Pre-harvest food safety diagnostics for *Salmonella* serovars. Part 1: Microbiological culture

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Nearly 1.5 million cases of human salmonellosis occur yearly in the United States, and 95% of these are foodborne.[1] *Salmonella* serovars rank second only to *Campylobacter* species in annual cases of bacterial foodborne disease, and are responsible for the largest proportion (30%) of deaths attributable to bacterial foodborne agents.[1]

Although recent reports suggest that only 3% of human *Salmonella* outbreaks of known etiology were attributable to pork products,[2] *Salmonella* serovars represent the bacterial foodborne pathogens of most importance for contamination of pork. This is a consequence of not only the risk to domestic public health and consumer confidence, but of competitiveness in export markets.

In response to large-scale foodborne outbreaks of salmonellosis associated with pork, both Denmark,[3,4] the major competitor of the United States for pork export markets, and other European Union (EU) pork producers have implemented "farm to table" *Salmonella* control programs. Demonstration of efficacious *Salmonella* control measures that reduce the contamination of pork products will be crucial for maintaining market share,[5,6] yet wholesale adoption of EU control programs may not be practical in the United States due to differences in production systems, industry structure, and regulatory organization.

In the United States, the approach to decreasing the risk of *Salmonella* contamination of meats has been focused on control measures during slaughter and processing. The Hazard Analysis Critical Control Point (HACCP)/Pathogen Reduction Act[7] established performance standards for *Salmonella* at slaughter and processing plants, which has resulted in decreased product contamination. It is expected that the salmonella standards at slaughter and processing will become more stringent, creating pressure from packers and processors for on-farm interventions to reduce the preharvest prevalence of *Salmonella*-positive swine.

A current challenge for *Salmonella* pre-harvest food safety is identification of a diagnostic tool that not only has desirable tests characteristics (eg, precision and accuracy, diagnostic sensitivity and specificity), but also reflects the risk of contamination of pork during slaughter and processing. In the United States, microbiological culture of feces or tissues has been the predominant diagnostic tool to establish *Salmonella* status of farms. This is most likely a combination of factors: microbiological culture is the "gold standard" diagnostic test for *Salmonella* serovars; regulatory monitoring at slaughter is based on microbiological culture; and there is an intuitive appeal to the idea that shedding of *Salmonella* organisms near the time of marketing contributes to the risk of contamination of carcasses during slaughter and processing.

In determining the status of *Salmonella* serovars (or that of any other infection) at the herd level, herd-level sensitivity depends on actual herd prevalence as well as on the sensitivity and specificity of the individual diagnostic tool, the herd-to-herd variability of sensitivity and specificity, and the number of animals tested.[8] Therefore, inherent characteristics of the diagnostic test and sampling strategies, and the investigator's understanding of the epidemiology of *Salmonella* serovars will both have an impact on interpretation of herd level diagnostic test results.

Epidemiology of *Salmonella* serovars

It has long been recognized that swine[9–18] can be asymptomatic carriers of S*almonella* serovars. In the United States, the frequency of the number of farms positive for *Salmonella* organisms ranges from 38.2 to 83.0%, and the frequency of the number of positive pigs ranges from 6.0 to 24.6%.[19,20]

Since microbiological culture of pathogenic salmonellae from the feces of swine depends on their shedding status, temporal variability of fecal shedding of salmonellae affects the herd-level test sensitivity. This variability may be extreme, both within a group of pigs and between marketing groups within the same farm. Funk et al[21] have reported significant changes in prevalence during the growing phase of pork production. Lo Fo Wong[22] reported that of 32 herds monitored longitudinally for 2 years, 62% changed their *Salmonella* status (categorized as positive or negative) at least once during the study. Gibson et al[23] also reported temporal variability in prevalence within US herds, estimated by lymph node culture. Current epidemiological investigations have been predominantly based on point-in-time evaluation of *Salmonella* prevalence, usually near the time of marketing (if sampled ante-mortem on farm) or at slaughter. Although it is attractive to believe that the S*almonella* status of a group of pigs close to the time of slaughter most closely reflects the risk of carcass contamination (and subsequent risk to human health), there is little data to suggest whether this accurately reflects the risk of contamination.

Another component of the epidemiology of salmonella shedding on swine farms that may be important to interpretation of diagnostic tests is that shedding of multiple serotypes (serovars) within a group of pigs

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is common.[21,24] A few studies have reported that individual pigs were shedding multiple serotypes simultaneously.[25] There does seem to be a certain group of *Salmonella* serotypes that are the "usual suspects" isolated from cases of foodborne disease in humans: *Salmonella* Enteriditis and *Salmonella* Typhimurium are the serovars most commonly isolated from human clinical cases.[26] Under the current HACCP/Pathogen Reduction Act standards, all pathogenic *Salmonella* isolates are considered of equal risk regardless of serotype. There is some evidence in the literature that different isolation methods may favor isolation of certain serotypes in samples containing more than one *Salmonella* serotype.[27]

