26.5, 55.1)	68.7 (55.1, 82.3)	7.3 (0, 15.3)	40.5 (25,55.9)

assess the efficacy of the addition to the pig feed of different products for the control of *Salmonella* infection. The pigs considered for the study belonged to six control groups (i.e. groups on which no interventions were carried out) included in those field trials (hereinafter batches A to E). Each batch of control pigs included ≈50 pigs. The farm was located in the NE of Spain and was known to be *Salmonella* positive.

Pigs had been individually identified by ear tags, and only those that had been blood sampled at 30 (30d), 60 (60d) and 90 (90d) days in the fattening unit and within 3 days before slaughter (approximately 1 month from last sampling), and for which a minimum of 25 g of faecal (FEC) samples were collected at slaughter, were considered for this study. In addition, 25 g of mesenteric lymph nodes (MLN) was also collected at slaughter from these pigs to determine their infection status.

Serum was obtained after blood clotting and kept at −20°C until analysed. The HerdCheck Swine *Salmonella* ELISA (IDEXX Laboratories, Westbrook, ME, USA) was used for detection of antibodies against *Salmonella* spp., and results expressed as OD% values following manufacturer's instructions. Bacteriology on FEC and MLN samples was performed according to the EN ISO 6579:2002/A1:2007 (Anonymous, 2007).

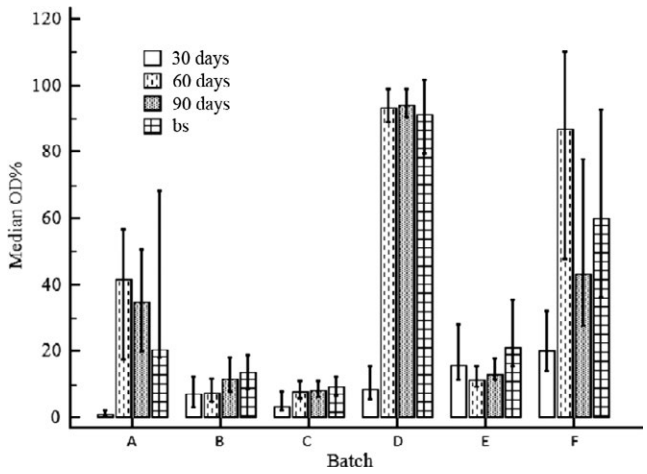

FIGURE 1 ELISA median OD% values and their corresponding 95% confidence intervals for pig serum samples collected on day 30 (30d), 60 (60d) and 90 (90d) on fattening and before slaughter (bs) for six batches of pigs (A–F)

2.2 | Statistical analysis

Median OD% values and their corresponding 95% confidence intervals were estimated for each sampling time and for each batch of pigs. Overall estimates of prevalence of shedding (FEC +) and infection (MLN +) at slaughter were also calculated for each batch.

The relationship between OD% values (log-transformed) at each sampling time (30d, 60d, 90d and before slaughter) and shedding at slaughter was assessed by logistic regression analysis after adjusting by batch. When a significant association was found, Receiver Operating Characteristic (ROC) curves were constructed, and the area under the curve (AUC) estimated. Estimates of probability of shedding *Salmonella* were calculated from the logistic regression equations. The relationship between being a MLN-positive pig and shedding at slaughter was also assessed by logistic regression analysis. All statistical analyses were performed with STATA software (STATA, StataCorp, LP, USA).

3 | RESULTS

3.1 | Overall estimates

Of a total of 306 pigs, 233 (76.1%) met the inclusion criteria and were included in the study. For 55 of them, no information was available on shedding at slaughter, and 18 lacked some serological data. The number of sampled pigs varied among batches, with a minimum of 25 (52%) pigs for batch A and a maximum of 49 (96%) for batch C (Table 1). Overall, a total of 101 (43.3%; 95%CI: 36.9, 49.8) pigs were shedding *Salmonella* spp. at slaughter, and in 97 (41.8%; 95%CI: 35.4, 48.2), *Salmonella* spp. was isolated from MLN. Results on prevalence of shedding and infection by batch are shown in Table 1. An overall positive significant relationship between being a MLN-positive pig and shedding *Salmonella* at slaughter was also found (OR = 4.2; CI95%: 2.02–8.57; $p < .001$).

