Semi-quantitative assessment of the distribution of *Salmonella* in the environment of caged layer flocks

Andrew Wales, Mark Breslin, Robert Davies

Department of Food and Environmental Safety, Veterinary Laboratories Agency, Woodham Lane, New Haw, Addlestone, Surrey KT15 3NB, United Kingdom

Running title: Quantifying *Salmonella* in layer flocks

Author for correspondence: Robert Davies

Telephone: 01932 357361

Fax: 01932 357595

E-mail: r.h.davies@vla.defra.gsi.gov.uk

SUMMARY

Aims: To evaluate a semi-quantitative technique for the enumeration of *Salmonella* in the environment of layer flocks, and to compare findings with those of a standard qualitative technique.

Methods and Results: Samples were taken from faeces, floor dust, dust on cages, feeders and egg belts. After mixing with buffered peptone water, serial dilutions were prepared and culture was performed using pre-enrichment, then plating on semi-solid selective and solid isolation media. Comparison with a qualitative pre-enrichment technique indicated a similar sensitivity for both methods despite smaller sample sizes. The numbers of *Salmonella* detected for a site or sample type did not correlate closely with the prevalence of positive samples.

Conclusions: The sensitive detection and quantification of *Salmonella* in the flock environment is practicable with the technique described. Quantitative data in many cases does not correlate with qualitative findings.

Significance and Impact of Study: The significance of certain environmental factors and interventions in the maintenance and dissemination of *Salmonella* in poultry houses may be over- or under- represented by prevalence data alone. The technique described allows the issue of poultry house contamination to be examined from a new perspective.

Keywords

Salmonella, poultry, layers, eggs, chickens, environment

INTRODUCTION

Salmonella enterica serovar Enteritidis came to prominence as a major food-borne pathogen in Europe and America during the 1980s (Hogue et al. 1997; Baumler et al. 2000; Velge et al. 2005). By 1997 it was implicated in over 70% of cases of human salmonellosis in England and Wales (Cogan and Humphrey 2003) and, despite a recent decline in incidence, it is the serovar most commonly isolated from gastrointestinal infections in the UK (Anon 2005) and remains amongst the most significant *Salmonella* serovars in public health elsewhere, including North America (CDC 2004). Poultry products, especially undercooked and raw eggs, have been a major risk factor for human infection with *S*. Enteritidis (Coyle et al. 1988; St Louis et al. 1988; Hogue et al. 1997; Palmer et al. 2000; CDC 2004; De Buck et al. 2004). *S.* Enteritidis, particularly phage type 4, has a particular affinity with the reproductive tract of the chicken and is more able to cause long term colonisation of the reproductive tract and become deposited within egg contents than other invasive serovars (Berchieri et al. 2001; Okamura et al. 2001; Amy et al. 2004; Guard-Bouldin et al. 2004). External contamination of the shell and bacterial penetration of the egg is also a concern (Messens et al. 2005).

Improved biosecurity and hygiene in the UK poultry industry and vaccination of the majority of commercial laying birds and broiler breeders, introduced in the mid to late 1990s, has been followed by a large reduction in reported incidents of *S.* Enteritidis in poultry and in humans (Anon 2000). Similar improvements have also occurred in some other countries (Wegener et al. 2003; Marcus et al. 2004; Mumma et al. 2004) but there is still a significant reservoir of infection in commercial laying flocks (Adak and Gillespie 2004; Crespo et al. 2005). Current controls implemented in the United Kingdom on breeding flocks should ensure that commercial day old chicks are free from *S.* Enteritidis when placed on farm. Therefore, persistence of contamination on commercial laying farms is

currently considered to be the predominant problem (van de Giessen et al. 1994; Davies and Breslin 2003a; Gradel et al. 2004). It is thought that selection for productivity and the immunosuppressive effect of the onset of lay may make laying birds especially susceptible to *S.* Enteritidis (van Eerden et al. 2004; Wigley et al. 2005). Vaccination and other interventions such as feeding competitive exclusion products do not reliably eliminate infection (Davies and Breslin 2003b; Davies et al. 2003), and their effectiveness is reduced where there is a heavy environmental challenge (Davies and Breslin 2003a, 2003b).

Environmental sampling has been shown to be an accurate indicator of the presence of *Salmonella* in poultry flocks (Poppe et al. 1992; Altekruse et al. 1993; Davies and Breslin 2001; Kinde et al. 2004) and there is good agreement between the level of environmental contamination and the prevalence of caecal infection, the level of internal egg contamination, and associated human disease (Altekruse et al. 1993; Henzler et al. 1994; Henzler et al. 1998). Whilst there have been several surveys examining the prevalence of *Salmonella* contamination of poultry houses, only occasional studies (Davies et al. 1998; Mallinson et al. 2000) have attempted to assess the numbers of *Salmonella* in the flock environment. Such an approach has potential benefits in further clarifying the relative importance of sources and reservoirs of contamination, and of evaluating the success of interventions. The present report examines the sensitivity, accuracy and practicality of a semi-quantitative cultural technique in laboratory and field studies.

MATERIALS AND METHODS

Sample collection

Caged layer flocks that had previously been identified, through the Zoonoses Order Database or by personal contact, as having *S.* Enteritidis were approached. When permission for intensive sampling had been obtained, the flocks were visited and

environmental samples were taken. For standard qualitative isolation (SS), samples were taken directly into 225 ml of buffered peptone water (BPW; Merck 1.07228) using gauze surgical swabs (Robinson Healthcare). Samples consisted of approximately 25 g faecal material, floor spillage from under cages and dust from within and around cages (10 to 15 g), surface swabs, rodent droppings (1 to 10 g) and flies from adhesive paper or contact insecticidal traps (1 to 2 g). Sterile swabs soaked in BPW were used to sample the surfaces (0.5 m^2) of egg belts and feeder troughs. Equivalent sites were sampled for semi-quantitative culture (QS) on the same occasion, with bulked faeces (approx. 30 g) and dry environmental samples being collected into dry pots, and surface swabs from 0.1 m^2 deposited in 100 ml chilled BPW.

All solid samples were returned to the laboratory under ambient conditions on the day of collection and processed immediately. Swab samples taken into BPW were kept in a cold box at below 10 °C and also processed on return to the laboratory. Mouse carcasses were collected as available from one house and transported to the laboratory where the liver, spleen and intestines (2 to 3 g per mouse) were removed aseptically for culture.