Salmonella organisms are rapidly disseminated after ingestion or inhalation. Reports suggest that salmonellae may be isolated from the cecum, ileum, lymph nodes, and feces of a pig within 30 minutes of oral exposure.[28] In the same investigation, all exposed pigs were *Salmonella*-positive by 6 hours post exposure to contaminated slurry. Fedorka-Cray et al[29] reported isolating salmonellae from the colons of pigs 3 hours after intranasal inoculation. The evidence for rapid infection is important, as lairrage (holding) pre-slaughter time is typically 2 to 3 hours in the United States, which does not include the time for transport from the farm to the slaughter facility. The implications are that exposure to salmonella during transport, lairrage, or both may result in an infection detectable by microbiologic culture at slaughter, but this may not reflect the *Salmonella* situation at the farm. This has obvious implications for the utilization of microbiological culture for *Salmonella* diagnosis. If the status of *Salmonella* serovars on the farm is the outcome of interest, pigs must be sampled on-farm if microbiological culture is the diagnostic test utilized.

Diagnosis of salmonellosis using fecal culture (the imperfect gold standard)

Fecal culture has the advantage of being available ante mortem, fecal samples are relatively easy to collect, and a *Salmonella* isolate is available for further identification (by serotype, phage type, genotype, or antibiogram, for example). Because a bacterial isolate can be definitively identified, microbiologic culture is assumed to have perfect specificity (no false positive results). Its weaknesses are well known: it is costly, time-consuming, and has poor sensitivity. False-negative results are common, ranging from 10 to 80%.[30–32] Fecal culture is also susceptible to sampling error if collection of samples does not coincide temporally with periods of shedding. As samples must be collected on-farm due to the risks of infection during transport and lairrage, fecal culture is disadvantageous from the standpoint of increasing biosecurity risk, as it requires on-farm visits and increasing costs of travel and labor expenses compared to sampling at a central location (eg, a slaughter facility).

Numerous studies have compared microbiologic techniques for isolating salmonella from a range of sources,[33] including swine feces.[32,34–39] In contrast to diagnosis of clinical salmonellosis, in which direct plating is often sufficient,[40] diagnosis of subclinical shedding typically requires specialized culture methods with several steps of selective enrichment. Two selective enrichment methods predominate in most epidemiological investigations of swine (Figure 1).[38] For Method 1, 10 g or more of feces is usually initially diluted in buffered peptone water (BPW). In Method 2, a 1-g sample of feces is initially diluted in tetrathionate broth. In a comparison of these two methods, Davies et al[38] found no statistical difference in the sensitivity for salmonella detection despite the differences in initial fecal sample weight. Funk et al[25] compared different fecal sample size (rectal swab, 1 g, 10 g, and 25 g) for the initial dilution in BPW for Method 1, and found increasing sensitivity with increasing fecal sample size. To the best of the author's knowledge, no one has published the effect of fecal sample size using Method 2.

Increased sensitivity has been achieved by using delayed secondary enrichment (DSE), which entails allowing one of the enrichment steps, usually Rappaport-Vassiliadis (RV) broth, to be stored at room temperature for several days, then aliquoting this inoculated broth at a 1:99 dilution in fresh RV and processing as before. Increases in sensitivity of approximately 25% have been described.[38]

