FINAL REPORT

Development of a rapid on-farm test for the detection of *Campylobacter*.

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GLOSSARY

ACMSF	Advisory Committee on the Microbiological Safety of Food
a_w	Water activity
BPW	Buffered peptone water
°C	Degree Celsius
mCCDA	Modified cefoperazone charcoal deoxycholate agar
cfu	colony forming units
ELISA	enzyme-linked immunosorbent assays
ESBL	extended spectrum ß lactamase
DEFRA	Department for Environment, Food and Rural Affairs
DH	Department of Health
EC	European Commission
EFSA	European Food Safety Authority
EQA	External Quality Assurance
FERA	Food and Environment Research Agency
FSA	Food Standards Agency
g	Gram
GLP	Good Laboratory Practice
h	Hour(s)
HPA	Health Protection Agency
IQC	Internal Quality Control
ISO	International Standards Organisation
LAMP	loop-mediated isothermal amplification
LFD	lateral flow device
LIMS	Laboratory Information Management System
mL	Millilitres
MRD	Maximum recovery diluent
NCP	National Control Plan
PHLS	Public Health Laboratory Services
QC	Quality Control
s	Seconds
SME	Small and medium-sized enterprises
TBX	Tryptone bile X- glucuronide

TSB	Technology Strategy Board
UKAS	United Kingdom Accreditation Service
UV	ultraviolet

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SUMMARY

Given the high prevalence of campylobacters on retail poultry the Food Standards Agency perceived a need for broiler farmers to be able to rapidly determine if their flocks have become infected. This would allow farmers to assess the impact of interventions introduced on farms to prevent *Campylobacter* infection, and refine them. Flock status could potentially be used to schedule removal of the birds to the abattoir, allowing the processing plants to minimise cross-contamination. This project aimed, in the first instance, to assess commercially available systems for the detection of *Campylobacter* spp. for their suitability for use on broiler farms. The detection system would require an appropriate level of sensitivity, be robust enough for use in typical farm environments, require minimal training for use by farm staff, and be cost effective.

The most appropriate type of sample to be taken on farm to allow campylobacters to be detected had also to be defined. A comparison of eight potential sample matrices from broiler farms for their ability to recover *Campylobacter* spp. was undertaken and bootswabs, specifically Tunika, were found to be the most effective. At the time this study was undertaken lateral flow devices (LFD) were seen as potentially meeting the detection system requirements, and were also proposed in a study undertaken by the Animal Health and Veterinary Laboratories Agency. Only two LFD were commercially available, but were both designed for medical use and when tested with material eluted from bootswab samples from broiler houses they were found to lack specificity and/or sensitivity. Both were therefore unsuitable for further use in this study.

Evaluation of DNA based systems found that the only loop-mediated isothermal amplification (LAMP) kit commercially available lacked selectivity, giving false positive results with *Arcobacter* spp. which are common in chickens. A real time polymerase chain reaction (qPCR) detection kit (QIAGEN) proved to be sensitive and specific, giving results equivalent to conventional microbiological culture for the detection of *Campylobacter* in bootswab samples, but the equipment and procedures required that it be used in an appropriate laboratory. However, a system whereby bootswab samples were taken in specific houses by farmers, and posted to the laboratory for analysis was successfully trialled. Results for specific flocks could be obtained and reported within 36 hours of receipt of samples.

Several trials were undertaken with the co-operation of the broiler industry, and the sample handling procedures steadily refined. Meetings with stakeholders showed broiler farmers and processors had very positive views of the sampling procedures, and rapid return of the results directly to farmers, supporting the project teams' adoption of the qPCR based analyses. Whilst most sampling involved the major processors, specific trials were also instituted to allow smaller, independent farmers, to evaluate sampling procedures and express their opinions. Again, the responses

were positive, leading to the adoption of a project dealing specifically with independent farmers.

The project team had kept the key stakeholders informed on the progress and refinements of flock sampling by bootswab and qPCR detection, and the conclusion that the methodology was successful, and the broiler industry interest led to a commercial service being established, based on the procedures developed in the project, thus indicating a successful outcome.

The success of the methodology, and the desire by the FSA to encourage research groups working in the field of *Campylobacter* to collaborate, led to AFBI providing flock testing as a service to other research institutes. The main aim of this work was to ensure that resources were not wasted working on samples from flocks which were presumed to carry campylobacters, but had remained uninfected. In addition newer on-farm detection procedures were compared with the methodology developed in this study, to assess their sensitivity and selectivity. This involved duplicate samples being taken for analysis by AFBI and the appropriate research organisation. During the timescale of this study no method was found which matched the cost efficiency, selectivity and sensitivity of the qPCR method.

A limited trial was also undertaken to evaluate the suitability of the qPCR method for the quantitative detection of campylobacters on broiler neckskins. This method is the FSA's chosen methodology for assessing contamination in retail chickens, and work was ongoing in this area within AFBI. A comparison of qPCR and conventional enumeration of *Campylobacter* spp. showed that the former method was unsuitable for detecting campylobacters at the 10^3 cfu/g level required by the FSA, and work in this area was discontinued.

Overall, at the time this study was undertaken no suitable, commercially available, methodology for the detection of campylobacters on-farm was found. However a successful, and commercially acceptable, sampling and analysis procedure, based on qPCR, was developed. The methodology required farmers to sample broiler houses using bootswabs, and dispatch these to a central lab for analysis. By engaging with stakeholders throughout the project the sampling, shipping and analytical procedures were rapidly optimised, leading to a successful outcome for the project.

BACKGROUND

Campylobacter species, especially *Campylobacter jejuni* and *Campylobacter coli,* are the main cause of human bacterial gastroenteritis in the developed world and it is estimated that in the UK there are in excess of 500,000 cases and 80,000 general practitioner consultations annually (Strachan et al. 2010, Tam et al. 2012). Chicken meat has been determined as a key food-borne vehicle for *Campylobacter* spp. infection by source-attribution studies, outbreak investigations and case-control studies (Tam et al. 2009, Danis et al. 2009, Friedman et al. 2004; Mullner *et al*. 2009, Sheppard et al. 2009). Cross-contamination from raw poultry meat, or the consumption of undercooked poultry, is believed to be an important vehicle of infection (EFSA, 2010). Raw chicken meat is frequently contaminated with *Campylobacter* (Moran et al. 2009) and a decrease in the exposure levels from this source is likely to reduce the number of human *Campylobacter* cases.

Accordingly the UK Food Standards Agency (FSA) and the poultry industry agreed to reduce *Campylobacter* contamination in raw chicken: The Joint Government And Industry Target To Reduce *Campylobacter* In UK Produced Chickens By 2015 (FSA 2010). As part of the work proposed a target in the food chain where campylobacters would be enumerated had to be determined, and the report noted that:

> *'Setting the target at the end of slaughter was the preferred option as it would take account of the majority of slaughterhouse interventions and allow feedback to farms on flock-level interventions. It is also a point at which samples can be collected with relative ease and low cost.'*

Hence, the FSA recognised that providing accurate and rapid feedback on *Campylobacter* contamination of specific flocks to farmers would be beneficial in achieving their goals of reducing *Campylobacter* contamination of retail chicken meat. Such information could be related to on-farm interventions and allow the improvement of biosecurity procedures. Subsequently a consortium of government funded bodies collaborated to produce the UK Research and Innovation Strategy for *Campylobacter*-in the food chain 2010-2015 (Available at: www.food.gov.uk/sites/default/files/multimedia/pdfs/campylobacterstrategy.pdf). The first of two overarching aims of the consortium was, 'Reduction of the incidence of *Campylobacter* infection in humans through reductions in the level of the bacterium

in farm-animal hosts'. To further the consortium's aims three categories of research priorities were identified, one of which was 'Development of novel detection and diagnostic tools, and resources for *Campylobacter* research'. The need to assist farmers in their efforts to control campylobacters was recognised and, under this category the first objective was: The development of a rapid, on-farm test for *Campylobacter*.

The FSA issued a call for the development of a rapid, on-farm test for the detection of campylobacters, but were mindful of the range of projects on-going on the topic of *Campylobacter* spp. on poultry farms, and their detection. It was also considered that as time was of the essence, and the study should centre on a methodology to be implemented on farms, then analytical procedures should be required to use 'off the shelf' materials, rather than undertake the basic science involved in detection methodology. The aim was therefore to adapt available technology with the aim of producing a detection methodology capable of working under on-farm conditions. The team working on the project was also required to collaborate with specific research teams active in related fields, to ensure effective use of resources, and avoid duplication of effort.

The project therefore proceeded sequentially though the objectives agreed with the FSA, see Appendix 1, and the report below follows this sequential pattern.

Project Technical Report

Objective 1. Produce detailed sample collection, transport and culture protocols suitable for the detection and enumeration of *Campylobacter* by industry in samples taken from poultry farms.

At the commencement of this study preliminary discussions with the poultry industry revealed that microbiological on-farm sampling was largely centred on *Salmonella* due to the National Control Plan (NCP). Further, the detection of *Campylobacter* from on-farm samples was not undertaken on an industry-wide basis and not only did sampling methodologies differ between companies, the media used to detect campylobacters also differed.

In order to ensure the principal project aim was met, meetings were held with poultry processors, and then individual poultry farmers, to discuss sampling procedures and determine which procedures had been used, and how their use had been perceived.

Processors views.

Four major processors participated in these discussions. The meetings commenced with a discussion, held on company premises, at which staff representing broiler production, laboratory analyses and animal welfare aspects were present. The aims and objectives of the project were explained and then comments from the company staff invited. The principal points arising from discussions with the processors were:

1. Most companies had at some time undertaken on farm sampling for campylobacters.
2. Most sampling trials were undertaken wholly within the company, although collaboration with university projects was not uncommon.
3. Across the companies visited a wide range of sampling methodologies had been used, and also several detection methods. However, the general experience was that the rates of detection varied widely.
4. No company had found a method of on farm sampling giving results which correlated with samples from the same flock subsequently taken in the plant. The latter samples were normally either caeca, or neck skins, and gave much higher rates of prevalence than any on farm samples.
5. At the time the meetings were held no processor had a regular on-farm monitoring scheme for *Campylobacter* in place, but *Campylobacter* prevalence was known from sampling in processing plants.

Meetings with farmers provided the following points:

1. On farm sampling for *Salmonella* was routine, and farmers were therefore used to a sampling methodology based on bootswabs. However, it was reported that individual farmers differed markedly in their willingness to comply with the sampling regime.
2. Removal of caeca from cull birds was acceptable to most, but not all, farmers. One noted that he had routinely checked caeca when screening for illness, but that he would like appropriate training to ensure effective sampling.
3. Cloacal swabs had been taken by some farmers and they were seen as a relatively simple sample to obtain.
4. Most farmers were kept busy by their flocks, therefore sampling should be simple, and not require any great commitment of time to complete.
5. On farm facilities varied greatly with the houses visited dating from the 1970s to less than a year old. Therefore any on-farm test would have to be suitable for housing in quite basic accommodation.

With the above viewpoints determined, discussions were undertaken with colleagues working in the field of on-farm sampling. At that time work was being undertaken which would lead to the publication of ISO 6887-6:2013(en) Microbiology of food and animal feed - Preparation of test samples, initial suspension and decimal dilutions for microbiological examination - Part 6: Specific rules for the preparation of samples taken at the primary production stage.

Sample collection and transport.

Based on the experiences of previous government studies conducted by the Animal Health and Veterinary Laboratories Agency (AHVLA) and other government sponsored bodies (e.g. projects B15003, B15004, B15014 and B15020) bootswabs were seen as appropriate for *Campylobacter* sampling. However, in the final report of Project B15005: ‘Sampling regimes and microbiological methods for detecting thermophilic *Campylobacter* spp. in poultry on the farm before slaughter’ (Corry et al. 2006), the authors reported that the terminology applied to swabs to be used on farm was open to interpretation and could therefore lead to very different materials being utilised.

