RESEARCH REPORT
PORK SAFETY

Title: *Determination of the Diagnostic Sensitivity and Specificity of a Reference Method for Salmonella sp. Detection in Swine Fecal Samples of Known Infection Status* - **NPB Project #05-053**

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Abstract:
This project was conducted in order to evaluate five commonly used culture methods (Methods 1, 2, 3, 4, and 5) for recovery of *Salmonella* species from swine feces, both for sensitivity of detection (ability to recover *Salmonella* from a positive sample), and for specificity (not to inadvertently identify an organism as *Salmonella* species in a negative sample). While these five methods have some overlap in terms of media used, there are different combinations of pre-enrichment media and growth media used. Fifty four negative samples and forty eight positive samples were processed using each of the five methods. All negative samples were negative for *Salmonella* species when cultured by all five methods (100% specificity). Two of the methods (Methods 1 and 4) resulted in the recovery of significantly less ($P<0.05$) *Salmonella* species when compared to the remaining three methods (Methods 2, 3, and 5). No one method was successful in recovering *Salmonella* species from all positive samples, although recovery with Method 2 was statistically similar to the total number of positive samples analyzed (44 versus 48 *Salmonella*-positive samples, $P>0.05$).

All of the fecal samples in this study were cultured in one laboratory. It is documented that inter-laboratory variability exists, so that when samples are divided and processed by more than one laboratory, different results are achieved. The results of this study can be used as baseline values for analysis of these methods when used in different laboratories, or when analyzing different culture methods for recovering *Salmonella* from naturally contaminated swine fecal samples.

Introduction:
There has been increasing concern in recent years over the human health risk posed by microbial pathogens in the food supply. Foodborne infections constitute a significant cause of morbidity and mortality in human populations, and *Salmonella enterica*, in particular, has been the focus of major concern. Because of the public health risk, several countries (most notably Denmark) have instituted surveillance programs to predict the risk of contamination of meat at the time of slaughter. Additionally, the European Commission is adopting the Danish protocol for pre-harvest testing of swine for *S. enterica* as mandatory as of 2008. This will undoubtedly increase the need for all countries that produce pork, such as the United States, to increase their monitoring and surveillance of prevalence of infection of swine by *S. enterica*.

Pre-harvest estimates of *S. enterica* prevalence are most commonly based on traditional culture methods, in spite of its shortcomings (such as lack of sensitivity and length of time to completion). Additionally, there is no single method for culture of *S. enterica*; in fact, there are probably more techniques and methods for culturing *S. enterica* than for any other bacterium. This fact was clearly illustrated by Waltman and Mallinson (1995), in a nationwide survey of methods used for culture of *S. enterica* from poultry samples. In the reported survey, no two participants used identical culture methods, and variation occurred in enrichment procedures, growing media, incubation temperatures and times, and multiplicity of culture attempts per sample.

