Detection and enumeration of zoonotic pathogens in agricultural effluents.

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Abstract
Livestock production is vital to the world economy. However, intensive animal production is known to require large inputs of raw materials and energy, whilst producing greater amounts and concentration of waste material than do the traditional methods of production. Although the wastes, in the form of slurries and manures, are high in plant nutrients and organic matter, they can also contain zoonotic pathogens including *Campylobacter, Salmonella* and *E. coli* O157:H7; presenting an important risk of the transmission of infectious diseases into the human population and other animal infection. Comprehensive investigations have been conducted specifically on the important human pathogen *Campylobacter*, both in its culturable, and its viable but non-culturable forms, using traditional cultivation and contemporary molecular techniques i.e. ImmunoMagnetic Separation (IMS) and Quantitative Real-time Polymerase Chain Reaction (qRT-PCR). Results show that 21.6% of surface water samples (n=180) taken at three dairy/beef cattle and sheep farms in Scotland were positive for *Campylobacter* using the culturing method as detailed below.

Introduction
The level to which livestock may contribute to human illness through contamination of watercourses is unknown. Water microbiology mainly concentrates on the safety of water sources for public supply; it is known, however, that agricultural activity results in the presence of enteric microorganisms in natural waters [1]. Livestock production is essential to the world economy, as well as providing food, employment and revenue for its population. In recent times this industry has become much more intensive, requiring large inputs of raw materials and energy, whilst producing greater – and geographically more concentrated - amounts of waste material than traditional methods of production. Animal waste materials are valuable sources of plant nutrients and organic matter which can benefit soil structure and fertility but they are known to diminish the qualities of soil, water and land, if used inappropriately. These materials may also contain pathogenic microorganisms including bacteria, viruses and protozoa e.g. *E.coli* O157:H7, *Campylobacter* and *Salmonella* [2] which may, following application to land, potentially result in microbiological contamination of the water environment .

The World Health Organisation named *Campylobacter* spp. as being responsible for the highest proportion of bacterial gastroenteritis in the world, noting that infection commonly follows the ingestion of undercooked poultry or contaminated water [3]. Their roles in human disease have only been recognised in the last 35 years, following Skirrow's development of a method to culture these fastidious organisms in the routine laboratory [4], and more recently their contribution to Guilain-Barré syndrome has become appreciated. These Gram-negative, helical rods are extremely motile owing to uni- or bi-polar flagella which are also involved in the invasion of the gastrointestinal tract and pathogenesis (i.e. secretion of virulence proteins, autoagglutination and the formation of microcolonies) [5]. The bacteria are widely found as part of the normal gut flora in domestic and wild animals, including sheep, cattle, birds and pigs [6].

Two morphologies of *Campylobacter jejuni* were described recently by Tangwatcharin *et al.* (2006) [7]; these were shown to be the routinely observed helical viable form and a viable but non-culturable (VBNC) modified coccoid form. This transformation may be initiated by response to environmental stress factors including nutrient reduction, osmotic shock, and changes to temperature and pH [8]. Some research has alluded to the fact that pathogens in the VBNC state may be more virulent than cells which are under no external stress [9]. There continues to be controversy regarding this VBNC state; some authors indicate that VBNC forms of *Campylobacter jejuni* are able to cause infection, and revert from the coccoid to spiral form in warm-blooded animals and birds, after passage through

the alimentary canal of the host [8]. Others do not provide such evidence of the resuscitative nature of VBNC cells and suggest that this form may be degenerative [10]. It is therefore important to understand the potentials that these VBNC forms in effluents might have in the infection of humans and animals.

Materials and Methods

The study focused on adapting established analytical methods in order to apply them to environmental samples and farm effluents that contain very high levels of commensal, pathogenic and environmental organisms, as well as particulate matter and other polluting substances. Scientific data were gathered from a number of specific agricultural sites, in Scotland, to provide information as to how the activities taking place may be contributing to the pathogenic bacterial flora in the environment. Comprehensive investigations have been conducted on the important human pathogen *Campylobacter jejuni*, which by its nature is very difficult to isolate, both from "clean" waters and from effluents in farms in Western and South-Western Scotland.