Since Objective 4 of this study aimed to deal directly with the question of the most appropriate sampling methodology, further consideration of this matter will be undertaken in the section pertaining to that objective.

To define the methodology for transporting samples to the laboratory consideration was given to the NCP under which all broiler houses are sampled for *Salmonella* using bootswabs, which are then sent to a laboratory for testing. For most poultry companies sampling was conducted by the farmers, who then sent the bootswabs, placed in a plastic bag, to a designated laboratory using Royal Mail. Whilst this method is relatively cheap and simple it is based on the survival of salmonellas in transit, at ambient temperature.

However, *Campylobacter* spp. are generally considered to be less tolerant of drying, and stress from being held at 12°C-20°C, than salmonellas, therefore they would be unlikely to tolerate the conditions that would be experienced during such simple sampling and shipping procedures. Therefore there was a need to experimentally determine the survival of *Campylobacter* spp. under such simulated shipping conditions. The aim was to present the broiler production industry with the simplest, and therefore cheapest, sample shipping methodology, therefore the effects of simulating the current *Salmonella* shipping procedures on the viability of campylobacters needed to be studied. Samples were also taken for non-cultural detection to determine if such methodologies are compromised by the shipping conditions.

Culture protocols suitable for detection and enumeration of *Campylobacter*.

The standard method for the detection of campylobacters is BS EN ISO 10272-1:2006, using of Bolton broth as enrichment medium and modified charcoal cefoperazone deoxycholate agar (mCCDA) as diagnostic medium. For the enumeration of *Campylobacter* (ISO-EN 10272-2:2006) mCCDA is used. To allow the detection of growth of *Campylobacter* in the shortest possible time samples can be plated directly onto mCCDA and then incubated appropriately. Such direct plating would also help to avoid a significant problem with Bolton broth, which has been compromised by the worldwide emergence of antibiotic resistant bacteria. For selectivity, Bolton broth contains four antibiotics, including cefoperazone, which is also present in mCCDA. Jasson et al. (2009), working with chicken meat in Belgium,

reported that extended spectrum ß lactamase (ESBL) producing *Escherichia coli* could proliferate in Bolton broth and subsequently mCCDA plates would show overgrowth of these organisms compromising the detection of campylobacters. In Northern Ireland, 90% of retail chicken sampled in 2008 (Moran et al. 2009) yielded campylobacters, with no discernible problems due to ESBL despite using Bolton broth. However, the following year the apparent *Campylobacter* prevalence in raw chicken fell below 50% (Moran et al. 2011), due to heavy growth of ESBL on mCCDA. However, later studies showed that the addition of clavulanic acid to Bolton broth was seen to eliminate the problem of ESBL (Moran et al. 2011).

Therefore direct plating of a processed bootswab sample onto mCCDA (and incubated appropriately) was seen as the simplest, and quickest, culture method for the detection of *Campylobacter*. Any significant increase in numbers of ESBL from those observed would compromise the sensitivity of the assay. Should this occur enrichment followed by non-culture detection may be utilised (Kawatsu et al. 2010). Tailoring of the choice of enrichment broth to the sample matrix may help recoveries (Kim et al. 2010), although that study was based solely on *C. jejuni* and *C. coli* is common in some flocks (Patriarchi et al. 2009).

Further details of the most appropriate methodology would be determined during studies to define the most appropriate sample matrix, Objective 4.

Objective 2. Contribute to “a review and consultation with industry on the practicality and cost of available technologies” to be undertaken by the AHVLA.

At the initiation of this study the AHVLA was undertaking the DEFRA funded project OZ0621: Science driven, industry informed feasibility study for the development of a rapid on-farm test for *Campylobacter* (Available at http://randd.defra.gov.uk/Document.aspx?Document=12756_01OZ0621-FinalReport01.12.11.pdf). In view of the obvious potential for collaboration a meeting was held with relevant AHVLA and AFBI staff, with the FSA Project Officer present. In addition AFBI staff completed the methodology assessment questionnaire which was part of OZ0621, and kept in touch informally by telephone to ensure full use was made of the deliberations of the AHVLA staff.

The final report for project OZ0621 was published in 2011 and noted that:

> 'The project has established a demand for a farm-based test that is simple to perform and should be able to identify flocks that are highly colonised with *Campylobacter*. The test should be rapid, providing results within 1-2 hours of sample collection, it should cost in the region of £5-£15 per test, and it should be ready for use on farms in the UK within the next year.'

Further, the report recommended the use of lateral flow devices (LFD) as being 'the most obvious method to meet the criteria for a simple test that is available now'. The authors also recommended 'continued development of the alternative methodologies discussed in this project, particularly the isothermal and biosensor systems' in case the promise shown by LFD was not fulfilled.

These recommendations were applied during this study and incorporated into the considerations reported below.

Objective 3. Consult with key providers (commercial) of rapid diagnostic technologies and appraise their products to determine applicability for rapid on farm testing.

This study was requested to use available technologies to detect campylobacters on-farm, in order to allow deployment within a relatively short period of time. The available systems were based primarily on two underlying methodologies of detection, using either antibodies or DNA. Considering each in turn:

Antibody based systems. The selectivity of antibodies can be utilised in enzyme-linked immunosorbent assays (ELISA), or the simpler LFD with the latter being recommended by AHVLA. However the complexity of utilising ELISA means that such assays are laboratory based procedures, whereas LFD are simple to use, with rapidly produced and easily read results (Ngom et al. 2010) making them prospective on-farm detection devices. Available LFD were:

ImmunoCard STAT! CAMPY test (Meridian Bioscience, Cincinnati, USA). Designed to detect *Campylobacter* in human stools during infection, and the use of which has been independently verified as sensitive and specific (Granato *et al*. 2010).

NH IC Campy (Nippon Meat packers Inc). Used to detect *Campylobacter* in chicken meat samples by sampling the enrichment broth cultures Kawatsu, *et al.* 2010).

SinglePath *Campylobacter* (Merck, Germany). Cited for use in detecting campylobacters in enrichment broth cultures.

In addition AFBI had developed monoclonal antibodies (mAb) and were working in conjunction with a local company which produced LFD. These mAb were also supplied to a group based at the Moredun Institute who were developing a LFD. It was envisaged that these LFD could be incorporated in to the programme of study.

It can be seen that none of the LFD were designed for use with the kind of materials likely to be found on-farm, apart from the ImmunoCard, which was designed to work with samples of faeces, albeit those from patients having a diarrhoeal illness.

DNA based systems. The rapid detection of specific bacteria can be undertaken using real time polymerase chain reaction (qPCR) methodology to detect target DNA in a relatively short time. The methodology is lab-based but with the potential for ruggedized systems to reach the civilian market it was possible that on-farm testing could be undertaken by trained staff using mobile systems. The methodology could also serve as the basis of a lab-based system, to be used should none of the on-farm systems under consideration prove suitable.

qPCR kits which were available to AFBI for consideration were:

Bio-Rad iQ-Check (Bio-Rad, Hemel Hempstead, UK).

mericon *Campylobacter* spp. kit. (QIAGEN Ltd, Manchester, UK).

mericon *Campylobacter* triple kit. (QIAGEN Ltd, Manchester, UK). This detects the specific presence of *C. jejuni*, *C. coli* and *C. lari*.

TaqMan *Campylobacter jejuni* detection kit (Applied Biosystems, UK)

Foodproof *Campylobacter* quantification kit (Biotecon Diagnostics, Germany)

In addition a loop-mediated isothermal amplification (LAMP) assay was available, and this required much less sophisticated equipment than qPCR:

Loopamp *Campylobacter* Detection Kit. (Eiken Chemical Co., Ltd, Tokyo Japan, but technology licensed to Mast Group Ltd, Bootle, UK).

Product appraisal. For all of the systems noted above the manufacturers had caveats regarding the sensitivity for their products for the detection of campylobacters and therefore most normally required that samples would be enriched overnight in a selective medium, principally Bolton broth. Such an incubation required microaerobic conditions and incubation at 42 °C. This was clearly unsuitable for an on-farm test, however some studies on LFD noted that their limit of detection was approximately 10^5 cfu/assay (Ngom et al. 2010), and qPCR assays had been seen to have a sensitivity below this value. Since chickens can be colonised to levels of 10^7 to 10^8 cfu/g of caecal contents (Hue et al. 2011) therefore obtaining sufficient inoculum without enrichment culture was considered a reasonable prospect. Accordingly none of the above systems were discounted at this stage.

Objective 4. Determination of appropriate on-farm sampling methodologies/matrices to use with a range of rapid detection approaches.

This study sought to evaluate eight potential matrices, all of which had been used in previous studies of bacterial populations in broilers, in terms of the numbers of viable campylobacters they carried when tested shortly after sampling. Accordingly this study was carried out in Northern Ireland, with the cooperation of a major poultry processor, so that analysis of all samples collected could be commenced within 2h of being taken. The matrices studied were: bootswabs, faeces, litter, caeca (from culled birds), ventral swabs, cloacal swabs, caecal droppings a dust. All had previously been used to detect campylobacters or *Salmonella* in broiler flocks.

Broiler farms studied. The 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, using analytical data from flocks, and the company scheduling of flocks for thinning (partial clearance of broiler houses) and final clearance. Since this study aimed to evaluate methods for detecting campylobacters the company representative ensured sampling took place in

previously thinned houses, which would shortly be subjected to final clearance. For biosecurity reasons only one farm was visited on a sampling trip. Sampling staff adhered to company biosecurity measures at all time. Once inside the house to be sampled staff used the normal equipment present to notionally divide the house into lanes, along which samples could be taken, delineated by the lines of drinkers and feeders present. The house could also be divided lengthwise based on the positioning of air ducts or lighting units, which were usually evenly spaced. Hence each house could be notionally divided into four roughly equal quadrants for sampling purposes. Officers prepared sketch plans of each house sampled, for subsequent reference.

Evaluation of sample matrices. Each broiler house sampled was notionally divided into four quadrants prior to collection of samples. Eight matrices were initially studied and samples taken in the four quadrants of a broiler house were placed in pre-labelled bags. On leaving the house samples were stored in a coolbox with ice packs prior to returning to the laboratory. Samplers wore a fresh pair of disposable gloves for each individual sampling exercise. Sampling used the following procedures for each matrix type:

Bootswabs. These were Tunika overshoes (Bowden & 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 and returned. The boot swabs were then placed in Seward closure bags (BA6041/CLR, Seward Ltd, Worthing, UK) and 10 cm^3 of maximum recovery diluent (MRD, CM733; Oxoid, Basingstoke, UK) added, and the bag sealed.

Faecal samples. The sampler walked a quadrant length in one lane and collected 5 ‘pinches’ of faecal 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 faecal samples.

Caeca samples. 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 containers. Once in the laboratory the birds were dissected and the caeca placed in Seward closure bags, and stored refrigerated until required. This is the only sample type where samples were not collected from 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 and five 'wipes' made with a fresh cellulose sponge using moderate pressure. The 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 labelled bag which was then sealed and stored.

Caecal droppings. 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 sample. Carcass swabs, as using in 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.

Bootswab storage. To assess the effect of storage on the recovery of campylobacters from Tunika bootswabs three farms were visited and a total of ten houses sampled, using the Tunika bootswab procedures described above. In each house the sampler walked the full length of the house and returned, seven times, wearing a fresh pair of bootswabs and a different lane each time. On return to the lab each set of seven pairs was assigned random numbers and based on these, pairs of swabs were allocated to one of seven treatments. One pair were 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 incubator were subjected to *Campylobacter* enumeration after 1, 2 and 4 days.

