Sensitivity of the ISO 6579:2002/Amd 1:2007 standard method for the detection of Salmonella spp. on mesenteric lymph nodes from slaughter pigs

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ABSTRACT

The ISO 6579:2002/Amd 1:2007 (ISO) has been the bacteriological standard used in the European Union for the detection of *Salmonella* spp. on 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 of different *Salmonella* prevalences (14 farms with \(\geq 20\%\) and 13 farms with <20\%) and through latent-class models in concert with Bayesian inference, assuming 100\% ISO specificity, and using an invA-based PCR as the second diagnostic method. The Se_{ISO} was estimated to be close to 87\%, while sensitivity of the PCR reached up to 83.6\% and its specificity 97.4\%. Interestingly, the bacteriological re-analysis of 33 potential false-negative (PCR positive) samples allowed to isolate 19 (57.5\%) new *Salmonella* strains, improving the overall diagnostic accuracy of the bacteriology. Considering the usual limitations of bacteriology regarding the 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 cost and time benefits of the molecular procedure.
**INTRODUCTION**

*Salmonella* is recognized as one of the major zoonotic pathogens in the European Union (EU) (13) and pigs one of the most important sources of infection for humans (30). In 2003, the EU initiated a process to monitor the control of *Salmonella* and other specified zoonotic agents transmitted by foods (EC Regulation No 2160/2003). For this purpose, several bacteriology-based baselines surveys were carried out to estimate the prevalence of *Salmonella* spp. in both fattening and breeding pigs within the EU Member States (MS) (11,12). To facilitate the comparison of results among MS, a harmonized sampling and bacteriological methods were used for the detection of *Salmonella*, according to the Annex I of the Commission Decision 2006/668/EC (10). The bacteriological method recommended for *Salmonella* spp. isolation from mesenteric lymph nodes (MLN) of finishing pigs was the ISO 6579:2002/Amd 1:2007 (hereafter ISO), since it was considered a thorough technique that yields 100% specificity (Sp) by including bacteriological confirmation of presumptive isolates by serotyping (24). However, no data on the sensitivity (Se) of this bacteriological protocol has been reported when performed on MLN.

It is well recognized that the Se of the 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 (2, 7, 8, 17, 32, 41). For instance, Se of bacteriology on fecal samples has been reported to be as low as 9% (17) or higher than 90% (32, 41). Thus, when more than one method is used within the same surveillance system, comparisons are likely to be biased (35).

A common limitation for estimating the diagnostic accuracy of *Salmonella* culture is the lack of proper infected and non-infected 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 (2, 8, 32). To overcome
this problem latent-class methods with Bayesian approaches have been used to obtain
unbiased estimates of Se of different culture protocols for the diagnosis of pig
salmonellosis on fecal samples under field conditions (26, 41).

In the present study, the advantage of this statistical methodology was used to
make an estimation of the Se of the ISO (Se_{ISO}) when performed on MLN 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 (14). 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 (27). The use of this
specific gene responsible for the invasion of *Salmonella* into enteric cells, either in a
conventional or quantitative real time (qRT-) PCR, has been reported to yield
diagnostic sensitivities ≥90% in different type of samples, after pre-enrichment in
proper media (16, 28, 33, 35, 38).

MATERIAL AND METHODS

1. Sampling and microbiological procedures. This study was carried out
within the frame 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
(38). 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* spp. isolation using the ISO as detailed previously (38). Briefly, fresh
MLN samples were defatted, weighed (25 grams/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 hours at 37±1°C. Thereafter, 3
drops (33µL each) of incubated BPW were inoculated into a Modified Semi-solid
Rappaport Vassiliadis (MSRV) medium and plates were incubated for 24±3 h at
41.5±1°C (negative samples were reincubated for additional 24 hours). One µl 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 indol reaction) and by serotyping in the National Centre for Animal Salmonellosis (Madrid, Spain) following the Kauffmann-White-Le Minor scheme (20).

In parallel, 1-mL aliquots of BPW air-liquid interface culture were stored at -20ºC to be used further for identification of *Salmonella* positive samples through the molecular PCR method described below, after selection of samples from high and low salmonellosis prevalence populations based on the ISO results (see below).

Since the main goal of this study was to assess the Se\(_{\text{ISO}}\), when discrepant PCR positive but ISO negative (hereafter PCR+/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* spp. culture following the steps as the ISO protocol described above.