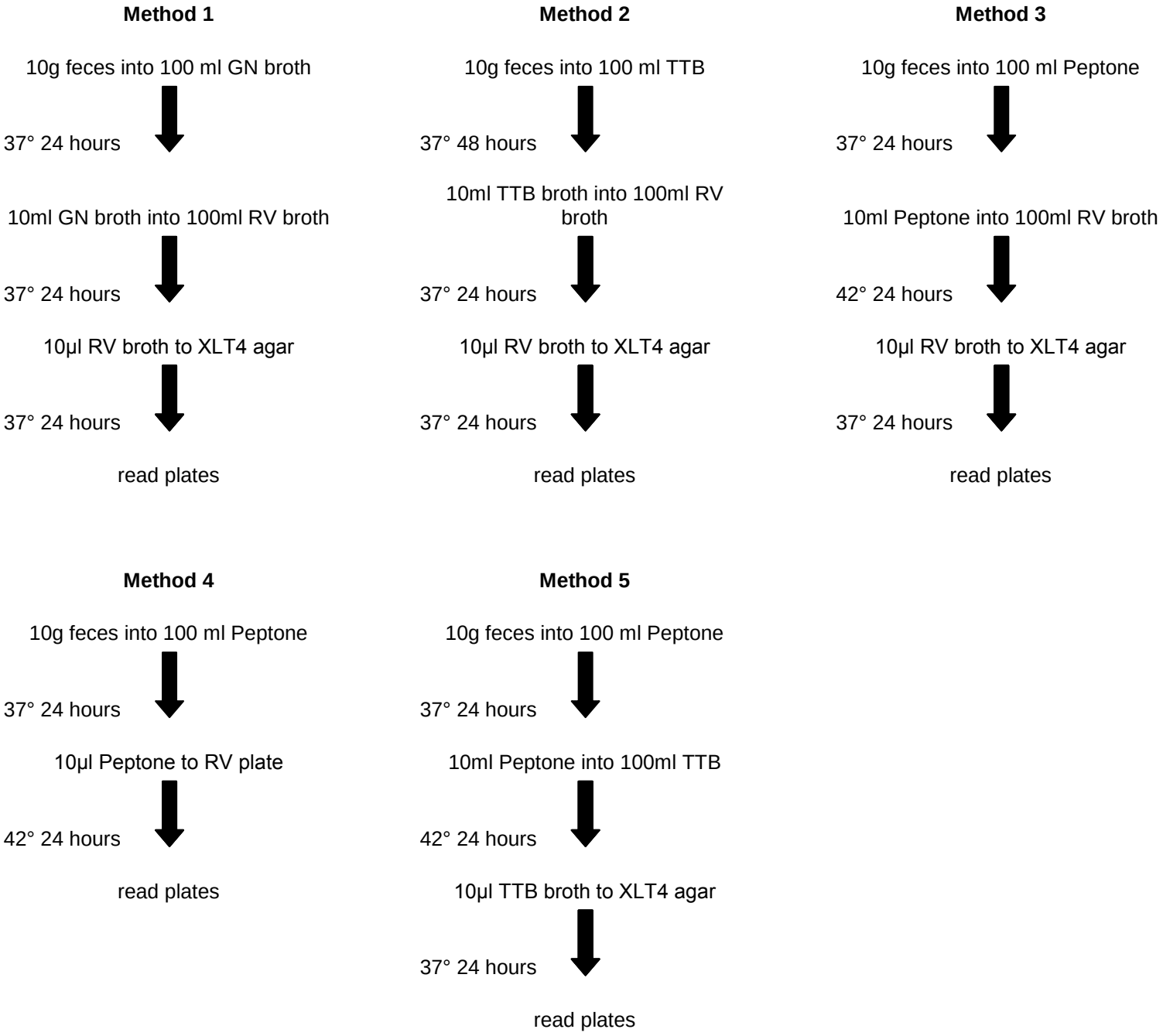
Several recent studies have focused on recovery of *S. enterica* from naturally-infected swine. In general, these studies show that "the more you look, the more you find", and that no one culture method is optimal for recovery of all serotypes of *S. enterica*. These studies used fecal samples from herds known to be infected; however, the status of the individual animals at the time of collection of the sample was unknown. As a result, these studies compared cultural methods in terms of relative sensitivity of isolation (i.e., *S. enterica* is isolated from a given sample more or less frequently by one method vs. another). Within an infected herd, the prevalence of shedding of *S. enterica* can vary dramatically over time. As a result, the true status of the individual fecal samples is unknown until at least one cultural method shows it to be positive. Consequently, the diagnostic sensitivity of any particular method cannot be determined.

While many studies have compared the relative sensitivity of one culture method to another, using samples from animals of unknown shedding status, a reference method for *S. enterica* isolation has not yet been defined in terms of diagnostic sensitivity when using swine fecal samples as the matrix. If this was to be defined, it would facilitate the comparison of one research project/publication to another. In addition, we hope to expand this project in subsequent years to include additional *S. enterica* culture and non-culture methods (detection), and to do a large-scale multi-laboratory comparison to determine the robustness of each method analyzed. This would greatly help in analyzing the outcomes of investigations conducted in different laboratories and different countries, contributing to our understanding of the ecology and epidemiology of *S. enterica* in the pre-harvest pork production chain. The scientific community, as well as the entire pork industry will benefit from this study with a predicted wide application of the parameters established.

Objectives of Research Project:

1) To determine the diagnostic sensitivity of commonly applied culture methods for the isolation of *Salmonella enterica* from swine fecal samples, and
2) To establish baseline values for future evaluation of additional methods (of isolation or detection of *S. enterica*), and inter-laboratory performance.

Materials and Methods:
Fecal samples (10g) from swine known to be shedding at least one serotype of *Salmonella enterica* (n=48), and from swine shown to be free of *Salmonella enterica* infection (n=54) were processed according to a set of five commonly used culture methods to isolate *S. enterica* from swine feces. A flow chart, summarizing the five culture methods, follows:



Identification of suspect colonies was performed by traditional biochemical reactions, agglutination using polyvalent *Salmonella* O antisera, and API strips (BioMerioux).

Statistical analysis of results is summarized in the following tables. The experimental design applied is of individually paired samples. Data analysis includes: comparison of proportions (McNemar's Chi-square test; significance level for inferences of $p<0.05$); determination of agreement between methods (Kappa statistic), and estimation of diagnostic sensitivity for each method (Thrusfield, 1995).