Serological results differed among batches. The OD% values for pigs from the batches B and C remained quite low (medians around or below 10%) for all sampling times. In contrast, for batches A, D and F, OD% values increased significantly after first sampling on day 30 (Figure 1). For batch E, OD% values remained similar along the fattening period with some increase in the last sampling.

No relationship was observed between OD% values and shedding at slaughter when serum samples were collected on day 30 on

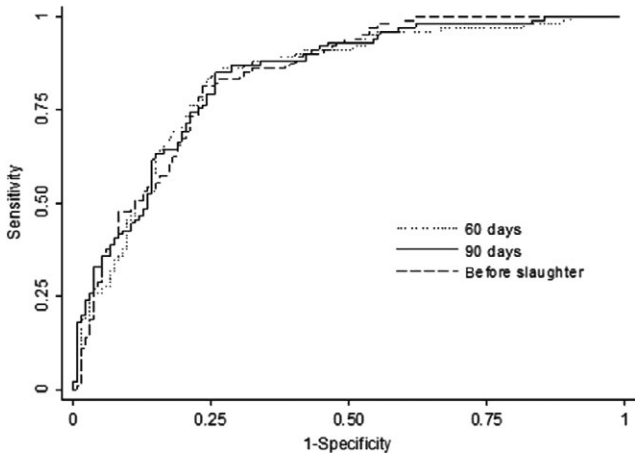

FIGURE 2 Receiver Operating Characteristic (ROC) curves estimated for prediction of shedding when an ELISA test was used on serum samples collected at 60 and 90 days on fattening and before slaughter

fattening (p = .79). However, a positive significant relationship was found between OD% values and shedding at slaughter for samplings on days 60, 90 and before slaughter (p values of .009, .004 and .004, respectively). Thus, the use of the ELISA at day 30 on fattening was not considered for further analysis.

3.2 | ROC analysis and estimates of probability of *Salmonella* shedding

Figure 2 depicts the ROC curves for the ELISA test with regard to shedding at slaughter when performed at 60 and 90 days on fattening and before slaughter. Table 2 shows the AUCs for these ROC analyses. No differences were observed regarding AUC among these three sampling times.

As batches B and C presented very low OD% values along the entire fattening phase, a further ROC analysis was performed only with those batches that showed some increase in OD% values along this period (A, D, E and F). Results remained similar although the AUC for the different sampling times increased somewhat (Table 2).

Estimates of the probability of shedding *Salmonella* spp. at slaughter with regard to OD% values for pigs sampled on day 90 of the fattening period from batch A are shown in Figure 3. When all batches were considered the probability of shedding *Salmonella* spp. for a pig showing an OD% = 10 was as high as 42.8%. When only batches that showed some increase in OD% values during the fattening period were included (batches A, D, F and E), this probability was slightly lower (39.7%; Figure 3).

4 | DISCUSSION

Pig salmonellosis is increasingly related to human salmonellosis (Snary, Swart, & Hald, 2016) being probably the most important zoonotic infection of pigs now. The main aim of any swine *Salmonella* control plan should then be to contribute to the reduction in the incidence of human salmonellosis. Had on-farm serology be useful for predicting *Salmonella* shedding at slaughter, then it may help to reduce the risk of abattoir contamination and the subsequent carcass contamination, which is the most likely source of contamination of pork and pork products.

Pig salmonellosis is an infection highly variable because depends on the concurrence of a large variety of factors related to the pig, the farm, the environment, the slaughter, etc., which may vary within and among years (Fosse, Seegers, & Magras, 2009). For that reason, in the current approach, national control programmes usually rely on a weighted mean seroprevalence to characterize pig farms after several consecutive samplings (Anonymous, 2008; Blaha, 2003; Hanssen et al., 2007; Méroc et al., 2012; Mousing et al., 1997). In this study, carried out along six almost consecutive years on a fattening unit known to be *Salmonella* positive and from an area of high prevalence of *Salmonella* in pigs (Vico et al., 2011), results among batches differed largely, representing this expected variability somehow. Serological results showed different on-farm pig *Salmonella* exposure experiences among batches. In batches B and C, the low OD% values

