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Sensitivity of the ISO 6579:2002/Amd 1:2007 Standard Method for Detection of *Salmonella* spp. on Mesenteric Lymph Nodes from Slaughter Pigs

R. C. Mainar-Jaime,[a] S. Andrés,[a] J. P. Vico,[a] B. San Román,[b] V. Garrido,[b] M. J. Grilló[b]

Centro de Investigación y Tecnología Agroalimentaria (CITA) de Aragón, Zaragoza, Spain[a]; Instituto de Agrobiotecnología (CSIC-UPNA-Gobierno de Navarra), Pamplona, Spain[b]

The ISO 6579:2002/Amd 1:2007 (ISO) standard has been the bacteriological standard method used in the European Union for the detection of *Salmonella* spp. in pig mesenteric lymph nodes (MLN), but there are no published estimates of the diagnostic sensitivity (Se) of the method in this matrix. Here, the Se of the ISO (Se_{ISO}) was estimated on 675 samples selected from two populations with different *Salmonella* prevalences (14 farms with a ≥20% prevalence and 13 farms with a <20% prevalence) and through the use of latent-class models in concert with Bayesian inference, assuming 100% ISO specificity, and an *invA*-based PCR as the second diagnostic method. The Se_{ISO} was estimated to be close to 87%, while the sensitivity of the PCR reached up to 83.6% and its specificity was 97.4%. Interestingly, the bacteriological reanalysis of 33 potential false-negative (PCR-positive) samples allowed isolation of 19 (57.5%) new *Salmonella* strains, improving the overall diagnostic accuracy of the bacteriology. Considering the usual limitations of bacteriology regarding Se, these results support the adequacy of the ISO for the detection of *Salmonella* spp. from MLN and also that of the PCR-based method as an alternative or complementary (screening) test for the diagnosis of pig salmonellosis, particularly considering the cost and time benefits of the molecular procedure.

Salmonella is recognized as one of the major zoonotic pathogens in the European Union (EU) (1), and pigs are one of the most important sources of infection for humans (2). In 2003, the EU initiated a process to monitor the control of *Salmonella* and other specified zoonotic agents transmitted by foods (EC Regulation 2160/2003). For this purpose, several bacteriology-based baseline surveys were carried out to estimate the prevalence of *Salmonella* spp. in both fattening and breeding pigs within the EU member states (MS) (3, 4). To facilitate the comparison of results among MS, harmonized sampling and bacteriological methods are used for the detection of *Salmonella*, according to Annex I of Commission Decision 2006/668/EC (5). The bacteriological method recommended for *Salmonella* sp. isolation from mesenteric lymph nodes (MLNs) of finishing pigs is ISO 6579:2002/Amd 1:2007 (ISO), since it is considered a thorough technique that yields 100% specificity (Sp) by including bacteriological confirmation of presumptive isolates by serotyping (6). However, no data on the sensitivity (Se) of this bacteriological protocol when performed on MLNs have been reported.

It is well recognized that the Se of bacteriological culture varies with regard to factors such as the sample of choice (feces, lymph nodes, or tonsils), the type of sample (single or pooled), the amount of sample processed, or the combination of culture media used for isolation of the bacterium (7–12). For instance, the Se of bacteriology on fecal samples has been reported to be as low as 9% (10) and higher than 90% (11, 12). Thus, when more than one method is used within the same surveillance system, comparisons are likely to be biased (13).

A common limitation for estimating the diagnostic accuracy of *Salmonella* culture is the lack of proper infected and noninfected "gold standard" populations. In fact, most studies estimate the Se of a given bacteriological technique relative to a combination of different but imperfect bacteriological methods (7, 9, 11). To overcome this problem, latent-class methods with Bayesian approaches have been used to obtain unbiased estimates of the Se's of different culture protocols for the diagnosis of pig salmonellosis on fecal samples under field conditions (12, 14).

In the present study, the advantage of this statistical methodology was used to estimate the Se of ISO (Se_{ISO}) when performed on MLNs from pigs raised under typical intensive-production conditions. The Bayesian method chosen is based on the use of two diagnostic tests applied to individuals from two different populations with different prevalences (15). For this purpose, a PCR method targeting the *invA* gene of *Salmonella* was used as a second diagnostic method. The *invA* gene-based PCR has been proposed as an alternative to bacteriology (16). The use of this specific gene, which is responsible for the invasion of *Salmonella* into enteric cells, in either a conventional or a quantitative real-time PCR (qRT-PCR) has been reported to yield diagnostic sensitivities of ≥90% in different type of samples, after preenrichment in the proper medium (13, 17–20).

