Culture methods differ on the isolation of *Salmonella enterica* serotypes from naturally contaminated swine fecal samples

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**Abstract.** Four culture methods (A, B, C, and D) were comparatively evaluated for their ability to isolate *Salmonella enterica* from pooled swine fecal samples (n = 100). None of the methods was able to isolate *Salmonella* from all positive samples. The relative sensitivity of the culture methods evaluated was 82%, 94%, 95%, and 78% for methods A, B, C, and D, respectively. The comparison of sensitivities showed that methods B and C performed significantly better (P < 0.05) than methods A and D. Although relative sensitivities of methods B and C were equal, from the 89 positive samples concomitantly detected by both, 35 (39.3%) had different serotypes (no match) isolated by each method. On the basis of the results of this study, it was concluded that culture methods differ on the isolation of *S. enterica* serotypes from naturally contaminated swine fecal samples. Depending on the objective(s) of investigations on the ecology and epidemiology of *S. enterica* in swine populations, a method or a combination of methods should be considered for more reliable results.

An incredible number of different culture methods for isolation of *Salmonella* have been published, but currently, there appears to be no consensus on what would be the best method.12 As a consequence, confusing and contradictory results are frequently found in the literature.

For years, 2 culture methods (methods A and B, described below) have been concomitantly applied in our laboratory routine for isolation of *Salmonella* from swine samples.13 A recent study3 compared these methods with another frequently applied culture method (preenrichment in buffered peptone water, followed by enrichment in Rappaport–Vassiliadis [RV] broth and isolation on Xylose-Lysine-Tergitol-4 [XLT-4] agar), reporting better results when isolation was conducted using method B. However, we felt that continuous improvement in the sensitivity of culture methods was necessary and should be persistently pursued. Therefore, culture methods C and D (described below) were developed and included in this study, whose objective was to comparatively evaluate 4 different culture methods (methods A, B, C, and D) and to assess their relative sensitivity for the isolation of *Salmonella enterica* from naturally contaminated swine fecal samples. The current knowledge of the effect of different culture methods on the isolation of different *S. enterica* serotypes from naturally contaminated samples is very limited. Most studies published to date have simply focused on the sensitivity of culture methods for the detection of positive samples presenting little information on the isolation of multiple serotypes. This information is crucial, particularly when trying to understand the complex ecology and epidemiology of *Salmonella* in livestock.

To compare the culture methods for the isolation of a wide range of serotypes, pooled fecal samples (n = 100) were collected from several abattoir holding pens, which had recently held market swine (for 2–4 hours before slaughter) from many different farms. To get as much diversity as possible of *S. enterica* serotypes, 5 samples were collected from 10 different pens on 2 different occasions.

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Table 1. Frequency distribution and agreement by sample of *Salmonella enterica* serotypes isolated using culture methods B and C.

<table>
<thead>
<tr>
<th>Serotypes</th>
<th>Method B</th>
<th></th>
<th>Method C</th>
<th></th>
<th>Agreement by sample (%)</th>
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<tr>
<td></td>
<td>Isolates</td>
<td>Samples</td>
<td>Isolates</td>
<td>Samples</td>
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<tr>
<td>Adelaide</td>
<td>22</td>
<td>14</td>
<td>45</td>
<td>24</td>
<td>58.3</td>
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<td>Agona</td>
<td>22</td>
<td>13</td>
<td>13</td>
<td>9</td>
<td>22.2</td>
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<tr>
<td>Anatum</td>
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<td>5</td>
<td>9</td>
<td>5</td>
<td>42.9</td>
</tr>
<tr>
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<td>2</td>
<td>3</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
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<td>85</td>
<td>46</td>
<td>68</td>
<td>32</td>
<td>34.5</td>
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<tr>
<td>Heidelberg</td>
<td>3</td>
<td>2</td>
<td>4</td>
<td>2</td>
<td>33.3</td>
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<tr>
<td>Infantis</td>
<td>4</td>
<td>2</td>
<td>5</td>
<td>3</td>
<td>25</td>
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<tr>
<td>Kinshasa</td>
<td>4</td>
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<td>22</td>
<td>9</td>
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<tr>
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<td>9</td>
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<tr>
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<td>2</td>
<td>17</td>
<td>8</td>
<td>25</td>
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<tr>
<td>Saint-Paul</td>
<td>60</td>
<td>33</td>
<td>14</td>
<td>8</td>
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<tr>
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<td>3</td>
<td>5</td>
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<tr>
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<td>3</td>
<td>0</td>
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<tr>
<td>Typhimurium (Copenhagen)</td>
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<td>10</td>
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<td>4</td>
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</table>

