UNIVERSITY OF LIVERPOOL

The role of wildlife in the epidemiology of campylobacteriosis, salmonellosis and VTEC infections of domestic cattle

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor of Philosophy by

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Declaration

Apart from the guidance and advice received from my supervisors and peer colleagues that I have acknowledged, the work in this thesis is my own.

Angela Lahuerta Marin

Esta Tesis va dedicada a la memoria de Doña Angela Bermejo Ayensa, mi abuela querida

"Mi secreto es muy simple: no se ve bien sino con el corazon; lo esencial es invisible a los ojos."
Antoine de Saint-Exupery, El Principito

"Hay una circulación comun, una respiración comun. Todas las cosas están relacionadas."

Hipocrates de Kos

" Llenósele la fantasía de todo aquello que leía en los libros, así de encantamientos como de pendencias, batallas, desafíos, heridas, requiebros, amores, tormentas y disparates imposibles; y asentósele de tal modo en la imaginación que era verdad toda aquella máquina de aquellas soñadas soñadas invenciones que leía, que para él no había otra historia más cierta en el mundo".

Miguel de Cervantes Saavedra, El Ingenioso Hidalgo Don Quijote de la Mancha

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Abstract

The role of wildlife in the epidemiology of campylobacteriosis, salmonellosis and VTEC infections of domestic cattle

Campylobacter, *Salmonella* and VTEC infections account for the largest proportion of reported gastroenteric disease cases in human beings caused by zoonotic bacteria in the UK. Domestic cattle are considered an important source of these pathogens and there is increasing evidence that wildlife can become infected with these bacteria as well. However, the role, if any, of wildlife in their epidemiology remains unknown.

The aims of this study were to investigate the existence, and if possible prevalence, of these pathogens in cattle, wild rodents, other wild mammals and wild birds; to determine any spatial or habitat clustering of infection, inter-species transmission and the risk factors associated with these pathogens in six cattle farms in Cheshire (UK). This was done by a cross-sectional study carried out from July 2004 until May 2005.

E. coli O157 was isolated only from cattle on the one beef farm in the study. The overall prevalence was approx 20% both inJuly 04 and March 05. Analysis of PFGE patterns showed 12 different restriction profiles (RP), but there was one predominant RP, isolated mainly from adult stock and calves. PFGE comparison was made with *E. coli* O157 strains previously isolated from cattle and wildlife animals on this farm during 2002, and this demonstrated similarities of 45- 80% between current and archived *E. coli* O157 isolates. This suggests that there has been a shift in the predominant *E. coli* O157 strain on this herd over time.

The use of a newly developed microarray test allowed a survey for of 45 *E. coli* virulence genes in a subset of 400 *E. coli* isolates from healthy cattle and wildlife animals. A total of 70% of isolates carried virulence genes. The *iss*, *iroN* and *astA* genes were the most frequent. In addition, wild birds may be a possible reservoir for the *iss-iroN-mchF* gene profile that is associated with APEC pathotypes. Furthermore, similar virulence profiles were carried by *E. coli* isolates from cattle and wildlife on the same farm suggesting that transmission may be possible.

A further 200 *E. coli* isolates were microarray tested for both virulence and antibiotic resistance genes. A high proportion of *E. coli* isolates from wildlife carried antibiotic resistance genes (59%, n=155). Virulence genes and antibiotic resistance gene profiles seemed to be carried independently by isolates. Moreover, antibiotic resistance gene profiles were similar in isolates from the same farms. The genes that were carried in highest frequency were the *tem1 (βlactams), aadA1 (aminoglycoside) and tetA (tetracyclines)* .

The *eae* gene followed by the *vt1* gene was the most common VTEC virulence-associated gene isolated from cattle and wildlife. Risk factors were determined by univariate analysis and Generalised linear models (GLM) . *E. coli* from wild birds associated with farm land had a higher probability of carrying the *eae* gene. On the other hand, the probability of rodents carrying this gene was independent of the species of rodent. Significant numbers of *E. coli* isolates that carried the *vt1* gene also carried the eae gene in cattle and small rodents. The farm was a risk factor variable for cattle, wild birds, small rodents and large mammals, suggesting that unknown differences between the six participating farms also influence the ecology of these virulence genes.

Salmonella serovars were isolated from eight faecal samples (n=2329). Six of these isolates (comprising *S.* London and *S.* Dublin) were isolated from domestic cattle at a prevalence of 1.2% (n=497). A putative *Salmonella* Typhimirium was isolated from a house sparrow. Furthermore, *S.* London was isolated from a calf and a badger on a farm where there had previously been an outbreak in the herd caused by *S.* London. No *Salmonella* was isolated from small rodents (n=1014) and rats (n=16).The prevalence of *Salmonella* in wildlife was low, suggesting that the probability of transmission between domestic cattle and wildlife, although possible, may be limited.

Campylobacter jejuni was the main species isolated from wild birds, rats and small rodents. GLMs were carried out where the number of infected hosts was sufficiently large to assess risks factors of infection. Bank voles (11.3%, n=194) had a significantly higher *C. jejuni* prevalence than wood mice (0.9%, n=658). Rodent species and farm were the only significant variables in the final GLM model. Moreover, there was a spatial cluster in rodent infection, whereby the highest prevalence was found in bank voles trapped in a hedge on the boundaries of a red meat abattoir. DNA sequences for the partial *groEL* gene in *Campylobacter jejuni* strains isolated from cattle, wild rodents and birds showed a possible host-adaptation, with the highest diversity of strains in bank voles. This suggests that although *Campylobacter jejuni* seems to be a multi-host bacterium in this study, the rate of transmission between wildlife and cattle may be low.

