A quantitative comparison of sample matrices for the detection of *Campylobacter* spp. in broiler houses

Received for publication, August 10, 2014
Accepted September 20, 2014

ROBERT H. MADDEN a, HYWEL BALL b, MIKE HUTCHISON c, FIONA YOUNG, a AND MALCOLM TAYLOR a
Food Microbiology Branch, AFBI, Newforge Lane, Belfast BT9 5PX, Northern Ireland a; Veterinary Sciences Division, AFBI, Stoney Road, Belfast BT4 3SD, Northern Ireland b; and School of Veterinary Sciences, Bristol University Langford, BS40 5DU, UK c.
Tel.: +44 (0)2890 255312; Fax: +44(0) 2890 255458; Email: Bob.madden@afbini.gov.uk
Corresponding author email: Bob.madden@afbini.gov.uk
Phone 44 (0)2890 255312
Fax 44(0) 2890 255458

Abstract
Most retail chicken in Europe, and in many other parts of the world, is contaminated with *Campylobacter* spp., a major cause of food poisoning. There is therefore a need to detect this pathogen on broiler farms in order to verify the efficacy of control measures which have been implemented, and determine which flocks are infected. In this study eight potential matrices from broiler houses were studied; bootswabs, cecal droppings, ceca, cloacal swab, dust, feces, litter, and ventral swab. They were compared in a quantitative manner to determine which was best for the detection of *Campylobacter*, using samples collected from commercial broiler houses. *Campylobacter* spp. recovered by the matrices were enumerated using ISO 10272-1:2006. Bootswabs consisting of plastic overshoes (Tunika) recovered the highest number of *Campylobacter* (p < 0.05) and showed the highest prevalence. When bootswabs were stored chilled no loss of *Campylobacter* viability was seen for up to 48h allowing for flexibility in sampling, and sample handling, since immediate analysis was not required. However, overgrowth of the selective medium, mCCDA, by non-campylobacters was seen if samples were stored at 22°C. Therefore transport of bootswabs cannot be undertaken at ambient temperature. Overall, in terms of *Campylobacter* recovery, ease of use and cost, bootswabs were the preferred sample matrix.

Keywords: *Campylobacter*, broiler, farm, sampling, bootswabs, enumeration.

1. Introduction
*Campylobacter* are the principal causes of gastro-enteritis world-wide (4, 19) with raw chicken an important source of *Campylobacter* spp. (7,20). Approximately 80% (3), to over 90% (9, 16, 17) of UK retail chicken carries *Campylobacter*. The economic costs of campylobacteriosis caused by broiler meat are estimated to be of up to €750 million in the European Community (6).

A Europe-wide study (5) found that 86% of broiler carcasses in UK abattoirs carried campylobacters. In order to reduce the load at slaughter, the prevalence of campylobacters on broiler farms must be decreased. In Denmark, intervention measures to achieve this aim have been proposed (22), with monitoring of broilers for *Campylobacter* initially undertaken at abattoirs, but subsequently extended to broiler houses (11, 23).

Historically, microbiological sampling of broiler houses was mainly developed to detect salmonellas, using qualitative procedures (8, 15). Qualitative detection of campylobacters on farms has been developed based on these sampling methods (10, 11, 21), with no single sampling methodology being universally applied. Accordingly, this study aimed to compare...
eight sample matrices to determine which one gave the highest recoveries of Campylobacter in broiler houses.

2. Material and Methods

Broiler farms studied

A major chicken processor supported the project by allowing access to broiler houses, and providing staff to assist during farm visits. Farms to be sampled were identified by company staff and took place in previously thinned houses, which would shortly be subjected to final clearance. For biosecurity reasons only one farm was visited on each sampling trip. Sampling staff adhered to company biosecurity measures at all times. Once inside the house to be sampled staff notionally divided the space into four equal quadrants, based on the distribution of the normal equipment present. Lengthwise, the lines of drinkers and feeders present provided lanes, along which samples could be taken. The house could be divided in two lengthwise based on the positioning of air ducts or lighting units, which were usually evenly spaced. Hence each house was divided into four approximately equal quadrants for sampling purposes. Officers prepared sketch plans of each house sampled, for subsequent reference.

