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Detection of *Campylobacter* and *Escherichia coli* O157:H7 from filth flies by polymerase chain reaction

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Abstract. Flies (Diptera: Muscidae) that breed in faeces and other organic refuse (filth flies) have been implicated as vectors of pathogenic bacteria including *Escherichia coli* O157:H7, which cause haemorrhagic colitis in humans, and *Campylobacter*, which is the principal causative agent of human enteritis. The potential role of filth flies in the epidemiology of these pathogens in the United States was investigated by examining the prevalence of *Campylobacter* spp. and *E. coli* O157:H7 from two Arkansas turkey facilities. Polymerase chain reaction was conducted on DNA extractions of individual *Musca domestica* Linnaeus, *Stomoxys calcitrans* (Linnaeus), *Hydrotaea aenescens* (Wiedemann), *Adia cinerella* Fallen and turkey faecal samples using primers specific for *E. coli* H7, O157 and *Campylobacter* spp. Culturing verified that the flies were carrying viable *Campylobacter* spp. and *E. coli* O157:H7. Results from this study indicated that *M. domestica*, *S. calcitrans*, *H. aenescens* and Anthomyids are capable of carrying *Campylobacter* in North American poultry facilities and that the *E. coli* O157:H7 is carried by house flies and black dump flies associated with poultry. This PCR method provided a rapid and effective method to identify *Campylobacter* spp. and *E. coli* O157:H7 directly from individual filth flies.

Key words. *Adia cinerella*, *Campylobacter*, *Escherichia coli*, *Hydrotaea aenescens*, DNA, house fly, molecular diagnostics, stable fly.

Introduction

Campylobacter species are one of the most common causes of bacterial diarrhoea in the United States, causing an estimated 2.45 million illnesses and 124 deaths each year in the United States (Mead *et al*., 1999). The main source of *Campylobacter* infections is the consumption of contaminated food, the most important of which is poultry (Deming *et al*., 1987; Craven *et al*., 2000). Epidemiological investigations have associated *Campylobacter* infection with eating chicken (Craven *et al*., 2000). *Escherichia coli* O157:H7 (EHEC O157:H7) has emerged as the leading cause of enterohaemorrhagic colitis and is becoming one of the most important food-borne human pathogens of animal origin (Altekrues *et al*., 1997). An estimated 73 480 illnesses, 62 458 hospitalizations and 61 deaths occur each year in the United States from this pathogen (Mead *et al*., 1999).

In poultry production facilities the occurrence of *Campylobacter* spp. (Montrose *et al*., 1985), *Salmonella* spp. (Cohen *et al*., 1994) and *E. coli* O157:H7 (Heuvelink *et al*., 1999) in faecal samples is well documented. Muscidae and Anthomyiidae dipteran species including the house fly, *Musca domestica* L., black dump fly, *Hydrotaea aenescens* (Wiedemann), stable fly, *Stomoxys calcitrans* (L.) and *Adia cinerella* Fallen, are commonly referred to as filth flies because of their requirement to breed in a substrate containing manure (house fly, stable fly, *A. cinerella*) or as in the black dump fly, acting as a predator of house fly larvae. *Salmonella* spp. have been detected on house fly and black dump fly in the United States and other parts of the world (Greenberg, 1971; Olsen & Hammack, 2000; Mian *et al*., 2002) and recently it has been documented that house flies carry *E. coli* O157:H7 in Japan (Iwasa *et al*., 1999; Moriya *et al*., 1999). House flies have also been documented as being able to transmit *Campylobacter jejuni* in laboratory

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studies (Shane *et al*., 1984), as well as *Campylobacter* spp. in Pakistan (Khalil *et al*., 1994) and Norway (Rosef & Kapperud, 1983). Recently, Mian *et al*. (2002) documented that filth flies associated with dairies and commercial egg layer poultry houses in San Bernardino County, California, U.S.A. were carrying *Salmonella* spp.

Polymerase chain reaction (PCR) methodology has been successfully employed to detect *Aeromonas caviae* from individual house flies (Nayduch *et al*., 2001) and *Borrelia lonestari* in the lone star tick, *Amblyomma americanum* (Linnaeus) (Bacon *et al*., 2003). Therefore, the objectives of this study were to detect pathogens carried by the house fly and other filth flies in turkey production facilities and establish their seasonal potential to spread these infectious organisms within and among turkey farms. In addition, we developed a molecular diagnostic procedure using PCR that identifies the presence of pathogens such as *Campylobacter jejuni* and *E. coli* O157:H7 carried by filth flies within 6 h of their collection in the field.