Semi-quantitative culture (QS)

Experimental evaluation. S. Enteritidis PT4 from a commercial cage layer farm was grown overnight in Luria-Bertani nutrient broth. A 1 ml aliquot of the broth was added to 9 ml of BPW, and a decimal dilution series was prepared by successively repeating this step twelve times, adding 1 ml of each consequent dilution to 9 ml BPW. The growth end-point of the dilution series was determined by pre-enrichment of the remaining 9 ml of each dilution at 37 °C for 18 h and then sequential culture of a 0.2 ml aliquot on Diasalm (Merck 1.09803) and Rambach (Merck 1.07500) agars, as described previously (Davies and Breslin 2003a). Aliquots (1 ml) of each of six dilutions (10^6- to 10^{11}-fold inclusive) of the broth were mixed with 10 g chicken faeces. The faeces were semi-quantitatively cultured using a dilution

series, pre-enrichment and culture on selective agar, as described below for environmental samples. In addition, for each artificially inoculated faeces preparation, direct plating onto Rambach and XLT4 (Oxoid CM1061) agars of 10 µl aliquots from each element in the QS dilution series was performed prior to the pre-enrichment incubation.

Environmental and wildlife samples. Faeces (10 g) were mixed with 90 ml BPW and the mixture was vortex-mixed until thorough mixing and dispersal of the solids within the BPW was achieved. A 10 ml aliquot of this primary preparation was dispensed into a universal container, to serve as the first in a decimal dilution series which was continued by taking 1 ml from the primary preparation, mixing it thoroughly as above with 9 ml BPW and successively repeating this step five times, adding 1 ml of each consequent dilution to 9 ml BPW. Similarly, 10 g of other solid/dry samples were mixed with 90 ml BPW and dilution series in BPW were prepared. The BPW bathing the surface swabs was serially diluted also.

For all samples a pre-enrichment incubation at 37 °C for 18 h was performed on a series of preparations comprising: the primary mixture in BPW (‘0’), a separate 10 ml aliquot of the same (‘1’), plus the decimal dilutions (‘2’ to ‘7’). After incubation, 0.1 ml of each of preparations ‘0’ and ‘1’ were inoculated onto modified semi-solid Rappaport-Vassiliadis agar with 0.01 % novobiocin (MSRV; Difco 218681) and incubated at 41.5 °C for 16 to 24 h. Preparations ‘3’ to ‘7’ were refrigerated. Where spreading opaque growth was seen on MSRV, a 1 µl loop from the edge of the growth was inoculated onto Rambach agar. Rambach and associated MSRV plates were incubated at 41.5 °C for 16 to 24 h. The plates were examined and any MSRV plates on which the growth had spread widely, but which were negative for *Salmonella* on the Rambach plates, were re-plated onto Rambach.

If *Salmonella* was isolated from either of the preparations ‘0’ or ‘1’ from any sample, then each element of the dilution series ‘2’ to ’7’ was cultured using the MSRV/Rambach method.

The likely density of *Salmonella* in a sample was quantified in tenfold bands by reference to the QS ‘score’, this being 1+ the designated number of the most dilute pre-enrichment broth that yielded a positive result.

For solid samples, the calculated relationship was:

Salmonella density ($cfu.g^{-1}$) = $10^{(QS\ score-2)}$ to $10^{(QS\ score-1)}$

For swabs, the relationship was:

Salmonella density ($cfu.0.1m^{-2}$) = $10^{(QS\ score-1)}$ to $10^{QS\ score}$

Where there was no growth in any dilution the QS score and *Salmonella* count were taken to be zero.

Standard culture (SS)

Samples in BPW were pre-enriched at 37 °C for 18 h and then cultured on selective and isolation media as for the QS technique. Presumptive *Salmonella* isolates were confirmed by complete serotyping at the *Salmonella* reference laboratory at VLA Weybridge according to the Kaufmann-White Scheme (Popoff 2001).

RESULTS

Evaluation of semi-quantitative culture

The broth culture yielded detectable growth up to and including the 10^9-fold dilution, indicating an initial density of around 10^9 $cfu.ml^{-1}$. The numbers of *Salmonella* in the inoculated faeces derived from this, plus the semi-quantitative culture results, are presented in Table 1. The findings indicate that, under experimental conditions, the QS technique had

a detection limit in chicken faeces of 1 cfu.g^{-1} on a 10 g sample, and it provided a semi-quantitative estimate either within the expected tenfold band or the one above. Direct plating of the QS dilution series failed to detect *Salmonella* at the concentrations tested, up to 10^2 cfu. g^{-1}

Environmental and wildlife sampling

The standard versus semi-quantitative sampling and culture results are presented in Tables 2 and 3, and in Figure 1. A single phage type of *S.* Enteritidis (4, 6 or 21B) was detected in most flocks, the exception being a flock (AL2/7) where two non-Enteritidis serovars were found. The overall prevalence of *Salmonella* in environmental samples (SS technique) ranged widely, from 7.9 to 95.7 % per flock, with a mean of 49.8 %. A similar average value of 50.8 % was obtained with the QS technique. Amongst the 62 sites sampled using both SS and QS, the techniques disagreed in the detection of *Salmonella* at nine (Figure 1).

For any sample site or type, a comparison can be made between the percentage of samples positive for *Salmonella* by the QS technique, and the maximum and arithmetical mean QS scores. This paired data for all 86 sites or wildlife samples examined by the QS method is plotted in Figure 2. It can be seen that, for any site, there tended to be an upper limit for the mean QS score, which correlated with the percentage positive score at that site. However, the mean QS score was often substantially below this limit at any particular site. One sample type with an exceptionally high mean QS score relative to prevalence was flies from flock HM3/4 (O), with a mean of 5.2 from 7/11 positive bulked samples.

The mean and maximum QS scores from all sites are illustrated in Figure 3, grouped by sampling site and with the order determined by the mean score from faeces. The

majority of minimum QS scores were zero. It can be seen that there is no close relationship between mean and maximum scores. The highest scores (seven, indicating 10^6-10^7 cfu per 2 g sample) were obtained from mouse faeces, mouse tissues at post-mortem, and flies. Scores of seven were also obtained for some pooled poultry faeces but this related to larger (10 g) samples.

DISCUSSION

The present study examined a semi-quantitative culture technique in the laboratory and the field, as there is potential value in quantitative data about *Salmonella* contamination at individual sites within the environment of poultry flocks. It was also possible in many cases to take paired samples for a comparison between the semi-quantitative and standard qualitative methods.