2. DNA extraction and PCR reaction. One 1-mL BPW aliquot of each frozen sample was submitted to DNA extraction by the rapid boiling procedure (29), consisting of: 1) centrifugation (13,000 rpm, 10 min); 2) resuspension of the pellet in 100 \(\mu\)L of distilled water; 3) boiling (99ºC, 20 min); 4) final centrifugation (4,000 rpm, 4 min); and 5) storage of the supernatant containing the bacterial DNA at 4ºC until its use. The primers Fw 5`-AGTGCTCGTTTACGACCTGAA-3`, and Rv 5`-TGATCGATAATGCCAGACGA-3` were designed to amplify a 229 bp DNA fragment.

The PCR mix was prepared with 5 \(\mu\)L of DNA and 20 \(\mu\)L of 0.4 mM each primer, 0.2 mM each dNTP, 0.5 U Taq DNA polymerase (Kappa Biosystems) and 1× buffer (containing 1.5 mM Mg\(^{2+}\)). After an initial denaturation step (94ºC, 5 min), the PCR was performed by 40 cycles of denaturation at 94ºC for 30 s, annealing at 55ºC for 30 s, extension at 72ºC for 20 s, and a final extension step at 72ºC for 10 min. Distilled water
and DNA extracted from *E. coli* ATCC 25922 (kindly provided by Dr. José Leiva, Clínica Universitaria de Navarra, Pamplona, Spain), *Salmonella* Enteritidis 3934 (from the IdAB collection, Pamplona, Spain) and *Salmonella* Typhimurium DT104 (kindly provided by Dr. Axel Cloeckaert, INRA, Tours, France) strains were used as negative and positive controls in each PCR reaction. The resulting PCR products were submitted to conventional electrophoresis in 1.5% (w/v) agarose gel and ethidium bromide staining.

3. **Statistical analysis.** McNemar’s $\chi^2$-test and the Kappa statistic ($k$) were used to test the level of agreement between the ISO and the invA-based PCR for the detection of *Salmonella* spp. McNemar’s $\chi^2$ was carried out first to test whether there was test bias (i.e. the proportion positive to each test differed) (9). Kappa and its 95% CI, was used further to measure the degree of agreement between the two tests after taking into account the probability of agreement by chance alone. Strength of agreement based on $k$ was judged according to the following guidelines: <0.2 = slight agreement; 0.2–0.4 = fair; 0.4–0.6 = moderate; 0.6–0.8 = substantial; >0.8 = almost perfect (9). The software Intercooler Stata 12.0 (StataCorp LP, College Station, TX) was used for these analyses.

A Bayesian approach was used to estimate the $\text{Se}_{\text{ISO}}$. This analysis also allowed for estimates of the $\text{Se}$ and $\text{Sp}$ of the PCR ($\text{Se}_{\text{PCR}}$, $\text{Sp}_{\text{PCR}}$) used as a secondary test. Bayesian methods rely on a combination of likelihood function (derived from the observed data) and prior distributions of the parameters under investigation, which are usually based either on expert opinion or peer-reviewed publications (14). The software Beta Buster (http://www.epi.ucdavis.edu/diagnostic-tests/) was used to obtain beta prior distributions for $\text{Se}_{\text{ISO}}$, $\text{Se}_{\text{PCR}}$, $\text{Sp}_{\text{PCR}}$ based on data from the published literature.

The reported $\text{Se}$ of different culture protocols on fecal samples varies widely, ranging from as low as 56% to as high as 90% (3, 8, 22, 23, 26, 32, 41). The authors are also aware of two studies assessing the $\text{Se}_{\text{ISO}}$ on fecal samples. The first study
reported a Se\textsubscript{ISO} of 98% on 288 samples from different animal species spiked with several \textit{Salmonella} strains (15). In the second study, the relative Se\textsubscript{ISO} was 100% when performed on 61 samples of cecal contents from pigs at slaughter and after comparing results with an \textit{inv}A real-time PCR through a frequentistic approach (6). These results suggested a high Se\textsubscript{ISO} (>95%), although these figures were likely overestimating the true Se\textsubscript{ISO} due to the origin of the samples (experimentally inoculated with \textit{Salmonella} spp.) in the first study, or the limited number of samples used in the latter. Accordingly, it was decided that the most likely value (mode) for Se\textsubscript{ISO} would be around 70%, with a 5th percentile as low as 40% (we were 95% sure that the Se\textsubscript{ISO} would be at least $\geq 40\%$). Hence, the beta distribution parameters for Se\textsubscript{ISO} were $a=6.33$ and $b=3.28$.

Specificity of the ISO (Sp\textsubscript{ISO}) was considered 100% as all positive samples were further serotyped, however due to unlikely event of laboratory errors (i.e. mislabeling, cross-contamination, etc.) beta distribution for Sp\textsubscript{ISO} was modeled as $a = 999$ and $b = 1$ (i.e. 1 error in 1000 analyses). The almost perfect Sp\textsubscript{ISO} helped to reduce the model uncertainty and the problem of identifiability that may have arisen (5, 14).