Samples of water, effluent, slurry and manure were taken at intervals over a period of 18 months at three farms in Western and South-Western Scotland housing dairy/beef cattle and sheep. Water and effluent samples were filtered through a 0.45µm membrane and then membranes were placed in an enrichment medium, Bolton's Broth, for 48 hours at 42ºC in a microaerobic atmosphere. Slurry and manure samples were homogenised in a stomacher for 10 minutes prior to dilution, filtration and enrichment. After 48 hours of enrichment, cultures were streak inoculated onto Modified Cefoperazone Charcoal Deoxycholate Agar and again incubated in a microaerobic atmosphere for 48 hours at 42ºC. Characteristic grey/buff colonies were presumptively identified as *Campylobacter* and confirmed by microscopy and biochemical testing using API Campy.

A further method was developed to detect and enumerate *Campylobacter jejuni* using quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) allowing enumeration of the total number of *C. jejuni*, including both the viable and VBNC form. To counteract the PCR inhibiting substances inherent in environmental and faecal samples, pre-treatment with tosylactivated Dynabeads M280 coated with Rabbit anti-*Campylobacter* Polyclonal antibodies (to isolate any target bacteria from the sample matrix) has also been assessed for suitability. Samples were processed as previously discussed and then the membranes were suspended in 10 ml STE Buffer (0.1M NaCl, 10mM Tris, 1mM EDTA at pH 7.6) and centrifuged at 15000 rpm for 30 minutes at 4ºC. The pellet was then resuspended in 1 ml Phosphate Saline Buffer. Genomic DNA was extracted from filtered water samples using DNeasy Blood and Tissue Kit (Qiagen) and DNA from filtered slurry and manure samples was extracted using QIAampDNA stool Minikit (Qiagen). The concentration of genomic DNA was assessed by measuring absorbance at A_{260}.

Quantitative PCR detection and enumeration of *C. jejuni* was carried out using previously published primers for the mapA gene which encodes for a membrane protein [11]; the primer sequence and annealing temperatures are detailed in Table 1. For quantification purposes, ten-fold dilutions were prepared using genomic DNA from a wild type *C. jejuni* isolated from one of the study farms ranging from ranging from $10^{0} – 10^{-9}$. Amplification was conducted in 25 µl reaction mixtures containing 12.5 µl PerfeCTa™ SYBR® Green FastMix™, 75 nM of each primer, 6 µl of DNase and RNase free deionised water and 5 µl of template DNA, in a Bio-Rad CFX96 Real-Time System.

Table 1: qPCR parameters for *C. jejuni* mapA gene.

Target	Primer sequence (5'-3')	Length (bp)	Annealing temperature	Amplicon size (bp)	Protocol for qPCR
C. jejuni mapA gene	5' -GGT TTT GAA GCA AAG ATT AAA GG 3' - AAG CAA TAC CAG TGT CTA AAG TGC	23 24	59ºC	94	95ºC for 15min , and 50 cycles of 94ºC for 15 s, 58ºC for 30 s, 72ºC for 30 s

Results

The findings of the study to date suggest that livestock rearing does result in the introduction of Faecal Indicator Organisms (FIO) and enteric pathogenic organisms into the water environment. *Campylobacter* spp. were regularly isolated from rivers running through agricultural land and demonstrated as not being naturally occurring as the pathogen has never been isolated from the spring the watercourse originates from i.e. 21.6% of surface water samples (n=180) taken at three dairy/beef cattle and sheep farms in Scotland were positive for *Campylobacter* using the culturing method as detailed above; the most prevalent species being *Campylobacter jejuni*.

Table 2: Levels of confirmed isolations of *Campylobacter* spp. at the study farms from March 2011 – November 2012 using mCCDA with enrichment.

Sample type	No. of samples taken	No. of positive isolations	% of positive isolations
Water / effluent	180	39	21.6
Slurry / manure	21	7	33.3

The qRT-PCR method which has been recently developed has shown promising results. A standard curve was produced which will allow enumeration of the total numbers of *C. jejuni* in water and other agricultural effluents in both the viable and VBNC form.

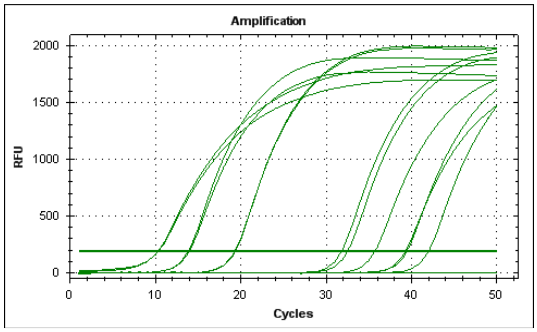

Figure 1: Amplification plot for 10- fold serial dilutions from 10^{0} – 10^{-7}.

