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Short communication

The impact of screening-test negative samples not enumerated by MPN

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ABSTRACT

In microbiological surveys, false negative results in detection tests precluding the enumeration by MPN may occur. The objective of this study was to illustrate the impact of screening test failure on the probability distribution of *Salmonella* concentrations in pork using a Bayesian method. A total of 276 swab samples in four slaughter steps (69 samples in each slaughter step: after dehairing, after singeing, after evisceration, and before chilling) were screened for *Salmonella* and enumerated by the MPN method. *Salmonella* contamination data were fitted to a lognormal distribution by using a Bayesian model that uses the number of positive tubes at each dilution in an MPN analysis to estimate the parameters of the concentration distribution. With *Salmonella* paired data, three data sets were used for each slaughter step: one that includes the positives in the screening test only, a second one that includes false negative results from the screening, and a third that considers the entire data set. The relative sensitivity of the screening test was also calculated assuming as gold standard samples with confirmed *Salmonella*. *Salmonella* was confirmed by a reference laboratory in 29 samples either by screening or MPN method. The relative sensitivity of the screening test was 69% (CI 95%: 52%–85%). The data set that included enumerations from screen-negative samples (false negative results) tended to have higher $\hat{\mu}$ and smaller $\hat{\sigma}$ in comparison with the data set that discards false negative results, suggesting that the lack of sensitivity of the screening test affects the distribution that describes the contamination across the population. Numerous surveys on fitting distribution methods of microbial censored data have been published and discuss source of bias due to fitting method. Results of this survey contribute with that discussion by illustrating another possible source of bias due to failure of the screening methods preceding the MPN.

1. Introduction

Quantitative microbiology risk assessment (QMRA) is a science-based approach which has been increasingly applied for food safety in developed countries (Pouillot et al., 2013). Important inputs for QMRA are the prevalence and the concentrations of a given pathogen in contaminated products (Lammerding and Paoli, 1997; Pouillot et al., 2013). These concentrations are frequently obtained by the most probable number (MPN) method, in particular if concentrations are expected to be low.

Since there is a need to describe prevalence and concentration data accurately, considerable attention has been given to methods of fitting statistical distributions to data on microbial detection and enumeration obtained from foods (Busschaert et al., 2010; Commeau et al., 2012; Duarte et al., in press; Lorimer and Kiermeier, 2007; Pouillot et al., 2013; Williams et al., 2013; Williams and Ebel, 2012). These studies describe different methods to fit a distribution to censored microbial sampling data.

In microbiological surveys of foods as well as of pork carcasses, pathogen enumeration is normally made in samples that were first tested positive in a screening test (Prendergast et al., 2008; Williams and Ebel, 2012). This screening test normally uses a larger sample volume and/or different enrichment media combinations to enhance detection of *Salmonella*, and is applied to save costs by not doing MPN tests with negative results only. Some models follow the protocol commonly applied in the routine of food laboratories, enumerating samples that tested positive in a screening test that is assumed to have high or perfect diagnostic sensitivity (Pouillot et al., 2013; Williams and Ebel, 2012). However, false negative results in detection tests precluding the enumeration by MPN may occur. Recently, Snabes et al. (2013) reported that laboratories fail to detect food pathogens in food samples more than 5% of the analyses.

The objective of this study was to evaluate the probability distribution of *Salmonella* concentrations in pork carcasses in a situation where all the samples were submitted to the MPN and illustrate the

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impact of screening test failure using a previously published method (Williams and Ebel, 2012) and a data set collected in Brazil.

2. Material and methods

2.1. Microbiological analysis

Enumeration of *Salmonella* spp. on pork carcasses was done using data from a previously published survey (Silva et al., 2012). Data from two slaughterhouses located in the largest pork producing region in Southern Brazil were used from that study. Briefly, 69 samples were obtained from pork carcasses (taken from the same pig) in each of the following slaughter steps: 1) after dehairing, 2) after singeing, 3) after evisceration, and 4) before chilling. A sponge was used to swab a 300 cm^2 area and then placed in 55 ml of buffered peptone water (BPW). For *Salmonella* isolation (screening test presence/absence in the sample), a 10 fold dilution (10 ml of the 55 in 90 ml) from the homogenized buffered peptone water was pre-enriched in BPW (37 °C overnight) and then transferred to Rappaport-Vassiliadis broth (RV; Merck) and tetrathionate broth (TT; Merck). After incubation at 42 °C for 24 h, a loop (10 μl) from each culture was streaked onto xylose lysine desoxycholate agar (XLD; Oxoid, Basingstoke, UK). For quantification, MPN was processed in parallel, independently of the screening test results. For this, homogenized aliquots (corresponding to 0.1, 1, and 10 ml of the original sample) were inoculated in triplicate into BPW and incubated at 37 °C for 18 h. The aliquots were transferred to RV (42 °C, 48 h) and then plated onto XLD (37 °C, 24 h). All the confirmed isolates were serotyped at the Brazilian *Salmonella* Reference Center (Fundação Instituto Oswaldo Cruz, Rio de Janeiro, Brazil).

The limit of detection (LOD) for the screening test, considering that 10 ml (volume of screen, v_{screen}) represents 54.55 cm^2, is 0.01833 CFU/cm^2 (−1.74 log CFU/cm^2) and the limit of quantification that includes volumes v_k (0.1, 1, 10) in three n_k tubes is 0.0055 CFU/cm^2 (−2.26 log CFU/cm^2). The LOD of the entire procedure, i.e., including both the screening test and MPN, is 0.0042 CFU/cm^2 (−2.37 log CFU/cm^2).

