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Do Microbial Interactions and Cultivation Media Decrease the Accuracy of *Salmonella* Surveillance Systems and Outbreak Investigations?

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ABSTRACT

Cultivation methods are commonly used in *Salmonella* surveillance systems and outbreak investigations, and consequently, conclusions about *Salmonella* evolution and transmission are highly dependent on the performance characteristics of these methods. Past studies have shown that *Salmonella* serotypes can exhibit different growth characteristics in the same enrichment and selective media. This could lead not only to biased conclusions about the dominant strain present in a sample with mixed *Salmonella* populations, but also to a low sensitivity for detecting a *Salmonella* strain in a sample with only a single strain present. The objective of this study was to determine whether cultivation media select preferentially for specific strains of *Salmonella* in heterogeneous cultures. In this study, four different *Salmonella* strains (one *Salmonella* Newport, two *Salmonella* Typhimurium, and one *Salmonella* Enteritidis) were competed in a broth-based experiment and a bovine fecal experiment with varied combinations and concentrations of each strain. In all experiments, the strain of *Salmonella* Newport was the most competitive, regardless of the starting concentration and cultivation protocol. One strain of *Salmonella* Typhimurium was rarely detected in competition, even when it was the only strain present in bovine feces. Overall, the probability of detecting a specific *Salmonella* strain had little to do with its starting concentration in the sample. The bias introduced by culture could be dramatically biasing *Salmonella* surveillance systems and hindering traceback investigations during *Salmonella* outbreaks. Future studies should focus on the microbiological explanations for this *Salmonella* interstrain variability, approaches for minimizing the bias, and estimations of the public health significance of this bias.

Salmonella enterica is a diverse bacterial species that is currently divided into six subspecies and more than 2,400 serotypes *(11).* Certain serotypes of *Salmonella enterica* can be important bacterial pathogens of humans and animals, with different serotypes having varying levels of host specificity *(3).* Foodborne transmission is considered to be the most important route for human infection. *Salmonella* is currently considered a potentially important bioweapon and is listed by the Centers for Disease Control and Prevention as a category B agent (http://www.bt.cdc.gov/agent/agentlist-category.asp).

Many research investigations focus on *Salmonella,* and entire national and international surveillance systems have been designed to track *Salmonella* infections. Cultivation methods, which are most often used to isolate, identify, and describe the bacterial strains in a sample, form the basis of most of these *Salmonella* endeavors and typically employ enrichment and selective media. In general, it is only after *Salmonella* has been recovered from a sample by using cultivation-based methods that specific isolates can be further characterized. Culture-based protocols are also used to estimate the approximate number of *Salmonella* cells in a sample, although this is performed less commonly in clinical settings. Finally, cultivation methods are used extensively during outbreak investigations, where the goal is to find potential sources of specific *Salmonella* strains that are genetically related to the strain that is causing the illness. Consequently, conclusions and inferences about *Salmonella* evolution and transmission are highly dependent on the performance characteristics of the cultivation-based methods.

Given the broad application of *Salmonella* cultivation-based methods, one might expect that there would be a harmonization of methods, at least within a specific project or surveillance system. In fact, there are many different protocols for *Salmonella* culture, most of which use one or more enrichment procedures. More than one method is often used in the same investigation or surveillance system. Studies on swine fecal samples *(5, 12)* demonstrated that different culture methods performed on the same set of samples yielded different *S. enterica* serotypes. Thus, comparisons among samples that were cultured with different methods are likely to be biased. Cultivation media clearly impose selection pressures on *Salmonella.* This pressure can vary by strain, as demonstrated by Harvey and Price's 1967 study *(7),* in which different *Salmonella* serotypes had different growth characteristics in the same selective broths.