This study, although sometimes limited, has provided novel results regarding the prevalence, distribution and genetic characteristics of these bacteria amongst cattle and wildlife on six farms in Cheshire (UK). This study could be a model for similar epidemiological studies with pathogens and the interfaces between domestic animals, wildlife andhuman beings, and for further research into the hypotheses generated by its results.

No pages 1-3 in original.

Chapter 1 General Introduction

Campylobacteriosis, salmonellosis and verotoxigenic *Escherichia coli* (VTEC) infections are regarded as the most important bacterial enteric zoonoses in the UK (Adak et al., 2002; DEFRA, 2006). Although domestic animals are known to be potential sources of these pathogens, very little is known about the role that wildlife might play in their epidemiology.

There is increasing evidence that wildlife can be infected with *Campylobacter*, *Salmonella* and VTEC (Kemp, 2005a; Kwan et al., 2008a; Liebana et al., 2003). Few studies have been carried out in wildlife, and very few in epidemiologically linked domestic animals and wildlife, enabling comparison of isolates. Although rodents have often been blamed for clinical salmonellosis in domestic livestock, direct evidence for rodents acting as a reservoir, or even source, of *Salmonella* infection is difficult to find.

1.1 Verotoxigenic E. coli (VTEC) and E. coli O157

Verotoxigenic *E. coli* (VTEC), also known as shiga toxigenic *E. coli* (STEC), are characterised by their capacity to produce distinctive toxin/s that have a marked cytotoxic effect on HeLa and vero cell lines (African green monkey kidney cells) . VTEC were first described in 1977 by Konowalchuck et al (Konowalchuk et al., 1977), and in human medicine comprise that group of *E.coli* known as enterohaemorrhagic *E. coli* (EHEC).

Although many VTEC strains have been identified, the most notorious serotype is *E. coli* O157:H7, because of the impact that it has in terms of severity of illness in human beings.

1.1.2 VTEC infections in human beings

VTEC in humans was first described in 1982 associated with a severe outbreak caused by *E. coli* O157:H7, associated with the consumption of undercooked burgers in restaurants from a fast food chain in the USA (Riley et al., 1983; Wells et al., 1983).

There were 1,216 laboratory confirmed cases of VTEC O157 in England and Wales in 2006, with a 5% increase compared with confirmed cases in 2005. Scotland accounts for the highest incidence rate within the UK, with approximately 4 per 100,000 people (DEFRA, 2007a). The reasons for this higher prevalence in comparison with the rest of the UK are not clear, but possible faecal contamination in water and contact with farm livestock could play a major role (Solecki et al., 2007).The high incidence of *E. coli* O157 in Scotland in comparison with the rest of the UK could be associated with other unknown factors such as differences in farming practices, differences in human exposures in Scotland, plus differences in surveillance practices.

So far there are no available data about disease cases produced by non-O157 VTEC strains in the UK (www.hpa.org.uk). It is known that other VTEC serotypes can produce disease in humans (Bettelheim, 2000), and the number of cases in the UK could be under-reported due to the lack of awareness. Some countries, such as the USA, have been running surveys in order to detect cases produced by non O157 VTEC (Elben, 2006) and in 1998 the Pennington group, recommended the establishment of such surveys in the UK (Pennington, 1998).

Although the number of people affected by VTEC O157 gastroenteritis is considered low compared with cases caused by other bacteria such as *Campylobacter* spp or *Salmonella enterica*, VTEC O157 infections have a high impact because of the severity of illness caused.

The infectious dose is low, the incubation period in humans is 12-72 hours, and average illness duration is 1-7 days (Griffin and Tauxe, 1991). The clinical signs include watery diarrhoea or hemorrhagic colitis with abdominal cramps. Some cases may develop haemolytic ureamic syndrome (HUS), with symptoms that include haemolytic anaemia, thrombocytopenia, renal failure, and death in extreme cases (Boyce et al., 1995; Chart, 2000; Hugh-Jones et al., 2000) (Griffin and Tauxe, 1991). Most cases that develop into HUS have involved small children and elderly people (Griffin and Tauxe, 1991)

Most clinical cases in the UK are sporadic (DEFRA, 2007a). Outbreaks affecting a high number of people are not uncommon (Strachan et al., 2001). Furthermore, person-to-person transmission is also possible (DEFRA, 2007a; Griffin and Tauxe, 1991; Seto et al., 2007; Willshaw et al., 2001)

Consumption of food such as meat, vegetables and water contaminated with faecal material is considered to be the main route of transmission to human beings (Maule, 2000; O'Sullivan et al., 2008; Parry and Palmer, 2000). Other sources of infection have also been described, such as drinking unpasteurized milk, recreational use of water, and direct contact with livestock, petting farms and wild birds in their environment (Caprioli et al., 2005; Chapman et al., 1993).

A seasonal pattern in human incidence has been observed, with a peak during summer in temperate countries (Willshaw et al., 2001). This coincides with the highest VTEC shedding in domestic cattle (Hancock et al., 1997; Paiba et al., 2002; Schouten et al., 2005).

In addition to verocytotoxin, VTEC strains carry other virulence factors that may play a role in the pathogenesis of disease in human beings. Such virulence factors can be carried by plasmids, phages or on pathogenicity islands (Caprioli et al., 2005).

Verotoxins

The verotoxin group contains two major immunologically non-cross-reactive groups, VT1 and VT2 toxins. The VT1 group is highly conserved, while the VT2 group is diverse, comprising several subgroups such as VT2c and VT2e, associated with porcine oedema disease (Mainil, 1999; Nataro and Kaper, 1998). The genes that encode these major verotoxins are mediated by temperate bacteriophages (Beutin, 2006; Nataro and Kaper, 1998; Scotland et al., 1983).