Standard methods for the detection and enumeration of *Salmonella* in food are of less use when applied to environmental samples, due typically to low numbers of *Salmonella* being present against a background of high numbers of other environmental and enteric bacteria (Cason et al. 1997, 2000; Cox and Berrang 2000; Yamane et al. 2000; Jorgensen et al. 2002). Current sensitive methods of culture and identification of pathogenic serovars of *Salmonella* from the environment utilise semisolid selective enrichment agars, such as MSRV, plus an isolation medium such as Rambach agar to separate and identify the organisms (Voogt et al. 2001). A pre-enrichment step of culture in BPW broth is used to increase the sensitivity of the method, which forms the basis of the standard qualitative sampling (SS) method described above, although in some cases *Salmonella* may fail to achieve detectable numbers in pre-enrichment due to overwhelming initial competition or

because of *Salmonella* strains which do not compete and grow well in mixed non-selective culture.

Quantitative culture can involve direct plating of the sample, or a diluted preparation thereof, with enumeration of colonies or detection of the growth end-point in a dilution series. In the present study, this approach proved insensitive when applied to chicken faeces, and a most-probable-number (MPN) based approach (Anon 1979; Dufrenne et al. 2001) was considered to be more appropriate. A semi-quantitative modification of the SS method, whereby a *Salmonella* growth end-point was determined by selective culture of a series of dilutions after they had undergone an 18 h non-selective pre-enrichment culture, was found to be suitable. This approach has also proved useful for the enumeration of *Salmonella* in poultry carcase rinses (Whittemore 1993), in pig faeces (Jensen et al. 2003), and in the environment of broiler-breeder houses (Davies et al. 1998). The QS 'score' thus derived is a logarithmic value indicating the likely concentration of *Salmonella* in the sample within a tenfold band. For samples with high numbers of organisms the range of a 3 or 5 tube MPN test will be exceeded (Smeltzer et al. 1980; Rigby 1982), but the present technique covers an overall range of zero to 10^7 cfu.g^{-1} for solids and zero to 10^8 cfu.0.1m^{-2} for swabs.

Even MPN based approaches are likely to provide a significant underestimation of the number of organisms in the environment (Roussanov et al. 1996) because of clustering in microcolonies and sample variability (Andrews et al. 1983; Jetton et al. 1992). By contrast, in some instances the QS technique over-estimated the concentration of *Salmonella* in experimentally-inoculated chicken faeces in the present study, which used dilution-enrichment. Dilution-enrichment is considered to be more accurate than total viable counts because of the inhibitory effects of the highly selective agars needed for direct counts on faecal and environmental material and loss of low numbers of *Salmonella*

through overgrowth by competitor organisms if selective enrichment is not used (Harvey and Price 1974) for determination of the density of *Salmonella* in the inoculum broth. The reason(s) for this over-estimation, by around 1 log unit, are not clear, but a possible cause might be the inclusion in early dilution transfers of large clusters of bacteria associated with organic particles, which were then dispersed in subsequent dilution steps. Bacterial aggregation and sublethal damage is likely to be more marked in environmental samples than in experimentally inoculated faeces, therefore the findings of the inoculation experiment cannot be taken as a precise guide to the QS technique's sensitivity and accuracy in the field. However, comparisons between the results from similar environmental samples can be made with reasonable confidence.

Any dilution-enrichment method involves a considerable labour requirement and this has previously been reduced by miniaturisation of the process in microplates (Humbert et al. 1997) or non-cultural confirmation of *Salmonella* (Pumfrey and Nelson 1991), but these methods present more potential for cross-contamination, overgrowth or non-specific reactions. In the current study considerable economy of resource was achieved by subculturing only the two initial dilutions in any series onto MSRV, and then subculturing the rest of the dilution series only if either of these two yielded *Salmonella*.

Although identical samples were not used from sites cultured by both SS and QS techniques, the sensitivity of both methods appeared to be similar, with only 9/62 sites producing differing results. A possible sensitivity advantage shown by the SS technique (Figure 1) may be due to the larger sample volume (25 g vs. 10 g) of faeces used, as the majority of SS+/QS– sites were bulked faeces/droppings boards. Comparing the whole SS and QS data sets, a very similar overall percentage of positive samples was obtained with each technique. The findings support the view that where QS is used instead of SS, the sensitivity of detection is likely to be maintained.

The logarithmic nature of the QS score scale facilitates the representation and assessment of data with a wide range of values, such as is commonly encountered with bacterial counts. Arithmetical mean QS scores for sampling sites are potentially useful as they average out variation at a site and allow trends to be examined more easily. However, as the scores are a logarithmic transformation of the underlying bacterial counts, the process of averaging over-represents the contribution of low counts and under-represents that of higher counts. Therefore the 'average' QS score of a site where the scores vary substantially (i.e. most sites) cannot be used as an indicator of the 'average' density of *Salmonella*, which is likely to be much higher. The apparent lack of correlation between maximum and mean scores in Figure 3 is probably a reflection of this relationship, plus intermittent shedding by long-term carrier birds (Gama et al. 2003; van Immerseel et al. 2004). An alternative calculation, involving the transformation of the scores back into non-logarithmic counts before attempting to derive an average, suffers from the inaccuracy of having to choose an arbitrary figure within the tenfold range represented by each QS score to map the log data to. However, such a method would probably generate more realistic overall counts for a site, were that desirable.

The generation of semi-quantitative scores from multiple samples at each site allows a comparison to be made between the QS score and the prevalence of positive samples at each site. Such a comparison addresses the question of whether the semi-quantitative score adds significantly to the value of the qualitative data obtained, for if the qualitative prevalence data can be used to predict the semi-quantitative scores then there is limited value in performing a quantitative assessment. Examination of Figure 2 shows that where the prevalence of positive samples is high, the mean QS scores varied substantially, from one to five. At a low prevalence, the mean QS scores were low and less varied. Therefore, with the current data, the ability to predict mean QS score from prevalence at any site

declines as the prevalence rises. Comparison of the prevalence of positive samples with the maximum QS score at a site (Figure 2) shows little correlation between the values even at low prevalences; for example the series maximum score (seven) was recorded against a prevalence of 50 % whereas lower scores of between one and three were recorded against a prevalence of 100 %. This suggests that the proportion of positive samples from a flock environment is not accurately predictive of either the overall or the localised numbers of *Salmonella* present, except perhaps when there is a low prevalence. Further work with flocks with a low prevalence of *Salmonella* contamination would be needed to clarify this latter point.

When mean and maximum QS scores are compared between flocks and between sampling sites (Figure 3), it can be seen that there is a trend of higher mean and maximum scores at several locations for some flocks compared with others. Moreover, when *Salmonella* was detected the range of QS scores derived was substantial, with maximum scores in the faeces, dust and feed categories varying between flocks by up to five, indicating a 10^5-fold difference in bacterial counts. In general, levels of *Salmonella* in dust were lower than those in faeces despite a higher prevalence of positive samples. This supports the usefulness of dust as a screening sample for identification of *Salmonella* in laying houses (Gast et al. 2004).