Upon arrival at the laboratory, the pooled fecal samples were individually homogenized, and 10 g of each sample was inoculated into each of the primary enrichment broths of the 4 culture methods evaluated. The first culture method evaluated (method A) consisted of a primary enrichment in 90 ml of GN-Hajna (GN) broth at (37 C/24 hours), followed by secondary enrichment in 10 ml of RV broth at (37 C/24 hours). The second method (method B) consisted of a primary enrichment in 90 ml of tetrathionate (TT) broth at (37 C/24 hours), followed by secondary enrichment in 10 ml of RV broth at (37 C/24 hours). The third method (method C) consisted of a primary enrichment in 90 ml of tetrathionate (TT) broth at (37 C/24 hours), followed by secondary enrichment in 10 ml of RV broth at (37 C/24 hours). The fourth method (method D) consisted of a primary enrichment in 90 ml of GN broth with 0.1% of Novobiocin (37 C/24 hours), followed by double secondary enrichment in 10 ml of RV broth and 10 ml of TT broth at 42 C/24 hours, and a combined postenrichment in 10 ml of M broth with 0.1% of Novobiocin (42 C/6 hours). After the secondary enrichment (methods A, B, and C) or postenrichment (method D) steps, a full loop was streaked on XLT-4 agar and incubated at 37 C/24 hours. From the XLT-4 plates, up to 3 suspect colonies were selected for identification. The suspect colonies were streaked on MacConkey agar, incubated at 37 C/24 hours, and biochemically identified. Selected colonies identified as *Salmonella* were transferred to tryptic soy agar slants and submitted for serotyping at the National Veterinary Service Laboratory of the US Department of Agriculture, in Ames, Iowa.

The experimental design applied was of individually paired samples, and the “gold standard” was defined as the total number of positive samples detected by at least 1 of the 4 culture methods applied in the study (i.e., total number of positive samples from a combination of all methods). The relative sensitivity of each culture method evaluated was defined as the proportion of positive samples detected from the total number of positive samples by at least 1 of the 4 methods (defined “gold standard”). The data analysis included frequency distributions analysis and calculation of the relative sensitivity for each culture method, as well as the comparison of proportions between methods (McNemar’s chi-square test). The statistical level of significance used was $P < 0.05$.

All 100 samples tested in this study were positive by at least 1 method. The relative sensitivity comparison between the evaluated culture methods showed that methods B and C (94% and 95%, respectively) performed significantly better ($P < 0.05$) than methods A and D (82% and 78%, respectively). None of the methods was able to isolate *Salmonella* from all samples.

Considering that bacteriologic culture methods are 100% specific and on the basis of their high relative sensitivity, both methods (B and C) proved to be suitable for isolation of *Salmonella* in epidemiological investigations in swine populations. However, it is important to note that, although these methods were very similar (differing only on the primary enrichment and incubation temperature), and were equivalent on the frequency of *Salmonella* isolation, different qualitative results were found, i.e., the frequency distribution of the serotypes isolated was different for each method (Table 1). Moreover, there was no agreement between both methods on the serotypes isolated from 39.3% of the samples (“no serotype match”). These observations indicate that although the culture methods applied were equal in their sensitivity to identify positive...
samples, results have to be carefully interpreted. If the objective of a study is just to classify pigs as *Salmonella* positive or negative, both methods are equal and adequate. Our results also indicate that both methods would be useful as enrichment for subsequent *Salmonella* detection using available assays (e.g., polymerase chain reaction, enzyme-linked immunosorbent assay, and others). However, if detailed information on the diversity and frequency distribution of serotypes is required, diverging results may be found, depending on which method is applied. The finding of different isolation frequencies, as well as the isolation of different serotypes from the same sample, clearly raises questions on what should be done to obtain reliable knowledge of the *Salmonella* ecology. Depending on the objective(s) of the study to be conducted, a method or a combination of methods should be considered for more reliable results.

Overall, 15 different *Salmonella enterica* serotypes were isolated. The culture method C isolated all 15 serotypes, whereas method B isolated 14 serotypes (Table 1). Combining results of method B and C, only 1 serotype was isolated from 32% of the samples. However, 2 different serotypes were isolated from 37% of the samples, 3 different serotypes were isolated from 25% of the samples, 4 different serotypes were isolated from 5% of the samples, and 5 different serotypes were isolated from 1 sample. Using culture method B, only 1 serotype was isolated from 69.1% of the samples, 2 serotypes from 26.6% of the samples, and 3 serotypes from 4.3% of the samples (from the total of 94 positive samples by this method). Using method C, only 1 serotype was isolated from 63.2% of the samples, 2 serotypes from 30.5% of the samples, and 3 serotypes from 6.3% of the samples (from the total of 95 positive samples using this method). The comparison of these proportions showed that there was no statistically significant difference (P < 0.05) between methods for the ability to isolate more than 1 serotype per sample. However, the frequency distribution of the serotypes isolated by both methods varied markedly (Table 1). The 5 most frequently isolated serotypes using method B were: Derby (36.2%), Saint-Paul (25.5%), Adelaide (9.4%), Agona (9.4%), and Typhimurium var. Copenhagen (3.4%), whereas using method C, Derby (28.8%), Adelaide (19.1%), Kinshasa (9.3%), Mbandaka (7.2%), and Saint-Paul (5.9%) were most frequently isolated (Table 1). In addition, from the 89 positive samples detected using both methods, 35 (39.3%) had different serotypes (no match) isolated by each method.