Verotoxins consist of two proteic subunits A (30-35kDa) and B (7-11kDa).These subunits have up to 57% common amino acid in VT1 and VT2.

Verotoxins inhibit the protein synthesis in certain animal cells. Moreover, the verotoxins bind to specific glicolipidic receptors, globotriosylceramide (Gb3), situated on the eukaryotic cell membranes, and those of relevance to human disease bind to erythrocytes and kidney cells. VT2 is believed to be more closely associated with the development of HUS in both O157 VTEC and non-O157 VTEC outbreaks in human beings. A particular VT2 subgroup, VT2e, associated with the porcine oedema disease, has high tropism for binding to another glicolipidic receptor, globotetraosylceramide (Gb4).This results in a completely different clinical onset in this animal species (Bonnet et al., 1998; Caprioli et al., 2005; Chart, 2000; Nataro and Kaper, 1998; Yoon and Hovde, 2008).

eae-intimin

VTEC also produce attaching and effacing (E/A) lesions. The bacteria attach to the wall of enterocytes using a 94 KDa outer membrane adhesion protein, intimin, encoded by the *eae* gene. The *eae* genes are themselves often part of a pathogenicity island known as Locus for Enterocyte Effacement (LEE). As well as intimin, LEE genes encode for a type III secretion system, secreted proteins ESP A, B and D that complement the type III secretion system and a translocated receptor for intimin or Tir.

The *eae* genes are heterogeneous in terms of aminoacid composition and antigenic diversity, resulting in different types of intimin classified in four major groups, based on antigenic variability, known as α, β , γ, and ε (Caprioli et al., 2005; Kaper, 1998).

The Tir on the host cell is responsible for the successful adhesion of the bacteria to the intestinal cell. Tir is injected into the host cell cytoplasm through a type III secretion system. The continued production and secretion of LEE-encoded proteins leads the host cell actin rearrangement resulting in formation of A/E lesions (Sinclair 2006 et all 2006, Boerlin 1998).

E. coli O157 and other VTEC serotypes such as O111-*eae* carriers have been responsible for HUS in humans. The presence of the *eae* gene was thought to be a necessary factor for the

colonization of the intestinal cells.There has been a cluster of HUS in humans produced by VTEC O113 not encoding *eae*, a sporadic case of HUS produced by VETC O48 without *eae*. It has been demonstrated that *E. coli*-verotoxin 2(VT2) carriers can express this toxin without the need for intestinal attachment (Kaper, 1998). This suggests that the role of *eae* in the pathogenesis of VTEC disease is not as well understood as previously thought (Paton et al., 1999).

Other virulence factors

Other VTEC virulence genes include *ehx*, which encodes for enterohaemolysin. This gene is carried in a 60 MD plasmid that also encodes for fimbriae, which appear to be involved in mediating attachment to intestinal cells (Levine, 1987; Mainil, 1999). STEC autoagglutinating adhesin (Saa) is encoded by *saa*, and is believed to play a virulence role in *eae* negative VTEC strains in humans. The *saa* gene has often been associated with *eae* negative cattle isolates of VTEC (Orden et al., 2005). Other important genes include *fliC* which encodes for the H7 antigen and O157 *rfb* which encodes for the O antigen, (Fratamico, 2005; OIE, 2004)

1.1.3 VTEC infections in domestic animals

Although certain serotypes such as O5, O8, O20, O26, O103, O111, O118 and O145 serotypes have been associated with diarrhoea in calves (DebRoy and Maddox, 2001; Mainil, 1999; Pearce et al., 2004; Wieler et al., 1998), VTEC, including VTEC O157, infections seem to be asymptomatic in ruminants, including sheep, goats and cattle. In particular, HUS has not been described in cattle, and this is believed to be because cattle lack specific receptors in the glomerular kidney cells (Mainil, 1999).

Pigs can develop oedema disease or *Escherichia coli* enterotoxemia affecting mainly piglets, normally associated with verotoxin vt2e (1998; Caprioli et al., 2005; Mainil, 1999).

VTEC in cattle

Ruminants and particularly cattle are considered to be one of the major sources for *E. coli* O157 and other VTEC (Bettelheim, 2000; Blanco et al., 2003; Borczyk et al., 1987; Griffin and Tauxe, 1991). The prevalence of infection on farms is considered low, although there is a lack of studies and surveys to determine the prevalence at farm level. The prevalence of excretion at the herd level varied between 1.1 and 51% with an average of 10% within herds in the UK (Paiba et al., 2003). Previous studies in a 100x100 km area of Cheshire (UK) determined the prevalence of *E. coli* O157 as 4-8% with a herd average prevalence of 32% (Kemp, 2005a; Robinson, 2004a). Moreover, differences in prevalence have been observed if the prevalence is measured on the farm or at slaughter (Milnes et al., 2007; Omisakin et al., 2003; Paiba et al., 2002; Paiba et al., 2003).

On farms, there is evidence that the prevalence and shedding patterns of VTEC amongst cattle are not homogeneous. It is known that age has an effect on the infection of animals with VTEC; calves and heifers tend to have higher prevalence than adult cattle (Blanco et al., 2003; Zhao et al., 1995).