The limited data from wildlife in the present study shows that a high (QS score seven) level of *Salmonella* was found in mouse faeces, flies and mouse carcases from all sample sets. Mouse faeces had the highest prevalence of positive samples but the highest mean QS scores were obtained from flies. The fly sample results from farm HM3/4 are notable because, despite 46% of 11 bulked samples being negative, the mean QS score (5.2) exceeded that of any other sample group in the study. This supports the view that flies can be carriers of relatively high concentrations of *Salmonella* (Mian et al. 2002), which

may be of particular significance in view of their movement within and between poultry houses, and of their established role in the transfer of pathogens (Olsen et al. 2000; Graczyk et al. 2001). Indeed, flies can be the only *Salmonella*-positive samples found in some flock environments (Davies and Breslin 2001). The role of mice in perpetuating *Salmonella* infection between flocks in cage-layer houses is well known (Garber et al. 2003).

Microbiological sampling of flocks and their environments for *Salmonella* has tended to concentrate on sensitive detection of the presence and distribution of the organism with, more recently, discriminatory typing of strains to facilitate analysis of spatial and temporal spread and persistence (Liebana et al. 2003). The actual number of organisms in samples has received less attention. The present study has demonstrated a sensitive and practical technique for the semi-quantitative enumeration of *Salmonella* in environmental samples, allowing meaningful comparison of levels of *Salmonella* between either solid or swab samples. The technique also provided similar data on the prevalence and distribution of the organisms when compared with a standard qualitative method. Furthermore, the data indicates that there is not a close relationship between the extent of environmental contamination and the actual numbers of *Salmonella* involved. This may yield further important insights into factors which determine the persistence and spread of this organism within and between poultry flocks.

ACKNOWLEDGEMENTS

This work was funded by Defra. The authors would like to thank farmers and their staff as well as VLA laboratory staff for their invaluable assistance with this study.

REFERENCES

Adak, G.K. and Gillespie, I.A. (2004) *Salmonella* epidemiology in the UK and Europe. In *Proceedings of Meeting 'Whither Salmonella', 9 December 2004.* London: Royal College of Physicians.

Altekruse, S., Koehler, J., Hickman-Brenner, F., Tauxe, R.V. and Ferris, K. (1993) A comparison of *Salmonella* Enteritidis phage types from egg-associated outbreaks and implicated laying flocks. *Epidemiol Infect* **110**, 17-22.

Amy, M., Velge, P., Senocq, D., Bottreau, E., Mompart, F. and Virlogeux-Payant, I. (2004) Identification of a new *Salmonella enterica* serovar Enteritidis locus involved in cell invasion and in the colonisation of chicks. *Res Microbiol* **155**, 543-552.

Andrews, W.H., Wilson, C.R. and Poelma, P.L. (1983) Improved *Salmonella* species recovery from nonfat dry milk pre-enriched under reduced rehydration. *J Food Sci* **48**, 1162-1165.

Anon (1979) *Methods for microbiological examination of food and animal feeding stuffs. Part 2.* BS 5763: Parts 2 and 3; ISO 4831-1978 and 4832-1978. UK: British Standards Institution.

Anon (2000) *Salmonella in Livestock Production 1999*. Ministry of Agriculture Fisheries and Food. Addlestone, Surrey: Veterinary Laboratories Agency.

Anon (2005) Trends in selected gastrointestinal infections - 2004. *The Communicable Disease Report Weekly* **15** (14), Health Protection Agency, UK. Available at http://www.hpa.org.uk/cdr/archives/2005/cdr1405.pdf (Accessed 13 July 2005).

Baumler, A.J., Hargis, B.M. and Tsolis, R.M. (2000) Tracing the origins of *Salmonella* outbreaks. *Science* **287**, 50-52.

Berchieri, A. Jr., Wigley, P., Page, K., Murphy, C.K. and Barrow, P.A. (2001) Further studies on vertical transmission and persistence of *Salmonella enterica* serovar Enteritidis phage type 4 in chickens. *Avian Pathol* **30**, 297-310.

Cason, J.A., Bailey, J.S., Stern, N.J., Whittemore, A.D. and Cox, N.A. (1997) Relationship between aerobic bacteria, salmonellae, and *Campylobacter* on broiler carcasses. *Poult Sci* **76**, 1037-1041.

Cason, J.A., Hinton, A. and Ingram, K.D. (2000) Coliform, *Escherichia coli*, and salmonellae concentrations in a multiple-tank, counterflow poultry scalder. *J Food Prot* **63**, 1184-1188.

CDC (2004) *Salmonella Surveillance: Annual Summary, 2003.* Atlanta, Georgia: Centers for Disease Control and Prevention, US Department of Health and Human Services.

Cogan, T.A. and Humphrey, T.J. (2003) The rise and fall of *Salmonella* Enteritidis in the UK. *J Appl Microbiol* **94**, 114-119.

Cox, N.A. and Berrang, M.E. (2000) Inadequacy of selective plating media in field detection of *Salmonella*. *J Appl Poult Res* **9**, 403-406.

Coyle, E.F., Palmer, S.R., Ribeiro, C.D., Jones, H.I., Howard, A.J., Ward, L. and Rowe, B. (1988) *Salmonella* Enteritidis phage type 4 infection: association with hen's eggs. *Lancet* **2**, 1295-1297.

Crespo, P.S., Hernandez, G., Echeita, A., Torres, A., Ordonez, P. and Aladuena, A. (2005) Surveillance of foodborne disease outbreaks associated with consumption of eggs and egg products: Spain, 2002 - 2003. *Eurosurveillance Weekly* **10**, 4-8. Available at http://www.eurosurveillance.org/ew/2005/050616.asp (Accessed 15 July 2005).

Davies, R. and Breslin, M. (2001) Environmental contamination and detection of *Salmonella enterica* serovar Enteritidis in laying flocks. *Vet Rec* **149**, 699-704.

Davies, R. and Breslin, M. (2003a) Observations on *Salmonella* contamination of commercial laying farms before and after cleaning and disinfection. *Vet Rec* **152**, 283-287.

Davies, R. and Breslin, M. (2003b) Effects of vaccination and other preventive methods for *Salmonella* Enteritidis on commercial laying chicken farms. *Vet Rec* **153**, 673-677.