Methods

Sample collection

House fly, black dump fly, stable fly and *A. cinerella* were collected within and among turkey finishing facilities at two turkey farms located in north-west Arkansas, U.S.A. from 26 June to 12 November 2002. The two farms were ≈ 0.5 km apart, with Farm 1 consisting of two 91.4×12.2 m brooder houses separated by 151.5 m from four 121.2×12.2 m finishing houses. Each brooder house was separated by 20 m, as were each of the finishing houses. Farm 2 consisted of one 91.4×12.2 brooder house connected directly to two 121.2×12.2 m finishing houses. On a weekly basis, flies were collected from the inside and outside of the turkey fishing houses using individual pocket folding aerial nets (BioQuip Products, Rancho Dominquez, CA, U.S.A.). The aerial nets were autoclaved at 121°C for 30 min and carryover DNA in the aerial nets was exposed to UV light using a CL-1000 crosslinker (UVP Inc., Upland, CA, U.S.A.) for 15 min to prevent PCR amplification and possible contamination between samples.

Field collections of filth flies and faecal samples

Flies collected in the aerial nets were killed by exposure to potassium cyanide in a killing jar immediately after field collection for 20 min and the net folded and placed in a zip-lock bag and stored in a chest cooler for transportation back to the laboratory. In the laboratory, flies were identified to species and stored at −80°C. Voucher specimens, preserved in 95% ethanol, are maintained at the Arthropod Museum, Department of Entomology, University of Arkansas, Fayetteville, AR, U.S.A.

Fresh turkey and beef cattle faecal deposits were sampled on an alternate-week basis from each facility. Two sterile cotton swabs that contained phosphate-buffered water were pressed into six fresh faecal deposits in each of six sections of each turkey facility (six fresh faecal samples at each end and the centre and both sides of the facility) and each two swabs were then placed in sterile disposable culture tubes and capped.

Microbiological methods

In the laboratory, one faecal swab was left in the culture tube and stored at −80°C until used in PCR tests. The second swab was removed and placed in tubes containing 5 mL of thioglycollate enrichment broth and antibiotics for 24 h at 42°C. Samples were contained in a 2 L zip-lock plastic bag using an evacuation-replacement technique (Shane & Harrington, 1998) to remove 66% of the air and a gas cylinder was used to provide a 1–10% CO_2 replacement to support *Campylobacter* growth. After 24 h the bacteria were loop-transferred to Difco Brucella blood agar plates containing sheep blood and antibiotics and incubated for 24 h at 42°C. Samples were presumptively identified as *Campylobacter* under phase microscopy based on shape and motility and confirmed as *Campylobacter jejuni* by loop-harvesting and strains were stored in Difco brain heart infusion containing 20% glycerin at −80°C for confirmation later using PCR.

Filth flies were identified, sexed and homogenized in pools of 15 flies of each species and sex in 0.5 mL of phosphate-buffered saline and centrifuged for 3 min at $3000 \times \mathbf{g}$. For detection of *Campylobacter*, 1.0 μL of the supernatant was placed in sterile tubes that contained 20 mL of thioglycollate enrichment broth (containing five antibiotics; amphotericin at 2.0 mg/L, polymyxin b at 0.322 mg/L, cephalothin at 15.0 mg/L, trimethoprim at 5.0 mg/L, vancomycin at 10.0 mg/L) and the tubes incubated for 24 h at 42°C in a plastic zip-lock bag for use in the evacuation-replacement technique described above. Next, the bacteria were loop-transferred to Difco Brucella blood agar plates and the procedures described for faecal samples used to determine the presence of *Campylobacter*.

For detection of *E. coli* O157:H7, after the flies had been homogenized in 0.5 mL of phosphate-buffered saline and centrifuged for 3 min at $3000 \times \mathbf{g}$, samples were inoculated onto CHROMagar O157 (CHROMagar, Paris, France) plates and incubated at 37°C for 24 h following the procedures of Sasaki *et al*. (2000). The colonies of EHEC *E. coli* O157 appear violet on these plates.