MATERIALS AND METHODS

Sampling and microbiological procedures. This study was carried out within the framework of a larger study developed between February 2008 and January 2010 to estimate the prevalence of pig salmonellosis in a high pig-producing area of Spain (20). In this study, an average of 25 fattening pigs per farm from 27 pig farms were randomly selected in the slaughter line, and MLN samples were obtained and submitted to *Salmonella* sp. isolation using ISO, as detailed previously (20). Briefly, fresh MLN sam-

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Address correspondence to M. J. Grilló, mariajesus.grillo@unavarra.es.

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ples were defatted, weighed (25 g/animal), externally decontaminated by dipping into absolute alcohol and further flaming, homogenized in 225 ml of buffered peptone water (BPW), and incubated for 18 ± 2 h at 37 ± 1°C. Thereafter, 3 drops (33 μl each) of incubated BPW were inoculated into a modified semisolid Rappaport Vassiliadis (MSRV) medium, and plates were incubated for 24 ± 3 h at 41.5 ± 1°C (negative samples were reincubated for an additional 24 h). One microliter of the presumptive *Salmonella* growth (detected by the halo generated in MSRV after 24 or 48 h) was transferred to two selective media (xylosine lysine deoxycholate [XLD] and brilliant green [BG] agars). Suspected colonies were confirmed biochemically (triple sugar iron [TSI] agar, urea agar, L-lysine decarboxylation medium, and indole reaction) and by serotyping at the National Centre for Animal Salmonellosis (Madrid, Spain) following the Kauffmann-White-Le Minor scheme (21).

In parallel, 1-ml aliquots of BPW air-liquid interface culture were stored at −20°C to be used further for identification of *Salmonella*-positive samples through the molecular PCR method described below, after selection of samples from populations with high and low prevalences of salmonellosis (P_A and P_B, respectively) on the basis of the ISO results (see below).

Since the main goal of this study was to assess Se_{ISO}, when discrepant PCR-positive (PCR^+) but ISO negative (ISO^-) samples were detected, the results were assessed with a 1-ml BPW aliquot that was defrosted, diluted 1:10 in BPW, and submitted to a second *Salmonella* sp. culture following the steps of the ISO protocol described above.

DNA extraction and PCR. One 1-ml BPW aliquot of each frozen sample was submitted to DNA extraction by the rapid boiling procedure (22), consisting of (i) centrifugation (13,000 rpm, 10 min), (ii) resuspension of the pellet in 100 μl of distilled water, (iii) boiling (99°C, 20 min), (iv) a final centrifugation (4,000 rpm, 4 min), and (v) storage of the supernatant containing the bacterial DNA at 4°C until its use. The primers Fw (5′-AGTGCTCGTTTACGACCTGAA-3′) and Rv (5′-TGATCGATA ATGCCAGACGA-3′) were designed to amplify a 229-bp DNA fragment. The PCR mix was prepared with 5 μl of DNA and 20 μl of 0.4 mM each primer, 0.2 mM each deoxynucleoside triphosphate, 0.5 U *Taq* DNA polymerase (Kappa Biosystems), and 1× buffer (containing 1.5 mM Mg^{2+}). After an initial denaturation step (94°C, 5 min), the PCR was performed by 40 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 20 s, with a final extension step at 72°C for 10 min. Distilled water and DNA extracted from the *Escherichia coli* ATCC 25922 (kindly provided by José Leiva, Clínica Universitaria de Navarra, Pamplona, Spain), *Salmonella enterica* serotype Enteritidis 3934 (from the IdAB Collection, Pamplona, Spain) and *Salmonella* Typhimurium DT104 (kindly provided by Axel Cloeckaert, INRA, Tours, France) strains were used as negative and positive controls, respectively, in each PCR. The resulting PCR products were submitted to conventional electrophoresis in a 1.5% (wt/vol) agarose gel and ethidium bromide staining.