Microbiological analysis. All sample blending used a Colworth 400 stomacher for 1 minute. All media were supplied by Oxoid (Basingstoke, UK) unless otherwise stated. Samples were prepared for the enumeration of *Campylobacter* as follows:

Bootswabs: Maximum recovery diluent (MRD) (90 cm^3) added and sample blended.

Faecal samples: samples thoroughly mixed, 10 g added to 90 cm^3 MRD and blended.

Litter sample: samples thoroughly mixed, 10 g added to 90 cm^3 MRD and then hand shaken for 15 s.

Caeca sample: sampled weighed, MRD added to give 1:9 dilution and caeca manually disrupted by hand squeezing, then blended.

Ventral and dust swabs: MRD (90 cm^3) added and sample blended.

Cloacal swabs and caecal 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 utilised as described in ISO EN 10272-2:2006 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 42°C in a microaerobic atmosphere (85% N2, 10% CO2 and 5% O2, all v/v) in a Don Whitley MACS workstation (Don Whitley Scientific, Shipley, UK). Typical colonies were enumerated and confirmed by determining morphology, motility and oxidase reaction, aerobic growth at 41.5°, and microaerophilic growth at 25°C.

Statistical analysis. The data was analysed using GenStat for Windows 11th Edition.

Results. One farm was visited and samples of the eight matrix types taken in four quadrants in two houses. Mean *Campylobacter* counts (Log_{10} cfu) for cloacal swab, litter, ventral swab, and dust were 6.40, 4.92, 3.37 and 2.78. Counts are per gram for litter samples, and per swab for the other three samples. No further sampling was undertaken with these sample matrices. A further eight farms were visited, and the remaining four matrices were sampled in each of four quadrants in twenty three houses to give an overall total of 100 samples per matrix. Bootswabs recovered the highest number of *Campylobacter*, $p<0.05$, of these four matrices, Table 1.

Table 1. Recovery of *Campylobacter* spp. 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 caeca and faeces results are log_{10} (cfu/g).

Sample matrix	*Campylobacter* log_{10} (cfu)	Standard deviation	Positive samples
Bootswab	7.61^{a}	0.69	95
Caeca	7.38^{b}	1.32	89
Caecal dropping swab	6.49^{c}	1.25	92
Faeces	6.41^{c}	0.87	92

When Tunika bootswabs were stored in a chiller the mean *Campylobacter* counts (log_{10} cfu/sample) after 0, 1, 2 and 4d 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. For the samples stored at 22°C considerable overgrowth of non-campylobacters was observed on the mCCDA plates, which prevented enumeration of *Campylobacter* colonies in many cases. After 1 d three samples could not be enumerated, after 2 d seven, and after 4 d only one sample could be counted. Figures are presented in Appendix II.

Conclusions. This study aimed to compare eight matrices to determine which would recover the highest numbers of *Campylobacter* from broiler houses. The initial enumeration of *Campylobacter* from eight matrices revealed that litter, cloacal swab, ventral swab, and dust gave the lowest recoveries. Given the sensitivity of

Campylobacter to desiccation (Kusumaningrum et al. 2003, Berrang and Northcutt, 2005) the low recoveries in dust are unsurprising. Cloacal swabs had been previously used to detect campylobacters (McDowell et al. 2008; Kudirkien et al. 2009) and swabbing the ventral area of the birds with carcass swabs was evaluated as a simpler method of sampling birds, more suitable for use by on-farm staff, but the latter method was seen to be ineffective. Since both litter samples and cloacal swabs yielded fewer campylobacters than the four remaining matrices they were dropped from the study.

Considering the remaining four matrices, the bootswabs recovered significantly higher numbers of campylobacters than the other matrices, $p < 0.05$, and also gave the highest number of positive samples. Their use was also relatively simple, and costs low. They were therefore selected as the most appropriate sample matrix for subsequent studies. The work undertaken to meet Objective 4 has subsequently been published (Madden et al. 2014). The bootswab sampling protocol for use on-farm is presented in Appendix III.

Objective 5. Undertake an evaluation of methods for the treatment of test samples to yield appropriate antigens or nucleic acid for detection methods.

When this objective was submitted to the FSA as part of the project proposal the project team considered that the limited sensitivity of LFD and some qPCR procedures might limit their utility in meeting the project aims. Accordingly equipment such as the Pick Pen (BioControl System Inc, UK) and Pathatrix Auto (Matrix Microscience, UK) was considered for their potential in recovering, and concentrating, *Campylobacter* spp. from sample matrices. By using such antibody capture systems it was considered that a significant number of campylobacters could be recovered, in a concentrated form, from sample matrices, and the quantity of potentially interfering material markedly reduced. Such enriched and partially 'cleaned' samples could potentially enhance the sensitivity of the assays chosen to detect campylobacters.

It was recognised that only the LFD would be suitable for on-farm use, due to their simplicity of use.

With regard to obtaining appropriate DNA from sample matrices, it was seen that no simple, robust, 'farmer proof' equipment for undertaking of DNA based analyses was currently available. Therefore DNA based work would only take place in an appropriate laboratory, and that efforts should concentrate on the potential for developing an appropriate service for farmers, using the guidelines established by the AHVLA in report OZ0621. Such a service would only be investigated if no suitable on-farm *Campylobacter* detection systems could be found. Since Mr Taylor's laboratory had significant experience with QIAGEN extraction chemistry, utilised in an automated QIAxtractor system, which was applied in a high throughput system to extract DNA from *Clostridium* spp., it was considered that this should serve as a reference system, against which other DNA extractions should be compared.

Objective 6. Undertake a preliminary evaluation of a range of detection methods (immunological and DNA-based) for the rapid detection of *Campylobacter* spp. in samples from farms.

A detailed study to determine the range of commercially available detection systems which could be applied to detect campylobacters on-farm was undertaken. Negotiations with suppliers were then undertaken to define which systems could be obtained for study within the project deadlines, and which would met the AHVLA proposed guidelines. Three categories of systems were found: LFD, qPCR and LAMP, and the systems are considered below.

Lateral flow devices. Only the Singlepath and ImmunoCard LFD devices were readily available when this trial was undertaken. Both the AFBI and Moredun Institute LFD devices were studied to a limited extent, but are not considered further in this report as, ultimately, neither proved suitable for commercialisation. Studies on the two commercially available LFD commenced with trials using pure cultures of *Campylobacter* spp. and other organisms found in chicken faeces to assess specificity and selectivity (listed in Appendix IV). Decimal dilutions of pure cultures, 10^5 to 10^7 cfu, were applied to the devices and the devices detected both *C. jejuni* and *C. coli* but *C. lari* gave weak, or no, results. No false positives were obtained with the non-*Campylobacter* challenge cultures used.

Samples of several matrices, obtained during the work required to meet Objective 04, and for which *Campylobacter* spp. enumeration data had been obtained were then investigated. Thirteen *Campylobacter* positive samples, consisting of bootswabs, faeces, caeca (culled birds) and caecal droppings were studied. No false negative results were found, with both LFD types giving positive results with all of the samples.

With Tunika boot swabs established as the sample matrix of choice (Objective 04, above) the material to be presented to the *Campylobacter* detection systems was defined as a sample of the 50mL of MRD added to the bootswabs, after blending in a stomacher. The samples were treated as required by the LFD manufacturer's instructions and applied to the LFD. A trial with 24 bootswab samples obtained from local broiler houses was then undertaken, and the ImmunoCard gave 11 false negative results (46%), when compared with the results of conventional enumeration. This was clearly unacceptable and this LFD could not be used in the on-farm study.

Once again the Singlepath LFD showed all of the samples as positive, as did the *Campylobacter* enumeration work. However, for three samples $<10^4$ cfu of *Campylobacter* would have been applied to the LFD, and as immunological assays of this type could not detect such low levels these are, in effect, false positives. This indicated a lack of selectivity with the sample matrix of choice and to confirm this a final trial using bootswab samples from negative houses was undertaken.

To obtain negative samples 24 houses containing young birds were visited, and the presence of campylobacters determined using conventional enumeration. Subsequently 21 houses were confirmed as *Campylobacter* negative. The ImmunoCard showed all of these samples to be negative, but the Singlepath gave six positive results. This confirmed that the Singlepath kit gave false positive results with samples of the matrix of choice, 29% in this case.

Overall both LFD were judged to be unsuitable for the uses they were put to in this study, and work on these devices was discontinued.

qPCR DNA detection systems.

The qPCR kits which were available to AFBI for consideration were:

Bio-Rad iQ-Check (Bio-Rad, Hemel Hempstead, UK).

mericon *Campylobacter* spp. kit. (QIAGEN Ltd, Manchester, UK).

mericon *Campylobacter* triple kit. (QIAGEN Ltd, Manchester, UK). This detects the specific presence of *C. jejuni*, *C. coli* and *C. lari*.

TaqMan *Campylobacter jejuni* detection kit (Applied Biosystems, UK)

Foodproof *Campylobacter* quantification kit (Biotecon Diagnostics, Germany)

At this time, and to the present day, AFBI provided a commercial service for the detection of psychrotrophic clostridia in samples submitted by the red meat industry, based on DNA detection. Given the high numbers of samples received daily automated liquid handling systems, including a DNA extraction system, were in place and in routine use. This system was based on QIAGEN equipment and reagents. Since the project resources were limited it was agreed with the Project Officer that the current equipment would be used to extract DNA, hence for all samples DNA was extracted using a QIAGEN QIAxtractor DX reagents (950107) and QIAxtractor DNA Plasticware (950037), according to the manufacturer's instructions.

Assessment of the mericon *Campylobacter* spp. kit.

Methods. For this preliminary assessment the kit was first studied on the basis of selectivity, which was assessed using the panels of pure cultures of campylobacters and non-campylobacters used in the assessment of the LFD (Appendix IV). A pure culture of *C. jejuni* was prepared as decimal dilutions containing from 2.40×10^6 to 2.40×10^1 cfu/mL to assess sensitivity. Subsequently samples (n=16) of four matrices used in the study described in Objective 4 above were analysed (bootswabs, caecal droppings, caeca from cull birds and faeces). To assess the reproducibility of the systems two DNA extractions from each sample were made, and analysed using the kit. Forty eight bootswabs for which enumeration data was available were analysed, and finally bootswabs used in the trials to determine the effects of storage temperature on the persistence of campylobacters were analysed.

Results. Using the panel of pure cultures all *Campylobacter* spp. were detected, and no false positives found with the non-campylobacters. The pure culture of *C. jejuni* showed that the threshold cycle (Ct) values had a linear relationship between 1.38 and 6.38 Log_{10} (cfu/mL) ($p < 0.001$):

$$Log_{10} (cfu/mL) = 13.15-0.314 \times Ct$$

For the four matrix types the numbers of *Campylobacter* found by enumeration also correlated significantly ($p < 0.001$) with the Ct values. The Ct values were also consistent between the two extractions undertaken, with the mean standard deviation between the duplicates (n=16) being less than 2%, and the highest value being less than 5%. For the 48 bootswabs a significant correlation between Log_{10} (cfu/mL) and Ct values was again seen ($p < 0.001$):

$$Log_{10} (cfu/mL) = 12.24-0.283 \times Ct$$

Accordingly the qPCR apparatus was programmed with a regression line to convert Ct values to Log_{10} (cfu/mL). Applying this to the bootswabs from the storage temperature trial (n=4, stored 0, 1, 2 and 4d) gave a result of 5.94 ± 0.23 Log_{10} (cfu/ml) for 4 ° C and 6.02± 0.27 Log_{10} (cfu/mL) for those stored at ambient temperature.