While there is a clear need for standardization of *Sal-*

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monella culture methods among laboratories, there is an even greater need to understand how the results of *Salmonella* cultivation methods should be interpreted. Culture techniques expose heterogeneous bacterial populations, which might also include heterogeneous *Salmonella* populations, to selection pressures unlike those found in the natural environment. Consequently, if one *Salmonella* strain grows more successfully in a specific selection or enrichment broth than does another *Salmonella* strain, the apparent abundance or importance of a particular strain could be over- or underestimated. Samples from sources such as retail meats, produce, animals, and the environment might possess heterogeneous populations of *Salmonella* and would therefore be affected by this potential bias. This would increase the difficulty of locating the source of an outbreak strain, thus prolonging the duration of the outbreak. This selection bias might not impact the culture of human-derived samples for *Salmonella,* as the majority of human infections are likely to contain a single strain of *Salmonella.*

The objective of this study was to elucidate the potential for cultivation media to select preferentially for specific strains of *Salmonella* in heterogeneous cultures. We hypothesized that the ratios of *Salmonella* strains isolated during culture would not match the ratios of the original strain concentrations added to the samples. An additional objective was to evaluate whether the probability of detecting a *Salmonella* strain in a sample with no other *Salmonella* would be the same among different *Salmonella* strains.

MATERIALS AND METHODS

Study design. This study was conducted in three phases. First, growth curves were estimated for the *Salmonella* strains used in the study. Next, a broth-based competition experiment was performed by using varied combinations and concentrations of the *Salmonella* strains. Finally, a competition experiment that used bovine feces inoculated with various *Salmonella* strains was conducted. In the fecal experiment, *Salmonella* strains were added in varied combinations and concentrations to bovine feces in which *Salmonella* was not detected, and four different cultivation protocols were compared.

Four different strains of *Salmonella* were used in this study: one strain of *S. enterica* serovar Newport, two strains of *S. enterica* serovar Typhimurium, and one strain of *S. enterica* serovar Enteritidis. The *Salmonella* Newport strain was previously isolated from the feces of a healthy cow. One *Salmonella* Typhimurium strain, known as *Salmonella* Typhimurium strain 798, is a clinical isolate from a pig and can cause persistent asymptomatic infections in pigs *(2).* The other *Salmonella* Typhimurium strain (*Salmonella* Typhimurium 35) was originally isolated from the feces of a healthy cow. Finally, the *Salmonella* Enteritidis strain was previously isolated from a healthy chicken. Each strain possessed a different combination of antibiotic resistance to the antibiotics nalidixic acid and streptomycin. The *Salmonella* Newport strain was resistant to both antibiotics, *Salmonella* Typhimurium 798 was susceptible to both antibiotics, *Salmonella* Typhimurium 35 was resistant to nalidixic acid and susceptible to streptomycin, and *Salmonella* Enteritidis was susceptible to nalidixic acid and resistant to streptomycin. It was determined through conjugation experiments that these resistances were not transferrable (data not shown). It is important to note that this study was not intended to model all potential salmonellae; we selected four strains with varied antibiotic resistance markers to evaluate the possibility of competition and differential selection in cultivation methods.

Growth curves. The growth characteristics of each strain were evaluated in broth culture. Each strain was grown overnight in 2.5 ml of tryptic soy broth (TSB) at 37°C. The turbidity values at 600 nm (OD_{600}) of 1/20 dilutions were measured spectrophotometrically. The overnight culture was then diluted in 125-ml flasks containing 25 ml of TSB to achieve a starting OD_{600} of 0.03. Cultures were grown at 37°C with shaking at $\sim 200 \times g$. After 70 min and then approximately every 30 min, growth was approximated by measuring the OD_{600}. When OD_{600} values became greater than 0.5, cultures were diluted to 1/10 concentration in additional TSB before determining the OD_{600} value. The final reading was taken at 4.5 h. Plate counts were performed by serially diluting a sample of each culture, and 50 μl of the 10^{-5} dilution was plated on duplicate tryptic soy agar (TSA) plates and grown overnight at 37°C.

Broth experiment. All four *Salmonella* strains were competed in varied combinations and concentrations by using a broth-only protocol. Each strain was grown overnight at 37°C in 8 ml of Luria-Bertani broth. The next day, the concentration of each strain was estimated by using OD_{600} measurements. The strains were diluted and mixed, and then added to tubes containing 9 ml of tetrathionate broth (TTB). The goal was to add a total of 4×10^3 *Salmonella* cells per tube of TTB. The specific combinations and strain ratios that were evaluated were as follows: two-strain competitions containing ratios of 75:25, 50:50, and 25:75; three-strain competitions containing ratios of 33:33:33; and four-strain competitions containing a ratio of 25:25:25:25. All possible combinations of strains were mixed in these ratios, resulting in 23 competitions (Table 1). All competitions were performed in duplicate. Because our pilot data indicated that the *Salmonella* Newport strain was more competitive than the others, we performed additional four-strain competitions by using lower concentrations of the *Salmonella* Newport strain. These competitions used ratios of 10:30:30:30 and 1:33:33:33, and were performed in duplicate. To confirm that each strain was capable of growing in the media, each strain was also grown individually with the same protocol in duplicate tubes.