Statistical analysis. McNemar's χ^2 test and the kappa (κ) statistic were used to test the level of agreement between the ISO and the *invA*-based PCR for the detection of *Salmonella* spp. McNemar's χ^2 test was carried out first to test whether there was test bias (i.e., whether the proportion positive by each test differed) (23). The kappa statistic and its 95% confidence interval (CI) were further used to measure the degree of agreement between the two tests after taking into account the probability of agreement by chance alone. The strength of agreement on the basis of κ was judged according to the following guidelines: <0.2, slight agreement; 0.2 to 0.4, fair agreement; 0.4 to 0.6, moderate agreement; 0.6 to 0.8, substantial agreement; >0.8, almost perfect agreement (23). The software Intercooler Stata (version 12.0; StataCorp LP, College Station, TX) was used for these analyses.

A Bayesian approach was used to estimate Se_{ISO}. This analysis also allowed estimates of the Se and Sp of the PCR (Se_{PCR} and Sp_{PCR}, respectively) when it was used as a secondary test. Bayesian methods rely on a combination of likelihood function (derived from the observed data) and the prior distributions of the parameters under investigation, which are usually based on either expert opinion or peer-reviewed publications (15). The software Beta Buster (http://www.epi.ucdavis.edu/diagnostictests/) was used to obtain beta prior distributions for Se_{ISO}, Se_{PCR}, and Sp_{PCR} based on data from the published literature.

TABLE 1 Se's and Sp's of some PCR techniques targeting the *invA* gene of *Salmonella* spp. when performed on feces or tissues from pigs

Se (%)	Sp (%)	Technique	Reference
100	100	qRT-PCR	28
100	96	PCR	31
100	75.5	PCR	34
99.4	100	PCR	32
98.9	97.3	qRT-PCR	33
97	96	PCR	18
91	88	PCR	14
90–96[a]	99	qRT-PCR	35

[a] Depending on whether or not the model included dependence parameters between qRT-PCR and bacteriology.

The reported Se's of different culture protocols on fecal samples vary widely, ranging from as low as 56% to as high as 90% (9, 11, 12, 14, 24–26). The authors are also aware of two studies assessing the Se_{ISO} on fecal samples. The first study reported a Se_{ISO} of 98% on 288 samples from different animal species spiked with several *Salmonella* strains (27). In the second study, the relative Se_{ISO} was 100% when performed on 61 samples of cecal contents from pigs at slaughter and after comparing the results with those of an *invA*-based real-time PCR through a frequentist approach (28). These results suggested a high Se_{ISO} (>95%), although these figures were likely overestimating the true Se_{ISO} due to the origin of the samples (samples experimentally inoculated with *Salmonella* spp.) in the first study or the limited number of samples used in the latter one. Accordingly, it was decided that the most likely value (mode) for Se_{ISO} would be about 70%, with a 5th percentile as low as 40% (we were 95% sure that the Se_{ISO} would be at least ≥40%). Hence, the beta distribution parameters for Se_{ISO} were *a* equal to 6.33 and *b* equal to 3.28.

The specificity of the ISO (Sp_{ISO}) was considered 100%, as all positive samples were further serotyped; however, due to the unlikely event of laboratory errors (i.e., mislabeling, cross-contamination, etc.), the beta distribution for Sp_{ISO} was modeled as *a* equal to 999 and *b* equal to 1 (i.e., 1 error in 1,000 analyses). The almost perfect Sp_{ISO} helped to reduce the model uncertainty and the problem of identifiability that may have arisen (15, 29).

On the basis of the ISO-positive results obtained, farms were classified as those with populations of high *Salmonella* sp. prevalence (14 farms showing ≥20% of pigs infected per farm [P_A]) or low *Salmonella* sp. prevalence (13 farms showing <20% of pigs infected per farm [P_B]). This cutoff value of 20% was considered appropriate to discriminate herds of high and low prevalence, given the mean *Salmonella* prevalence of 29% observed in Spain (3), and was therefore sufficient to satisfy the required model assumption of different population prevalences (30). Because of the perfect Sp_{ISO}, prior estimates of the minimum expected prevalences for P_A and P_B were readily available. Thus, we were 95% sure that the prevalence for P_A was about 50% (see Table 2); therefore, a reasonable mode could be set at 70%. For P_B, the minimum expected prevalence was about 6% and the mode was set at 15%. Corresponding beta priors were *a* equal to 13.32 and *b* equal to 6.28 for P_A and *a* equal to 3.04 and *b* equal to 12.56 for P_B.