E. coli O157 is not detectable in the majority of cattle groups, but it has been observed that a small proportion of individuals shed high numbers of bacteria in their faeces ($> 10^4$ CFU/g), so called 'super-shedders' (Omisakin et al., 2003; Robinson et al., 2004). It is not known why certain animals within a herd excrete large quantities of VTEC compared with other animals raised in similar conditions. It is believed that this phenomenon could be due to a combination of different factors such as exposure, genetic predisposition, diet, management and stress levels. It has been observed that these 'super shedders' represent a risk for other pen mates and carcase contamination at slaughter (Cobbold et al., 2007).

The shedding of *E. coli* O157 from infected bovine animals seems to be intermittent and varies over time (Besser et al., 2001; LeJeune et al., 2004; Robinson et al., 2004). Thus, the prevalence of *E. coli* O157 has been observed to be higher during summer and early autumn compared to winter (Hancock et al., 1997; Paiba et al., 2002; Schouten et al., 2005).

Production systems may have an effect on the prevalence of *E. coli* O157. Studies to determine differences in prevalence in different cattle production systems have produced different results. For example, Fegan *et al* did not find significant differences in prevalence between grass-fed and lot-fed cattle herds (Fegan et al., 2004), but Cobbold *et* al stated than beef herds tend to have higher prevalence of VTEC than dairy or feed-lot herds (Cobbold et al., 2004).

Molecular studies have demostrated that *E. coli* O157 strains are highly clonal and stable. Thus, similar isolates can be found in places separated by large distances. It seems that there is a predominant *E. coli* O157 clone on any farm, and this strain can be very stable in the animals and their environment (Akiba et al., 2000; Caprioli et al., 2005; LeJeune et al., 2004; Liebana et al., 2003; Liesegang et al., 2000). Other genetically different, but closely related, isolates can also be isolated from the same farm and even from the same animal (Akiba et al., 1999) and mutation can lead to the emergence of new types (LeClerc et al., 1996; Robinson, 2004a). Furthermore, new *E. coli* O157 can be introduced in the herds. Although, most epidemiological and molecular studies have focused on *E. coli* O157, it is known that other VTEC can be implicated in gastroenteric disease in humans. In this respect little is known about their distribution, genetic characteristics and frequency of infection in domestic cattle. Studies have found a higher proportion of cattle excreting non-O157 VTEC compared to O157 VTEC and a higher diversity of VTEC is more common in young calves than adult animals (Blanco et al., 2003; Hinton et al., 1982) . For example, VTEC O118 has been described as the most prevalent VTEC in calves in Belgium and Germany (Wieler et al., 1998) and also associated with human outbreaks (Beutin et al., 2000).

Environment

It is well documented that VTEC can survive in animal faeces, waste and soil on farms and agricultural land for long periods of time. *E. coli* O157 has been isolated from cattle troughs, water supplies, ropes and livestock food stores. VTEC are also able to survive in waste produced in sheep and cattle abattoirs (Caprioli et al., 2005; Fremaux et al., 2007a; Fremaux et al., 2007; Hepburn et al., 2002; LeJeune et al., 2001).

Calves are known to be exposed to *E. coli* O157 and other VTEC early in life, especially where VTEC loads are high in the environment (Besser et al., 2001; Laegreid et al., 1999). Furthermore, *E. coli* O157 may persist on the surface of pens being cleaned but not disinfected (Lahti et al., 2003).

The environment may play an important role in the epidemiology and especially of the transmission of VTEC from animal to animal, animal to humans and faecal contamination of water, vegetables and fruit production. The presence of VTEC strains in the environment has been proposed as one reason why *E. coli* O157 can persist for long periods of time on some farms (Caprioli et al., 2005). High levels of VTEC in the farm environment may also be a route of exposure for wild animals.

1.1.4 VTEC infections in wild animals

A limited number of studies have been conducted to determine the prevalence, carriage and host interactions between domestic livestock and wild animals in terms of VTEC transmission.

Wild ruminants can be infected with VTEC O157. For example this bacterium has been isolated from wild deer in the UK and the USA. Moreover, deer have also been implicated in a human disease outbreak (Mainil, 1999; Rice et al., 1995). In addition, *E.coli* O157 has also been isolated from other large wild mammals such as wild boar from Sweden (Boqvist et al., 2003).

A VTEC O157 human case transmitted by rabbits after a city farm visit has been previously described (Pritchard et al., 2001).VTEC strains were isolated from wild European rabbits in a number of studies, and this species are considered a potential reservoir for *E. coli* O157 (Bailey et al., 2002; Kemp, 2005a; Leclercq and Mahillon, 2003).

VTEC have also been isolated from wild birds such as tree sparrows (*Passer montanus*), barn swallows (*Hirundo rustica*), pigeons (*Columba livia*) and rats (*Rattus norvergicus*) (Nielsen et al., 2004a) . Pigeons in urban areas have been shown to carry VT and other VTEC virulence determinants that have been linked to human disease (Schmidt et al., 2000) (Dell'Omo et al., 1998; Pedersen et al., 2006).

The presence of VTEC from gulls varies. For example no VTEC serotypes were isolated from gulls in Sweden (Boqvist et al., 2003). In contrast, VTEC was isolated from gulls in Japan and in the UK where VTEC O157 was isolated from gull droppings at an urban landfill in the UK (Makino et al., 2000; Wallace et al., 1997). An outbreak in children caused by O157 VTEC was associated with faeces from rooks that previously had contact with cattle waste, as the human and bird *E. coli* strains were identical (Ejidokun et al., 2006). At present

there is not enough evidence to suggest that wild birds could act as natural reservoirs instead of merely being a vector for VTEC or amplifiers of VTEC virulence genes(Wallace et al., 1997).