Davies, R., Bedford, S. and Wray, C. (1998) A semi-quantitative study of the effectiveness of disinfection of broiler breeder houses contaminated with *Salmonella*. In *Proceedings of 4th World Congress on Foodborne Infections and Intoxications, 7-12 June 1998.* Berlin. pp.414-424.

Davies, R., Liebana, E. and Breslin, M. (2003) Investigation of the distribution and control of *Salmonella enterica* serovar Enteritidis PT6 in layer breeding and egg production. *Avian Pathol* **32**, 225-237.

De Buck, J., Van Immerseel, F., Haesebrouck, F. and Ducatelle, R. (2004) Colonization of the chicken reproductive tract and egg contamination by *Salmonella*. *J Appl Microbiol* **97**, 233-245.

Dufrenne, J., Ritmeester, W., Delfgou van Asch, E., van Leusden, F. and de Jonge, R. (2001) Quantification of the contamination of chicken and chicken products in the Netherlands with *Salmonella* and *Campylobacter*. *J Food Prot* **64**, 538-541.

Gama, N.M.S.Q., Berchieri, A. Jr. and Fernandes, S.A. (2003) Occurrence of *Salmonella* sp. in laying hens. *Rev Bras Cienc Avicola* **5**, 15-21.

Garber, L., Smeltzer, M., Fedorka-Cray, P., Ladely, S. and Ferris, K. (2003) *Salmonella* enterica serotype Enteritidis in table egg layer house environments and in mice in U.S. layer houses and associated risk factors. *Avian Dis* **47**, 134-142.

Gast, R.K., Mitchell, B.W. and Holt, P.S. (2004) Evaluation of culture media for detecting airborne *Salmonella enteritidis* collected with an electrostatic sampling device from the environment of experimentally infected laying hens. *Poult Sci* **83**, 1106-1111.

Graczyk, T.K., Knight, R., Gilman, R.H. and Cranfield, M.R. (2001) The role of non-biting flies in the epidemiology of human infectious diseases. *Microbes Infect* **3**, 231-235.

Gradel, K.O., Sayers, A.R. and Davies, R.H. (2004) Surface disinfection tests with *Salmonella* and a putative indicator bacterium, mimicking worst-case scenarios in poultry houses. *Poult Sci* **83**, 1636-1643.

Guard-Bouldin, J., Gast, R.K., Humphrey, T.J., Henzler, D.J., Morales, C. and Coles, K. (2004) Subpopulation characteristics of egg-contaminating *Salmonella enterica* serovar Enteritidis as defined by the lipopolysaccharide O chain. *Appl Environ Microbiol* **70**, 2756-2763.

Harvey, R.W.S. & Price, T.H. (1974) Isolation of Salmonellas, Public Health Laboratory Service, HMSO, London, 2-17

Henzler, D.J., Ebel, E., Sanders, J., Kradel, D. and Mason, J. (1994) *Salmonella* Enteritidis in eggs from commercial chicken layer flocks implicated in human outbreaks. *Avian Dis* **38**, 37-43.

Henzler, D.J., Kradel, D.C. and Sischo, W.M. (1998) Management and environmental risk factors for *Salmonella* Enteritidis contamination of eggs. *Am J Vet Res* **59**, 824-829.

Hogue, A., White, P., Guard-Petter, J., Schlosser, W., Gast, R., Ebel, E., Farrar, J., Gomez, T., Madden, J., Madison, M., Mcnamara, A.M., Morales, R., Parham, D., Sparling, P., Sutherlin, W. and Swerdlow, D. (1997) Epidemiology and control of egg-associated *Salmonella* Enteritidis in the United States of America. *Rev Sci Tech* **16**, 542-553.

Humbert, F.S., Salvat, G., Lalande, F. and Colin P. (1997) Miniaturized most probable number and enrichment serology technique for the enumeration of *Salmonella* spp. on poultry carcasses. *J Food Prot* **60**, 1306-1311.

Jensen, A.N., Sorensen, G., Baggesen, D.L., Bodker, R. and Hoorfar, J. (2003) Addition of novobiocin in pre-enrichment step can improve *Salmonella* culture protocol of modified semisolid Rappaport-Vassiliadis. *J Microbiol Methods* **55**, 249-255.

Jetton, J.P., Bilgili, S.F., Conner, D.E., Kotrola, J.S. and Reiber, M.A. (1992) Recovery of salmonellae from chilled broiler carcasses as affected by rinse media and enumeration method. *J Food Prot* **55**, 329-332.

Jorgensen, F., Bailey, R., Williams, S., Henderson, P., Wareing, D.R.A., Bolton, F.J., Frost, J.A., Ward, L. and Humphrey, T.J. (2002) Prevalence and numbers of *Salmonella* and *Campylobacter* spp. on raw, whole chickens in relation to sampling methods. *Int J Food Microbiol* **76**, 151-164.

Kinde, H., Castellan, D.M., Kass, P.H., Ardans, A., Cutler, G., Breitmeyer, R.E., Bell, D.D., Ernst, R.A., Kerr, D.C., Little, H.E., Willoughby, D., Riemann, H.P., Snowdon, J.A. and Kuney, D.R. (2004) The occurrence and distribution of *Salmonella enteritidis* and other serovars on California egg laying premises: a comparison of two sampling methods and two culturing techniques. *Avian Dis* **48**, 590-594.

Liebana, E., Garcia-Migura, L., Clouting, C., Clifton-Hadley, F.A., Breslin, M. and Davies, R.H. (2003) Molecular fingerprinting evidence of the contribution of wildlife vectors in the maintenance of *Salmonella* Enteritidis infection in layer farms. *J Appl Microbiol* **94**, 1024-1029.

Mallinson, E.T., De Rezende, C.E., Tablante, N.L., Carr, L.E. and Joseph, S.W. (2000) A management technique to identify prime locations of *Salmonella* contamination on broiler and layer farms. *J Appl Poult Res* **9**, 364-370.

Marcus, R., Rabatsky-Ehr, T., Mohle-Boetani, J.C., Farley, M., Medus, C., Shiferaw, B., Carter, M., Zansky, S., Kennedy, M., Van Gilder, T. and Hadler, J.L. (2004) Dramatic decrease in the incidence of *Salmonella* serotype Enteritidis infections in 5 FoodNet sites: 1996-1999. *Clin Infect Dis* **38** (Suppl 3), S135-141.

Messens, W., Grijspeerdt, K. and Herman, L. (2005) Eggshell penetration by *Salmonella*: a review. *Worlds Poult Sci J* **61**, 71-85.