In general, PCR-based methods targeting the *Salmonella invA* gene usually yield very high Se and Sp values (Table 1), with variations attributed mostly to the use of different primer pairs for gene detection, different DNA extraction methods, the type of matrix analyzed, or the use of more sensitive techniques, such as qRT-PCR (31, 35). However, some authors have reported lower Sp_{PCR}s after observing an important number of false-positive results, suggesting that the Sp of this PCR would be lower than 100% (31, 34). Thus, after considering all these studies, it was de-

TABLE 2 Cross-classification of results obtained by *invA*-based PCR and ISO protocol alone or after second culture of the 33 discrepant samples from P_A and P_B[a]

	No. of samples				
	P_A		P_B		
PCR result	ISO^+	ISO^-	ISO^+	ISO^-	Total
Positive	137 (155)[b]	27 (9)	16 (17)	6 (5)	186
Negative	22	146	5	287	460
Total	159 (177)	173 (155)	21 (22)	293 (292)	646

[a] Discrepant samples were PCR^+ and ISO^-. The kappa statistic value considering exclusively the ISO results was 0.77 (95% CI, 0.72, 0.83).
[b] Values in parentheses represent the number of samples with the indicated result after a second culture.

cided that a reasonable prior for Se_{PCR} would have a mode of 90% and a 5th percentile as low as 80% ($a = 42.57$ and $b = 5.61$). Similar beta parameters were considered realistic for Sp_{PCR}.

Both bacteriological and molecular diagnostic tests were considered conditionally independent with regard to their specificities, as Sp_{ISO} is equal to 100% (36). Regarding Se, bacteriology is based on the detection of live (viable) *Salmonella* organisms and the PCR technique is based on the detection of a specific genetic sequence of *Salmonella*. Thus, it was initially assumed that the Se_{ISO} and the Se_{PCR} were conditionally independent; i.e., the probability of a positive result for culture is the same regardless of the result obtained for the PCR and vice versa. However, since both tests were performed on the same BPW-enriched samples, some degree of Se correlation between tests was not unexpected (40). Hence, a conditional independence model for two tests was initially carried out in two populations (29) using Winbugs software (http://www.mrc-bsu.cam.ac.uk/bugs/). Further, a conditional dependence model for two tests in two populations (29) was also performed, and the results were compared to those obtained with the first model to assess whether the assumption of independence held.

The influence of prior information on the estimates was assessed after performing several models using noninformative (diffuse) priors (i.e., $a = 1$, $b = 1$) alternatively for Se, Sp, and population prevalences (30). Posterior inferences were based on 100,000 iterations after a burn-in phase of 5,000 iterations. Model convergence was assessed by visual checking of the kernel density and trace plots for each parameter and running of multiple chains from dispersed starting values and was further estimated by use of the Gelman and Rubin statistic (37).

RESULTS

From the 646 pigs analyzed, a total of 332 belonged to P_A and 314 belonged to P_B. The cross-classification of the results provided by the ISO and *invA*-based PCR methods for each population is shown in Table 2. ISO showed *Salmonella* sp. prevalences of 47.9% for P_A and 6.7% for P_B, while PCR showed prevalences of 49.4% and 7% for P_A and P_B, respectively. McNemar's χ^2 test indicated the absence of test bias ($\chi^2 = 0.417$, 1 degree of freedom; $P = 0.51$), and therefore, the overall kappa statistic was further calculated. The level of agreement between ISO and PCR was considered substantial ($\kappa = 0.77$).

Overall, ISO failed to detect 33 of the 186 PCR-positive samples (17.7%; 27 from P_A and 6 from P_B). When these 33 samples were submitted to a second BPW nonselective enrichment, 19 new *Salmonella* sp. isolates (18 from P_A and 1 from P_B) were obtained, suggesting that ISO failed to detect *Salmonella* spp. in at least 57.5% of PCR-positive samples (Table 2, data in parentheses). Considering these 19 new *Salmonella* isolates, the kappa statistic suggested an almost perfect agreement between the two tests ($\kappa = 0.85$; 95% CI, 0.80, 0.89).