Based on the ISO positive results obtained, farms were classified as population of high \textit{Salmonella} spp. prevalence (14 farms showing $\geq 20\%$ of pigs infected/farm, hereafter P\textsubscript{A}) or low \textit{Salmonella} spp. prevalence (13 farms showing <20\% of pigs infected/farm, hereafter P\textsubscript{B}). This cut-off value of 20\% was considered appropriate to discriminate herds of high and low prevalence given the mean \textit{Salmonella} prevalence of 29\% observed in Spain (11) and therefore sufficient to satisfy the required model assumption of different population prevalences (37). Because the perfect Sp\textsubscript{ISO}, prior estimates of the minimum expected prevalence for P\textsubscript{A} and P\textsubscript{B} were readily available. Thus, we were 95\% sure that the prevalence for P\textsubscript{A} was around 50\% (Table 2), therefore a reasonable mode could be set at 70\%. For P\textsubscript{B} the minimum expected prevalence was around 6\% and the mode was set at 15\%. Corresponding beta prior were $a=13.32$, $b=6.28$ for P\textsubscript{A}, and $a=3.04$, $b=12.56$ for P\textsubscript{B}.
In general, PCR-based methods targeting the *Salmonella invA* gene usually yield very high Se and Sp (Table 1), with variations attributed mostly to the use of different primer pairs for the gene detection, different DNA extraction methods, the type of matrix analyzed, or the use of more sensitive techniques such as quantitative real time (qRT-) PCR (1, 25). However, some authors have reported lower Sp\(_{\text{PCR}}\) after observing an important number of false-positive results suggesting that the Sp of this PCR would be lower than 100% (1, 42). Thus, after considering all these studies it was decided that a reasonable prior for Se\(_{\text{PCR}}\) 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\(_{\text{PCR}}\).

Both bacteriological and molecular diagnostic tests were considered conditionally independent with regard to their specificities as Sp\(_{\text{ISO}}\)=100% (18). Regarding Se, bacteriology is based on the detection of live (viable) *Salmonella* organisms and PCR technique on the detection of a specific genetic sequence of *Salmonella*. Thus, it was initially assumed that the Se\(_{\text{ISO}}\) and the Se\(_{\text{PCR}}\) 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 (41). Hence, initially a conditional independence model for two tests, in two populations (5) was carried out using Winbugs software (http://www.mrc-bsu.cam.ac.uk/bugs/). Further, a conditional dependence model for two tests in two populations (5) was also performed and results compared to the first model to assess whether the assumption of independence held.

The influence of prior information on the estimates were assessed after performing several models using non-informative (diffuse) priors (i.e. a = 1, b = 1) alternatively for Se, Sp and population prevalences (37). 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 Kernel density and trace plots for each parameter, and running multiple chains from dispersed starting values and further estimate the Gelman and Rubin statistic (36).

RESULTS

From the 646 pigs analyzed, a total of 332 belonged to P_A and 314 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. The ISO showed a Salmonella spp. prevalence of 47.9% for P_A and 6.7% for P_B, while the PCR showed prevalences of 49.4% and 7% for P_A and P_B, respectively. The McNemar’s $\chi^2$ test indicated the absence of test bias ($\chi^2 = 0.417, 1$ df; $P = 0.51$) and therefore the overall Kappa statistic was further calculated. The level of agreement between the ISO and the PCR was considered substantial ($k = 0.77$).

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

The posterior medians obtained with the different Bayesian models and their respective 95% probability intervals (95% PI) for Se_{ISO}, Se_{PCR}, Sp_{PCR}, and prevalence 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 remained also very similar to those from the same models assuming independent tests (data not shown). The posterior estimate of correlation between tests’ sensitivities included zero, 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 Typhimurium in both populations, followed by 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 it 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 the Se_{ISO} since different serotypes may exhibit different growth characteristics in the same enrichment and selective media (8, 32, 34). However, no differences were expected with respect to Se_{PCR} since the *invA* gene is considered present in all *Salmonella* spp. (31). 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 (26). Hence, it was decided to check the assumption of constant test accuracy across populations by running separate Bayesian analyses of the two populations (19). Results from these independent analyses showed slightly different Se_{ISO} between populations (88% in P_A and 85% in P_B), but same Se_{PCR} (82%). The Sp_{PCR} also varied to some extend between populations P_A (91%) and P_B (97%).