Conclusion. The qPCR system showed no problems during the selectivity and sensitivity trials, and the Ct values of duplicate samples of four matrices were consistent. The three sets of statistically significant regression data suggested that the mericon *Campylobacter* kit was fit for purpose, and merited further study.

Assessment of the BioRad iQ-Check *Campylobacter* spp. kit.

This kit was used with DNA extracted as above, in conjunction with a BioRad CFX96 qPCR system. However that machine failed whilst in use and due to constraints of time subsequent analyses were performed using an ABI 7500. The assessment was as used for the mericon kit, above, and similar results were obtained (results not shown). However, the manner in which the reagents were used was not compatible with the automated handling systems available in AFBI. Accordingly, whilst the

BioRad kit appeared to be fit for the purpose of meeting the aims of this study, the constraints on resources meant that no further work was undertaken with it.

Loop-mediated isothermal amplification (LAMP) of DNA

An alternative to conventional PCR methods, which has been applied to *Campylobacter* detection, is LAMP (Yamazaki et al. 2008; 2009). The latter paper of Yamazaki et al. noted 'Compared with *C. jejuni-C. coli* isolation using the conventional culture test, the LAMP results showed 98.5% (67/68) and 97.4% (74/76) sensitivity and specificity, respectively, and the positive and negative predictive values were 97.1% (67/69) and 98.7% (74/75), respectively. The study was based on detecting campylobacters in Preston broth used to enrich samples of chicken meat, hence high numbers of campylobacters will have been present. The inherent nature of primer design for LAMP reactions has been claimed to increase specificity in comparison with conventional PCR reactions, and the LAMP reactions are performed under isothermal conditions, ranging from 60-65^{o}C, thus obviating the need for sophisticated thermal cycling equipment. The reaction takes place in a single reaction tube with a result available in less than one hour. Furthermore, interpretation/detection can be based on visual inspection of turbidity in the reaction tube, or by incorporation of a fluorescent marker. Therefore LAMP detection is potentially a rapid and relatively simple method to detect *Campylobacter* DNA, provided enough is present, and has scope for being deployed out with a laboratory setting. Only one commercial LAMP kit for *Campylobacter* detection was available for evaluation at the time of this study: the Loopamp *Campylobacter* Detection Kit, (Eiken Chemical Co Ltd) obtained from MAST Diagnostics, Bootle, UK.

The kit was used as per the manufacturer's instructions, available at: http://www.mast-diagnostica.com/MASTD_dt/PCR-Fly-67LMP721-Loopamp-Campylobacter-e.pdf.

Preliminary investigations visualised the products of the Loopamp kit using both gel electrophoresis, and using visible fluorescence of the amplification product in its reaction tube, under ultraviolet (UV) light. However, study of some of the stock

isolates used to determine specificity showed adverse results, Appendix VI. One culture of *C. lari* was not detected, but of greater concern was the detection of three *Arcobacter* spp.: *A. butzleri* 32824, *A. butzleri* NCTC 10828 and *A. skirrowii* LMG 9880. Arcobacters are common in poultry (Scullion et al. 2006) hence such false positive reactions would be expected to occur commonly with samples from broiler farms.

As a final trial of the methodology samples from bootswabs which had been confirmed as being free of campylobacters, or contaminated, using both conventional culture and qPCR were analysed using the Loopamp kit. Twenty one negative samples were analysed and LAMP identified one as positive whilst fourteen of the 23 positive samples were positive using LAMP.

Despite studies to ensure the assay was used optimally the specificity and sensitivity problems remained and therefore this assay was adjudged to be unsuitable for the purposes of this study.

Objective 7. Produce a report on objectives 1-6.

A report on the findings of the work undertaken to meet the relevant objectives was submitted to the FSA Project Officer and accepted.

Objective 8. Undertake an evaluation of a range of detection methods (informed by the VLA review) for the rapid detection of *Campylobacter* spp. on farm.

The principal findings of the study up to this point were:

1. Poultry producers and poultry farmers required a simple and robust methodology to detect campylobacters on farm. Evisceration of cull birds was not acceptable to some farmers, but a routine task to others.
2. A comparison of eight potential broiler house matrices found that Tunika bootswabs yielded the highest numbers of campylobacters, and the procedure was familiar to all broiler farmers due to sampling for *Salmonella* under the NCP. However, the bootswabs used for the NCP were inferior to Tunika in terms of the total number of bacteria collected.

3. A review by AHVLA staff recommended LFDs to detect campylobacters on farm, but the only commercially available devices lacked sensitivity and/or selectivity when examined during this study.
4. DNA detection using real time PCR (qPCR) was seen to be both selective and sensitive for the detection of campylobacters in broiler farm matrices.
   A basic study with contaminated bootswabs showed that after four days of storage at ambient temperature no change in the qPCR signal for campylobacters was seen. Thus it should be possible for farmers to ship bootswabs to a central facility for qPCR analysis.

At the outset of the study it was envisaged that several methodologies would prove to be potential candidates for the task of rapidly detecting campylobacters on broiler farms. Therefore it was considered that a comparison, in some detail, would be required to evaluate the methods and produce a ranked list, with the method(s) chosen for further study being agreed following discussion the FSA, and appropriate stakeholders. Thus, at the outset, the aim of Objective 8 was planned to be a comparative study of the methodologies assessed as acceptable for the role of on-farm campylobacter detection. As noted above, the LFD kits were not acceptable, and neither was the only available LAMP assay.

Based on the foregoing it was agreed with the FSA Project Officer that studies should concentrate on the use of Tunika bootswabs for the sampling of boiler houses, followed by the detection of campylobacters on the bootswabs using qPCR in the form of the mericon kit.

Accordingly subsequent studies were to designed to fully evaluate the potential use of bootswabs for the sampling of broiler houses for campylobacters, with their subsequent analysis using qPCR at a remote facility. The manner in which bootswabs would be sent to the facility had yet to be determined, although a precedent existed, and would have to be designed and validated.

Objective 9. Undertake validation of combined sampling, processing and detection methodologies using field samples.

This objective had been proposed when the study was designed, and when it was assumed that a range of methodologies for assessment would be available to the

study team, therefore it was intended to cover trials designed to assess the ability of all of the available methodologies to cope with field samples, as opposed to laboratory cultures. The studies presented above show how bootswab sampling on-farm, with subsequent qPCR analysis in a laboratory, appeared to be the best methodology to progress the overall aims of the study. Thus these two methodologies would require validation The manner in which bootswabs would be made available to farmers, protocols for their on-farm use, and the methods by which they would be transported to the laboratory all had to be defined, and tested. All work relating to the bootswabs would have to be approved as appropriate by the relevant stakeholders.

The laboratory procedures for sample reception and preparation, DNA extraction and the qPCR analysis itself also required validation.

On-farm sampling. Tunika bootswabs had previously been used in FSA projects B15003, B15004, B15014 and B15020, but in the final report of Project B15005 (Corry et al. 2006), the authors noted that the terminology applied to swabs to be used on farm was open to interpretation, possibly leading to very different materials being utilised. As part of the NCP for *Salmonella* bootswab sampling was routinely used on broiler farms but discussions with producers showed that the most commonly used swabs, which were commercially available from Technical Service Consultants Ltd, were of light construction, and easily torn (Appendix VII). The physical weakness of the TSC bootswab was adversely commented on by some of the farmers interviewed during this study.

A limited comparison of the Tunica and TSC bootswabs was undertaken (Appendix VII) and showed that the Tunica bootswab recovered higher numbers of bacteria than did the TSC. Therefore given the more reliable nature of the Tunika bootswab, and the results obtained in this study it was confirmed as the most appropriate methodology for use in the on-farm sampling programme.

With the bootswabs selected procedures for delivering the swabs to farmers for preliminary trials were agreed with a local processor, and instructions for their use, were required. For the preliminary trials it was agreed with processors that the sampling materials required (Appendix III) would be supplied in a tear proof envelope (Mail Lite Tuff, 270 X 360mm) with instructions (Appendix VIII. NB these instructions

are those supplied in later studies, hence references are made to posting the samples directly to the laboratory). Boxes of kits would be supplied to the advisory staff of the producer, who would distribute the kits to the farmers. The advisory staff would also collect samples from the farmers.

For the preliminary trial, a total of 22 farms were involved and a total of 60 houses sampled and the study aimed to find mainly negative samples, for use in the assessment studies of LFD and LAMP, discussed above, as well as qPCR. Samples (n=21) obtained at a flock age of 28 days also had *Campylobacter* enumerated by ISO 10272-2:2006 to confirm the qPCR results.

Sample analysis by qPCR. A series of investigations to validate the qPCR system were undertaken, and these were mainly based on the DNA extraction and qPCR procedures described below as a single process. These studies validated the use of this process to detect *Campylobacter* spp. in this study (Appendix IX). In the laboratory bootswab closure bags were opened and 50mL maximum recovery diluent, MRD, (*Oxoid* CM733) added. The sample was then blended in a stomacher (Seward 400) for 1 min, and a sample passed to a QIAgility liquid handling unit for addition of the DNA extraction reagents. DNA was extracted using a QIAGEN QIAxtractor with DX reagents (950107) and QIAxtractor DNA Plasticware (950037), then a separate QIAgility machine used to aliquot the qPCR reagents. *Campylobacter* were detected using the QIAGEN mericon *Campylobacter* spp. detection kit for real-time PCR (290035) in an Applied Biosystems ABI7500 using 7500 fast systems sequence detection software Version 1.4.0.27. All reagents and equipment were used as per manufacturer's instructions.

Results and discussion

Overall 22 farms were involved, and a total of 60 houses sampled with 175 samples being submitted during the study. All samples submitted were analysed on the day they were collected by AFBI, with qPCR results available by 4pm. On average 51 out of a potential 60 samples were returned for each sampling day indicating that further discussions with the relevant farmers were required to ensure sampling regimes were adhered to. Other issues with the on farm sampling included the fact that while each sampling kit requested appropriate sample information this was frequently not provided by farmers. Failure to accurately record sampling date, and farm or house

information, contributed to inaccurate sample identification. In addition where the information was provided some handwriting was difficult to read.

The results obtained, as determined by qPCR are presented on Table 2. All 21 samples enumerated for campylobacters were negative.

Table 2 Prevalence of *Campylobacter* in broiler flocks as determined by RTPCR of bootswab samples of houses (n=175).

	Flock age		
	25d	2d pre-thin	4d post-thin
Location	**Percentage *Campylobacter* positive**		
Farms (n=17)	17	47	88
Houses (n=51)	12	33	82

All samples confirmed as *Campylobacter* negative by culture had Ct values in excess of 37, which was in agreement with pure culture studies undertaken with this system, and the results of analysis of samples analyse as described above. The results showed that most houses, 77%, were negative two days before thinning, but over 80% had campylobacters four days after thinning.

Conclusions. Follow up discussions with farmers found that the materials supplied were regarded as fit for purpose, and the sampling methodology was acceptable, and not onerous. Since farmers have the daily task of walking through the broiler houses then the sampling procedure could be readily incorporated into a pre-existing routine, and would not require the addition of a completely new task to the farmers workload.

In the laboratory the practical difficulties with reading information provided by the farmers indicated that changes would be required to ensure samples were readily identified, with pre-printed labels proposed to obviate problems with illegible handwriting. This would have the benefit of minimising the farmer's input and ensure samples could be readily identified on receipt in the lab. Further, incorporating the use of bar codes on sample identification materials would allow more automation of sample reception processes, and further reduce the possibilities of errors. The use of processor advisory staff could not be sustained if sampling was increased, hence

farmers would have to submit samples to the laboratory directly, using the Royal Mail, as with NCP salmonella samples.