The posterior medians obtained with the different Bayesian models and their respective 95% probability intervals (PIs) for Se_{ISO}, Se_{PCR}, Sp_{PCR}, and prevalences for P_A and P_B are shown in Table 3. Under the assumption of conditional independence between tests and when prior information was used for all parameters (model I, fully informative), posterior medians were 87.6% (Se_{ISO}), 83.6% (Se_{PCR}), 97.4% (Sp_{PCR}), 56.7% (P_A prevalence), and 7.9% (P_B prevalence). These results remained virtually without modification when uninformative priors were used for the different parameters under the independence assumption (models II to IV) (Table 3). Under the conditional dependence assumption, results also remained very similar to those from the same models assuming independent tests (data not shown). The posterior estimate of the correlation between the tests' sensitivities included 0, suggesting that there was no evidence of conditional dependence between Se_{ISO} and Se_{PCR}.

The distribution of the *Salmonella* serotypes and serogroups isolated in each population is presented in Table 4. The most prevalent serotype was *Salmonella* Typhimurium in both populations, followed by *Salmonella* serotype Rissen in P_A and the monophasic variant of *S.* Typhimurium in P_B. Overall, in both populations, the most prevalent serogroup was B, followed by C1 (Table 4). Although there did not appear to be large differences regarding the *Salmonella* serogroups between P_A and P_B, a higher variability was observed regarding serotypes, which might somewhat influence Se_{ISO}, since different serotypes may exhibit different growth characteristics in the same enrichment and selective media (9, 11, 38). However, no differences with respect to Se_{PCR} were expected, since the *invA* gene is considered present in all *Salmonella* spp. (32). Regarding the Sp_{PCR}, it has been suggested that it may be affected by the proportion of potential cross-reacting bacteria in the guts of the animals sampled in each population (14). Hence, it was decided to check the assumption of constant test accuracy across populations by running separate Bayesian analyses of the two populations (39). Results from these independent analyses showed slightly different Se_{ISO}s between populations (88% in P_A

TABLE 3 Results from different Bayesian models of Se_{ISO}, Se_{PCR}, and Sp_{PCR} for detection of *Salmonella* spp. in MLN samples from P_A and P_B[a]

	Median % (95% PI)				
Model	Se_{ISO}	Se_{PCR}	Sp_{PCR}	P_A prevalence	P_B prevalence
I	87.6 (81.7, 92.7)	83.6 (78.4, 88.1)	97.4 (95.2, 98.8)	56.7 (51.1, 62.2)	7.9 (5.1, 11.3)
II	87.9 (81.9, 92.9)	83.7 (78.5, 88.1)	97.3 (95.1, 98.8)	55.9 (50.1, 61.6)	7.6 (4.8, 11)
III	88.7 (82.7, 94)	83.7 (78.5, 88.1)	97.3 (95, 98.8)	56.5 (50.8, 62)	7.9 (5.1, 11.3)
IV	85.7 (79.6, 90.9)	82 (75.9, 87.1)	99.1 (97.1, 99.9)	57.7 (52.2, 63.2)	8.4 (5.6, 11.9)

[a] Conditional independence between tests was assumed. Model I, fully informative; model II, noninformative (1, 1) priors for P_A and P_B; model III, noninformative (1, 1) priors for Se_{ISO}; model IV, noninformative (1, 1) priors for Se_{PCR} and Sp_{PCR}.

TABLE 4 Distribution of *Salmonella* sp. serotype and serogroup strains isolated in pig MLN samples from P_A and P_B[a]