DNA extraction, PCR and DNA sequencing

Of the remaining homogenate, 2 mL was centrifuged at $14\,000 \times \mathbf{g}$ for 5 min and DNA extracted from the bacterial pellet for PCR. Total genomic DNA was extracted from individual filth flies, faecal samples and bacterial pellets using the Puregene DNA extraction kit (Gentra, Minneapolis, MN, U.S.A.). Extracted DNA was

re-suspended in 50 μL Tris:EDTA pH 8.0 and frozen at −20°C. Genomic DNA for *Campylobacter jejuni* ssp. *jejuni* (Jones *et al.*) Veron and Chatelain (ATTC number 33560D), was obtained from the American Type Culture Collection (Manassas, VA, U.S.A.). *Escherichia coli* serotype O157:H7 genomic DNA was obtained from the USDA ARS National Animal Disease Center (Ames, IA, U.S.A.).

The MD16S1 and MD16S2 PCR primers amplified a 857 bp region of the mtDNA 16S gene from *Campylobacter* spp. (Denis *et al.*, 2001), RfbF and RfbR yield a 292 bp amplicon for *E. coli* serotype O157 (Hu *et al.*, 1999), and $FLIC_{h7}$-F and $FLIC_{h7}$-R amplify a 625 bp portion of the *E. coli* H7 flic gene (Gannon *et al.*, 1997). Two different PCR reactions were conducted, the first using the *Campylobacter* spp. and *E. coli* H7 PCR primer sets in a multiplex PCR assay and the second one using PCR primers for the *E. coli* O157 serotype. PCR was conducted following Szalanski *et al.* (1997) with a total reaction volume of 50 μL. Briefly, each PCR reaction consisted of 5.0 μL of 10 *Taq* DNA polymerase buffer (Promega, Madison, WI, U.S.A.), 4.0 μL of dNTPs (100 μM, Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.), 0.5 μL of each PCR primer (20 μM), 0.4 μL *Taq* DNA polymerase (2.0 units, Promega), 36.6 μL nanopure H_2O and 2.0 μL DNA template. A negative control was made by using sterile water instead of template DNA. The PCR profile consisted of a denaturation step of 94°C for 2 min, followed by 35 cycles of 94°C for 45 s, 48°C for 45 s and 72°C for 1 min, with a final extension step of 72°C for 5 min. PCR product were visualized using 1% agarose gel electrophoresis and diagnostic PCR amplicons were visualized and documented using a UVP biodoc-it system (UVP Inc., Upland, CA, U.S.A.). To test if the DNA extractions were yielding sufficient DNA for PCR, DNA extractions from 40 samples that were not positive for the *Campylobacter* or *E. coli* PCR primers were subjected to PCR using the invertebrate rDNA internal transcribed spacer 1 (ITS1) primers rDNA2 and rDNA1.58S (Szalanski & Owens, 2003). PCR product for the ITS1 amplicon was obtained for all 40 samples, indicating that sufficient DNA was present in the samples for PCR.

The PCR products from the *Campylobacter* and *E. coli* H7 and O157 primer sets were identified to *C. jejuni*, *E. coli* H7 and *E. coli* O157, respectively, by subjecting the PCR product from three separate filth fly amplifications to DNA sequencing. Amplified DNA was purified and concentrated using Microcon-PCR Filter Units (Millipore, Bedford, MA, U.S.A.). Samples were sent to The University of Arkansas DNA Sequencing Facility (Fayetteville, AR, U.S.A.) for direct sequencing in both directions using an ABI Prism 377 DNA sequencer. A BLAST search (http://www.ncbi.nlm.nih.gov/BLAST/) was applied to determine the bacterial species and/or serotype closest to the sequencing results based on DNA sequence similarity. Then, the GAP 1 program of GCG was used for sequence alignments and determination of the percentage of DNA similarity between the DNA sequence and the closest bacterial species and or serotype. The EMBL-GenBank accession numbers used for the sequence comparisons were as follows: *E. coli* serotype O19ab:H7, AF228495; *E. coli* serotype O53:H7, AF228496; *E. coli* serotype O157:H7, AF061251; *E. coli* serotype O157:H7, AF005429; *C. jejuni* strain B99/206, AF550630; and *C. jejuni* strain H99/240, AF550628.

Results

A total of 3603 filth flies were collected from the turkey finishing houses from June to November 2002 with representatives of four species: *S. calcitrans*, *A. cinerella*, *H. aenescens* and *M. domestica* (Table 1). Standard culture tests, as previously described, validated that viable *Campylobacter* spp. and *E. coli* O157:H7 DNA was being detected in the filth flies by PCR diagnostics using pools of 15 house flies each, taken from weekly collections, homogenized and cultured. In addition, samples of the bacteria were collected from the culture plates and PCR was used to specifically identify the bacteria. *Campylobacter* spp. and *E. coli* O157:H7 were detected by PCR of DNA extracted from whole, frozen flies (Fig. 1).