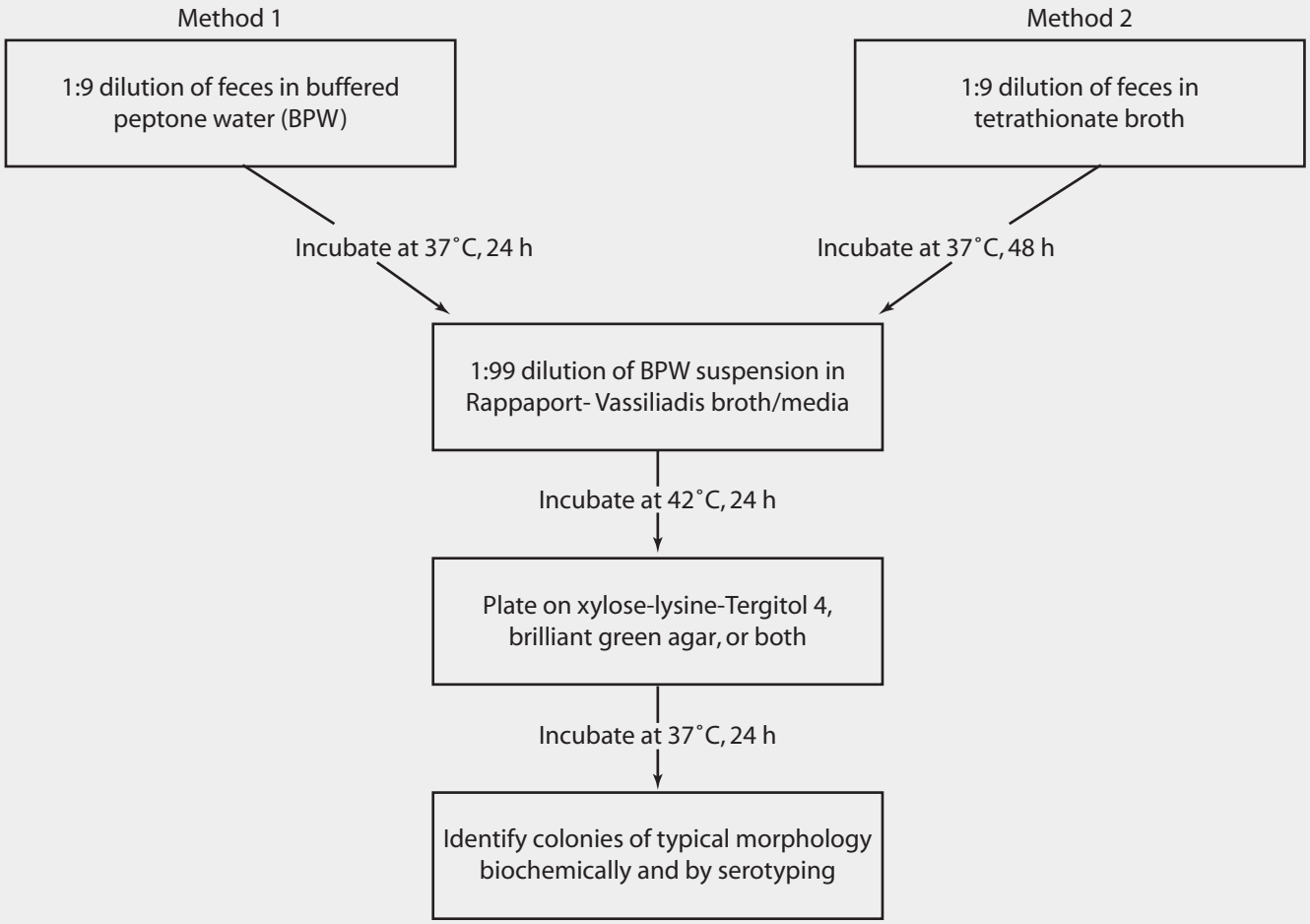
From a practical standpoint of the effect of handling and storage of feces prior to culture, refrigeration for 6 days did not significantly reduce the sensitivity of culture compared to same-day processing of fecal samples, but freezing of fecal samples at -15°C for 14 days resulted in statistically significant decreases in *Salmonella* isolation rates.[37]

Implications for sampling strategies on farm

Given the challenges associated with the epidemiology of salmonellae, the limited sensitivity of fecal culture, and the balancing of economic limitations for investigative efforts, on-farm sampling strategies must take into account the diagnostic goal. In many epidemiologic investigations, estimation of group prevalence, as well as identification of the serotypes present on the farm, are important. Criteria for determining the proportion of the herd to sample for epidemiological studies of *Salmonella* serovars usually do not consider the likely presence of more than one serovar in a herd, let alone in an individual animal. However, if the objective of a study is to characterize the prevalence and serovars of *Salmonella* in herds, some consideration is warranted. Various approaches for identifying the presence of multiple serotypes (serovars) in samples have been discussed.[41] Some possibilities at the herd level include serotyping multiple isolates per plate, use of multiple enrichment broths (and time and temperature of enrichment) and plating media, culturing multiple samples per pig, or sampling more animals per herd. In an investigation by Funk et al,[25] sampling more animals per group, which maximizes the diversity of the source material while providing the benefit of more accurate estimation of prevalence, was the more efficient approach compared to serotyping more colonies per positive fecal sample. In addition, as suggested by investigation of the dynamics of bacterial growth in selective enrichment broths,[27] selective enrichment may result in asynchronous growth curves due to differing susceptibilities among serotypes to the restrictive components of the media. Therefore, selection of more than one colony for serotyping may not be as efficient as sampling more animals or utilizing delayed secondary enrichment techniques.[36,37,41]

In situations where only the herd level status is important (positive or negative) and the expected salmonella prevalence is low, pooling individual fecal samples for microbiological testing increases the herd level

Figure 1: Flow diagrams for two predominant methods of microbiological culture for *Salmonella* serovars.



sensitivity of the test,[42] while potentially decreasing the cost of sampling and microbiological methods. However, this method may underestimate the number of serotypes present on a farm, and is not beneficial if on-farm prevalence is much higher than 5%.

The one consistent aspect of a review of the literature involving sampling and diagnostic strategies for *Salmonella* serovars is that increased effort, either through more intensive sampling or the use of multiple microbiological broths or plating media, increases the sensitivity of fecal culture methods.[33,38,41] Balancing the benefits of different sampling strategies and microbiological methods with economic limitations, while still meeting the diagnostic goal, is a challenge for epidemiological monitoring of salmonellae on farms.

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