Mian, L.S., Maag, H. and Tacal, J.V. (2002) Isolation of *Salmonella* from muscoid flies at commercial animal establishments in San Bernadino County, California. *J Vector Ecol* **27**, 82-85.

Mumma, G.A., Griffin, P.M., Meltzer, M.I., Braden, C.R. and Tauxe, R.V. (2004) Egg quality assurance programs and egg-associated *Salmonella* Enteritidis infections, United States. *Emerg Infect Dis* **10**, 1782-1789.

Okamura, M., Kamijima, Y., Miyamoto, T., Tani, H., Sasai, K. and Baba, E. (2001) Differences among six *Salmonella* serovars in abilities to colonize reproductive organs and to contaminate eggs in laying hens. *Avian Dis* **45**, 61-69.

Olsen, A.R. and Hammack, T.S. (2000) Isolation of *Salmonella* spp. from the housefly, *Musca domestica* L., and the dump fly, *Hydrotaea aenescens* (Wiedemann) (Diptera: Muscidae), at caged-layer houses. *J Food Prot* **63**, 958-960.

Palmer, S., Parry, S., Perry, D., Smith, R., Evans, M., Nehaul, L., Roberts, R., Walapu, M. and Wright, D. (2000) The role of outbreaks in developing food safety policy: population based surveillance of *Salmonella* outbreaks in Wales 1986-98. *Epidemiol Infect* **125**, 467-472.

Popoff, M.Y. (2001) *Antigenic formulas of the Salmonella serovars,* WHO Collaborating Centre for Reference and Research on *Salmonella.* Paris: Institut Pasteur.

Poppe, C., Johnson, R.P., Forsberg, C.M. and Irwin, R.J. (1992) *Salmonella* Enteritidis and other *Salmonella* in laying hens and eggs from flocks with *Salmonella* in their environment. *Can J Vet Res* **56**, 226-232.

Pumfrey, L. and Nelson, C.E. (1991) Use of a most probable number method modified with a deoxyribonucleic acid probe to monitor control by food preservatives of natural *Salmonella* contamination in animal meat meals. *Poult Sci* **70**, 780-784.

Rigby, C.E. (1982) Most probable number cultures for assessing *Salmonella* contamination of eviscerated broiler carcasses. *Can J Comp Med* **46**, 279-282.

Roussanov, B., Hawkins, D.M. and Tatini, S.R. (1996) Estimating bacterial density from tube dilution data by a Bayesian method. *Food Microbiol* **13**, 341-363.

Smeltzer, T., Thomas, R. and Collins, G. (1980) Salmonellae on posts, hand-rails and hands in a beef abattoir. *Aust Vet J* **56**, 184-186.

St Louis, M.E., Morse, D.L., Potter, M.E., Demelfi, T.M., Guzewich, J.J., Tauxe, R.V. and Blake, P.A. (1988) The emergence of grade A eggs as a major source of *Salmonella* Enteritidis infections. New implications for the control of salmonellosis. *Jama* **259**, 2103-2107.

Van Eerden, E., Van Den Brand, H., Parmentier, H.K., De Jong, M.C.M. and Kemp, B. (2004) Phenotypic selection for residual feed intake and its effect on humoral immune responses in growing layer hens. *Poult Sci* **83**, 1602-1609.

Van De Giessen, A.W., Ament, A.J. and Notermans, S.H. (1994) Intervention strategies for *Salmonella* Enteritidis in poultry flocks: a basic approach. *Int J Food Microbiol* **21**, 145-154.

Van Immerseel, F., De Buck, J., Pasmans, F., Bohez, L., Boyen, F., Haesebrouck, F. and Ducatelle, R. (2004) Intermittent long-term shedding and induction of carrier birds after

infection of chickens early posthatch with a low or high dose of *Salmonella* Enteritidis. *Poult Sci* **83**, 1911-1916.

Velge, P., Cloeckaert, A. and Barrow, P. (2005) Emergence of *Salmonella* epidemics: The problems related to *Salmonella enterica* serotype Enteritidis and multiple antibiotic resistance in other major serotypes. *Vet Res* **36**, 267-288.

Voogt, N., Raes, M., Wannet, W.J., Henken, A.M. and Van De Giessen, A.W. (2001) Comparison of selective enrichment media for the detection of *Salmonella* in poultry faeces. *Lett Appl Microbiol* **32**, 89-92.

Wegener, H.C., Hald, T., Lo Fo Wong, D., Madsen, M., Korsgaard, H., Bager, F., Gerner-Smidt, P. and Molbak, K. (2003) *Salmonella* control programs in Denmark. *Emerg Infect Dis* **9**, 774-80

Whittemore, A.D. (1993) Research note: a modified most probable number technique to enumerate total aerobes, enterobacteriaceae, and *Salmonella* on poultry carcasses after the whole carcass rinse procedure. *Poult Sci* **72**, 2353-2357.

Wigley, P., Hulme, S.D., Powers, C., Beal, R.K., Berchieri, A. Jr., Smith, A.and Barrow, P. (2005) Infection of the reproductive tract and eggs with *Salmonella enterica* serovar Pullorum in the chicken is associated with suppression of cellular immunity at sexual maturity. *Infect Immun* **73**, 2986-2990.

Yamane, Y., Leonard, J.D., Kobatake, R., Awamura, N., Toyota, Y., Ohta, H., Otsuki, K. and Inoue, T. (2000) A case study on *Salmonella* enteritidis (SE) origin at three egg-laying farms and its control with a *S.* enteritidis bacterin. *Avian Dis* **44**, 519-526.

TABLES

Table 1: Results of semi-quantitative culture of chicken faeces artificially inoculated with *Salmonella* Enteritidis

Salmonella in 10 g artificially inoculated faeces (cfu)	Values derived by QS technique in 3 replicates (log cfu/sample)	Detection by direct plating of dilutions before pre-enrichment
10^3	3-4, 3-4, 4-5	No
10^2	2-3, 2-3, 2-3	No
10^1	1-2, 2-3, 2-3	No
10^0	0-1, 0-1, 0-1	No
10^{-1}	No isolation	No

Table 2: Results of standard qualitative versus semi-quantitative environmental sampling and culture for *Salmonella* in layer houses