After 48 h in TTB, 0.5 ml was transferred from each TTB tube to 4.5 ml of Rappaport-Vassiliadis R-10 medium (R-10) and incubated for 24 h at 37°C. Dilutions were then made of each R-10 tube, and 50 μl of each 10^{-3}, 10^{-4}, and 10^{-5} dilution was plated onto xylose-lysine-deoxycholate (XLD) agar plates. Plates were incubated overnight at 37°C. The next day, 48 colonies from the dilution plate that contained 200 to 300 well-isolated colonies were picked and transferred to a grid on a fresh XLD plate. These plates were incubated overnight at 37°C. The next day, these colonies were transferred to 96-well plates containing 100 μl of Luria-Bertani broth in each well, using a replica plater. The microtiter plates were incubated overnight at 37°C. The next day, the plates were stamped onto the following agar plates: TSA containing 32 μg/ml streptomycin, TSA containing 100 μg/ml nalidixic acid, XLD, and brilliant green agar. These plates were incubated overnight at 37°C, and the next day, the resistance profile of each colony on each plate was recorded to determine which of the four strains was present.

Fecal experiment. The goal of this experiment was to determine how well the *Salmonella* strains competed in the presence of other bacteria as well as nutrients and inhibitors naturally present in bovine feces. For this experiment, four different cultivation

TABLE 1. *Colony counts of each strain in the broth experiment for the different competition combinations*

Competition[a]	Ratio[b]	Replicate[c]: 1	2
N:T798	75:25	26:22	36:12
	50:50	36:12	29:19
	25:75	22:26	15:33
N:E	75:25	45:3	47:1
	50:50	44:4	44:4
	25:75	45:3	39:9
N:T35	75:25	48:0	48:0
	50:50	48:0	48:0
	25:75	48:0	48:0
T798:E	75:25	40:8	43:5
	50:50	37:11	43:5
	25:75	39:9	35:13
T798:T35	75:25	47:1	45:3
	50:50	38:10	36:12
	25:75	40:8	35:13
E:T35	75:25	44:4	47:1
	50:50	44:4	48:0
	25:75	43:5	37:11
N:T798:E	33:33:33	44:2:2	34:12:2
N:T798:T35	33:33:33	25:21:2	28:17:3
N:E:T35	33:33:33	44:3:1	47:1:0
T798:E:T35	33:33:33	29:13:6	37:7:4
N:T798:E:T35	25:25:25:25	44:4:0:0	46:2:0:0
	10:30:30:30	29:16:2:1	22:20:4:2
	1:33:33:33	18:26:3:1	6:35:7:0

[a] N, *Salmonella* Newport; E, *Salmonella* Enteritidis; T798, *Salmonella* Typhimurium 798; T35, *Salmonella* Typhimurium 35.
[b] The ratio reflects the relative starting concentration of each strain inoculated into the broth.
[c] Results are shown for each replicate, and the results represent the number of colonies of each strain that was observed.

protocols were used. Only strains *Salmonella* Newport, *Salmonella* Typhimurium 35, and *Salmonella* Enteritidis were used. *Salmonella* Typhimurium 798 was susceptible to both of our antibiotic markers; therefore, it would have been extremely difficult to screen for this strain among the bacteria in the feces that could potentially grow.

As described previously, strains were grown overnight in Luria-Bertani and were mixed so that the final concentration added to each 9-ml TTB tube was 4×10^3 *Salmonella* cells. One gram of fresh feces from a dairy cow was also added to each TTB tube. The fecal sample was tested for the presence of *Salmonella* by using a previously published *invA* PCR protocol *(13).* Based on five independent PCR assays from the same fecal sample, we determined that the sample used in this experiment was negative for *Salmonella.*

Each strain was grown individually in six replicate tubes with 1 g of bovine feces (Table 2). The specific combinations and strain ratios that were evaluated were as follows: two-strain competitions containing ratios of 75:25, 50:50, and 25:75; and three-strain competitions containing a ratio of 33:33:33. All possible combinations of strains were mixed in these ratios, and all competitions were performed in six replicates (Table 3). This resulted in 60 cultures in each of the four protocols.

