OPTIMISATION OF POOLED FAECAL SAMPLES FOR THE ISOLATION OF SALMONELLA FROM FINISHER PIGS IN GB.

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Abstract Pooled pen floor faecal sampling represents a simple and non-invasive method to measure Salmonella infection in pigs. We extended an existing model of the sensitivity of detection of Salmonella in individual samples to create a mathematical model of the sensitivity of pooled sampling. Parameters for the model were estimated using data obtained by collecting 50 faecal samples from 2 pig farms. Individual samples of 0.1, 0.5, 1, 10 and 25g were tested and pools of 5, 10 and 20 samples were created from these. For individual samples, the highest test sensitivity (90%) was found at 10g whilst the 25g test sensitivity was 83%. Sensitivity reduced with sample weight for samples of less than 10g. Incubation for 48 hours produced a more sensitive test than incubation for 24 hours. Model results indicated that for pooled samples, the more samples included in the pool the higher the expected mean pooled test sensitivity.

Introduction Surveys in GB have demonstrated that up to 23% of finisher pigs slaughtered at quality assured abattoirs may carry Salmonella in their caecal contents (Davies et al 2004). These represent a potential source of some human cases of salmonellosis and in response, the industry launched the Zoonoses Action Plan Salmonella Monitoring Programme (ZAP) in June 2002, which aims “to reduce the prevalence of Salmonella in assured pigs at slaughter by 25%” in 3 years (British Pig Executive, 2003). ZAP utilises a meat juice mix-ELISA (MJ ELISA) system to detect antibodies against Group B and C1 Salmonella (van der Heijden, 2001). Farms are assigned a ZAP score of 1 (<65% of samples positive), 2 (65% - 85% of samples positive) or 3 (>85% of samples positive) and those that receive a ZAP 2 or 3 score must act to reduce the prevalence of MJ ELISA positive pigs or face loss of their quality assured status (Armstrong, 2001).

The MJ ELISA measures life time exposure to Salmonella. However, in studies to measure the impact of intervention measures, we have found it more useful to use microbiological isolation to detect infection. We prefer to collect pooled pen samples because:
1. stress to the pig is minimised
2. Salmonella excretion is intermittent and a negative sample may be obtained from an infected individual pig;
3. there is a reduced cost, since sampling is quicker and farm staff can feasibly trained in sample collection for large scale studies.

There is little work in the literature on quantitative estimates of the sensitivity of pooled pen faecal samples for Salmonella in pigs. A study by Funk et al. (2000) examined the impact of sample weight on the sensitivity of the test for individual samples. Their results may not be applicable to GB since there are differences in the serovars of Salmonella encountered and secondly there are differences in the methods used for isolation – for example, Funk et al (2000) used a selective enrichment broth whereas we use buffered peptone water. Extrapolation of the results of single sample sensitivity into theoretical models of pooled sampling (e.g. Evers and Nauta, 2001) generally assumed that there was no effect of pooling on the sensitivity of the test. However, there may be unpredictable effects from mixing samples. Firstly, there is a dilution effect from combining Salmonella positive and Salmonella-free samples. Secondly, there may be various inhibitory factors in one or more of the individual samples that are combined in a pool that affect growth of Salmonella. These include the presence of other organisms that compete for nutrients and that may release metabolic products that inhibit growth of Salmonella, e.g. colicins (Harvey and Price, 1974). Moulds and yeasts may produce antibacterial substances and alcohols through fermentation. Copro-antibodies and cytokines secreted into the gut by some infected pigs may also be present at varying concentrations and some samples may contain bacteriophages. As the culture of a sample progresses the medium becomes progressively more acidic and this, too, inhibits Salmonella growth (Davies et al., 2000).

We produced a mathematical model of the sensitivity of pooled pen faecal samples for Salmonella in pigs by extending an individual sample model (Arnold et al 2005; Cannon and Nicholls, 2002). The model was used to investigate the optimal number of individual faeces to include in a pooled pen sample.
Materials and Methods The VLA procedure for pooled pen faecal samples comprised pre-enrichment in buffered peptone water (BPW) for 18 hours at 37°C and selective enrichment in Diasalm agar plates (Merck) at 41.5°C for 48 hours. Selected colonies are inoculated onto a Rambach agar plate (Merck) for 24 hours at 37°C. Suspect Salmonella colonies were subjected to a slide agglutination test using a range of typing sera and to the minimum phenotypic criteria for identification to Salmonella species (Davies et al., 2001). A subculture of each confirmed Salmonella isolate was submitted for full serotyping and phage typing, where applicable.

Our mathematical model was based on the following assumptions:

- Salmonella is clustered in faeces at the rate of C clusters/g,
- After mixing, Salmonella organisms are homogenously distributed and multiply
- The final concentration of Salmonella organisms in the BPW depends upon:
  1. The growth rate of the serovar
  2. The growth rate of other organisms
  3. The concentration of inhibitory substances, which is directly proportional to the sample weight
  4. The carrying capacity of the BPW
- To detect Salmonella, it must grow in the Diasalm-Rambach system, which depends on the ratio of the number of Salmonella clusters in the faecal sample and the sample weight (representing the inhibitory factors)
- In a pooled sample, an equal mass of faeces is collected from each pig
- The number of Salmonella clusters/g (C) is the same for all infected pigs, irrespective of serovar and faecal consistency
- In a pooled faecal sample C is directly proportional to the prevalence of infected faeces in the sample.
- The test sensitivity for a pooled faecal sample with \( \pi_{pool} \) positives out of \( n_{pool} \) samples, \( \theta \), is

\[
\theta(\pi_{pool}, n_{pool}) = 1 - \exp\left(-\frac{Cw\pi_{pool}}{n_{pool}}\left(1-e^{-\frac{n_{pool}}{w}}\right)\right) \quad \text{(Arnold et al., 2005)}
\]

In order to parameterise this formula, we conducted experiments to estimate C and to study pooling.