Campylobacter spp. was detected from 10.9% of the house flies, 23.3% of the black dump flies, 3.8% of *A. cinerella* and a single stable fly using PCR (Table 1). The *E. coli* H7 antigen was detected from 4.5% of the house flies, 1.3% of *A. cinerella* and 3.7% of the black dump flies (Table 1).

Table 1. Occurrence of *Campylobacter* spp., *Escherichia coli* H7 and *E. coli* O157 from filth flies and turkey fecal samples collected from two turkey facilities in Arkansas, between 26 June and 12 November 2002, using polymerase chain reaction.

Origin	Species/sample	*n*	*Campylobacter* (*n*, %)	*E. coli* H7 (*n*, %)	*E. coli* O157 (*n*, %)	*E. coli* O157:H7 (*n*, %)
Farm 1	*Stomoxys calcitrans*	10	1, 10.0%	0, 0%	0, 0%	0, 0%
Farm 2		0	0, 0%	0, 0%	0, 0%	0, 0%
Farm 1	*Adia cinerella*	167	6, 3.6%	2, 1.2%	0, 0%	0, 0%
Farm 2		73	3, 4.1%	1, 1.4%	0, 0%	0, 0%
Farm 1	*Hydrotaea aenescens*	552	141, 25.5%	20, 3.6%	10, 1.8%	2, 0.4%
Farm 2		315	61, 19.4%	12, 3.8%	4, 1.3%	4, 1.3%
Farm 1	*Musca domestica*	1426	181, 12.7%	69, 4.8%	18, 1.3%	9, 0.6%
Farm 2		1060	89, 8.4%	42, 4.0%	14, 1.3%	8, 0.8%
Farm 1	faecal sample	50	24, 48.0%	12, 24.0%	13, 26.0%	12, 24.0%
Farm 2	faecal sample	56	45, 80.4%	5, 8.9%	7, 12.5%	5, 8.9%

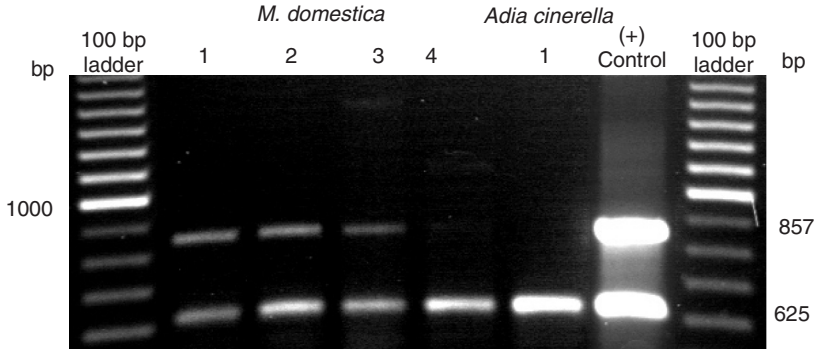

Fig. 1. Polymerase chain reaction assay for *Campylobacter* (857 bp. product) and *Escherichia coli* H7 (625 bp product), resolved on a 1% agarose gel. *Musca domestica* 1–3 are positive for both *Campylobacter* and *E. coli* H7; *Adia cinerella* 1 and *M. domestica* 4 are positive for only *E. coli* H7.

The Rfb primer set, which will produce a PCR product for the O157 antigen, was positive for 1.3% of the house flies and 1.6% of the black dump flies, all of which were also positive for the H7 serotype. The other flies positive for H7 but not for O157 were probably carrying another H7 serotype, which could include O22:H7, O159:H7 or O76:H7 (Hu *et al*., 1999). No genetic variation was observed among the three Rfb and FliC amplicons that were sequenced. Both the H7 and O157 serotypes showed high percentages of DNA similarity to DNA sequences on GenBank used for the GAP analysis, with a 100.0% DNA similarity to the two *E. coli* O157:H7 Rfb genes and 100.0 and 99.5% similarity to the *E. coli* O19ab:H7 and O53:H7 fliC genes, respectively. *Campylobacter* spp. was detected from all four fly species, with the black dump fly having the greatest proportion of infected flies (Table 1). All three of the *Campylobacter* 16S amplicons sequenced in this study were identical to each other and, based on the GAP analysis, were 99.6% similar to *C. jejuni* strains B99/206 and H99/240.