Serotype (no. of strains)	Serogroup (no. of strains)	No. of strains					
		P_A			P_B		
		Total	PCR^-	ISO^-	Total	PCR^-	ISO^-
Typhimurium (77)	B (99)	70	6	7	7	1	0
4,12:i:− (17)		11	0	0	6	2	0
Wien (2)		2	1	0	0	NA	NA
Bredeney (1)		1	1	0	0	NA	NA
Derby (1)		0	NA	NA	1	0	0
Paratyphi B (var. Java) (1)		1	0	0	0	NA	NA
Rissen (24)	C1 (40)	24	5	0	0	NA	NA
Oranienburg (4)		4	1	0	0	NA	NA
Mikawasima (4)		1	0	0	3	0	0
Thompson (1)		1	0	0	0	NA	NA
6,7:−:1,5 (3)		3	0	0	0	NA	NA
Infantis (3)		2	0	0	1	1	0
Montevideo (1)		0	NA	NA	1	0	0
Newport (9)	C2 (11)	8	3	0	1	0	0
Muenchen (2)		2	0	0	0	NA	NA
9,12:−:− (1)	D1 (1)	1	0	0	0	NA	NA
London (12)	E1 (15)	12	0	3	0	NA	NA
Anatum (2)		2	1	0	0	NA	NA
Give (1)		1	0	0	0	NA	NA
Szentes (5)	I (6)	5	2	0	0	NA	NA
Gaminara (1)		1	0	0	0	NA	NA
Toulon (2)	K (2)	2	1	0	0	NA	NA
Umbilo (2)	M (2)	0	NA	NA	2	1	1
Arizonae 48:z4,z23:− (22)	Y (23)	22	1	7	0	NA	NA
Arizonae 48:z4,z23,z32:− (1)		1	0	1	0	NA	NA
Total[b] (199)		177	22	18	22	5	1

[a] According to the number of total positive samples in either the *invA*-based PCR (PCR) or ISO protocol (total) and to PCR- or ISO-discrepant results. ISO^- samples were detected after a second culture of 33 samples with discrepant results PCR^+ and ISO^-. PCR^- and ISO^-, PCR and ISO negative, respectively; NA, not applicable.
[b] Total for all 25 serotypes and 8 serogroups.

and 85% in P_B) but the same Se_{PCR} (82%). The Sp_{PCR} also varied to some extent between populations P_A (91%) and P_B (97%).

DISCUSSION

ISO has been chosen as the reference bacteriological method to be used for assessing the prevalence of *Salmonella* sp. infection in fattening pigs in the EU countries (3, 4). This bacteriological method is a thorough culture protocol that is considered sensitive enough and suitable for comparing results among MS (5). In fact, according to the results of two previous studies, this culture method would yield a good overall Se when performed on fecal samples (27, 28). When used on MLNs, this technique would be expected to yield a better Se than when used on feces because of the competitive flora and/or other inhibitory substances in feces that could interfere with bacteriological isolation of *Salmonella* spp. (5). However, there are no published studies assessing Se_{ISO} on MLNs.

In the absence of a gold standard, latent-class analyses allowed us to obtain unbiased estimates of Se_{ISO}. The analysis performed in this study included three main assumptions that may significantly influence the posterior estimates obtained (30), i.e., different population prevalences, conditional independence between tests, and a constant Se and a constant Sp across populations. Here, the assumption of different population prevalences was easily met since one of the tests (ISO) was considered 100% specific and populations were defined on the basis of its results. The large difference between population prevalences found contributed to obtain a better precision (i.e., a smaller 95% PI) of test estimates (30).

Regarding the assessment of the conditional independence between the two tests, the different models performed under this assumption yielded results very similar to those from the conditional dependence models (data from the dependence models not shown), supporting the lack of a significant correlation between the sensitivities of the tests (39) and thus indicating that the conditional independence model (Table 3) could be used.

It has been described that the absence of a constant test Se across populations biases the overall test Se results toward the estimate supported by the population with the highest disease prevalence (30). Thus, Se_{ISO} may be somewhat lower than 87.6%, and this figure should be interpreted as an estimate of the average Se_{ISO} across populations (29). Likewise, Sp_{PCR} was higher for P_B, and the overall Sp_{PCR} obtained was likely biased toward the estimate supported by the population with the lower disease prevalence; thus, Sp_{PCR} would be somewhat lower than 97.4%.

Finally, estimates from the different models (models I to IV in Table 3) remained very close, regardless of the use of informative or diffuse priors, therefore showing the consistency of the results and suggesting that these estimates did not much depend on the priors but did depend on the observed data. Since data were obtained from a large number of field samples, these results may be more representative of the diagnostic accuracy of the ISO than that obtained from experimental studies.