TABLE 2 Area under the curve (AUC) from the ROC analyses assessing the accuracy of ELISA test results for predicting shedding at slaughter when serum was collected at three different sampling times along the fattening period

Day of serum sampling	All batches		Only batches A, D, E and F	
	AUC	95%CI (AUC)	AUC	95%CI (AUC)
60 days on fattening	0.829	0.77, 0.88	0.844	0.78, 0.91
90 days on fattening	0.835	0.78, 0.89	0.860	0.80, 0.92
Before slaughter	0.835	0.78, 0.89	0.876	0.82, 0.93

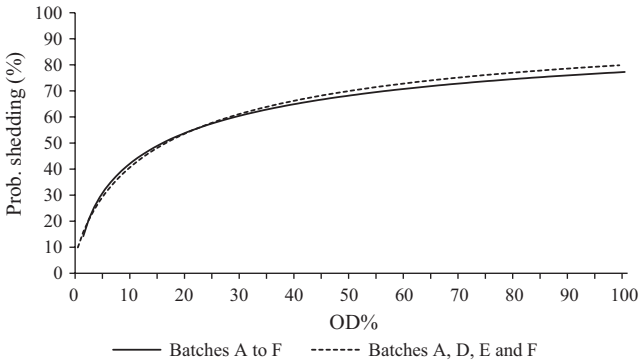

FIGURE 3 Estimated probability of shedding at slaughter for a pig with regard to the OD% value on serum when pigs from batch A were blood sampled on day 90 of the fattening period. Estimates are calculated for all the batches together (A–F) and only for those batches that showed increasing OD% values along the fattening period (A, D, E and F)

observed suggested that *Salmonella* hardly circulated within the unit. Interestingly, the proportion of pigs shedding *Salmonella* at slaughter was relatively high or very high (21.4% in B and 69.4% in C), and the prevalence of infection also followed the same pattern (18.5% and 40.8%, respectively). It appears that pigs in batch B, and particularly in batch C, would have been infected at the end of the fattening period, i.e. within the last 10–15 days before slaughter, or during transport or lairage, with no time to develop detectable antibodies (IgG) (Nielsen, Baggesen, Bager, Haugegaard, & Lind, 1995; van Winsen et al., 2001). This would explain why pigs remained seronegative but many were MLN-positive and also shed *Salmonella* at slaughter. As pigs usually shed *Salmonella* right after the initial infection and for some days and then become intermittent shedders (Beloeil et al., 2003; Nielsen et al., 1995; Scherer et al., 2008), the use of on-farm serology at any time point during the fattening period would have been virtually useless in these two batches.

In contrast, pigs from batches A, D and F experienced a significant increase in OD% values after the first sampling on day 30, which was compatible with the circulation of *Salmonella* on the farm and pigs being exposed to the bacterium. In all these batches, the proportion of both infected and shedding pigs was high (Table 1). In this scenario, shedding at slaughter would have been associated not only to early on-farm infections likely exacerbated by the stress induced by the transport to the slaughterhouse and the lairage (Argüello et al., 2013), but also to some new infections occurring close to the date of slaughter. All together would explain the overall positive highly significant association observed between infected pigs (i.e. MLN-positive) and *Salmonella* shedding at slaughter (OR = 4.2; CI95%: 2.02–8.57; $p < .001$). Pigs from batch E kept OD% values relatively low and fairly constant along the fattening period, which was consistent with moderate levels of infection and shedding at slaughter (7.3% and 9.7%, respectively). In this latter situation, few new cases of infection seemed to have occurred during transport or lairage.

To explore the potential that on-farm serology may have to predict *Salmonella* shedding at slaughter considering this variability in scenarios, this study considered all the batches in a first step. Logistic regression and ROC analyses were used for assessing the relationship between on-farm serological results obtained from pigs at different time points during the fattening period and shedding at slaughter. A variable number of pigs were analysed within each batch due to the different availability of complete serological and microbiological information from the pigs in each batch, i.e. from a minimum of 52% of the pigs with complete information in batch A to a maximum of 96% in batch C. The number of pigs included in each batch and the lack of association between the inclusion criteria and the pig serological status (data not shown) suggested that the sample used was representative of the whole batch.