The results of the qPCR analyses, and the validation procedures, when combined with those obtained in other parts of this study suggested it was effective in determining which samples carried campylobacters. The analytical process could be completed in a working day allowing the prospect of results being returned to farmers the day after samples were posted.

Discussions with the industrial collaborators found great enthusiasm for extending the on-farm sampling and qPCR analysis, therefore, in conjunction with the poultry producers, and the Project Officer, further studies aimed at defining the utility of the on farm sampling procedure, combined with the qPCR assay, were planned.

Objective 10. Configuration of complete “best approach” methodology into a convenient easy-to-use ‘farm-proof’ format.

In conjunction with the Project Officer and stakeholders from the broiler industry it was agreed that at that time no methodology to detect campylobacters which could be placed on-farm was yet available. The only currently feasible approach was to establish a qPCR system in an appropriate laboratory and arrange for farmers to take bootswab samples and ship them to the laboratory as required by the processor.

Objective 11. Laboratory validation of easy-to-use ‘farm-proofed’ protocol/device (‘On-Farm test’) using farm samples.

In the light of the observations discussed in Objective 10 above, this objective became redundant. Since only off-farm detection of campylobacters showed any prospect of success, no ‘farm proofing’ of methodology was required. However, refining the systems and materials to ensure farmers received sampling packs at an appropriate time, and could use the bootswab sampling kits effectively, required input from the project team. Due to close cooperation with the poultry industry, and the simple nature of bootswab sampling, effective sampling was soon established.

Objective 12. On-farm feasibility study of easy-to-use 'On-Farm test' using farm samples.

This objective was also rendered redundant by the adoption of an 'on farm sampling but off-farm analysis' protocol.

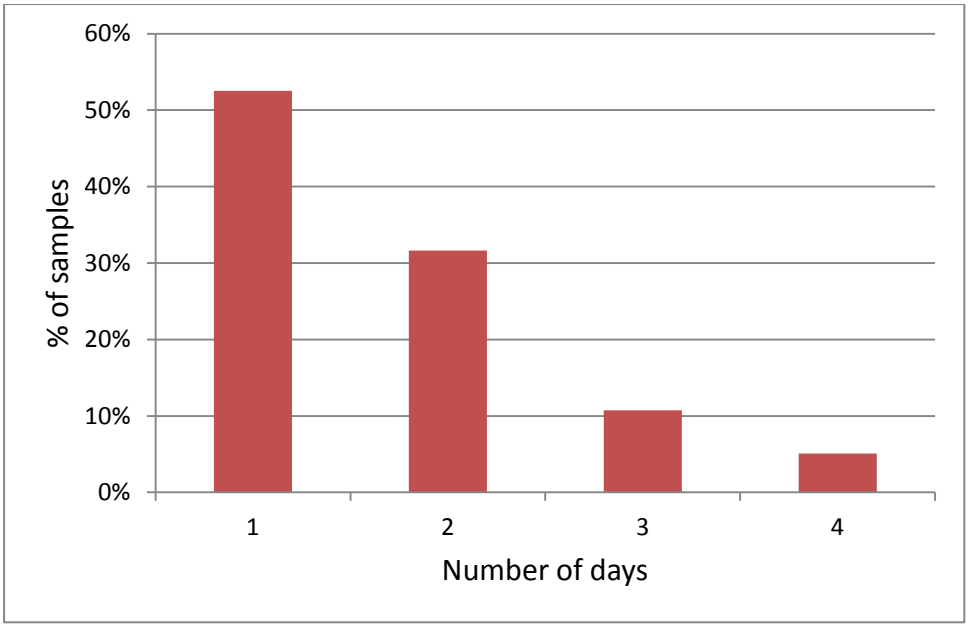
Objective 13. Model Farms studies: Practicality of using *Campylobacter* real time PCR boot swab assays to provide farmers with results prior to slaughter.

With the sampling and analysis protocols defined and seen to be effective the industrial collaborators were consulted in order to undertake larger scale trails. For this trial the flocks chosen were those kept on model farms run by the processors, where best practices were maintained. The staff were also familiar with testing regimes and were therefore seen as most likely to comply with the requirements of the trial.

The study involved AFBI supplying bootswab test kits to the processor for distribution to the farm staff, and the resulting samples being sent by Royal Mail to the laboratory, for qPCR analysis. The aim was to provide results to the staff of the relevant farms within 36h of samples being delivered to AFBI. It should be noted that AFBI staff only work on Mondays-Fridays, and no mail is delivered on Saturdays. Therefore samples sent on a Friday would be delivered on the following Monday.

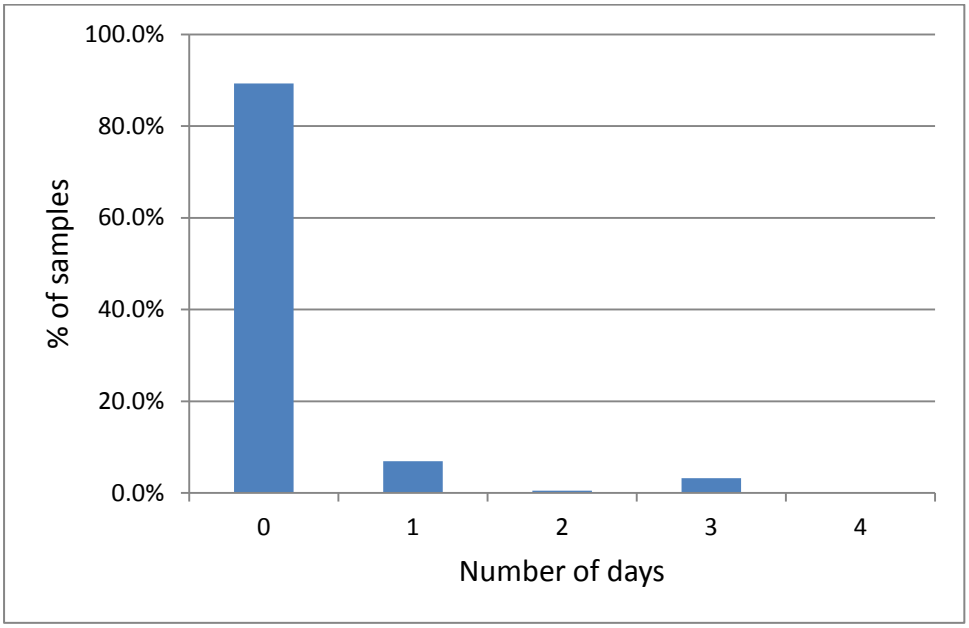
Eight farms were selected for participation and a total of 440 bootswab samples received and analysed over the period of the trial. Using the sampling date supplied by the farmer the time taken for a subset (n=178) of the samples to reach the lab and be recorded is shown on Fig. 1. Note that samples may not have been posted immediately after having been taken, or may have uplifted from post boxes the day after posting. Overall 84.2% of these samples were delivered to the laboratory within 2 days of sampling.

Figure 1. Time elapsed between sampling of broiler houses and reception of samples in the laboratory (n=187).



Once delivered to the laboratory most samples were analysed on the day on which they were received, Fig 2.

Figure 2. Time elapsed, in days, between receipt of samples in the laboratory and analysis being undertaken (n=187).



The results of the analysis on the whole sample set are presented in Table 3, below. Overall, 52.0% of samples were positive and 1.1% of samples required the analysis to be repeated.

Table 3. Results of qPCR analysis of bootswabs (n=440) from the model farm (n=8) study. Overall, for 1.1% of samples the analysis had to be repeated to obtain a valid result.

Farm	Bootswabs	Positive	Fail[1]
A	141	44.0%	1.4%
B	65	81.5%	1.5%
C	92	16.3%	2.2%
D	44	81.8%	0.0%
E	31	100.0%	0.0%
F	30	53.3%	0.0%
G	24	66.7%	0.0%
H	13	0.0%	0.0%

[1]Samples were required to be re-analysed to obtain a result.

All of the samples requiring a second analysis during this study came from only three out of a total of eight farms, and the failure rate for the remaining five farms was 0%. No overt cause for the latter observation could be discovered by the project team. Subsequently, on scaling up the analytical process to meet commercial rates of analysis, failure rates were set the target of remaining below 5%.

Almost 99% of samples were successfully analysed at the first attempt and the results were then submitted to farmers by email and/or text message. When re-analysis was required results were provided within 36h of sample reception. The delays due to AFBIs Monday-Friday working schedule could not be readily overcome but were adjudged by the participants to be within acceptable limits.

Discussions with the processors and the Project Officer were held, and the trial was seen by both the processors and participating farmers as highly successful. The use of Royal Mail first class post proved economical and efficient with most samples being delivered within an acceptable timeframe. Further, the use of email and text messaging allowed the target time for the return of results, within 36h of sample reception, to the participants to be met. The success of this trial led to requests from specific processors for the sampling and analysis study to be expanded.

Objective 14. National Farmer Union Farms studies: Practicality and initial assessment of any effect on behaviour of using *Campylobacter* real time PCR boot swabs assays sourced from NFU farms.

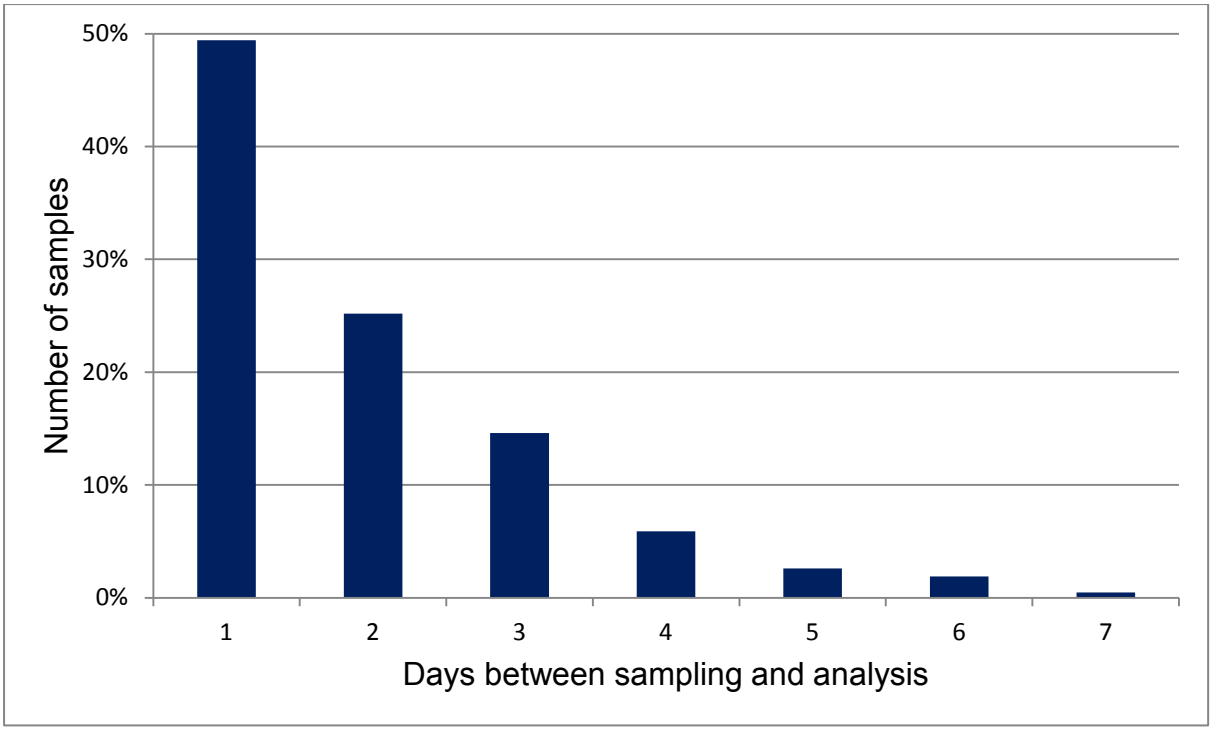
When this study was initiated the aim was to reach the maximum number of broiler farms and therefore contact was initiated with the larger poultry processors in the UK. As the work was progressing presentations were made to relevant stakeholders, including farmers who were not directly employed by poultry processors. These independent farmers were members of the National Farmers Union and responsible for a relatively small proportion of UK broiler production. However, representatives approached the project team requesting that consideration be given to involving some of the independent farmers in a feasibility study, to assess the procedures that had been developed in Objective 11 above.