Farm / Flock (label*)		Bulked faeces / Droppings boards		Floor spillage under cages / corridors		Feeders		Egg belts		Dust/cages		**Totals for SS (%)**
		SS	QS	SS	QS	SS	QS	SS	QS	SS	QS	
AL2/7	(A)	1/25 e	0/5	4/18 de	1/5	0/5	0/5	0/5	0/5	0/10	0/5	**5/63 (7.9) de**
CK9/2	(B)	4/12 b	0/5	0/24	1/5	NS	0/5	NS	0/5	3/24 b	1/5	**7/60 (12.7) b**
CK11/3	(C)	6/10 b	3/10	3/5 b	NS	NS	0/5	NS	1/5	6/10 b	6/10	**15/25 (60.0) b**
CK14/4	(D)	9/10 c	4/5	5/10 c	3/5	3/4 c	3/5	2/5 c	1/5	3/6 c	3/5	**22/35 (62.9) c**
CC2/11	(E)	8/10 a	4/10	4/6 a	2/5	9/10 a	2/5	8/10 a	4/4	4/4 a	5/5	**33/40 (82.5) a**
CC3/3	(F)	9/9 b	5/10	9/9 b	5/5	6/6 b	3/5	4/6 b	3/5	5/5 b	4/5	**33/35 (94.3) b**
CT4/3	(G)	1/11 b	0/5	5/14 b	0/5	NS	0/5	NS	0/5	3/14 b	2/5	**9/39 (23.1) b**
CT5/4	(H)	6/10 b	5/10	7/10 b	7/9	NS	2/5	NS	4/5	4/10 b	1/1	**17/30 (56.7) b**
CT6/2	(I)	6/7 b	3/5	4/7 b	2/5	NS	2/5	NS	3/5	2/7 b	3/5	**12/21 (57.1) b**
CT6/5	(J)	7/7 b	4/5	7/7 b	5/5	NS	3/5	NS	4/5	3/7 b	5/5	**17/21 (80.1) b**
CT9/8	(K)	4/20 b	1/5	10/10 b	5/5	NS	NS	NS	4/5	10/15 b	5/5	**24/45 (53.3) b**
CT8/1	(L)	1/6 b	0/3	5/6 b	3/3	NS	1/2	3/6 b	2/2	2/6 b	1/2	**11/24 (45.8) b**
HM1/2	(M)	5/6 a	5/5	12/12 a	5/5	NS	4/5	NS	4/5	5/5 a	5/5	**22/23 (95.7) a**
HM2/6	(N)	5/6 a	5/5	NS	5/5	4/5 a	2/5	3/6 a	3/5	NS	5/5	**12/17 (70.6) a**
HM3/4	(O)	16/24 a	5/5	8/12 a	4/5	7/10 a	3/5	8/10 a	5/5	8/10 a	5/5	**47/66 (71.2) a**
HM7/3	(P)	3/10 a	0/5	8/10 a	4/5	0/6	0/5	0/6	0/5	NS	4/5	**11/32 (34.4) a**
SG7/C	(R)	1/6 a	0/10	1/6 a	0/5	NS	NS	0/6	0/5	0/6	NS	**2/24 (8.3) a**
Totals		**92/189**	**44/108**	**92/166**	**52/82**	**29/46**	**25/72**	**28/60**	**38/81**	**58/139**	**55/78**	**299/600 (49.8)**

SS - standard qualitative sampling; **QS** semi-quantitative sampling; NS - not sampled. *Label for Figure 3
Salmonella serotypes: a - *S.* Enteritidis (SE) PT4; b - SE PT6; c - SE PT21B; d - *S.* Agama; e - *S.* Mbandaka

Table 3: Qualitative (SS) and Semi-quantitative (QS) data from wildlife samples

Farm / Flock (label)		Mouse faeces		Mouse tissues	Rat faeces	Flies	
		SS	QS	QS	SS	SS	QS
AL2/7	(A)	0/2					
CK9/2	(B)	0/1			1/1		
CK14/4	(D)		1/1 (2; 2.0)				
CT4/3	(G)				5/5		
CT6/2	(I)				2/2		
CT6/5	(J)				2/2		
CT8/1	(L)						1/1 (6; 6.0)
HM2/6	(N)		17/17 (1-7; 2.6)	6/32 (0-7; 0.6)		0/3	
HM3/4	(O)	2/2					7/11 (0-7; 5.2)
Totals		**2/5**	**18/18**	**6/32**	**10/10**	**0/3**	**8/12**

Values are: number of positive samples/total number of samples at each site.
Numbers in parentheses are the range and arithmetical mean QS scores at that site. Conversion of individual QS scores to *Salmonella* per bulked (1 to 10 g) sample is as follows: '0'- 0, '1'- 1 to 10, '2' - 10 to 10^2, '3' - 10^2 to 10^3, '4' - 10^3 to 10^4, '5' - 10^4 to 10^5, '6' - 10^5 to 10^6, '7' - 10^6 to 10^7.

FIGURES

Figure 1: Detection of *Salmonella* by standard (SS) and semi-quantitative (QS) methods in matched environmental sites

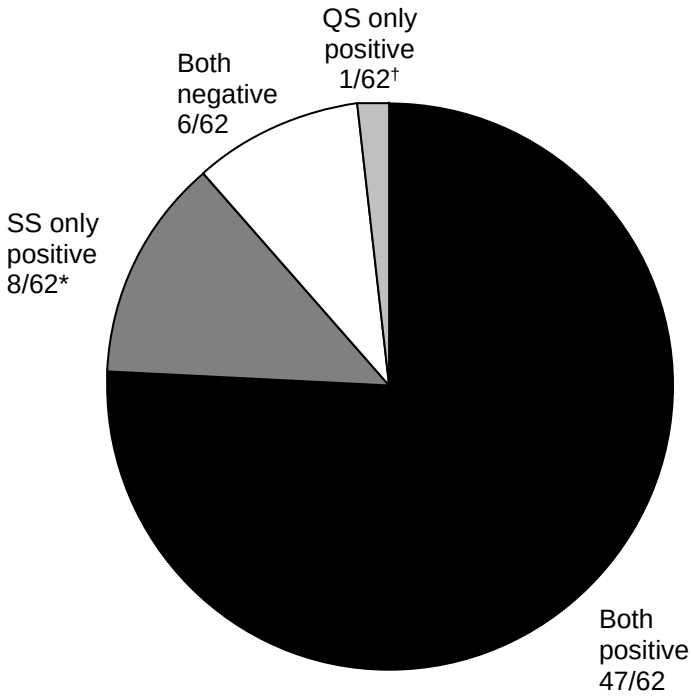


Figure 2: Percentage of *Salmonella*-positive samples versus mean and maximum quantitative score for each of 86 sites or wildlife samples examined with the semi-quantitative method

