RESEARCH REPORT

PORK SAFETY

Title: Comparative Evaluation of Rapid Methods for *Salmonella* Detection in Pork – **NPB #08-120**

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Industry Summary: The goal of this study was to develop a single-step assay which combined all of the steps of conventional culture of *Salmonella* into a single well of a 48-deep well microtiter plate. The second goal was to screen hog carcass samples and ground pork to continuously refine the assay as it was being developed. Finally, the specificity and sensitivity of the assay was compared to conventional microbiological *Salmonella* testing methods. Conventional culture begins with pre-enrichment, followed by transfer of an aliquot of this nonselective enrichment to a selective broth containing novobiocin, and then plating to a selective agar, such as XLT-4, on which *Salmonella* appear as black colonies. The components used for conventional *Salmonella* isolation were consolidated into each well (5 ml/well capacity): XLT-4 was first added to each plate and allowed to solidify after which Modified Semisolid Rappaport-Vassiliadis (MSRV) with novobiocin was added followed by the selective broth. The 10% suspension of the sample to be screened was the final upper layer. Plates were sealed, incubated (42C, 48 hrs), and scored as positive if the lower XLT-4 indicator agar layer turned black. The assay is called the RX plate because it incorporates Rappaport-Vassiliadis modified semisolid agar (R) and for XLT-4 agar (X). Pure cultures were initially screened to ensure that the assay could detect *Salmonella*. Next, hog carcass lymph nodes harvested after the deep chill were screened (n=264). This was done to ensure that non-specific blackening of the wells due to the tissue itself did not occur. Swine lymph nodes harvested prior to the chiller, hog fecal samples (n=80) and retail pork sausage (n=240) were also evaluated. The reliability of the RX plate was based on specificity and sensitivity estimates, which compared RX plate results with those obtained by conventional bacteriological isolation. The RX format, while theoretically attractive, failed to achieve the either the specificity or sensitivity of conventional microbiological culture.

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Scientific Abstract: The goal of this study was to develop an assay which combined all of the steps of conventional culture of *Salmonella* into a single well of a 48-deep well microtiter plate. The second goal was to screen hog carcass samples and ground pork to refine the assay as it was being developed. Finally, the specificity and sensitivity of the assay was compared to conventional microbiological *Salmonella* testing methods. Conventional culture includes pre-and selective enrichments followed by plating to a selective agar, such as XLT-4, on which *Salmonella* appear as black colonies. The components used for conventional *Salmonella* isolation were consolidated into each well (5 ml/well capacity): XLT-4 (400ul) was first added to each plate and allowed to solidify followed by addition of Modified Semisolid Rappaport-Vassiliadis (MSRV) with novobiocin (800ul) and the selective enrichment broth (900 ul). The 10% suspension of the test sample to be screened was added as the final upper layer (100 ul). Plates were sealed, incubated (42C, 48 hrs) and scored as positive if the lower XLT-4 agar layer turned black. The assay is called the RX plate because it incorporates Rappaport-Vassiliadis modified semisolid agar (R) and XLT-4 agar (X). Pure cultures were initially screened to ensure that the assay could detect *Salmonella*. Hog carcass lymph nodes harvested after the deep chill were screened (n=264) to ensure that non-specific blackening of the wells due to the tissue matrix did not occur. Lymph nodes harvested prior to the chiller (n=733), hog fecal samples (n=80) and retail pork sausage (n=240) were also evaluated. The original RX tube method offered the potential to replace conventional culture in a single tube with a reported 96.9% sensitivity and 93.1% specificity and a limit of detection of 1 *Salmonella* even in the presence of competing microflora. The RX microtiter format achieved 100% specificity and sensitivity only for pure cultures and the carcass swine lymph nodes recovered post deep chill. For retail purchased ground pork (n=210 samples), when compared to conventional culture, the estimated sensitivity (67%) and specificity (80%) of the RX reflect the unacceptable high number of false-positives. Despite repeated modifications, including incorporating antibiotics specifically inhibiting *Pseudomonas*, *Citrobacter*, and *Proteus*, the number of false positive reactions persisted. The time needed to transfer the RX blackened wells to XLT-4 for *Salmonella* confirmation realized little time savings when compared with conventional bacteriological isolation. The percentage of *Salmonella* positive samples detected in the BAX real-time PCR format was as expected slightly higher than that achieved by culture. In conclusion, the RX format, while theoretically attractive, failed to achieve the either the specificity or sensitivity of conventional microbiological culture.