There is also limited information on VTEC in rodents. Hancock *et al* could not isolate any VTEC in 300 rat samples in the USA but other studies carried out in the Czech Republic were able to isolate VTEC O157 in rat samples collected in a cattle barn (Cizek et al., 1999; Hancock et al., 1998; Rice et al., 2003). Another study found similar results, with two out of ten rats infected with *E. coli* carrying the vt1 gene and VTEC isolated from one house sparrow. PFGE profiles from cattle isolates indistinguishable those of isolates from cows, suggesting that domestic animals can act as a source for this bacteria for wildlife in close contact with infected livestock (Nielsen et al., 2004a).

VTEC and antibiotic resistance

The use of large amounts of antimicrobial substances in modern farming has created a reservoir of resistance bacteria in food animals. There is evidence that antibiotic resistance appears to circulate and spread freely amongst different hosts (Hammerum and Heuer, 2009). This is especially worrying for bacteria such as VTEC. This pathotype has domestic cattle as main reservoir and can cause severe disease in humans sometimes with fatal outcomes.

There is limited information about associations between virulence and antibiotic resistance genes in both VTEC strains isolated from humans and from livestock. Although the use of antimicrobials has been contraindicated as a treatment for HUS cases as their use may increase severity of the onset, the risk and implications that resistant VTEC strains could pose for human health is currently unknown.

Recent studies showed that antibiotic resistance has been found in VTEC from cattle, soil and diverse environments such as rivers (Diarra et al., 2009; Ram et al., 2009). Moreover some VTEC serotypes, that have previously been associated with human disease, have been found

to be resistance to multiple antibiotics. Information about virulence and antibiotic resistance characterisation of VTEC strains isolated from wildlife living close to domestic cattle is non existent.

1.2 Campylobacter spp

1.2.1 General characteristics

Campylobacter was first described by Escherich in 1886 in the faeces of children with diarrhoea (Engberg, 2006). Smith and Taylor (1919) described and named it as *Vibrio fetus* isolated from bovine abortions and Vernon and Chatelain identified *Vibrio fetus* as *Campylobacter fetus* for the first time in 1973 (cited by Vernon and Chatelain) (Veron, 1973). Jones, in 1931 (cited by Engberg) described a new species, *Vibrio jejuni*, and its association with intestinal disorders in cattle (cited by Engberg) (Engberg, 2006). *Campylobacter* has been well known in the veterinary field for a long time, although it has only recently been associated with human disease.

Campylobacter belongs to the Class V. *Epsilonproteobacteria*, Order I. *Campylobacterales*, Family I. *Campylobacteraceae*, genus I. *Campylobacter*. These bacteria are Gram negative spiral rods 0.2-0.8 x 0.5-5 μm. *Campylobacter* spp are microaerophilic and require an oxygen concentration between 3-15% and a CO_2 of 35%. There is a thermophilic subgroup within the genus *Campylobacter* that grows in temperatures between 42-45°C. The *Campylobacter species* within this group most relevant to this studyare *C. jejuni, C. coli, C. upsaliensis, C. lari , C. intestinalis and C. fetus* (Brenner D.J., 2005).

Campylobacter spp can be found in the digestive tracts of birds and mammals and are sensitive to a variety of environmental stressors such as the UV light, disinfectants and heat

treatments(Wang et al., 1983). *Campylobacter* spp have developed strategies in order to cope with such stressors, including producing proteins such as GroELS and DanK in response to heat shock (Alter and Scherer, 2006). *Campylobacter* is unable to grow at temperatures of 4°C or below and freezing can reduce their viability.

1.2.2 Campylobacter spp in humans

Campylobacter have only relatively recently been identified as human pathogens (Butzler et al., 1973; Skirrow, 1977). In the last 20 years, *Campylobacter* has become the leading reported cause of bacterial gastroenteric disease in developed countries.

Most cases of human campylobacteriosis are sporadic (DEFRA, 2006; Fussing et al., 2007; Potter et al., 2003). There were nearly 200,000 cases reported in the EU during 2005, with an overall incidence of 51.6 cases per 100, 000 people. The incidence in the UK was slightly higher than the average: 88 per 100,000 people in 2005 (Anonymous, 2007b).

Campylobacter jejuni and *C. coli* account for most of the outbreaks but other *Campylobacter* spp have also been implicated in human outbreaks at a smaller scale such as *C. lari*, *C. hyointestinalis*, *C. fetus* and *C. upsalinesis*. Current statistics could be biased as 61% of *Campylobacter* confirmed cases in Europe from 1994 to 2004 were not characterized at species level (Anonymous, 2007b).

Campylobacteriosis in humans can be produced by a low infective dose (<500 cells) and tends to be a self-limiting disease (Robinson, 1981). The incubation period varies between 2 and 7 days. Clinical signs include bloody diarrhoea, abdominal pain, nausea and vomiting. Although rare, ulcerative colitis, bacteraemia and sometimes even death can occur(Blaser et al., 1983; Skirrow, 1977). Campylobacteriosis also has been linked to other chronic

syndromes such as the Guillain-Barre syndrome, the Miller-Fisher syndrome and reactive arthritis (WHO, 2000).

The incidence of campylobacteriosis in humans from industrialized countries such as the UK is completely different from the incidence in developing countries. Reasons suggested include high incidence of gastrointestinal disease in children, extreme poverty, high prevalence of HIV infection, war and post-war conflicts, diet and livestock densities (Altekruse et al., 1999; Blaser et al., 1983; Mdegela et al., 2006; Uzunovic-Kamberovic, 2001).

The main transmission routes are thought to be consumption of contaminated food and water (Adak et al., 2002) and the consumption and handling of poultry products and undercooked food are also important risk factors(Altekruse et al., 1999). Furthermore, person to person transmission can also occur, although it is uncommon.