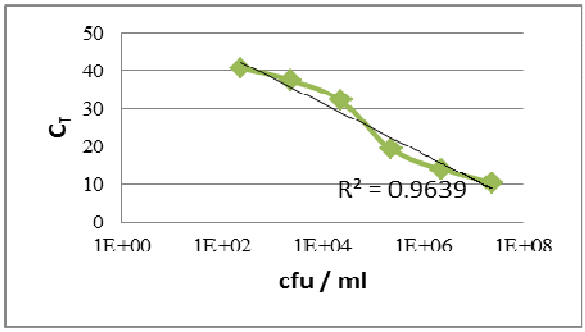

Figure 2: Standard curve derived from ten-fold serial dilutions.

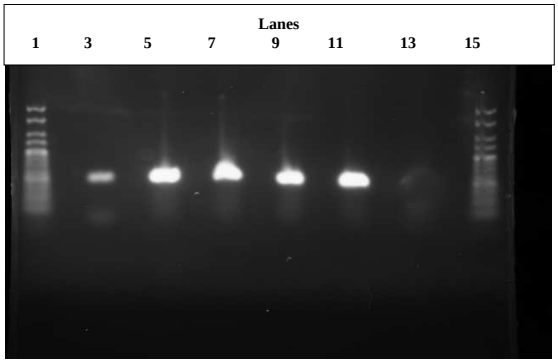

Figure 3: 2% Agarose gel showing 94bp PCR product.

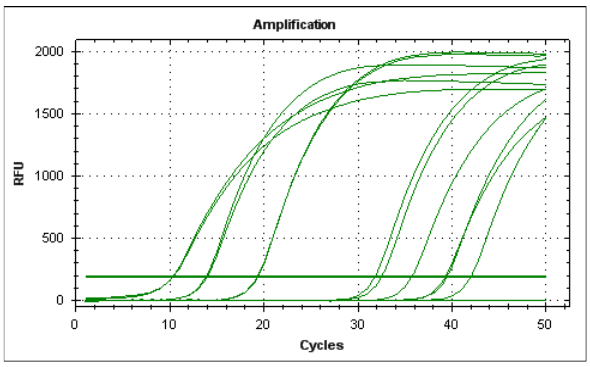

Figure 4: Amplification plot showing amplified product in positive controls and from farm water samples.

Figure 1 shows an amplification plot used in the construction of the standard curve as shown in Figure 2. The qRT-PCR method provided much greater sensitivity and the ability to now gather data on *Campylobacter jejuni* levels, of both forms, in considerably less time than traditional culturing methods which will enable rapid detection and/or quantification of pathogens in a sample. Figure 3 is a photograph taken of a 2% Agarose gel electrophoresis (120V for 1 hour) of the 94 bp PCR product as encoded by the mapA gene within the *C. jejuni* genome. Lanes (1) & (15) contained 6µl of Hyperladder V (Bioline), (3) & (5) contained DNA extracted from *C. jejuni* isolated from river water on two separate days, (7) contained DNA extracted from *C. jejuni* obtained from faeces from 7 day old lambs, (9) contained DNA extracted from *C. jejuni* isolated from 2 day old calves, (11) contained

DNA from *C. jejuni* NCTC 11322 and (13) was the negative control. From the results, it is apparent that the fragment was amplified from all the samples and confirmed the presence of the *C. jejuni*. The amplification plot in Figure 4 demonstrates a positive result for the presence of *C. jejuni* DNA in water samples after filtration, DNA extraction and qRT-PCR (without the requirement of prior enrichment).

Conclusion and perspectives

Results obtained from culturing *C. jejuni* by the traditional viable count method can be compared with that from the qRT-PCR method to obtain a total number of genome copies including those numbers in the VBNC state, providing valuable data which will provide a more accurate assessment of the health risk and microbial pollution attributed to agricultural activity.

This research will increase our knowledge of the microbiology associated with livestock production, and provide data that will inform the industry, and encourage the adoption of good waste management and animal husbandry practices in order to reduce the potential of the transfer of zoonotic organisms to the environment and so lessen the risks to human and animal health.

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