Introduction: In response to the ongoing USDA-FSIS national initiative to reduce human salmonellosis by 50% by the year 2010 there is a need to develop rapid, economical, and simple techniques for detection and ultimately enumeration of *Salmonella* to assess the effectiveness of pathogen reduction strategies both on the farm, during processing, and at the retail level. The goal of this project is to develop and evaluate a new method, the RX plate method, for detecting *Salmonella* in pork. The **RX** assay relies on, first, the motility of *Salmonella* in semi-solid Rappaport-Vassiliadis (**R**V) and, second, ability of *Salmonella* to generate H_2S, which forms a black precipitate on Xylose lysine tergitol 4 (**X**LT-4) agar. The RX microplate method, a modification of the RX tube designed by Gailey *et al.* (unpublished observation), consolidates pre-enrichment (upper layer; buffered peptone water or GN Hajna), selective enrichment (middle layer; modified semisolid **R**V; MS-RV), and plating to a selective agar (bottom layer; **X**LT-4) into a single well of a 48-deep well plate. Wells are inoculated with a 10% suspension of homogenate (ground pork, porcine lymph nodes, carcass swabs, or excised carcass sample) prepared in buffered peptone water, which is then added to the upper layer. The assay includes both negative (BPW only) and positive (*Salmonella enterica* serotype Abaetatuba in BPW) control wells. Plates are sealed and incubated (48-72 hrs, 42C). After incubation, *Salmonella*-positive samples are identified by a blackening of the XLT-4 agar in the well. The procedure can screen 24 samples in duplicate using a 48-well format in 48 hours and is an attractive alternative to the more tedious steps in conventional *Salmonella* isolation.

Objectives:

(i) Optimize the RX microtiter well system for detection of *Salmonella* using known concentrations of pure cultures, and irradiated ground pork seeded with *Salmonella*;

(ii) Screen retail purchased ground pork using the four protocols: RX microtiter, commercial BAX real-time system, an in-house real-time PCR assay, and traditional culture:

(iii) Evaluate the sensitivity and specificity of the RX microtiter to commercial BAX real-time system, an in-house real-time PCR assay, as well as traditional culture, which will serve as the reference method for comparison.

Materials and Methods

The RX single tube format was converted to a 96-well plate and ultimately to the 48-deep well plate format while maintaining the initial proportions. The larger size of the well in the 48-well format (5 ml/well, Axygen Scientific, Union City, CA) accommodated a larger sample size, and was easier to inoculate than the 96-well format (1 ml/well). The components of the well consisted of the lower layer of XLT-4 (400 ul, Difco, Sparks, MD), overlayered with modified semisolid Rappaport-Vassiliadis (MSRV, Difco, Sparks, MD) media supplemented with 20 ug novobiocin/ml (800 ul) and an upper layer (900 ul) of enrichment broth, such as Tetrathionate, GN broth, or GN-TSB broths (Difco, Sparks, MD). When all of the layers had solidified, a 10% suspension of the sample prepared in buffered peptone water (BPW, Oxoid, Hampshire, England) was added (100 ul) to duplicate wells. *S. enterica* serotype Abaetatuba was added to duplicate to positive control wells; an aliquot of BPW (100ul) was added in duplicate to the negative control wells. A plastic film was used to seal the plates and the plates incubated (42C). Reactions were read at 24,48 and 72 hrs using a microtiter mirrored plate reader. A reaction was scored as "positive" based on blackening of the XLT-4 indicator at the bottom of the well. Green or yellow wells were scored as negative.