The FSA project officer held a meeting to discuss the approach, and requested that a trial was established. The study commenced with three farms, comprising 20 houses, being sent sampling kits by AFBI, and with the results, simply described as positive or negative, being returned by text message. The initial success of the work led to more farms being incorporated and the work was expanded to include a total of 19 farms, comprising 108 houses.

AFBI refined the reporting system by adopting software to allow bulk text message dispatch from the laboratory PC, to meet the increased sample throughput.

Overall 432 samples were analysed, with 61.2% of samples being positive. Over 95% of samples were analysed and the results returned within 4 days of sampling (Figure 3).

Figure 3. Time elapsed between sampling of broiler houses and analysis of samples in the laboratory (n=432).



Conclusions

The procedures developed in this study were applicable to independent farmers, who considered that the results were useful to them in controlling campylobacters in broilers. This reinforced earlier opinions presented at project meetings acknowledging the contribution *Campylobacter* testing made to farmer engagement and heightened awareness of farm / house bio-security .

In view of the success of this work as a feasibility study some team members contributed to the proposals subsequently funded by the FSA as FS101123 Title: On-farm *Campylobacter* testing of independent broiler farms Oct 2014-Jan 2016 (Report available at: https://www.food.gov.uk/science/research/foodborneillness/b14programme/b14projlist/fs101123)

Objective 15. Research project farm screenings: Provision of *Campylobacter* screening of broiler houses in the context of FSA funded and industry *Campylobacter* slaughterhouse studies including the TSB funded lamp rapid test.

With the qPCR detection of *Campylobacter* in bootswabs working effectively the FSA encouraged research groups researching campylobacters in broilers to work co-

operatively. Therefore the AFBI team's ability to rapidly determine whether broiler flocks had become infected with *Campylobacter* was seen as a valuable tool by other research groups who then approached AFBI to integrate rapid flock screening into aspects of their research programmes. Teams at Bristol University and Campden BRI approached AFBI in order to have specific flocks tested to ensure that the flocks which would be the subject of their analyses in slaughterhouses were *Campylobacter* positive, or negative as appropriate. This saved the application of significant resources, only to find that the presumed flock status was incorrect and hence all of the work undertaken on birds taken from the flock, gave results which were, in effect, useless.

This flock screening work, to assure research teams that they would sample flocks of the appropriate *Campylobacter* status, involved AFBI working in close collaboration with the project leaders. The aim was to dispatch test kits to the specific farms involved in a timely manner, and report the results of the qPCR analysis as soon as was possible. The two projects to which this service was provided were:

Campden BRI Project 129567: Investigation of the efficacy, practicality and cost effectiveness of modified atmosphere packaging on *Campylobacter* numbers on raw chicken intended for retail. *FSA Funded as FS101038.*

FSA project FS241063, conducted by Bristol University: Investigations into changes of *Campylobacter* numbers on broiler carcasses during and following processing. *FSA Funded*. (https://www.food.gov.uk/science/research/foodborneillness/b15programme/b15projects/fs241063).

Whilst this study (M01060) had the aim of using readily available testing procedures to detect *Campylobacter* the Technology Strategy Board (TSB) funded a project for the 'development of in-farm diagnostic tests for *Campylobacter*' (http://gtr.rcuk.ac.uk/projects?ref=101134). As part of that project one of the tests which was investigated, by the Food and Environment Research Agency (FERA), was a new commercial LAMP test. In view of their experiences in this study AFBI was approached to undertake a brief comparison of the sensitivity of the LAMP test

with AFBI's qPCR procedures. This involved AFBI supplying a major broiler processor with 200 bootswab sampling kits with the aim of duplicate samples being taken in broiler houses and then supplied to AFBI and FERA for analysis. AFBI then supplied the results of qPCR analyses directly to FERA, and also copied them to the FSA project officer of this project.

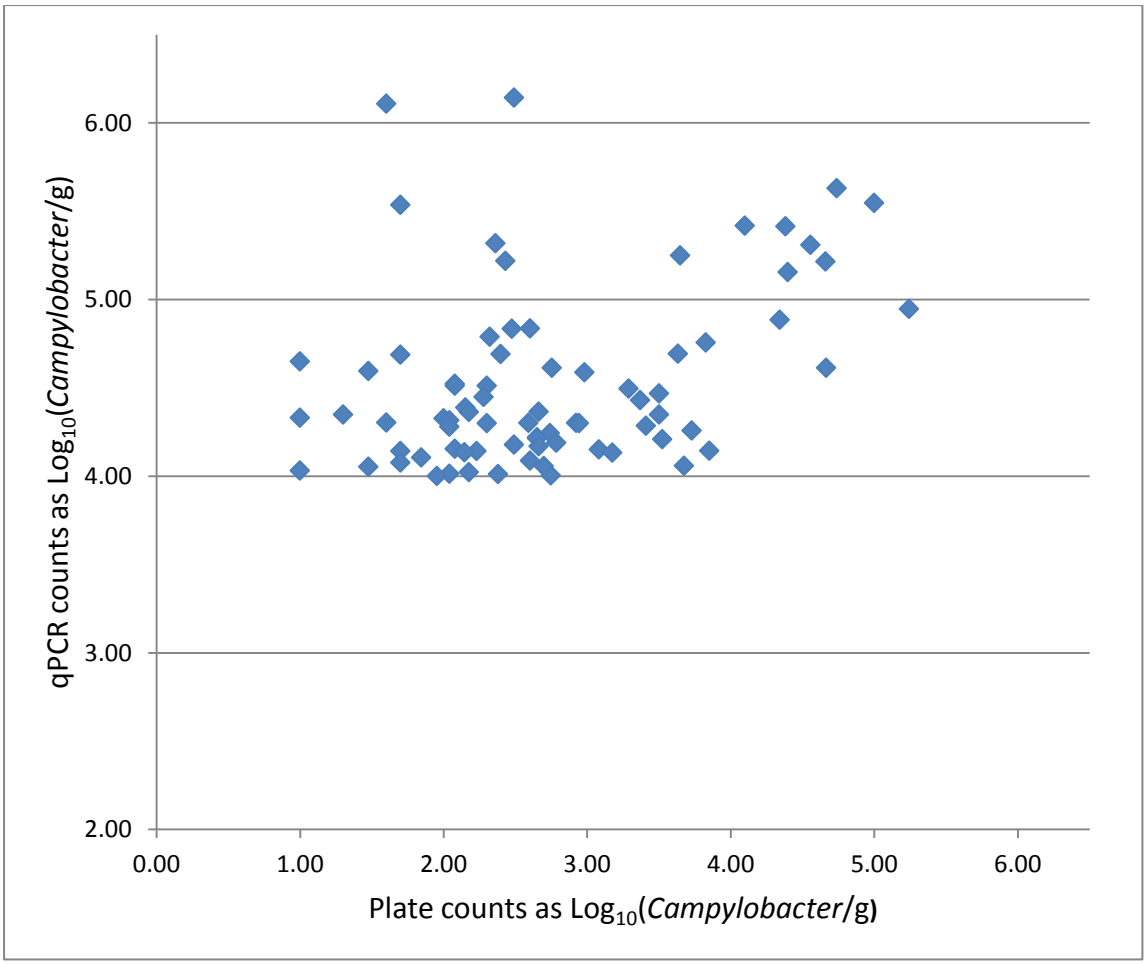
FERA did not supply their results to AFBI but informed the FSA project officer that the sensitivity of the LAMP test was inferior to the AFBI qPCR and that further refinements of the analytical procedures, such as immunocapture, would therefore be undertaken. AFBI was not informed of subsequent findings of the TSB project.

Objective 16. Application of *Campylobacter* real time PCR assay to chicken skin samples to investigate the correlation with campylobacter culture enumerations.

During the time that this project, M01060, was in progress Food Microbiology staff were also undertaking the enumeration of campylobacters on the neck skins of retail broiler chickens, as part of the Food Standards Agency survey (http://www.food.gov.uk/sites/default/files/multimedia/pdfs/board/board-papers-2013/fsa-130904.pdf). Subsequently the survey was continued as FSA Project FS241044 (http://www.food.gov.uk/sites/default/files/campylobacter-retail-survey-final-report.pdf). In view of the success of the qPCR detection procedures, using bootswabs as the matrix, resources were made available for a brief study into the possibility of the qPCR methodology being applied to quantify the campylobacters present on the neck skin samples.

Accordingly samples (n=339) of neck skin homogenate (1:9 in maximum recovery diluent) were supplied by staff undertaking the survey of retail broilers, and subsequently prepared for qPCR analysis in the same manner as bootswab diluent. However, the qPCR assay was seen to lack sensitivity. Using the samples for which qPCR predicted a result of Log_{10} 4 cfu/g of *Campylobacter* or greater, Fig. 4, it can be seen that qPCR could not be used to predict the numbers of campylobacters on neckskin samples. Accordingly, given the limited resources available, and with the agreement of the FSA project officer, no further work on this Objective was undertaken.

Figure 4. Comparison of plate counts and qPCR estimates of *Campylobacter* numbers for neck skin samples (n=76).



Objective 17. Final report detailing the performance and practicality of developed methods for use as a rapid on-farm diagnostic to determine flock colonisation status by *Campylobacter*.

This report constitutes Objective 17 and the outputs from this study, Objective 18, are listed below.

Outputs.

- Madden, R.H., Ball, H.J., Hutchison, M., Young, F.J., Taylor, M.J. (2014). A quantitative comparison of sample matrices for the detection of

Campylobacter in broiler houses. Romanian Biotechnological Letters, 19(5):9785-9791

- Madden, R.H., Ball, H.J., Hutchison, M., Young, F.J., Taylor, M.J. (2013). Determination of the most appropriate matrix for use in detecting *Campylobacter* in broiler houses. 17th International Workshop of *Campylobacter, Helicobacter* and Related Organisms, AECC, Aberdeen, Scotland, pp 126-126, September 2013.
- Taylor, A. J., Ball, H. J., Hutchinson, M., Madden, R. H., Young, F. J. (2013). Development of a rapid on-farm test for campylobacter. 2013 *Campylobacter* Strategy Workshop (BBSRC, DEFRA, FSA), Belfry, BT76 9PR, March 2013.
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Appendix I

Project Objectives.