Four different laboratory protocols were assessed for each competition: protocol 1 used the same protocol as in the broth experiment, with 48-h TTB and 24-h R-10 incubations; protocol 2 used a 48-h TTB incubation with no R-10; protocol 3 used a 24-h TTB incubation that was followed by a 24-h R-10 incubation; and protocol 4 used a 24-h TTB incubation with no R-10. Depending on the protocol, dilutions of each TTB or R-10 tube were then made, and 50 μl of each 10^3, 10^{-4}, and 10^{-5} dilution was plated onto XLD agar plates. The remaining cultivation steps were identical to those described for the broth experiment.

Statistics. To determine the odds of detecting one strain versus another as a function of the initial concentration of each strain, a generalization of logistic regression to three and four outcomes, termed ''polytomous regression'' *(9),* was used. For the broth experiment, let p_1, p_2, p_3, and p_4 be the probability of strains 1, 2, 3, and 4, respectively; let x_1, x_2, x_3, and x_4, be the initial concentrations of each strain. Treating strain 1 as the baseline, a polytomous regression model for the four-strain broth experiment stipulates

$$\log\left(\frac{p_j}{p_1}\right) = \beta_{0j} + \beta_{1j}\log(x_1) + \beta_{2j}\log(x_2) + \beta_{3j}\log(x_3) + \beta_{4j}\log(x_4)$$

for strain $j = 2$, 3, and 4. Note that there are $5 \times 3 = 15$ parameters total, describing the relationship between the initial concentration vector (x_1, x_2, x_3, and x_4) and the probabilities (p_1, p_2, p_3, and p_4). Both of these vectors must sum to unity. For the fecal experiment, only three strains were included in the polytomous regression model. In the fecal experiment, a separate model was created for each cultivation protocol, and then a model was created that combined all four protocols and added a categorical variable for protocol. Interactions between protocol and strain were assessed in this model. Goodness of fit of all models was checked via Pearson's chi-square test and deviance statistics aggregating over the distinct initial concentration values. Models were fit via maximum likelihood by using the LOGISTIC procedure in SAS, version 9.1.4 (SAS Institute Inc., Cary, NC). For competitions in which one or more strains were not included, a value for x_j, the initial concentration of the strain, was set to 10^{-14}, effectively producing a structural zero in the contingency table; results were

TABLE 2. *Colony counts of each strain in the fecal experiment for the different protocols when grown individually over six replicates*

Protocol (h)[a]	*Salmonella* serovar: Newport	Enteritidis	Typhimurium 35
24	5, 35, 10, 4, 0, 12	0, 6, 0, 0, 0, 0	0, 0, 0, 0, 0, 0
24–24	25, 7, 27, 17, 2, 24	5, 4, 5, 13, 3, 10	0, 0, 0, 3, 0, 0
48	9, 8, 5, 8, 4, 3	2, 7, 1, 3, 0, 0	0, 0, 0, 0, 0, 0
48–24	16, 25, 24, 22, 1, 25	2, 3, 14, 2, 7, 5	0, 0, 0, 0, 0, 0

[a] 24, 24 h in TTB; 24–24, 24 h in TTB and 24 h in R-10; 48, 48 h in TTB; 48–24, 48 h in TTB and 24 h in R-10.

TABLE 3. *Colony counts of each strain in the fecal experiment for the different competition combinations, using the 48-h TTB–24-h R-10 protocol*

Competition[a]	Ratio[b]	Replicate[c]: 1	2	3	4	5	6
N:E	75:25	27:0	20:0	32:0	42:0	NA	38:0
	50:50	34:2	45:2	35:0	40:0	32:0	35:0
	25:75	16:4	19:0	14:1	24:3	6:0	16:2
N:T35	75:25	38:1	24:0	27:1	11:0	41:0	NA
	50:50	45:0	33:0	43:0	25:0	11:3	32:0
	25:75	34:0	22:0	27:0	31:0	39:0	34:0
E:T35	75:25	13:0	12:0	2:0	4:0	13:0	7:0
	50:50	4:0	3:0	2:0	8:1	4:0	4:0
	25:75	1:0	9:0	14:0	10:0	20:2	6:0
N:E:T35	33:33:33	19:0:0	17:0:0	21:2:0	37:0:0	24:2:0	28:0:0