To determine the limit of detection, either pure cultures of *Salmonella enterica* serotype Abaetatuba or pure cultures of *S. enterica* serotype Abaetatuba seeded into irradiated pork were serially diluted (10^0 to 10^{-16}) and added to duplicate wells. Plates were incubated (42C) and read at 24, 48 and 72 hrs.

Field samples screened with the RX format included swine carcass lymph nodes obtained after the deep chill (-60F, n=264), lymph nodes obtained prior to the deep chill (n=733), hog feces (n=80), and retail purchased ground meat (pork) samples (n=240). In all cases, a 10% (wt/vol) suspension was prepared in BPW and 100 ul added in duplicate to the RX wells. Continuous improvements to the RX plate based on results of screening field samples included replacing a modified BGS agar as the indicator agar, using V-bottom wells, and replacing the plastic film with a pierceable silicon mat to reduce condensation and eliminate potential cross contamination. Most recently the highly specific GN broth replaced BPW and tetrathionate in the enrichment layer. To assess the reliability of the RX format, conventional bacteriological *Salmonella* isolation consisting of enrichment in BPW (37C, 24 hrs), subcuture to RV with novobiocin (20 ug/ml, 24 hrs, 42C), transfer to RV (24 hrs, 42C) and streaking to XLT-4 agar (24 hrs, 37C) was incuded for comparison. In addition, a 1 ml aliquot of the BPW after incubation (24 hrs, 37C) was added to Tetrathionate broth (9 ml), incubated (42C, 24 hr) and plated to XLT-4 (24, 37C). Presumptive *Salmonella* (black) colonies were verified by agglutination (*Salmonella* O antiserum, poly A-1 & Vi, Difco, Sparks, MD).

Three methods were initially compared for recovery of *Salmonella* from retail purchased ground pork samples (n=210, ~5 gram samples): Conventional bacteriological isolation; the RX method; and the Dupont Qualicon BAX format (Wilmington, DE). The RX method involved inoculating a 10% ground pork suspension in duplicate wells, incubation (42C, 72 hrs), and scoring blackened wells as positive. For confirmation, an aliquot from the blackened wells was plated to XLT-4 (24, 37C), and resultant presumptive *Salmonella* colonies (black) confirmed by latex agglutination. *Salmonella* isolates were submitted to USDA-NVSL for serotyping using the PremiTest PCR-based assay (DSM Nutritional Products, Geleen, The Netherlands). The BAX format was included for comparison because it is the commercially available PCR method used by USDA-Food Safety and Inspection laboratories. Because of the quality controls in place by the manufacturer and ease of use, the BAX was ultimately selected as the real-time PCR method for these studies. Specificity and sensitivity estimates were used to compare the reliability of the RX plate with conventional bacteriological culture.

Results

Objective 1. For pure cultures the level of detection was 2.5 *Salmonella*/ml and positive wells were easily identified by 48 hours. When pure cultures of *Salmonella* were seeded into irradiated pork, the results were less clear, but nevertheless readable, with wells inoculated with the higher dilutions questionably black/gray.

Objective 2. None of the swine carcass lymph nodes obtained after the deep chiller room (n=264) were positive either by conventional culture or by the RX plate. Generic *E. coli* counts (<100 cfu/ml) and total aerobic plate counts (<300 cfu/ml) determined for a subsample of these carcass lymph nodes affirmed the bactericidal effect of the deep chiller room. *Salmonella* prevalence estimates in a subset of lymph nodes collected prior to entering the chiller room were higher by RX format (18.6%) than by conventional culture (13.6%). However, prolonging incubation beyond 48 hrs, resulted in overgrowth of competing microbes which darkened the XLT-4 indicator layer, leading to an unacceptable level of false positive samples.

Trials were conduced to determine the suitability of the RX micotiter method for screening hog feces. When fecal samples from a small number of piglets (~21 day old) raised at our facility (n=12) were screened by the RX format, blackened wells only appeared in the positive control wells inoculated with *Salmonella*. Feces from the piglets remained negative (green color in wells) by the RX format and by conventional culture. This seemed to indicate that the RX protocol was adequate for depressing bacterial contaminants. However, a larger study of feces taken from market-weight conventionally reared adult hogs (n=80) indicated unacceptable estimated sensitivity (57%) and specificity (93%) for the RX method when compared with conventional isolation.