2.2. Distribution fitting and data analysis

Salmonella contamination data were fitted to a lognormal distribution by using a Bayesian model that employs the number of positive tubes at each dilution in an MPN analysis to estimate the parameters of the concentration distribution (Williams and Ebel, 2012). In the Bayesian model we assume that the average concentrations of bacteria in each sample i are lognormally distributed [$\lambda_i \sim \text{lognormal}(\mu; \sigma)$]. Since no information on *Salmonella* concentration in pork carcasses is available in Brazil, vague prior distribution that describes the concentration (λ) was assumed in the model: $(\mu \sim \text{normal}(-2.5,\ \text{var} = 10)$ and $^1/_{\sigma^2} \sim \text{gamma}(0.1, 0.1))$, as suggested by Williams and Ebel (2012). The influence of the prior for the data sets at prechill was checked based on data made in prechill carcasses reported by Duggan et al. (2010) and van Hoek et al. (2012) assuming a mean of $\mu = -2.0$ log CFU/cm^2 and $^1/_{\sigma^2} \sim \text{uniform}(0.05, 1)$.

The model assumes that MPN is applied in screen-test positive samples. The Bayesian OpenBUGS code contains two loops, one including three serial dilutions with three replicas each, corresponding to MPN made in screen-test positive samples and the other for the screen-test negative samples. The first loop estimates the posterior distribution for the λ_i in screen-test positive samples i that have followed enumeration via the MPN. The probability of observing x organisms at a dilution k for a sample i (S_{ik}) is obtained from a binomial $\left(n_{ik}, 1-e^{(-v_k\lambda_i)}\right)$ distribution, where n_{ik} is the number of tubes from sample i at a dilution k and v_k is the volume of dilution. The loop for screen-test negative samples, in which no MPN is performed, includes only the likelihood of observing growth in the screening test, which occurs with probability $1-e^{(-v_{screen}\lambda_i)}$ where v_{screen} is the volume taken for screen. The model provides posterior estimates $\hat{\mu}$ and $\hat{\sigma}$ for the lognormal distribution that describes the contamination across the population, as well as individual estimates of the concentration λ_i for each sample in the data set.

If the screen test and MPN are combined, four types of result can occur (Fig. 1): both positive (PP), positive screening and negative MPN (PN), negative screening and positive MPN (NP) and both negative (NN). A sample was considered positive if *Salmonella* was isolated in at least one method (screen test and/or MPN: PP, PN and NP). Screen-test false negative results correspond to samples with *Salmonella* enumeration that are negative in screening test (NP). The relative sensitivity of the screening test was obtained by assuming as true positive samples the ones in which *Salmonella* was confirmed by the reference laboratory by either screening and/or MPN (using software SAS version 9.2):

$$\text{Relative sensitivity} = (\text{PP} + \text{PN})/(\text{PP} + \text{PN} + \text{NP}).$$

Three sets of data were run in the model to assess estimates $\hat{\mu}$ and $\hat{\sigma}$ in the sample: 1) MPN_Pos (PP and PN), 2) MPN_FN (PP, PN and NP), and 3) MPN_All (PP, PN, NP and NN) (see Fig. 1). The original OpenBUGS code described above, using the data set MPN_Pos, was run considering performing MPN in screen-positive samples only, as it is normally done in microbiology surveys. Additionally, a second data set (MPN_FN) including MPN results from samples that were screen-test negative, but where *Salmonella* was subsequently detected in MPN (i.e., from screen test false negative), was also tested using the same code. For the third data set (MPN_All), the original code of Williams and Ebel (2012) was adapted to account for the MPN performed in all the pork carcass samples (screen-positive and screen-negative). In this model, the screen-negative loop was excluded from the OpenBUGS code and all samples were included. Results of the screening test were included in the MPN calculation as an additional dilution step with one replica, in all samples. Here, as well as in the original code, the screening test serves the same purpose as a single ($n_{i0} = 1$) additional "tube" in the MPN method with sample volume v_{screen} (Williams and Ebel, 2012). Appendix A provides the OpenBUGS code and an example data set for the model adapted used in the MPN_All.

Models were run separately for each of the four steps during slaughter and the mean value estimates of the posterior distribution of the parameters $\hat{\mu}$ and $\hat{\sigma}$ obtained in the MPN_Pos, MPN_FN, and MPN_All data sets were compared using ~N ($\hat{\mu}$; $\hat{\sigma}$). Results were expressed as log CFU/cm^2.

3. Results

A total of 276 swab samples were screened for *Salmonella* and enumerated by the MPN method, 69 samples in each slaughter step. Overall, *Salmonella* was confirmed in 29 samples either by screening or MPN method (Table 1). In nine samples *Salmonella* was detected only by the MPN technique; in seven of them, tube result triplets from these contaminated carcass were low (0–1–0, 1–0–0 or 0–0–1). The most common serotypes were Typhimurium and Derby, and seven isolates were nontypeable. The relative sensitivity of the screening test in this specific situation was 20/29 (69%, CI 95%: 52%–85%).

Posterior distributions of the parameters $\hat{\mu}$ and $\hat{\sigma}$ in the population differed between the three data sets (Table 2). Probability distributions of *Salmonella* concentrations ~N ($\hat{\mu}$; $\hat{\sigma}$) using the mean value for $\hat{\mu}$ and $\hat{\sigma}$ are shown in Fig. 2. A general trend of lower $\hat{\mu}$ and higher $\hat{\sigma}$ was found with the MPN_All, while the data set that also included enumerations from screen-negative samples (MPN_FN) tended to have higher $\hat{\mu}$ and smaller $\hat{\sigma}$ in comparison with the other two data sets for all the steps. The differences between MPN_FN and MPN_Pos data sets were more pronounced when the proportion of samples successfully enumerated