Evaluation of sample matrices

Eight matrices were initially studied and samples, as described below, taken in the four quadrants of a broiler house were placed in pre-labeled bags. On leaving the house samples were stored in a coolbox with ice packs prior to returning to the laboratory within two hours. Samplers wore a fresh pair of disposable gloves for each individual sampling exercise. The sampling procedures used were:

Bootswabs. These were Tunika overshoes (Bowden and Knights, Thetford, UK). Firstly, the sampler put on a fresh pair of disposable Tyvek overshoes (Arco, Hull, UK) to prevent their footwear from contaminating the bootswabs. The Tunika bootswabs were then put on over the clean overshoes and the sampler walked down the designated quadrant in one lane and returned by another. The boot swabs were then placed in Seward closure bags (BA6041/CLR, Seward Ltd, Worthing, UK), 10 cm³ of maximum recovery diluent (MRD, CM733; Oxoid, Basingstoke, UK) added, and the bag sealed.

Fecal samples. The sampler walked the length of a quadrant in one lane and collected 5 ‘pinches’ of fecal material in a sealable bag, then returned by a second lane repeating the collection procedure. Litter samples. These were collected using the same procedures as the fecal samples.

Ceca. A company representative walked through the house and selected four birds meeting the normal criteria for culling. These were killed and placed in individual large plastic bags, and transported to the laboratory in insulated coolbox containers with ice packs. Once in the laboratory the birds were dissected and the ceca placed in Seward closure bags, and stored refrigerated until required. This is the only sample type that was not collected from the designated quadrants.

Ventral swab. Four birds per quadrant were sampled using pre-moistened (MRD) standard cellulose carcass sampling swabs (TS/15-B:PSD. TSC, Heywood, UK). A bird was caught, inverted to expose the ventral area which was subjected to five ‘wipes’ using one side of a fresh cellulose sponge. This sponge was then used to sample a further three birds, using each side twice. The swab was placed in a Seward closure bag which was sealed and stored.

Cloacal swab. After the ventral area of a bird had been swabbed a company staff member inserted a transport swab (Amies charcoal, TS/5-10. TSC, Heywood, UK) into the cloaca and turned it. The swab was then returned to its tube. After four birds had been sampled, each with a fresh swab, the swabs were placed in a labeled bag which was then sealed and stored.
Cecal droppings. These were differentiated from normal feces by their characteristic appearance; dark brown and glossy. Four individual droppings per quadrant were sampled by sweeping a transport swab through a fresh dropping, using a fresh swab for each of the four droppings.

Dust. Cellulose carcass sampling swabs, as used for the ventral swab, were used to swab the upper surfaces of feeder and water lines. One side of the swab was used to wipe the chosen line whilst walking down a quadrant, and a different line was swabbed using the other surface of the swab on the return walk.

**Microbiological analysis.**

All samples were blended using a Seward 400 stomacher (Seward Ltd, Worthing, UK) for 1 min. Samples were prepared for the enumeration of *Campylobacter* as follows:

**Bootswabs:** MRD (90 cm$^3$) added and sample blended.

**Fecal samples:** Pooled fecal samples were thoroughly mixed, and 10 g of the feces was added to 90 cm$^3$ MRD and blended.

**Litter:** This was again thoroughly mixed, and 10 g was added to 90 cm$^3$ MRD. To prevent sample loss from punctured bags the sample was hand shaken for 15 s.

**Ceca:** These were weighed, then MRD added to give 1:9 (w/w) dilution and ceca manually disrupted by hand squeezing, before being blended.

For ventral and dust swabs, MRD (90 cm$^3$) was added and the sample was blended.

**Cloacal swabs** and swabs of cecal droppings: sets of four swabs were added to 10 cm$^3$ MRD in a sterile plastic universal and vortex mixed for 10 s.

**Enumeration of Campylobacter.** The sample suspensions prepared as above were utilized as described in ISO EN 10272-1:2006 Microbiology of food and animal feeding stuffs-Horizontal method for detection and enumeration of *Campylobacter* spp.. Briefly, the suspensions were used to prepare decimal dilution series in MRD. Each dilution was plated out, in duplicate, with 0.1 cm$^3$ being applied to modified charcoal cefoperazone deoxycholate agar (mCCDA; Oxoid CM0739 plus SR0155). Plates were incubated at 41.5°C in a microaerobic atmosphere (85% N$_2$, 10% CO$_2$ and 5% O$_2$, all v/v) in a Don Whitley MACS workstation (Don Whitley Scientific, Shipley, UK). Typical colonies were enumerated and confirmed by determination of cell morphology, characteristic motility in a hanging drop, oxidase reaction, aerobic growth at 41.5°, and microaerophilic growth at 25°C.