[a] N, *Salmonella* Newport; E, *Salmonella* Enteritidis; T798, *Salmonella* Typhimurium 798; T35, *Salmonella* Typhimurium 35.
[b] The ratio reflects the relative starting concentration of each strain inoculated into the feces.
[c] Results show the colony counts of each strain in each competition for each replicate.

not sensitive to the choice of this arbitrarily small number. Probabilities were then estimated from the model coefficients by using the formula

$$p_j = \frac{\left(\frac{p_j}{p_1}\right)}{1 + \left(\frac{p_2}{p_1}\right) + \left(\frac{p_3}{p_1}\right) + \left(\frac{p_4}{p_1}\right)}$$

when four strains were competed, as in the broth experiment.

RESULTS

Growth curves. Over the 4.5-h evaluation, all four strains grew at approximately the same rate in pure TSB. The *Salmonella* Typhimurium 35 strain grew at a slightly slower rate than did the other three (Fig. 1), but the final estimated concentrations of all four strains were very similar; the final concentrations ranged from 6.8×10^8 to 1.2×10^9 CFU/ml, with the order being *Salmonella* Typhimurium 798 with the greatest concentration, which was followed by *Salmonella* Typhimurium 35, then *Salmonella* Newport, and finally, *Salmonella* Enteritidis.

Broth experiment. Every duplicate competition produced more than the 48 *Salmonella* colonies that were picked for identification. The data for the counts of each strain in each competition (Table 1) were then analyzed in the polytomous logistic regression model. The model fit the data well ($P > 0.5$ for Pearson and deviance statistics), and the actual odds matched well with the predicted odds. By using the final model, the predicted probability of detecting each strain when in competition with every other strain was estimated and graphed (Fig. 2). The *Salmonella* Newport strain was the most dominant strain of the four. Except for the *Salmonella* Newport competition against *Salmonella* Typhimurium 798 at the 75:25 ratio, *Salmonella* Newport had a higher-than-expected probability of detection against all strains for all initial concentrations. When competed one-on-one against *Salmonella* Newport, *Salmonella* Ty-

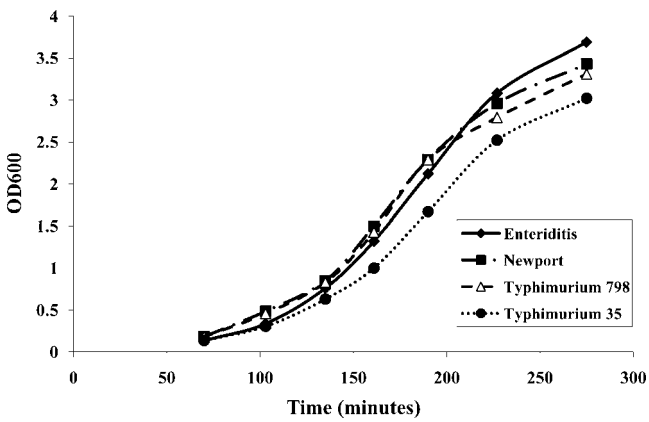

FIGURE 1. *Growth curves of the four* Salmonella *strains in TSB at 37°C. Strain growth was measured with* OD_{600}.