The origin of one in four human outbreaks in Europe is unknown (Takkinen et al., 2003). The development of molecular techniques such as Multilocus Sequence Typing (MLST) has made possible the comparison of *Campylobacter jejuni* isolates from different origins (Kwan et al., 2008a; Manning et al., 2003). This has highlighted that sources of infection other than food and water might contribute more than previously thought to human infection, and the assumption that poultry are more frequent sources of human infection than ruminants has recently been questioned (Wilson et al., 2008).

Factors such as contact with farm stock, recreational use of water, overseas travel and consumption of milk contained in bottles pecked by birds, are considered of risk for campylobacteriosis (Robinson and Jones, 1981a). In addition, this bacterium has been isolated from a high variety of environmental sources including cattle troughs, soil, sewage and mud (Kemp et al., 2005). It is believed that domestic cattle are continuously exposed to and excrete *Campylobacter* spp into the environment and this can be a source of

contamination for recreational waters situated close to areas with high stock densities (Jones, 2001).

Weather seems to have an effect on the number of human outbreaks. Thus, there is a marked seasonality in temperate countries with a higher peak during spring and late summer and less incidence during autumn and winter months (Anonymous, 2007b).

1.2.3 Campylobacter spp in domestic animals

Campylobacter spp are well known in the veterinary field. Traditionally campylobacteriosis in animals has been associated with *Campylobacter fetus* sbp *fetus* and *C. fetus* sbp *veneralis*. Both produce infertility and abortions in cattle and sheep (1998).

Gastrointestinal campylobacteriosis caused by thermophilic *Campylobacter* is recognized in animals such as dogs, cats, calves, sheep, ferrets and mink(1998; Fox et al., 1987). In contrast, most farm animals tend to carry *Campylobacter* spp in an asymptomatic state.

Poultry is considered the major reservoir for thermophilic *Campylobacter*. Furthermore, this bacterium is considered to be a common commensal of poultry intestines. Some toxigenic and invasive strains of *C. jejuni* can cause enteritis and death in hatched chicks (1998).

The prevalence of this bacterium in domestic flocks and poultry meat is thought to be high. In the UK, non-randomised studies have been conducted at a small scale in poultry flocks and concluded that prevalence could vary between 30-90% (DEFRA, 2006). Moreover, an annual survey carried out in poultry at the slaughter point indicated that the UK prevalence for thermophilic *Campylobacter* was 54.6% in 2005 (DEFRA, 2006).

A six month survey conducted to determine the carriage of *Campylobacter* in poultry meat in the UK, found *Campylobacter* were isolated from 62% of chicken meat samples, 36% of turkey meat samples and 42% of game fowl samples in 2004 (DEFRA, 2005). These data

support the idea that one of the main risk factors for human campylobacteriosis is handling and consumption of poultry products (Altekruse et al., 1999; Stafford et al., 2007)

The prevalence of *Campylobacter* in poultry shows a distinct seasonality pattern with a peak in spring and summer months (Anonymous, 2005; Meldrum et al., 2004). Meldrum *et al* observed that rates of isolation from fresh retail chickens followed the same seasonality pattern as human campylobacteriosis cases in Wales (Meldrum et al., 2005).

There is evidence that domestic cattle are a natural reservoir for multiple *Campylobacter* spp such as *Campylobacter jejuni, C. coli, C. fetus, C. hyointestinalis, C. lalineae* and *C. lari,* as they have been isolated from dairy and beef animals and their products (Enokimoto et al., 2007; Garcia et al., 1985; Inglis et al., 2004; Minihan et al., 2004; Robinson and Jones, 1981a; Stanley and Jones, 2003; Wesley et al., 2000). Moreover, some *Campylobacter* isolates from cattle have been indistinguishable by molecular typing from *Campylobacter* isolates from human clinical cases (Karenlampi et al., 2007; Nielsen et al., 2000).

It appears that the dynamics of *Campylobacter* infection in domestic cattle may be complex. There is a lack of information about the numbers and species type of *Campylobacter* that domestic cattle can carry as part of their normal flora: indeed several studies of the intestinal flora of cattle have not been able to characterize a proportion of confirmed *Campylobacter* to a species level (Inglis et al., 2004; Minihan et al., 2004). Moreover mixed infection of different *Campylobacter spp* in the same animal has also been described (Enokimoto et al., 2007; Inglis et al., 2004).

The shedding of *Campylobacter* in bovine faeces is associated with the age of the animals. Calves are born *Campylobacter* free but most start shedding it at four days old. Stanley *et* al found that calves can excrete 100 times more *Campylobacter* spp than finisher beef animals. (Stanley et al., 1998)Other factors such as overcrowded calves in pens can be a risk factor for an increase in shedding *Campylobacter* spp in calves (Wesley et al., 2000).

It is known that *Campylobacter* shedding in adult cattle is intermittent and the existence of 'super-shedders' or small numbers of animals within a herd that excrete the bacteria in high quantities, has been suggested (Inglis et al., 2004; Stanley and Jones, 2003). Moreover, *Campylobacter* can be isolated from both the cattle and their environment on the farm. The presence of campylobacter in the environment may be a source of reinfection for infected animals; a primary source for non-infecting cattle and also a possible contamination route for wildlife species (Kemp et al., 2005; Kwan et al., 2008a; Minihan et al., 2004)

A study in US dairy herds found an animal prevalence of *C. jejuni* to be 38% and 1.8% for *C. coli* (Wesley et al., 2000). Recent studies have found 31% prevalence in cattle in Finland (Hakkinen et al., 2007) and 52% in Italy. In general there is a lack of epidemiological studies carried out on healthy cattle on farms (Acik and Cetinkaya, 2005). A study of 61 cattle farms in Cheshire (UK) determined an overall prevalence of *Campylobacter* spp of 55% and of those 20% were *Campylobacter jejuni* (Kemp, 2005a).