**Bootswab storage**

To assess the effect of storage of bootswabs on the recovery of campylobacters three farms were visited and a total of ten houses sampled, using the Tunika bootswab sampling procedures described above. In each house the sampler walked the full length of the house and returned. This conducted seven times, wearing a fresh pair of bootswabs and in a different lane each time. Previous work had shown that such replicate samples did not differ significantly, p < 0.05, in terms of *Campylobacter* enumeration. On return to the lab each pair of bootswabs was assigned a random number, based on which they were allocated to one of seven treatments. One pair was immediately subjected to *Campylobacter* enumeration whilst three pairs were placed in a chill (3°C) and three in an incubator (22°C). One pair of bootswabs from the chill, and one from the incubator, was subjected to *Campylobacter* enumeration after 1, 2 and 4 d. sampling was replicated in 10 different houses.

**Statistical analysis**

Prior to analysis the *Campylobacter* counts for each of the matrix samples were transformed by taking a logarithm to base ten for each sample measured. Variation in the measured count was attributable to either the farm, house or quadrant from which the sample was taken. Additionally for the ceca samples this could be split into farm, house and bird.
In order to ascertain the percentage variation attributable to each of the factors listed above a REML variance components model was fitted to each count in turn. For the boot swab, feces and dropping counts, quadrant nested within house nested within farm was fitted as the random term in the model. A similar model was fitted for the ceca counts with bird replacing quadrant in this case. Individual variance components for the factors fitted to each count were saved for each variable and used to calculate the percentage variation for each factor in the model in question.

As the method of analysis carried out above requires that each variable being analyzed follows a normal distribution, this was assessed using the Anderson-Darling, Cramer-von Mises and the Watson statistic in every case.

All analyses were carried out using GenStat for Windows Release 14.1. For all analyses, a value of \( p < 0.05 \) was considered significant.

3. Results and Discussions

One farm was visited and samples of the eight matrix types taken in two houses, in four quadrants. Mean \( \text{Campylobacter} \) counts (\( \log_{10} \text{cfu} \)) for cloacal swab, litter, ventral swab, and dust were 6.40, 4.92, 3.37 and 2.78 respectively. Counts were per gram for litter samples, and per swab for the other three samples. Only three dust samples yielded campylobacters. Campylobacters were enumerated from all of the other matrices. No further sampling based on cloacal swabs, litter, ventral swabs, or dust was undertaken. A further eight farms were then visited, and the remaining four matrices were sampled in twenty three houses, in each of four quadrants, to give an overall total of 100 samples per matrix. Compared with the other matrices, significantly higher numbers of \( \text{Campylobacter} \) were recovered from boot swabs, \( p<0.05 \). In addition, boot swabs had the highest number of positive samples, Table 1.

To investigate the persistence of \( \text{Campylobacter} \) on boot swabs samples were stored in a chill, and at 22°C to mimic ambient temperature. For samples stored at 3°C the mean \( \text{Campylobacter} \) counts (\( \log_{10} \text{cfu/sample} \)) after 0, 1, 2 and 4 d were 7.31 ±0.67, 7.06 ±0.67, 7.29 ±0.56 and 6.52 ±0.62, respectively. Each result is the mean of 10 samples. Results are not presented for the samples stored at 22°C as considerable overgrowth by non-campylobacters was observed on some of the mCCDA plates, which prevented enumeration of \( \text{Campylobacter} \) colonies; after 1 d three samples could not be enumerated, after 2 d seven, and after 4 d nine samples could not be counted.

This study aimed to compare eight matrices to determine which would recover the highest numbers of \( \text{Campylobacter} \) from broiler houses containing infected birds. The lowest recoveries were from cloacal swabs, litter, ventral swabs, and dust. Since \( \text{Campylobacter} \) are highly susceptible to desiccation (2,13) the low recoveries from dust are not surprising. Poor recoveries of \( \text{Campylobacter} \) from litter were previously reported (1), supporting the decision to discontinue work with this matrix.