**DISCUSSION**

The ISO has been chosen as the reference bacteriological method to be used for assessing the prevalence of *Salmonella* spp. infection in fattening pigs in the EU countries (11, 12). This bacteriological method is a thorough culture protocol that is considered sensitive enough and suitable for comparing results among MS (10). In fact, according to the results of two previous studies, this culture method would yield a
good overall Se when performed on fecal samples (6, 15). When used on MNL, this technique would be expected to yield better Se than when used on feces, because the competitive flora and/or other inhibitory substances present in feces that could interfere with bacteriological isolation of *Salmonella* spp. (10). However, there are no published studies assessing the Se_{ISO} on MLN.

In the absence of gold standard, latent-class analyses allowed us to obtain unbiased estimates of the Se_{ISO}. The analysis performed in this study included three main assumptions that may influence significantly on the posterior estimates obtained (37), i.e. different population prevalences, conditional independence between tests, and constant Se and Sp across populations. Here, the assumption of different population prevalences was easily met since one of the tests (the ISO) was considered 100% specific and populations were defined based on its results. The large difference found between population prevalences contributed to obtain better precision (i.e. smaller 95% PI) of test estimates (37).

Regarding the assessment of the conditional independence between the two tests, the different models performed under this assumption yielded very similar results to those from the conditional dependence models (data from the dependence models not shown), supporting the lack of significant correlation between tests’ sensitivities (19) and thus indicating that the conditional independence model (Table 3) could be used.

It has been described that the absence of constant test Se across populations bias the overall test Se results towards the estimate supported by the population with the highest disease prevalence (37). Thus, the 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 (5). Likewise, the Sp_{PCR} was higher for P_{B}, and the overall Sp_{PCR} obtained was likely biased towards the estimate supported by the population with the lower disease prevalence, thus the Sp_{PCR} would be somewhat lower than 97.4%.
Finally, estimates from the different models (I to IV in Table 3) remained very close regardless the use of informative or diffuse priors, therefore showing the consistency of the results, and suggesting that these estimates did not depend much on the priors but 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 those obtained from experimental studies.

Overall, these results confirmed that the $Se_{ISO}$ was somewhat below 87.6%, rather high for a culture protocol, and higher than most of the reported results from other studies using different culture protocols and matrices (3, 8, 17, 22, 23, 26). Both the large amount of sample (25 grams) and the repeated enrichment and subculturing in different media used by the ISO would have had an effect on the recovery of $Salmonella$ organisms (2, 17, 32) and would explain this high overall $Se_{ISO}$.

Considering the usual limitations of bacteriology regarding Se, our results support the adequacy of the ISO for the detection of $Salmonella$ spp. from pig MLN, but they also mean that around 13% of the infected pigs would be overlooked when this standard method is strictly (i.e. without re-culturing negative samples) applied.

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 up to 88.6%, which is in line with other studies suggesting that the addition of a second enrichment medium may help to increase the $Se_{ISO}$ (40).

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. In these conditions, the $Se_{PCR}$
appeared to be lower than expected according to previous studies (Table 1). Main
differences between this study and prior studies lay mostly on 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 (1, 28, 31) and another a much lower number of samples (26). 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 (21). Finally, observations
from our laboratory (data not shown) suggest that working with fresh samples would
likely improve the overall $\text{Se}_{\text{PCR}}$. However, due to requirements of the experimental
design (i.e. to select samples from high and low prevalence populations based on ISO
results) this study used previously frozen BPW samples for all the PCR analyses and
second cultures, diminishing the Se of both methods. Thus, the 14 samples that
remained PCR+/Culture- after the second culture could also indicate a lack of $\text{Se}_{\text{ISO}}$
due to the presence of dead bacteria after frozen of BPW samples, but they could also
indicate a lack of $\text{Sp}_{\text{PCR}}$. This latter possibility has been associated with the use of
some primer sets (42) and the presence of non-*Salmonella* intestinal bacteria such as
*E. coli* that may present similar DNA sequences to *invA* (1). Regarding the primers
used here for PCR-*invA*, the 14 PCR products showed the expected size in gel
electrophoresis (PCR+), thus, suggesting the lack of $\text{Se}_{\text{ISO}}$. This is also suggested by
the fact that interfering bacteria are less likely in MLN and defrozen BPW samples than
in intestinal content matrix. Whether the PCR products were truly the expected *invA*
fragments it was not further investigated, since it was not required for the statistical
analysis. In fact, the potential lack of $\text{Sp}_{\text{PCR}}$ is already included in the underlying
Bayesian approach when the priors for $\text{Sp}_{\text{PCR}}$ were considered. The $\text{Sp}_{\text{PCR}}$ remained
quite constant and high (97.4%) in all the analyses performed, in correspondence with
the average $\text{Sp}_{\text{PCR}}$ showed by other published reports (Table 1).
This is the first study assessing the SeISO for *Salmonella* spp. detection in MLN. According to these results, around 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 had to 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 EFSA in 2008 (11).

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, the 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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