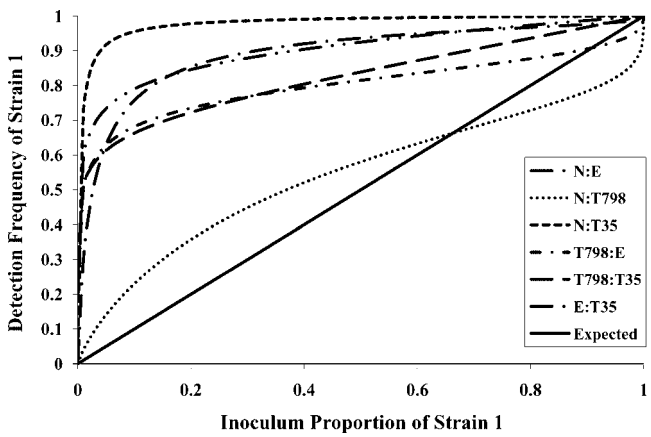

FIGURE 2. *Estimated probability of detection in the broth experiment. Using the polytomous logistic regression model from the broth experiment, estimated detection frequencies of one strain versus another in two-way competitions are graphed for a full range of inoculum proportions. For each curve, the first strain listed in the legend is considered strain 1 (e.g., in the N:E competition,* Salmonella *Newport is strain 1). The Expected line represents the null hypothesis that strains will grow at a frequency that matches their relative inoculated proportion. N,* Salmonella *Newport, E,* Salmonella *Enteritidis; T798,* Salmonella *Typhimurium 798; T35,* Salmonella *Typhimurium 35.*

phimurium 35 was never detected. There was a clear order in dominance to the four strains: *Salmonella* Newport, *Salmonella* Typhimurium 798, *Salmonella* Enteritidis, and *Salmonella* Typhimurium 35. In the four-strain competitions, *Salmonella* Newport stood out, particularly when its initial concentration was 10 or 1%. In the four-strain competitions, approximately 50 and 25% of the recovered colonies were *Salmonella* Newport when its starting concentrations were 10 and 1%, respectively.

Fecal experiment. Unlike the broth competition experiments, most of the competitions in this experiment failed to produce the 48 *Salmonella* colonies that were targeted for identification (Tables 2 and 3). The number of *Salmonella* colonies that grew varied by the specific strains being competed and the cultivation protocol being used. In the tubes that contained only a single strain, there were major differences in the growth characteristics among the three strains and among the four protocols (Table 2). Recovery of *Salmonella* improved with the addition of the R-10 step.

The 24-h TTB protocol yielded very few colonies. The *Salmonella* Typhimurium 35 strain was not detected in any of the six replicates, and the *Salmonella* Enteritidis strain was detected in one of the six, with a total of six colonies. In the 48-h TTB–24-h R-10 protocol, the *Salmonella* Typhimurium 35 strain was again not detected in any of the six replicates that contained only *Salmonella* Typhimurium 35. The *Salmonella* Enteritidis strain was detected in all six replicates, with 2 to 14 isolated colonies present on the agar.

The data for the number of colonies of each strain in each competition for each cultivation protocol were then analyzed in separate polytomous logistic regression models. The models fit the data well ($P > 0.4$ for Pearson and deviance statistics for all four models), and again, the actual odds matched well with the predicted odds. The predicted probabilities of detecting each strain when in competition with every other strain for the 48-h TTB–24-h R-10 protocol are shown in Figure 3. The *Salmonella* Newport strain was the most dominant strain, as in the broth experiment. The *Salmonella* Enteritidis strain was again more dominant than the *Salmonella* Typhimurium 35 strain. In the logistic regression model that included the data from all four protocols, the variable for protocol was highly significant ($P < 0.0001$), and there was a significant interaction between *Salmonella* Enteritidis and protocol ($P < 0.001$), implying that the performance of *Salmonella* Enteritidis in competition varied by protocol. More *Salmonella* Enteritidis colonies were recovered in both of the protocols that included R-10. Even though *Salmonella* Newport was detected much more frequently than was *Salmonella* Enteritidis in all competitions, *Salmonella* Enteritidis was more competitive when R-10 was part of the cultivation protocol. In the competitions with *Salmonella* Typhimurium 35, very few colonies of either *Salmonella* Typhimurium 35 or *Salmonella* Enteritidis grew in the protocols that lacked R-10.