Obj. No.	Objective Description
01	Produce detailed sample collection, transport and culture protocols suitable for detection and enumeration of *Campylobacter* by industry in samples taken from poultry farms.
02	Contribute to "a review and consultation with industry on the practicality and cost of available technologies" to be undertaken by the VLA.
03	Consult with key providers (commercial) of rapid diagnostic technologies and appraise their products to determine applicability for rapid on farm testing
04	Determination of appropriate on-farm sampling methodologies/matrices to use with a range of rapid detection approaches
05	Undertake an evaluation of methods for treatment of test samples to yield appropriate antigens or nucleic acid for detection methods.
06	Undertake a preliminary evaluation of a range of detection methods (immunological and DNA-based) for the rapid detection of *Campylobacter* spp. in samples from farms.
07	Produce a report on objectives 1-6.
08	Undertake an evaluation of a range of detection methods (informed by the VLA review) for the rapid detection of *Campylobacter* spp. on farm.
09	Undertake validation of combined sampling, processing and detection methodologies using field samples.
10	Configuration of complete "best approach" methodology into a convenient easy-to-use 'farm-proof' format.
11	Laboratory validation of easy-to-use 'farm-proofed' protocol/device ('On-Farm test') using farm samples.
12	On-farm feasibility study of easy-to-use 'On-Farm test' using farm samples.
13	Model Farms studies: Practicality of using *Campylobacter* real time PCR boot swab assays to provide farmers with results prior to slaughter
14	National Farmer Union Farms studies: Practicality and initial assessment of any effect on behaviour of using *Campylobacter* real time PCR boot swabs assays sourced from NFU farms.
15	Research project farm screenings: Provision of *Campylobacter* screening of broiler houses in the context of FSA funded and industry campylobacter slaughterhouse studies including the TSB funded lamp rapid test
16	Application of *Campylobacter* real time PCR assay to chicken skin samples to investigate the correlation with *Campylobacter* culture enumerations.
17	Final report detailing the performance and practicality of developed methods for use as a rapid on-farm diagnostic to determine flock colonisation status by *Campylobacter*.
18	Produce peer-reviewed publications

Appendix II. Figures illustrating the effect of storage of bootswabs on persistence of campylobacters.

Figure A1. Total *Campylobacter* counts obtained per pair of bootswabs (n=10), during storage for up to four days in chill (4°C). Note that the y axis shows cfu/pair of bootswabs.

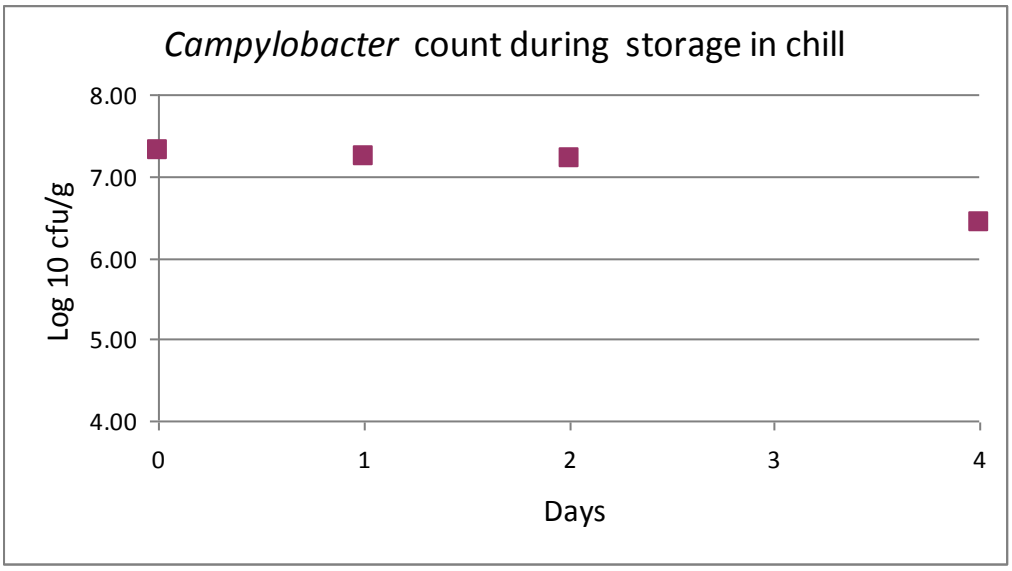
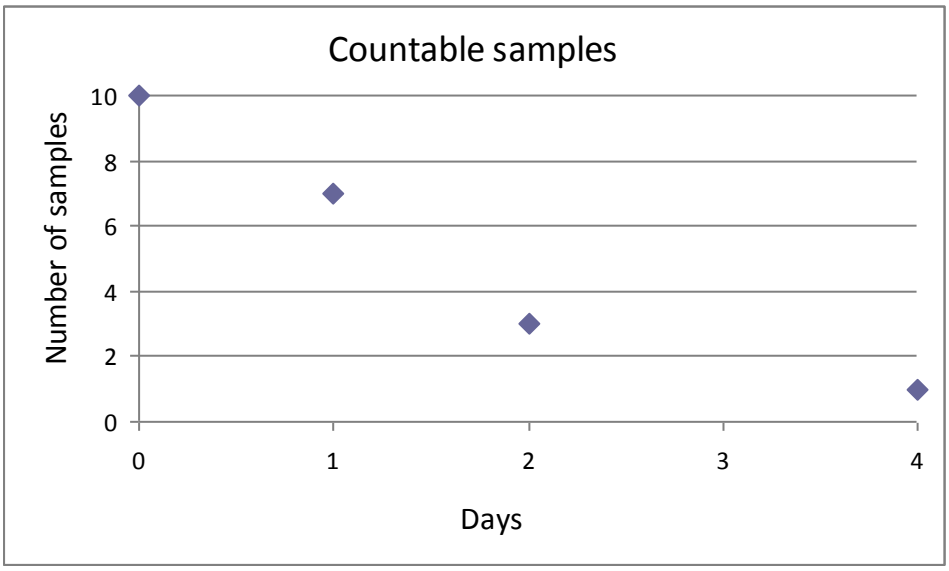


Figure A2. Number of bootswab samples stored at 20°C from which *Campylobacter* could be counted. Numbers of bootswab samples fall due to overgrowth by antibiotic resistant mesophilic bacteria.



APPENDIX III.

DRAFT POULTRY HOUSE SAMPLING PROTOCOL FOR *CAMPYLOBACTER* USING BOOTSWABS.

Materials required per sample to be collected:

2x pairs of disposable Tyvek overshoes

(Arco, Hull, UK. Reference No. 1703700)

1x pair of bootswabs (Tunika, Bowden and Knights, Thetford, GB)

1x sealable commercially sterile plastic bag

1xcontainer of sample buffer (10 ml maximum recovery diluent)

On farm.

1. Observing the normal biosecurity protocols, the sampler will arrive at the step-over barrier in the anteroom and prepare to enter the house.

The sampler **should NOT use the foot dips.**

2. A pair of clean, disposable Tyvek overshoes must be placed over the existing footwear of the sampler.
3. The sampler will then enter the house and a second pair of clean, disposable Tyvek overshoes will be placed over the first pair.
4. Disposable gloves will then be put on and a pair of bootswabs (Tunika), shall be placed over the outer overshoes.
5. The sampler will then walk the full length of the house and return to where he started. The two bootswabs will be carefully removed, and placed in the sealable plastic bag. The container with the sample buffer will be opened and the entire contents added to the bootswabs. The sealable bag will then be securely closed and safely stored.
6. The sampler will remove the outer pair of overshoes, change gloves and refit a fresh pair of overshoes followed by fresh bootswabs if another sample is required for that house.

7. Only after all sampling has been completed will the sampler proceed to the ante room.
8. The outer pair of overshoes with be removed before leaving the house observing normal biosecurity procedures.

Sample shipping.

If the company has a laboratory in the vicinity of the farm, the samples should be kept cool and delivered to the lab within 2 hours of sampling. If the laboratory is not in the locality, once collected the sample should be immediately put into an insulated cool box containing frozen freezer blocks or crushed ice. This is to keep the samples cold but not frozen, therefore samples should not come in contact with the coolant. Keeping samples chilled at 0^{o}C to 4^{o}C will help prevent unwanted bacteria from multiplying and improve the survival rate of the campylobacters during transport. Samples should be sent within 2 h of sampling, to arrive at the laboratory within 22 h of despatch. Sample testing at the lab should commence a maximum of 24 h after sample collection.

Sample analysis.

Bootswabs should be transferred to a stomacher bag and 40ml of maximum recovery diluent added. The sample should be blended in a Colworth stomacher, or similar, for 1 minute at medium power. Two separate aliquots of 0.1ml should be plated onto modified charcoal cefoperazone deoxycholate agar (mCCDA) and incubated in a microaerobic atmosphere (41.5°C, 44h±4h). The presence of *Campylobacter* spp. will be confirmed as per ISO-EN 10272-2:2006.

For confirmation, take from each plate of mCCDA at least one colony considered to be typical or suspected as being *Campylobacter* and a further four colonies if the first is negative. In summary the confirmatory procedures are:

1. Streak individual colonies onto Columbia blood agar plates and incubate in a microaerobic atmosphere at 41.5 °C for 24 h to 48 h.
2. Use the pure cultures for examination of morphology, motility, microaerobic growth at 25 °C, aerobic growth at 41.5 °C and the presence of oxidase.

Campylobacter spp. should not grow at 25 °C, or in air at 41.5 °C. They should exhibit characteristic curved/spiral morphology and “corkscrew” motility and a positive oxidase reaction.

Appendix IV. Cultures used to assess sensitivity and selectivity of two LFD devices.

Both *C. lari* isolates gave faint positive results with the ImmunoCard LFD, but the Singlepath kit gave a negative result for RC513.

Name	**Code**	**Source**
C. *jejuni*	RC203	Poultry
C. *jejuni*	RC135	Poultry
C. *jejuni*	68285	Clinical
C. *jejuni*	69884	Clinical
C. *coli*	RC200	Poultry
C. *coli*	70273	Clinical
Atypical C. *coli*	RC271	Poultry
C. *lari*	RC376	Poultry
C. *lari*	RC513	Poultry

Organism	**AFBI code**	**Source**
A. *butzleri*	328 24	Poultry
A. *butzleri*	NCTC 10828	Type Culture
A. *butzleri*	350 48	Poultry
A. *butzleri*	265 48	Poultry
A. *skirrowii*	LMG 9880	Type Culture
H. *pullorum*	NCTC 13155	Type Culture
H. *canadensis*	NCTC 13241	Type Culture
Unidentified	H2C1 19/08/11	Caecal Swab

Appendix V. Methodology for the use of the QIAGEN mericon Pathogen Detection *Campylobacter* spp. kit (290035).

Sample preparation procedures

1. Add 1040µl of Multiplex PCR master mix to the mericon Assay tube.
2. Mix by pipetting up and down several times. The assay is stable at 4^{o}C for 1 month.
3. Reconstitute positive control DNA by the addition of 200 µl of QuantiTech nucleic acid dilution buffer.
4. Using the QIAGEN QIAgility automated platform pipette 5 µl of mericon *Campylobacter* spp Pathogen detection assay PCR mix into each reaction tube.
5. Add 5 µl of extracted DNA or negative control or E positive control avoiding bubbles at the bottom of the wells, centrifuge if necessary.
6. Cycle at 5min at 95 $^{\circ}$C, then 40 cycles of 15 sec for 95 $^{\circ}$C , 25 sec at 60 $^{\circ}$C followed by 10sec at 72 $^{\circ}$C using FAM (*Campylobacter* target) and VIC (Internal positive control IPC) data collection.

Appendix VI. Specificity of the Loopamp *Campylobacter* Detection Kit

Table A1. Specificity of LAMP assay for *Campylobacter* spp.

Species	AFBI Code	Source	Log_{10} cfu/ml	Agarose gel visualisation
C. *jejuni*	RC203	Poultry	4.41	+[1]
C. *jejuni*	RC135	Poultry	7.16	+
C. *jejuni*	68285	Clinical	7.55	+
C. *jejuni*	69884	Clinical	7.38	+
C. *coli*	70273	Clinical	8.51	+
Atypical C. *coli*	RC271	Poultry	8.92	+
C. *lari*	RC376	Poultry	8.41	+
C. *lari*	RC513	Poultry	8.22	-[2]

[1]Positive, [2]Negative.