Most studies that isolated *Salmonella* from different types of samples, including feces, were on the basis of the selection and identification of a single *Salmonella* suspect colony per sample. In this study, selecting up to 3 suspect colonies per plate for identification and combining results from methods B and C, it was observed that most of the samples analyzed had more than just 1 serotype (2–5 different serotypes per sample were found in 68% of the samples). If only 1 suspect colony had been selected for identification, a distorted picture would be presented on the frequency distribution of serotypes, with serious implications for ecological and epidemiological investigations. In this study, pooled samples were analyzed, increasing the probability of finding multiple serotypes. However, previous studies have reported the isolation of multiple serotypes from individual pigs. Several approaches for identifying the presence of multiple serotypes in samples (or animals) are possible. Some possibilities include serotyping multiple isolates per plate, use of multiple enrichment broths and plating media, culturing multiple samples per pig, or sampling more animals per herd or studied groups. Research on the dynamics of bacterial growth in selective enrichment broths suggests that selective enrichment may result in asynchronous growth curves among serotypes, because of differing susceptibilities to the restrictive components of the media, with a consequent difference in recovery rates of different serotypes, as demonstrated in our study. Therefore, if only 1 culture method is used, selection of more than 1 colony per sample for identification or sampling more animals per group may not be as efficient as using multiple methods for isolation of different serotypes. Our results indicate that if more than 1 culture method is used, chances are increased that multiple serotypes would be easily found in most of the samples. However, although logical, this approach was not scientifically proved to be the best choice yet.

In several laboratories (including ours), methods A and B have been applied (concomitantly) for isolation of *Salmonella* from several types of swine samples, including feces. The superiority of method B has been noticed, but method A has been recommended for isolation of *Salmonella Choleraesuis*. Although accepted by most researchers, the results of this study question its use, especially if the objective is to isolate *Salmonella* serotypes other than *Salmonella Choleraesuis*, as is common in food safety studies. In addition, satisfactory isolation of *Salmonella Choleraesuis* using RV broth and no inhibitory effect by TT broth for this serotype have been reported. As noted by others and on the basis of our experience in previous investigations, results reported here corroborate with the observation that method A is significantly (P < 0.05) less efficient than method B, indicating that it is not a good choice for food safety studies.

From the results presented and discussed, it can be concluded that culture methods for the isolation of *Sal-
monella have great effect on ecological and epidemiological studies, justifying more attention and studies. Although conventional culture methods constitute the basis for Salmonella ecological and epidemiological investigations, there is still a need for standardization of the methods applied, as well as continuous improving and comparative investigations to establish parameters for comparison of results from different studies. Research and diagnostic laboratories need to weigh the costs of increased diagnostic effort, be it through more intensive sampling or the use of multiple enrichment broths or plating media (or both), against the expected gain in sensitivity of S. enterica serotypes isolation in relation to their objectives.

Sources and manufacturers

a. Becton Dickinson Microbiology Systems, Sparks, MD.
b. BBL-Crystal Enteric/Non-Fermenter biochemical ID system, Becton Dickinson Microbiology Systems, Sparks, MD.

References


Actinobacillus capsulatus septicemia in a domestic rabbit (Oryctolagus cuniculus)

David K. Meyerholz, Joseph S. Haynes

Abstract. A 5-year-old pet rabbit (Oryctolagus cuniculus) died after a 3-day history of anorexia and depression. At necropsy, the stomach was distended with dough-like ingesta and hair consistent with gastric stasis syndrome. The lungs had multifocal, raised red nodules with circumferential hemorrhage. Microscopic examination showed pulmonary hemorrhage with intravascular fibrin thrombi and bacterial colonies, which were present in lesser amounts in the kidney, heart, and liver. Bacterial culture of the lung produced a heavy pure growth of Actinobacillus capsulatus. Acute septicemia is a novel presentation for this pathogen. This is the first documented case of A. capsulatus disease in the contiguous United States and may represent an underdiagnosed to emerging disease of lagomorphs.

Recently, a 5-year-old domestic pet rabbit (3 kg, Oryctolagus cuniculus) with no previous history of illness was presented to Iowa State University College of Veterinary Medicine’s (ISU-CVM) Community Ser-