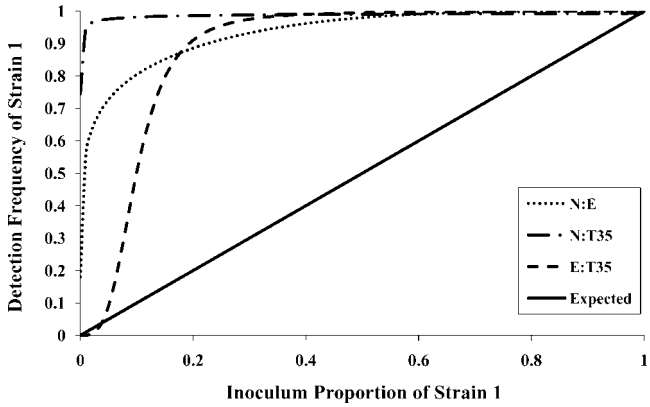

FIGURE 3. *Estimated probability of detection in the fecal experiment. Using the polytomous logistic regression model from the fecal experiment and the 48-h TTB–24-h R-10 protocol, estimated detection frequencies of one strain versus another in two-way competitions are graphed for a full range of inoculum proportions. For each curve, the first strain listed in the legend is considered strain 1 (e.g., in the N:E competition,* Salmonella *Newport is strain 1). The Expected line represents the null hypothesis that strains will grow at a frequency that matches their relative inoculated proportion. N,* Salmonella *Newport; E,* Salmonella *Enteritidis; T35,* Salmonella *Typhimurium 35.*

DISCUSSION

This study demonstrates several critical aspects of *Salmonella* cultivation methods typically used in surveillance and outbreak investigations. First, not all strains grew equally well in media and with all protocols. Even though this has been known for decades *(7),* few studies have investigated the factors that influence this variability or the impact that this bias has on the probability of detecting all *Salmonella* strains in a sample. Second, we observed variability in cultivation results and inconsistent success in detecting *Salmonella* in the sample among replicates, strains, and protocols. This finding is similar to the interlaboratory variation that has been observed in other studies *(12)* and that has plagued multicenter surveillance systems *(15).* This variability was also observed in the fecal experiment tubes containing a single strain of *Salmonella,* suggesting that the factors influencing the variation are not solely due to competition among *Salmonella* strains. Finally, the observed frequencies of each *Salmonella* strain deviated significantly from the expected frequencies, given the starting concentration of each strain; certain strains were more competitive in culture than were other strains. To ensure a high probability of detecting all strains present in a sample, it is important to know the number of colonies on an agar plate that should be characterized. Previous studies have developed mathematical models for determining this sample size *(1, 14).* Unfortunately, an assumption of these models is that the ratios of different *Salmonella* strains that grow on the plate are representative of the initial concentrations of each strain in the original sample. As we have shown in this study, the expected strain frequencies—based on the starting concentration of each strain—did not match with the actual observed strain frequencies on the agar plates. In fact, *Salmonella* Newport was a dominant strain, even when its concentration was 10% or less of the total *Salmonella* in the sample. These results indicate that surveillance systems could be severely biased and outbreak investigations

dramatically hindered by the differential selection probability of *Salmonella* in complex samples.

In the fecal experiment conducted in this study, different cultivation protocols had a variable selection on the different *Salmonella* strains. In the polytomous logistic regression model that included the fecal experiment data from all four protocols, there was a significant interaction between protocol and *Salmonella* Enteritidis. This strain grew significantly better when R-10 was included in the protocol. As has been noted in other studies, such as by Harvey and Price *(8),* different media can bias toward the selection of certain *Salmonella* serovars. This result seems to highlight the need for a standardized *Salmonella* cultivation protocol, particularly within a single project or surveillance system. For example, Global Salm-Surv, a global network of laboratories and individuals in *Salmonella* surveillance, has identified the lack of standardized data collection techniques for *Salmonella* as a weakness of the network (http://www.who.int/salmsurv/en/). Given that (i) different strains compete differentially, (ii) this competition could be affected by sample type, and (iii) every country or region is likely to have different strains of *Salmonella* circulating, it is not clear that a harmonized cultivation protocol would reduce the bias inherent in *Salmonella* culture. More work needs to be done to understand the factors that influence the cultivation bias, the extent to which bias exists for different *Salmonella* strains and sample types, and which protocols most effectively minimize this bias for specific sample types.