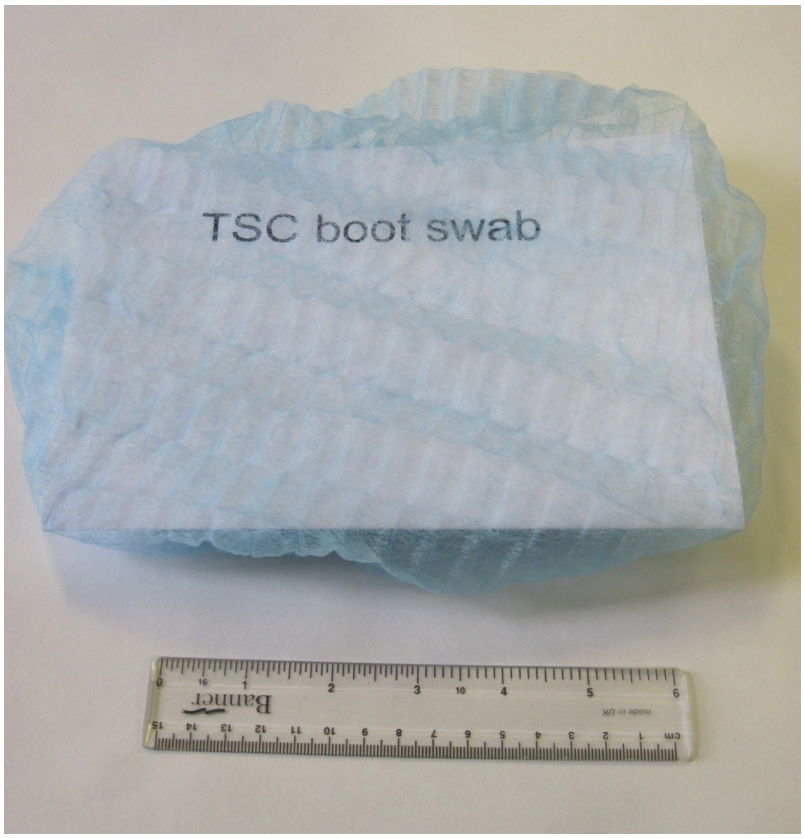
Table A2. Specificity of LAMP assay for non-*Campylobacter* spp.

Species	AFBI Code	Source	Log_{10} cfu/ml	Agarose gel visualisation
A. *butzleri*	328 24	Poultry	7.74	+
A. *butzleri*	NCTC 10828	Type Culture	7.33	+
A. *butzleri*	350 48	Poultry	8.08	-
A. *butzleri*	265 48	Poultry	7.86	-
A. *skirrowii*	LMG 9880	Type Culture	7.55	+
H. *pullorum*	NCTC 13155	Type Culture	7.77	-
H. *canadensis*	NCTC 13241	Type Culture	8.60	+
Unidentified	H2C1 19/08/11	Caecal Swab	8.92	-

Appendix VII. Technical Service Consultants bootswabs.

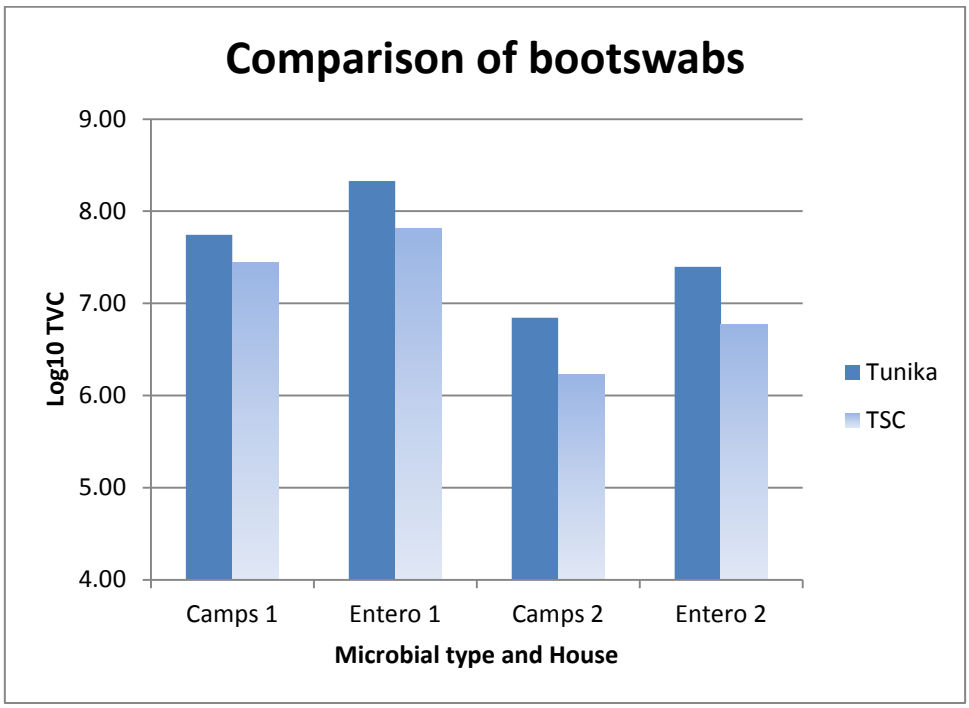
Bootswabs available from TSC at http://tscswabs.co.uk/Products/167/151/TS-BSW5-B-Poultry-Boot-Swab-5-Pairs-of-pre-moistened-blue-Boot-Swabs-including-overboots

Figure A3 Picture of a TSC bootswab showing its lightweight structure.



Tunika and TSC bootswabs were compared for their ability to recover *Campylobacter* and *Enterobacteriaceae* from two houses. In each of two houses the operator walked the full length of the house and returned, wearing two TSC swabs (as per manufacturer's instructions) on one foot and one Tunika bootswab on the other. This was repeated five times.

Figure A4. Recoveries of two types of bacteria, *Campylobacter* (Camps) and *Enterobacteriaceae* (Entero), on two types on bootswab (Tunika and TSC). Sampling was undertaken in two houses (1 and 2). Each bar is the mean of five samples.



Appendix VIII. Instruction sheet supplied to broiler farmers with bootswab sampling kit.

Boot swab sampling protocol for *Campylobacter* detection in poultry houses

Kit contents:
1x addressed postage paid large letter tear proof envelope

1x pair Tunika boot swabs

1x labelled sample bag

1x instructions

Equipment required not in the kit
Plastic overshoes

Pen for completing label on sample bags with house number etc

When to sample

1. Sample all houses no earlier than 24hrs before 1st thin. Results will be texted to the farm mobile number within 24hr of laboratory receipt of samples.
2. If a house was **negative before thin (or if no pre-thin result has been received then always resample)**, re-sample that house no sooner than 24hrs before clearing.

Instructions per house

1. Clearly complete the label on the sample bag with:
    a. Farm name
    b. house number
    c. date sampled

    Results will be reported together with the farm, house and sampling date.
2. The sampler should observe the normal biosecurity protocols up to the step-over barrier in the anteroom and prepare to enter the house. **The sampler should NOT use any foot dips in the anteroom.**

3. After crossing the step-over barrier into the house, and once footwear for wearing inside the house has been put on, a pair of clean, disposable plastic bag overshoe should be put on top (not included in the kit).
4. The pair of boot swabs present in the sampling pack should then be placed over the overshoes.
5. The sampler will then walk the full length of the house and return. Remove the two boot swabs (one per foot) carefully, place in the labelled sealable bag provided.

6. As much air as possible should be removed from the bag before sealing (as flat as possible) and before being placed in the pre-addressed / postage paid envelope.
   Check the sample bag is labelled before sealing the envelope.
7. This large letter should be posted by Royal Mail on the day of sampling (normally before 3.00pm to meet that day's postal pickup). This should ensure the sample arrives at the laboratory the following day enabling results to be reported within 24hr. If the postal collection is missed, houses may be automatically re-sampled resulting in increased sampling costs.

Any questions or queries should be directed to Malcolm Taylor (Food Hygiene Unit, Agri-Food and Biosciences Institute, Newforge lane Belfast BT9 5PX tel 02890255313, 07533064599, malcolm.taylor@afbini.gov.uk)

Thank you for taking the time to collect these samples.

Appendix IX. Assessments of the DNA extraction procedures and qPCR to detect campylobacters.

Initial qPCR studies reported Ct values but, for consistency and ease of reporting, all runs undertaken included a dilution series prepared from material in which campylobacters had been enumerated. Samples containing from approximately 10^1 to 10^6 cfu/ml were included and the system software used to derive a calibration curve with results presented as $\log_{10}$(cfu/ml). This enabled all results to be presented as $\log_{10}$(cfu/ml). The slope of the line under ideal conditions would be 3.32 cycles / $\log_{10}$(cfu/ml) with values greater than 90% of this, i.e. 2.99, but less than 110% (3.65) being considered acceptable by the equipment manufacturer: https://www.qiagen.com/resources/download.aspx?id=d6191d0e-701b-4eb1-bafa-d7ab7677875f&lang=en

Thus all runs of the DNA extraction and qPCR system had their efficiency confirmed as being acceptable.

Selectivity was assessed using a panel of 75 pure cultures, referred to as Panel B, and which comprised 30 *Campylobacter* strains (including 20 *C. jejuni*, 10 *C. coli* and 5 *C. lari*), 10 *Arcobacter* spp. and 10 *Helicobacter* spp. along with 20 other poultry associated non-*Campylobacteraceae*. All *Campylobacter* spp. were detected, but no non-campylobacters.

To assess repeatability DNA extracted from 8 bootswab samples was split into 10 sub-samples and analysed, Table A3.

Table A3. Repeatability of qPCR using eight bootswab samples analysed 10-fold.

	$\log_{10}$(cfu/mL)		
Bootswab	**Mean[1]**	**stdev**	**% stdev**
1	5.71	0.15	2.6%
2	5.37	0.11	2.0%
3	6.57	0.09	1.4%
4	6.24	0.15	2.4%
5	5.30	0.08	1.5%
6	6.46	0.10	1.6%
7	6.46	0.07	1.0%
8	6.76	0.08	1.1%

[1]Determinations

The mean standard deviation was 1.7%, indicating very consistent results were obtained. The same bootswab samples then had DNA extracted 10 times and the qPCr was undertaken, Table ?

Table A4. Repeatability of DNA extraction for eight bootswab samples each extracted 10 times, then subjected to qPCR.

	Log10(cfu/mL)		
Bootswab	**Mean[1]**	**stdev**	**% stedev**
1	5.79	0.12	2.0%
2	5.36	0.07	1.3%
3	6.75	0.08	1.2%
4	6.38	0.11	1.7%
5	5.34	0.11	2.1%
6	6.53	0.12	1.8%
7	6.53	0.12	1.9%
8	6.82	0.23	3.4%

[1]Determinations

The mean standard deviation was 1.9%, again showing consistent results.

In addition a comparison of qPCR results using DNA from 92 bootswabs (replicate samples) found no significant difference between the results (p=0.306, Students t test), as did analysis of 40 bootswab samples in duplicate (p=0.342, Students t test). A significant correlation ($p < 0.001$) was observed between Log_{10}(cfu/mL) for bootswabs (n=82) derived by ISO standard enumeration and the qPCR method.

As more analyses were undertaken a retrospective study of 338 results for the ability of the two tests applied (qPCR and conventional enumeration) to detect if a sample was positive or negative with respect to campylobacters was undertaken. Using the unconditional test of equivalence for difference of two related binomial proportions, and applying the null hypothesis that the difference in these proportions is greater than 5%, the exact p-value is P=0.0002702, hence two methods are producing equivalent results. Therefore the mericon qPCR kit, as applied in this study, gave equivalent results for the detection of campylobacters as did BS EN ISO 10272-2:2006.