Most of the studies carried out in domestic cattle have been carried out at slaughter. In the UK, an abattoir survey showed a prevalence of thermophylic *Campylobacter* spp at slaughter in cattle of 54.6%, of those 81% were *C. jejuni* (DEFRA, 2005; Milnes et al., 2007). It seems that both the prevalence at slaughter and on farms were similar. However, more surveys should be undertaken at the farm level in order to determine the prevalence and dynamics of this pathogen in their production habitat and not under highly stressful conditions such as transport and point of slaughter.

In the UK, it has been recorded that the prevalence of *Campylobacter* in cattle is influenced by seasonality with two maximum peaks in spring and autumn, and a decline in winter (Blaser et al., 1983; Stanley et al., 1998).

1.2.4 Campylobacter spp in wildlife

Campylobacter spp have been isolated from a wide range of wildlife species. The lack of standard methods of isolation and characterization, and the high genetic diversity of *Campylobacter* spp makes it difficult to compare different studies. Furthermore, most studies carried out in wildlife were not epidemiologically structured (Tables 1- 4).

Most prevalence studies have focused on wild birds because of the high risk of infection in domestic poultry. Waldenstrom *et* al determined the prevalence of *Campylobacter* in migrating birds in Sweden as 22% and most prevalent isolates were *C. jejuni, C.coli* and *C.lari.* They observed that the prevalence was not homogeneous throughout the different bird species, and possible risk factors that explained the differences in prevalence could be feeding habits, increased body mass in different species and habitat. Thus, wild birds that forage along the sea shoreline and terrestrial ground feeders had a higher prevalence of *Campylobacter* than granivores, arboreal and reed-bed insectivores (Broman et al., 2002). Indirectly, outbreaks in human beings have been associated with contact with water contaminated with geese faeces, and milk pecked by wild birds, suggesting that wild birds can carry zoonotic *Campylobacter* spp (Broman et al., 2002; Southern et al., 1990).

There is a lack of epidemiological studies that determine prevalence in terrestrial wild mammals. *Campylobacter fetus* has been isolated from small rodents in the wild from different habitats. Fernie *et* al isolated *Campylobacter* from bank voles but from no other wild rodents including wood mice and field voles in the England, suggesting that bank voles could be a possible *Campylobacter* reservoir, in contrast (Fernie and Healing, 1976; Fernie and Healing, 1977) Corbel *et* al did not isolate *Campylobacter fetus* sbsp *veneralis* from bank voles experimentally inoculated with this bacterium via different routes (Corbel and Redwood, 1978).

Wild rodents are capable of shedding *Campylobacter* for long periods of time. For example an experimental study in which water voles were orally inoculated with *Campylobacter jejuni* showed that these rodents excreted *Campylobacter* over a number of weeks (Pacha et al., 1987).

Table 1. Previous published studies carried out in terrestrial wildlife and *Campylobacter*.

Author & Year	Region of Study	Wildlife spp. *Campylobacter* was isolated	Wildlife spp. *Campylobacter* was not isolated	Duration of the study	Type of study
Fernie et al, 1976	Reading and Hampshire (UK)	Bank vole, Rat *	Rabbit*, guinea pig*, hamster*, field vole, Wood mouse	3 months	Observational laboratory and field survey
Fernie et al, 1977	Berkshire and Hampshire (UK)	Bank vole	Wood mouse Field vole	3 months	Observational field survey
Corbel et al, 1978	UK	(Bank vole)	Bank vole	7-14 days	In-vitro experimental inoculation
Skirrow et al, 1980	UK	Gulls, monkey	_____	_____	Comparison of biochemical characteristics from different *C*. strains from different hosts
Leuchtefeld et al, 1981	USA	Primates, wild ruminants, felids, Reptiles, birds, Wild pigeons	unknown	12 months	Observational survey among zoo clinical - healthy cases
Pacha et al, 1985	USA	Musk rat	_____	16 months	Observational field survey
Rosef et al, 1985	Norway	Blue hares	Moose, reindeer, roedeer, bank vole, wood mouse	9 months	Observational field survey
Pacha et al, 1987	USA	Bear, water vole*	Small rodents, rabbit, elk	24 months	Observational field survey, in-vitro inoculation
Cabrita et al, 1992	Portugal	Black rats, sparrow, ducks	unknown	12 months	Observational field survey

Table 2: The major findings and testing methods used in the 17 previously published papers about isolation of Campylobacter in wild mammals