To estimate C, we collected 50 individual faecal samples from each of two farms (A and B). These were divided into 0.1, 0.5, 1, 10 and 25g samples, each of which was tested for Salmonella. The data were used to estimate C using a Markov chain monte carlo (MCMC) method in WinBUGS 3.1.

The same samples were used to create a set of pooled samples – 5x5g, 10x2.5g, 20x1.25g. Thirty-five replicates of each pooled set were produced from each of the two farms, giving a total of 210 sets. Samples were randomly allocated to each set without prior knowledge of the individual sample results. A binomial model was fitted to the pooled testing data using an MCMC method in Winbugs.

Results Of the 100 individual faecal samples cultured for detection of Salmonella at sample weights of 0.1, 0.5, 1, 10 and 25g, 44 were positive for at least one sample weight after 24 hours of incubation, and 48 were positive after 48 hours incubation. The increase in detected positives was largely due to the increased sensitivity of the 25g sample test, which increased from 35 positives after 24 hours incubation to 40 positives after 48 hours incubation. The corresponding number of 10g sample positives increased from 42 to 43. There was no observed increase in the number of detected positives for lower sample weights.

The pooling experiment gave the following results:

- 5x5g Pools There were 18 pools with no samples positive by any of the individual 48 hour tests. Four of these gave positive tests, indicating that some positive samples were not detected by the individual sample tests. Of the remaining 52 pools, 39 gave positive tests after 24 hours of incubation and 41 gave positive tests after 48 hours of incubation.

- 10x2.5g Pools One of 6 pools with no samples positive by any of the individual sample 48 hour incubation tests tested positive after 48 hours of incubation. Of the remaining 64 pools, 44 were positive after 24 hours of incubation and 49 were positive after 48 hours of incubation.
20x1.25g Pools Both pools with no samples positive by any of the individual tests after 48 hours of incubation tested negative. Of the remaining 68 pools, 45 were positive after 24 hours of incubation, and 55 were positive after 48 hours of incubation.

The median value of $C = 6.7$ (2.5 and 97.5 percentiles: 5.1-8.7). The expected test sensitivity of a 25g pooled pen sample from a pen of 50 pigs was estimated for the national prevalence of 25% (Davies et al. 2004). This resulted in a test sensitivity of approximately 70% with 20 samples in the pool. Results indicated an increase in test sensitivity as the number of individual faeces included in the pool is increased, especially for the first 5 individual faeces. This is due to the increased probability of capturing positive faeces in the pool as the number of individual faeces is increased.

Discussion The individual faecal test results showed a higher test sensitivity for the 10g sample than the 25g sample. The difference between the 25g and 10g sample sensitivity was significant after 24 hours incubation ($p=0.02$) but not after 48 hours ($p=0.63$). This was an unexpected finding and further experiments are needed to clarify whether this was a random event in this study or whether it is related to the procedures used. Other authors have reported a detrimental effect of increasing sample weight if a large number of competing microorganisms are present (Leifson, 1936; Harvey and Phillips, 1955). Inhibition of Salmonella growth in the 25g samples is also suggested by the higher rate of positives after 48 hour selective enrichment with these samples. This finding conflicts with the EU Reference method for Salmonella testing, ISO 6579:2002, which specifies a 25g sample and 24 hour selective enrichment.

Results from this pooling study indicate close to 100% sensitivity for pools with greater than 50% prevalence of positive faecal samples. This was higher than that observed for the individual 25g samples (approximately 80%). In this study we made the assumption that every sample contained an equal amount of Salmonella inhibiting factors. To our knowledge, very little research has been done on bacterial growth inhibiting properties in a faecal sample. If local immunological factors such as copro-antibodies were usually associated with infection in the animal, samples from infected pigs would contain more inhibiting factors than samples from a healthy pig. Other microorganisms may have adapted to the presence of Salmonella in infected pigs and thus be more competitive to Salmonella than organisms from non-infected pigs. If this is the case, diluting samples from infected pigs with samples from non-infected pigs may increase the possibility of isolating Salmonella. This may explain the higher sensitivity in pooled samples compared to individual samples but more work is needed to elucidate these speculations.

The most frequent serovars isolated from pigs in GB are S. Typhimurium and S. Derby. However, by chance in this study, the main serotypes were S. Reading (Farm A) and S. Enteritidis (Farm B). These serotypes have infrequently been identified in British pigs and S. Enteritidis is typically associated with poultry. We intend to conduct a further study during current fieldwork, selecting farms known to have a S. Typhimurium infection.

Conclusions Pooled faecal sampling represents a feasible and valid approach for measuring the prevalence of infected pens of pigs. In collecting such samples, the optimum method is to ensure the maximum number of individual faeces contribute to a total pool weight of 25g and this should then be cultured for 48 hours. Further work is needed to confirm these findings with common serovars. Research into inhibitory substances in faecal material would also be valuable.

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References


Arnold, ME; Cook, AJC & Davies, RH (2005) A modelling approach to the interpretation of pooled faecal samples for isolation of Salmonella from UK finisher pig farms In review