Serological results at the beginning of the fattening period, i.e. on day 30 of fattening, appeared to be useless for the objective of predicting shedding at slaughter. This was an expected result as many pigs at 30d may not have getting in contact with *Salmonella* yet, and some seropositive pigs at this time may have become seronegative before arriving to the slaughter (van der Wolf et al., 2001). Thus, sampling pigs on day 30 of fattening seems to be too early in order to get a proper picture of what the shedding status of the batch would be at slaughter. However, and despite serological and microbiological results from batches B and C, an overall significant positive correlation was observed between serology when serum samples were collected at 60, 90 days on fattening or just before slaughter and *Salmonella* shedding at slaughter.

Analysing pig serum in any of the last three sampling times yielded similar results as indicated by their corresponding AUC. Pigs with higher OD% values at these point times would have higher risk of shedding when arriving to slaughter (Figure 3). When all batches were considered in the analysis, the probability of shedding *Salmonella* at slaughter for a pig from batch A, sampled on day 90, and presenting an ELISA OD% value of 10 was around 43%. This risk increased significantly up to 65% if the pig had an OD% of 40. When the analysis was carried out only with the batches that experienced an increase in OD% values after the first sampling (i.e. excluding batches B and C), the risk of shedding tended to be slightly lower for low OD% values (39.7% for an OD% = 10) and remained similar for OD% values of 40 (Figure 3). These latter estimates appeared to be somewhat more realistic as those batches of pigs that were likely infected close to the date of slaughter were not included. As for these pigs serology is not able to predict *Salmonella* shedding at slaughter, then it is reasonable to expect that the analysis with all the batches will yield biased results towards increasing the risk of shedding for pig showing low OD% values. Had infection during transport and lairage be prevented, the ability of on-farm serology to predict shedding at slaughter would have surely improved.

Bearing in mind the limitations of this study, which was restricted to a single farm sampled in different years, its results should be taken with caution. Overall, they suggest that for infections occurring time ahead of the slaughter date (between 15 and 45 days before), on-farm serology may be of help to determine to some extent the risk of *Salmonella* shedding at slaughter. To achieve this objective, a representative sample of pigs should be selected from the batch of interest. Once the potential risk of shedding has been determined for a batch, on-farm interventions could then be scheduled ahead of time to try to mitigate the probability of shedding when pigs arrive to slaughter. For example, the addition of organic acids to the feed, water or both may help to reduce *Salmonella* shedding (Calveyra et al., 2012; Creus, Pérez, Peralta, Baucells, & Mateu, 2007; Lynch et al., 2017), but this strategy will surely require long periods of treatment, at least 4 weeks, before any positive effect is detected (Casanova-Higes, Andrés-Barranco, & Mainar-Jaime, 2017b). Thus, on-farm serum sampling could be performed any time after 60 days on fattening and until 2–3 days before slaughter, but as sooner the serum is collected and analysed, more time will be available to implement this type of strategy and higher the likelihood of obtaining some positive effects. In addition, being the slaughter aware of the risk, additional interventions could be implemented such as special transport, separate lairage and logistic slaughter. A combined farm/slaughterhouse approach would likely have cumulative benefits (Swart et al., 2016). This approach would also benefit

from a more precise serological characterization of the pig farms as proper representative serum samples of the pigs in the batch will be collected. Farms presenting low OD% values on day 90 would be expected to remain so for the rest of the fattening period if nothing wrong happens during the time remaining before slaughter. But as pigs showing consistent seronegative results during the fattening period may end up shedding *Salmonella* at slaughter if they are exposed to highly contaminated environments (Casanova-Higes et al., 2017a), appropriate disinfection of trucks and lairage areas should be guaranteed for these pigs to try to prevent late infections and further shedding. A large-scale study to confirm the potential of this approach to reduce *Salmonella* shedding at slaughter is warranted.

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CONFLICT OF INTEREST

None.

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