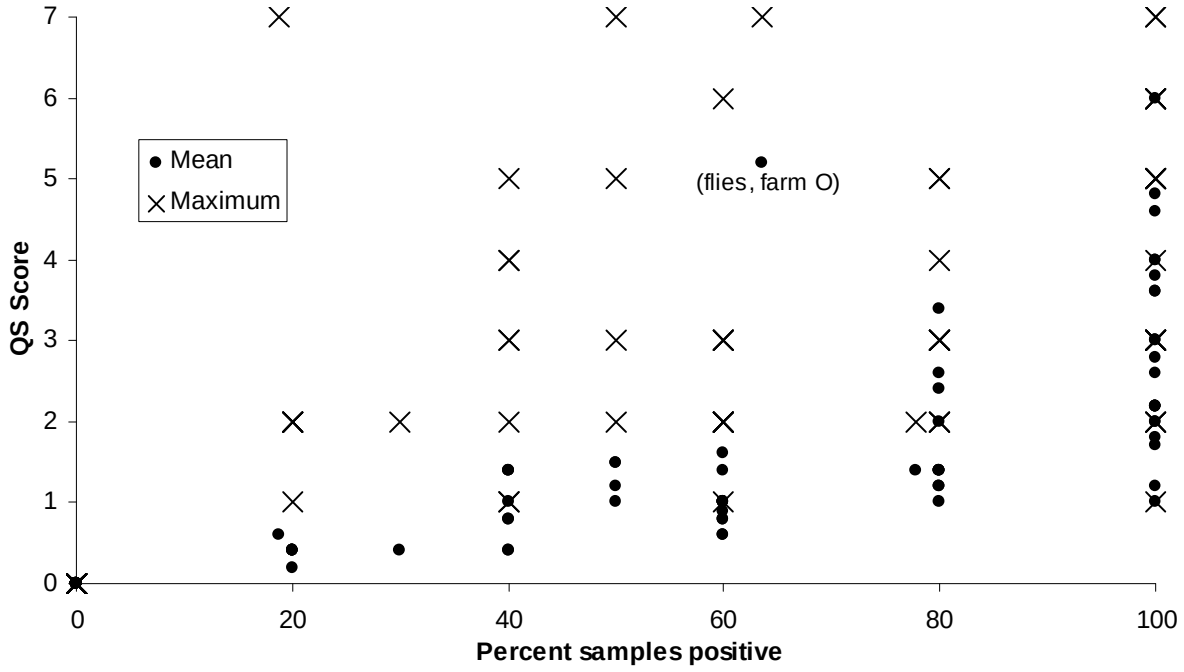
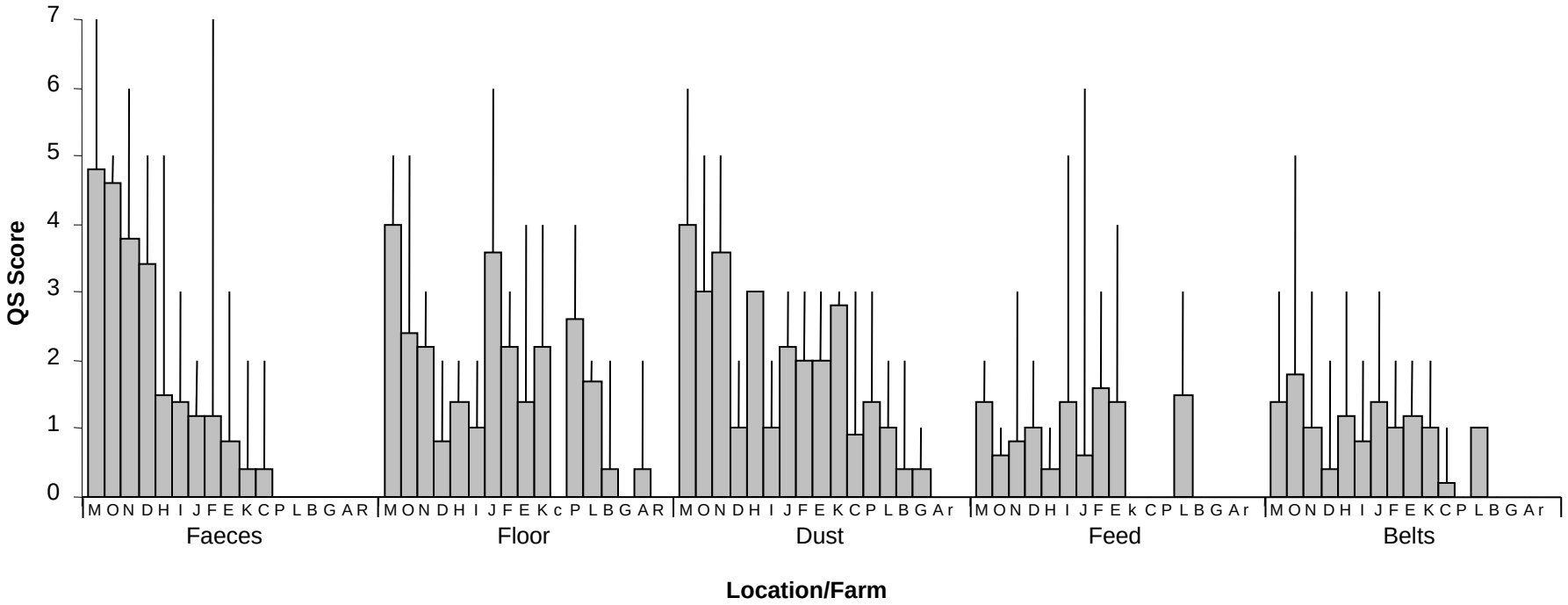


Figure 3: Mean and maximum quantitative scores by location and farm



LEGENDS TO FIGURES

Figure 1
* 6/8 faeces, 2/8 floor spillage. † floor spillage site.

Figure 2
Note: there is considerable superimposition of data points.

Figure 3
Columns indicate arithmetical mean QS scores. Lines indicate maximum QS score.
For solids (faeces, floor spillage, dust), QS score conversions to *Salmonella* per gram are: '0'- 0, '1' - <1, '2' - 1 to 10, '3' - 10 to 10^2, '4' - 10^2 to 10^3, '5' - 10^3 to 10^4, '6' - 10^4 to 10^5, '7' - 10^5 to 10^6.
For swabs (feeders and egg belts), conversions to *Salmonella* per $0.1m^2$ are: '0'- 0, '1'- 1 to 10, '2' - 10 to 10^2, '3' - 10^2 to 10^3, '4' - 10^3 to 10^4, '5' - 10^4 to 10^5, '6' - 10^5 to 10^6.
For key to farm identifier letters (A to R), see Table 2. Lower-case letters indicate there is no data available from that farm at that location.