Of the 174 turkey faecal samples collected and cultured, 88 and 67% collected from the Farm 1 brooder and finishing houses, respectively, were found to be positive for *Campylobacter*, whereas 42 and 22% were found to be positive for *E. coli* O157, respectively. Turkey faecal samples from Farm 2 were 100 and 83% positive for *Campylobacter* and 50 and 42% positive for *E. coli* in the brooder and finishing houses, respectively. PCR analysis of duplicate faecal samples confirmed the culture results and allowed specific identification of *Campylobacter jejuni* and *E. coli* O157:H7 serotypes (Table 1).

Discussion

This study provides the first documented evidence that stable fly, house fly, black dump fly and *A. cinerella* carry *Campylobacter* spp. in turkey production facilities in the United States. In addition, this study provided the first evidence that *E. coli* O157:H7 is carried by house flies and black dump flies in the North American turkey production environment. Recently, Mian *et al*. (2002) documented that filth flies associated with dairies and commercial egg production facilities in San Bernardino Country, CA, U.S.A. were carrying *Salmonella* spp. using culturing to verify the presence of the pathogen. The presence of cattle in the immediate area of the turkey facilities was shown to provide a source of *Campylobacter* and *E. coli* O157:H7 in faecal droppings that could be a source of bacterial contamination by filth fly activity. Previous research has shown that house flies carry *E. coli* O157:H7 at cattle farms in Japan (Iwasa *et al*., 1999) and that the flies were capable of carrying and mechanically transmitting the pathogen from cattle farms to humans (Moriya *et al*., 1999) and to food (Sasaki *et al*., 2000).

Cattle produced on Farms 1 and 2 were between 6 and 9 months old. Twenty-four swab samples were taken from fresh faeces between 17 September and 19 November 2002 and 42% of the culture samples were positive for *E. coli*, whereas no *Campylobacter* was detected in the samples from animals of this age. However, 18 swabs of fresh faeces were taken from mature cows (> 4 years old) on farms immediately adjacent to Farms 1 and 2 and 56% of the samples were positive for *Campylobacter* and no samples from mature cows were positive for *E. coli* O157:H7 during this time period. Wells *et al*. (1991) reported that *E. coli* O157:H7 was recovered from 2.3% of calves, 3% of heifers but only one of 662 adult cows (0.15%) in Washington, U.S.A. In similar dairy cattle studies, Wesley *et al*. (2000) showed that *C. jejuni* was found in up to 53% of mature dairy cattle. PCR analysis of bacteria harvested from plates cultured in our study confirmed the *E. coli* O157:H7 serotypes and that the *Campylobacter* was *C. jejuni*.

It is interesting to note that 23.3% of the black dump flies were found to be carrying *Campylobacter* spp. and 1.6% were carrying *E. coli* O157:H7. This is important because the larvae of these flies have been reported to have potential as predators that could be used to control house flies in the United States and in various parts of Europe (Nolan & Kissam, 1985; Turner *et al*., 1992). Further investigation of the relationship between black dump fly and *Campylobacter* spp. is warranted. The information obtained in this study shows that the flies were carrying specific pathogens, but

did not provide information relative to the pathogenicity of the bacteria. The results of the PCR assay specifically identified pathogens that the flies had been exposed to inside or outside the turkey production facilities. Given the availability of PCR diagnostics tests for *Bacillus anthracis* (Qi *et al.* 2001), *Shigella* spp. (Houng *et al.*, 1997; Lindqvist, 1999) and many other bacterial pathogens, the molecular diagnostic technique involving PCR of pathogen DNA from insect DNA extractions presented in this study allows rapid detection of specific pathogens that filth flies are carrying. After flies have been collected at field sites, the DNA diagnostic test described here can be completed within 6 h after filth flies have reached the laboratory. Rapid pathogen detection in filth fly species provides a warning of animal or human premises contamination as well as pathogen distribution in the animal and human ecosystem. Based on our study, it is clear that filth flies, especially the house fly, are carriers of *E. coli* O157:H7 and *Campylobacter* spp. within the turkey, cattle and human components of the agro-ecosystem. Filth flies have a high potential for the distribution of many pathogens into the human population living in close proximity to animal production facilities that harbour fly populations. Future studies should be implemented to determine the dispersal of filth flies among the production animal and human components of the agro-ecosystem.

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