In preliminary studies of retail purchased pork samples (n=30), none of the pork samples were positive by the RX plate whereas 9 of 30 samples yielded *Salmonella* after conventional culture. When these initial studies were conducted, the incubation period for the RX plates was 48 hrs, with longer incubation resulting in false positive readings. Thus prolonged incubation was not used. Three methods were compared for recovery of *Salmonella* from retail purchased ground pork samples (n=210, ~5 gram samples): Conventional bacteriological isolation; the RX method; and the BAX real-time PCR assay. *Salmonella* isolates obtained by conventional culture were submitted to USDA-NVSL for serotyping employing the PCR-based Premitest and were identified as *Salmonella enterica* serovar Infantis. For *Salmonella* confirmation of darkened RX wells, an aliquot from the blackened wells was plated to XLT-4, and resultant presumptive *Salmonella* colonies (black) confirmed by latex agglutination. As seen in Table 1, two scores are provided for the RX plate: The percentage of darkened wells (RX) prior to confirmation and the percentage of *Salmonella* subsequently recovered after plating an aliquot from the darkened RX wells to XLT-4 (RX to XLT-4). The comparative recovery of *Salmonella* from retail purchased pork using these methods is summarized in Table 1. Whereas conventional culture detected *Salmonella* in 0.95% of samples, the RX format, based on blackening of the wells, yielded nearly 25% *Salmonella*-positive samples. When blackened wells were plated to XLT-4 agar and only black colonies picked and confirmed with an agglutination assay, 0.5% of pork samples yielded *Salmonella*. By BAX, a real-time PCR based assay, 1.43% of the samples were positive. The higher percentage is expected since the assay detects *Salmonella* DNA and does not differentiate between living or dead cells as does conventional culture.

Table 1. Recovery of *Salmonella* by culture, RX and BAX formats.

	Culture	RX	RX to XLT-4	BAX
Number Positive	2/210	52/210	1/210	3/210
Percent positive (%)	0.95	24.8	0.5%	1.43%

Objective 3. Evaluate the sensitivity and specificity of the RX microtiter to commercial BAX real-time system, an in-house real-time PCR assay, as well as traditional culture, which will serve as the reference method for comparison.

The RX microtiter format achieved 100% specificity and sensitivity only for pure cultures and for the carcass swine lymph nodes recovered post deep chiller room. As summarized in Table 1, for retail purchased ground pork (n=210 samples), when compared to conventional culture, the estimated sensitivity (67%) and specificity (80%) of the RX only format prior to plating to XLT-4 reflects the high number of false-positives (Table 1).

Discussion: The original RX tube method offered the potential to replace conventional culture in a single tube with a reported 96.9% sensitivity and 93.1% specificity and a limit of detection of 1 *Salmonella* even in the presence of competing microflora (Gailey *et al*., unpublished observation). The RX microtiter format achieved 100% specificity and sensitivity only for pure cultures of *Salmonella* and for the carcass swine lymph nodes recovered post deep chill. For retail purchased ground pork (n=210 samples), when compared to conventional culture, the estimated sensitivity (67%) and specificity (80%) of the RX only format prior to plating to XLT-4 reflects the high number of false-positives (Table 1). Despite repeated attempts, including incorporation of antibiotics specifically inhibiting *Pseudomonas*, *Citrobacter*, and *Proteus*, optimization of media, modifying the volume of suspension inoculated into the well, and adjusting the time and temperature of incubation, the number of false positive reactions persisted in the RX only format (RX). The time needed to transfer the RX blackened wells to XLT-4 (RX to XLT-4) for *Salmonella* confirmation realized little time savings when compared with conventional bacteriological isolation. The percentage of *Salmonella*-positive samples detected in the BAX real-time PCR format was, as expected, slightly higher than that achieved by culture since the assay detected DNA from either living or dead *Salmonella*. In conclusion, the multiwell RX format, while theoretically attractive, failed to achieve the either the specificity or sensitivity of conventional microbiological culture