Cloacal swabs have been used for the qualitative detection of campylobacters in broilers (12, 14), but with the involvement of veterinarians. Routine sampling with cloacal swabs would require staff training, and could cause stress to the birds. However, similar numbers of campylobacters were recovered from fecal samples (Table 1), which were much easier to collect, therefore sampling with cloacal swabs was discontinued. Ventral swabbing was evaluated as a simpler, non-invasive alternative to cloacal swabs, but recovered approximately 1000-fold fewer campylobacters per sample and hence sampling was discontinued.
Table 1

<table>
<thead>
<tr>
<th>Sample matrix</th>
<th>Campylobacter $\log_{10}$ (cfu)</th>
<th>Standard deviation</th>
<th>Positive samples (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bootswab</td>
<td>7.61$^a$</td>
<td>0.69</td>
<td>95</td>
</tr>
<tr>
<td>Ceca</td>
<td>7.38$^b$</td>
<td>1.32</td>
<td>89</td>
</tr>
<tr>
<td>Cecal dropping swabs</td>
<td>6.49$^c$</td>
<td>1.25</td>
<td>92</td>
</tr>
<tr>
<td>Feces</td>
<td>6.41$^c$</td>
<td>0.87</td>
<td>92</td>
</tr>
</tbody>
</table>

Table 1. Mean recovery of campylobacters, as $\log_{10}$ cfu, from four sample matrices. Numbers with different suffixes are significantly different, $p < 0.05$. Samples ($n=100$) were collected from four discrete areas (quadrants) in each of twenty five broiler houses. For swab samples results are $\log_{10}$ (cfu) per set of swabs, whilst for ceca and feces results are $\log_{10}$ (cfu/g).

Of the four remaining matrices, bootswabs recovered significantly higher numbers of campylobacters than the other three matrices, $p < 0.05$, and also gave the highest number of positive samples (Table 1). They were also relatively cheap and simple to use, and allowed sampling to be undertaken quickly. A further advantage of boot swabs is that farmers routinely walk the broiler houses several times per day to remove injured or deceased birds and samples can be collected as part of that practice. For these reasons bootswabs have been used previously in several UK studies (8). Reactions regarding the practicality of bootswabs from poultry farmers and production staff observing the trial were favorable.

For the remaining matrices, it was noted that removal of the ceca from cull birds required the use of trained staff. Further, although high numbers of campylobacters were recovered from these samples, 6% of samples were false negatives, with regards to the house status. Increasing the sensitivity of the method would require that more birds were culled, further reducing the acceptability of this matrix. In addition shipping of the birds to the laboratory would be costly due to their weight. Alternatively farm staff would have to be taught how to routinely eviscerate the birds and remove the ceca for shipping, which could prove problematic.

Recoveries of campylobacters in fecal samples and cecal droppings were not significantly different, $p > 0.05$ (Table 1). However, collecting feces was a relatively simple procedure whereas locating the characteristic cecal droppings required careful examination of the litter surface. This was difficult in houses with no natural daylight. As birds neared the maximum stocking density floor space was minimized and birds kicked litter over the droppings and obscured them. Therefore sampling of feces would be the preferable method. However, 3% of fecal samples gave false negative results with regard to the presence of campylobacters in the house, and Campylobacter counts were 93% lower than those obtained using bootswabs. It should be noted that a fecal sample comprised 10 discrete ‘pinches’ of material, whilst in a typical broiler house the bootswabs of a sampling officer were estimated to make contact with approximately 2.5m² of litter.

Since bootswabs were clearly the best sampling matrix the effect of shipping them from a farm to the laboratory, using chilled containers and at ambient temperature, was investigated. At chill temperature there was no significant loss of viability of campylobacters for up to 48 h, allowing for flexibility in the times of sampling, shipping and laboratory analysis. However, ambient temperature storage resulted in a proliferation of non-campylobacters which subsequently prevented the enumeration of Campylobacter spp. on mCCDA. Similar Romanian Biotechnological Letters, Vol. 19, No. 5, 2014 9789
overgrowth was found to be due to the proliferation of antibiotic resistant E. coli, able to degrade cefoperazone (18). Since E. coli grows readily above 10°C, but not below 6°C (24), it is probable that similar organisms were causing the overgrowth observed. Hence it was essential that samples were kept chilled when being shipped to the laboratory.

To control Campylobacter spp. in broilers it is essential that the colonization status of a broiler house is determined accurately so that the efficacy of biosecurity measures can be determined, and the appropriate fate of the infected birds in the food chain chosen. Bootswabs gave the highest recoveries of Campylobacter spp., in terms of numbers and prevalence, of eight matrices studied. They were seen to be cheap, simple and quick to use, and samples could be stored for up to 48h with no loss of campylobacter viability. They are therefore the recommended sampling matrix.

Acknowledgements
This project was funded by the Food Standards Agency as project M01060. The authors thank various members of the British Poultry Council for donations of staff time and farm access.

References
A quantitative comparison of sample matrices for the detection of Campylobacter spp. in broiler houses