Table 1 – Frequency of *Salmonella*-positive samples by culture method evaluated and comparison of proportions (Chi-square, $P<0.05$)

Method	Frequency	%	Comparison of proportions
1	3/48	6.25	a
2	44/48	91.67	b, d
3	22/48	45.83	c
4	0/48	0	a
5	38/48	79.17	b
Any ("Standard")	48/48	100	d

Table 2 – Frequency of *Salmonella*-positive samples, based on the combination of culture methods evaluated

Combinations	Frequency	%
Any one method	10/48	20.83
Any two methods	19/48	39.58
Any three methods	17/48	35.42
Any four methods	2/48	4.17
Any five methods	0/48	0
At least one method	48/48	100

Table 3 – Relative sensitivity of culture methods evaluated for the isolation of *Salmonella* from naturally contaminated swine fecal samples

Method	Relative sensitivity
1	6.3%
2	91.7%
3	45.8%
4	0%
5	79.2%

Table 4 – Agreement between culture methods evaluated (Kappa statistic) on the isolation of *Salmonella* from naturally contaminated swine fecal samples

	Method 1	Method 2	Method 3	Method 4	Method 5
Method 1	-	7.7%	11.4%	0%	9.7%
Method 2	7.7%	-	40.5%	0%	71.6%
Method 3	11.4%	40.5%	-	0%	58.7%
Method 4	0%	0%	0%	-	0%
Method 5	9.7%	71.6%	58.7%	0%	-

While a single serogroup of *Salmonella* species was most commonly recovered from any given sample if more than one method was positive, occasionally one method resulted in recovery of one serogroup while another method resulted in recovery of a different serogroup (i.e., mismatch between methods applied). This type of differences between methods, as well as the ability to recover any *Salmonella* species on a sample-by-sample basis, constitutes a fundamental topic for evaluation in future research projects. The baseline information generated in this research can also be used to analyze additional culture methods, or in determining inter-laboratory variability in these five methods.

Discussion:
These results show that there can be marked differences in the ability to recover *Salmonella* species when different culture methods are employed. In general, methods that used one particular pre-enrichment broths (i.e., non-selective media in the first step of the recovery process) did not perform adequately. The only culture method with satisfactory recovery rate of *Salmonella* from naturally contaminated swine fecal samples was Method 4, which included a primary enrichment in the first step of the recovery process. This observation demonstrates the importance of suppressing competitor bacteria in the original sample to allow *Salmonella* to grow up to the level of detection by the plating media applied.

While these animals were not clinically ill, it can be assumed that the methods would perform similarly when used on fecal samples from clinically ill animals, although this assumption has not been tested yet. This could have a direct impact on the ability to diagnose the cause of diarrhea in sick animals, as well as the ability to recognize clinically normal animals that are shedding *Salmonella* species as they enter the food chain. If the United States begins to implement culture-based pre-slaughter surveillance for *Salmonella* species, such as other countries have done, the outcome of culture could be greatly skewed by use of different culture methods.

Lay Interpretation:
Salmonella enterica is a bacterium that causes disease in many animals, including swine and human beings. This organism can be transmitted from swine to human beings through direct contact, or through indirect contact such as by eating pork products that have been contaminated with the organism (i.e., foodborne infections). Many European countries have implemented surveillance methods to detect pigs that are shedding *Salmonella* species as they go to slaughter, and these standards may eventually be adopted by the United States. In addition, diagnostic methods to detect *Salmonella* species are used to determine cause of illness in sick pigs. The traditional way to detect *Salmonella* species is to perform bacterial culture in a laboratory. This method, while traditional, widely used and somewhat standardized, can consist of many variations. If the sample probably contains very few bacteria, there can be a pre-enrichment step, where all organisms are allowed to grow to high numbers, followed by a selective enrichment step where the *Salmonella* are allowed to grow but other types of bacteria are inhibited. Finally, the samples are put onto a solid medium that contains biochemicals which help to identify *Salmonella*. This is especially useful when the sample is fecal material or intestinal tract, which contains many different types of bacteria.

It has been shown that variations in the steps used, or the type of media used for each of these steps in the culture procedure can greatly affect the amount and types of *Salmonella* recovered. It has also been shown that the same protocol, used in different laboratories, can result in differences in recovery of *Salmonella*. This project was conducted in order to evaluate five different culture protocols, used in one laboratory, on samples collected from pigs of known infection status (positive or negative). This research project generated information that can then be used to compare additional culture methods, besides of demonstrating how different culture methods perform for the recovery of *Salmonella* from naturally contaminated swine fecal samples.

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