Overall, these results confirmed that the Se_{ISO} was somewhat below 87.6%, which is rather high for a culture protocol and higher than most of the results reported from other studies using different culture protocols and matrices (9, 10, 14, 24–26). Both the large amount of sample (25 g) and the repeated enrichment and subculturing in different media used by ISO would have had an effect on the recovery of *Salmonella* organisms (7, 10, 11) and would explain this high overall Se_{ISO}. Considering the usual limitations of bacteriology regarding Se, our results support the adequacy of ISO for the detection of *Salmonella* spp. from pig MLNs, but they also mean that about 13% of the infected pigs would be overlooked when this standard method is strictly applied (i.e., when negative samples are not recultured).

Interestingly, when the 33 discrepant (PCR^+/ISO^-) samples were resubmitted for bacteriological analysis, 57.6% new isolates were detected, highlighting the inherent limitations of the ISO and suggesting the adequacy of the *invA*-based PCR as a complementary method to bacteriology. In fact, when these new results were incorporated into the Bayesian analysis (data not shown), the overall Se of this bacteriological approach (i.e., a second culture of frozen BPW samples) increased to 88.6%, which is in line with other studies suggesting that the addition of a second enrichment medium may help to increase the Se_{ISO} (12).

Although estimating the diagnostic accuracy of the *invA* gene-based PCR used here was not the main goal of this study, the Bayesian analysis provided information regarding the overall accuracy of this technique. Under these conditions, Se_{PCR} appeared to be lower than expected according to previous studies (Table 1). Main differences between this study and prior studies lay mostly in the origin of the samples and their number, which may introduce important analytical bias. Three of these studies used reference *Salmonella* strains or experimental infections instead of field samples (18, 31, 32), and another used a much lower number of

samples (14). Another three studies used qRT-PCR instead of PCR (Table 1). The qRT-PCR method is based on an increase in fluorescence that indicates the presence of the target more accurately than the common gel electrophoresis analysis of the PCR (41). Finally, observations from our laboratory (data not shown) suggest that working with fresh samples would likely improve the overall Se_{PCR}. However, due to requirements of the experimental design (i.e., to select samples from high- and low-prevalence populations on the basis of ISO results), this study used previously frozen BPW samples for all the PCR analyses and second cultures, diminishing the Se's of both methods. Thus, the 14 samples that remained PCR^+ and culture negative after the second culture could also indicate a lack of Se_{ISO} due to the presence of dead bacteria after BPW samples were frozen, but they could also indicate a lack of Sp_{PCR}. The latter possibility has been associated with the use of some primer sets (34) and the presence of non-*Salmonella* intestinal bacteria, such as *E. coli*, that may present DNA sequences similar to those of *invA* (31). Regarding the primers used here for the *invA*-based PCR, the 14 PCR products showed the expected size in gel electrophoresis (PCR^+), thus suggesting the lack of Se_{ISO}. This is also suggested by the fact that interfering bacteria are less likely in MLNs and defrozen BPW samples than in an intestinal content matrix. Whether the PCR products were truly the expected *invA* fragments was not further investigated, since it was not required for the statistical analysis. In fact, the potential lack of Sp_{PCR} is already included in the underlying Bayesian approach when the priors for Sp_{PCR} were considered. The Sp_{PCR} remained quite constant and high (97.4%) in all the analyses performed, in correspondence with the average Sp_{PCR} shown by other published reports (Table 1).

This is the first study to assess Se_{ISO} for *Salmonella* sp. detection in MLNs. According to these results, about 13% of the infected pigs would be overlooked when this culture technique is strictly performed. Being aware of the limitations related to this diagnostic technique will help to estimate adjusted values of prevalence and calculate appropriate sample sizes when the prevalence of pig salmonellosis must be estimated in large-scale studies. In particular, the true mean *Salmonella* prevalence in fattening pigs in EU countries would be 11.8%, instead of the unadjusted 10.3% observed by the European Food Safety Authority in 2008 (3).

As found in other studies, the diagnostic accuracy of the PCR technique was reasonably good. Since PCR is quicker and cheaper than culture methods, it could be used as an alternative or complementary (screening) test for the diagnosis of pig salmonellosis. Thus, a trade-off between the purpose of the diagnosis, diagnostic accuracy, and cost should be sought. In general, bacteriology may be used further on all PCR-positive samples in order to confirm positive results and, when required, to identify and characterize them.

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