Many factors other than the media could also be influencing the variation of detecting *Salmonella* strains in culture. One obvious possibility is the competition among the *Salmonella* strains. In the broth experiment, there was a clear order to the four strains tested. This order was identical in the fecal experiment, regardless of the media protocol used. Given that the strains did not grow fully in the fecal experiment when cultured individually, there must be additional factors influencing their growth. Because of the complex matrix of a bovine fecal sample and diversity of microbiota within the sample, many factors could have influenced growth rates. While it has been speculated that multidrug antibiotic resistance could potentially make an organism less fit *(4),* the *Salmonella* Newport strain was multidrug resistant and was the best competitor in all experiments. Another recently identified factor that could be influencing growth rates is the presence of bacteriophages. Muniesa et al. *(10)* found that the presence of bacteriophages in *Salmonella* can bias the distribution and diversity of *Salmonella* cultures. Finally, previous studies have shown that the amount of sample that is added to the culture can influence the probability of detecting specific *Salmonella* strains *(6).* Therefore, future work on cultivation bias should continue to assess the impact of sample volume on the probability of detection in complex samples.

This study was intended to serve as a proof-of-concept model to demonstrate a bias that can potentially impact public health systems; accordingly, there are a couple of limitations to this study. First, we only tested a small number of strains. To effectively gauge the effect of this cultivation bias, more strains need to be tested so that a wide range of growth characteristics in varied culture conditions can be assessed. Another limitation of the study was the use of cattle feces as the natural sample. Only two of the strains were cattle derived, so there is the possibility that this substrate artificially influenced the results. It should be noted, though, that one of these two cattle strains (*Salmonella* Newport) was the most fit, while the other, *Salmonella* Typhimurium 35, was the least fit. In the competitions, the strains were actively growing and inoculated into the media. This is unlikely to reflect the real condition of *Salmonella* strains when being cultured from samples. In addition, *Salmonella* strains in natural samples can be severely stressed by the time they are added to cultivation media. For these reasons, the performance of strains from natural samples in cultivation media could differ substantially from the results of this study.

The bias that we have quantified could dramatically impact *Salmonella* surveillance systems and outbreak investigations, particularly if contamination levels are low. Surveillance systems often report *Salmonella* trends over time. It is generally assumed that the most prevalent strains, particularly from human infections, are the ones representing the greatest public health burden. This study raises questions such as (i) Could the most prevalent *Salmonella* strains or serotypes actually be more of a reflection of dominance in culture rather than of public health importance? and (ii) Could major clonal shifts seen in *Salmonella* over time actually be the result of the emergence of a specific strain that is more competitive in culture? For outbreak investigations, the results of our study present an additional difficulty with linking cases to each other and with tracing cases to a source. For example, a salmonellosis case might be caused by a homogenous infection with a *Salmonella* strain. Based on the results of this study, we would not necessarily expect a 100% probability of detecting this strain. If the strain were to be identified, however, there is no guarantee that a match will be made to the source. If the sample from which the strain was derived possesses a heterogeneous population of *Salmonella,* only the strain that is the most competitive, and not necessarily the most abundant or most relevant to public health, will be detected. Could this partially explain the challenge of finding the source of the *Salmonella* Saint Paul outbreak that affected more than 1,000 people in the United States in 2008 (http://www.fda.gov/oc/opacom/hottopics/tomatoes.html, http://www.cdc.gov/Salmonella/saintpaul/)? Many surveillance systems and outbreak investigations analyze a single colony from a sample, exacerbating the problem of correctly characterizing heterogeneous samples. In our study, observed strain ratios did not match with the inoculated strain concentrations. In systems that analyze only a single colony, there is a high probability that the selected strain might not reflect the strain of public health importance. Only molecular assays that are specific to the serovar or to the strain of interest would be capable of detecting the target strain if it is always outcompeted in culture. Dramatic increases in the number of colonies analyzed might circumvent some of the bias, but with a large concomitant increase in cost.

In summary, this study has quantified the disconcerting phenomenon that the probability of detecting a specific *Salmonella* strain in a sample might have very little to do with its concentration in the sample, but more to do with its ability to compete in the cultivation media and with the specific mixture of *Salmonella* strains present in the sample. The potential impact of this finding on epidemiological investigations and public health systems has been discussed for decades *(7, 16),* but it has never been assessed formally. The bias introduced by culture could be skewing the relative distribution of *Salmonella* strains such that certain strains appear to be less significant than they are under natural conditions. More work needs to be done to determine the microbiological explanations for this *Salmonella* interstrain variability, to determine approaches for minimizing the bias, and to estimate the public health significance of this bias.

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