Author & Year	Microbiological Method of Isolation	Characterization Method	Data Analysis	Major findings
Fernie et al, 1976	**Filtration, CAB**	**Catalase, nitriate , H2S, glycine, Electrophoresis APS**	**Descriptive**	***Campylobacter fetus*** **isolated from bank voles in the wild and lab rodents**
Fernie et al, 1977	**Filtration, CAB**	**Catalase, nitriate , H2S, glycine, Electrophoresis APS**	**Descriptive**	***C. fetus sbsp. veneralis*** **isolated from wild bank voles , possible reservoir to domestic anmals**
Corbel et al, 1978	**Experimental inoculation**		**Descriptive**	***C. fetus sbp.veneralis*** **was not isolated or excreted. Bank voles were asymptomatic after inoculation.**
Skirrow et al, 1980	**BA**	**Oxidase, catalase, selenite reduction,sensitivity to nalidixic acid, NaCL, metonidazole**	**Descriptive**	**Similar biochemical profiles were seen with isolates from gulls, cattle and sheep.**
Leuchtefeld et al, 1981	**Tryptose plus blood agar with amphotericin, cephalothin,polymixin, methoprim, vancomycin**	**Serotyping passive hemagglutination**	**Descriptive**	***C. jejuni*** **is widely distributed in wildlife spp. in zoo, among clinical and healthy animals. Serotypes are heterogeneous.**
Pacha et al, 1985	**CEB, CAB**	**Motility, Gram, catalase, oxidase, Hippurate , NaCL, H2S**	**Descriptive**	**Musk rat could act as reservoir of Campylobacter and a contamination source of water.**
Rosef et al, 1985	**CAKA**	**Motility, catalase, oxidase, H2S, hippurate, nalixidic acid susceptivility**	**Descriptive**	***C.*** **was isolated from hare but not from different cervids and wild rodents in Norway.**
Pacha et al, 1987	**CEB, CAB**	**Motility, Gram, catalase, oxidase, Hippurate , NaCL, H2S**	**Descriptive**	**Small incidence of infection found among wild rodents. Water vole experimentally infected and shedding *Campylobacter* for weeks,reservoir?.**
Cabrita et al, 1992	**Selective medium described by Skirrow**	**Oxidase, catalase, Gram, Hippurate, sensitivity to nalidixic acid, byotyping, plasmid screaning**	**Descriptive, chi-square test with Yate's correction**	**High prevalence of *Campylobacter* among rats, sparrows and ducks, possible reservoir. Plasmid carriage found in c. isolates from domestic and wild animals and humans, possible antibiotic resistance.**
Broman et al, 2000	**CAB plus polymixin B and vancomycin**	**Catalase, hippurate, *flaA* -PCR, PFGE**	**Descriptive**	***C. jejuni*** **isolated from penguins possible introduced in that earth free area**
Petersen et al, 2001		**Serotyping, PCR-RFLP, PFGE**	**Descriptive, chi-square test**	**Hedgerows potential reservoir for campylobacter in humans. Wildlife isolates had not much similarities with poultry and humans isolates**
Rosef et al, 2001	**Selective blood free agar**		**Descriptive**	***Campylobacer*** **was not isolated from Eurasian Beaver (*Castor fiber*). Beaver does not seem to be involved in water contamination.**
Brown et al, 2004	**CEB, CBFA, CA**	**PCR's**	**Fisher scoring algorithm , Markov random field model**	***C. jejuni*** **isolates from cattle, water and wildlife were indistinguishable *C. lari* prevalent in cattle and wild bird faeces. Non spatial dependence was found.**
French et al, 2005	**CEB, CBFA, CA**	**MLST**	**Arithmetic mean (UPGMA) dendrogram, generalized additive model**	**Important ST isolates in human disease isolated from wildlife and water including new STs.**
Fearnhead et al,2005		**MLST**	**Likelihood methods, recombination models**	**Evidence of recombination in *C. jejuni* from different source was found.**
Lillehaug et al, 2005	**CBFA plus cef, amphoB,teicoplanin**	**Catalase, hippurate**	**Descriptive**	**Only isolated from roe deer, not from cervids in Norway. Not a reservoir**
Leatherbarrow et al, 2007	**-**	**MLST**	**Descriptive**	***C. lari*** **is wide spread in host and environment. Spatial clustering between cattle, rabbits and badgers isolates.**

Table 3. Previous published studies carried out in wild birds and *Campylobacter* and

Table 4: The major findings and testing methods used in the 21 previously published papers about isolation of Campylobacter in wild birds

Author & Year	Region of Study	Wildlife spp. *Campylobacter* was isolated	Wildlife spp. *Campylobacter* was not isolated	Duration of the study	Type of study
Smibert et al, 1969	USA	Pigeons, blackbirds, starlings, sparrows	------_____	Unknown	Biochemical comparison of different campylobacter strains from different hosts
Leuchtefeld et al, 1980	USA	Shoveler,pintail,american widpigeon, mallard, gadwall, green-winged teal	_____	3 months	Observational field survey afte hunting waterfowl season
Rosef et al, 1982	Norway	Unknown	_____	_____	Biochemical comparison of different strains
Kapperud et al,1983	Norway	5 spp. in urban areas, 12 spp. in rural areas	26 species of birds	14 months	Observational urban-rural surve
Kapperud et al, 1983a	Norway	Puffin	_____	2 months	Observational field survey populations with different mortalities
Ito et al, 1988	Japan	Eastern,turtledove,bulbul,pigeon,crow,gray starling, blue magpie	Tree sparrow, pheasant, Chinese bamboo pheasant	8 months	Observational survey
Pacha et al, 1988	USA	Migratory ducks, Canada geese , sandhill crame	_____	2 months	Observational field survey
Yogasundram et al , 1989	USA	Psittaciformes, Galliformes, Anseriformes, Falconiformes,Columbiformes	Passeriformes,Strigiformes,,Ciconiifromes, Gruiformes,Pelecaniformes, Musophagiformes, Piciformes, Struthioniformes	6 months	Observational survey on dead wild birds
Casanovas et al, 1995	Spain	Pigeons	_____	12 months	Observational urban survey
Oyarzabal et al, 1995	USA	Emu, hawk, ,ostrich,parrot,	Black bird, cockatiel,goose, ,dove,duck, house finch, lovebird,owl, pigeon,quail,Rhea, swan	6 months	Observational survey on dead wild birds
Fernandez et al, 1996	Chile	Yellow-billed pintail,kelp gull,olivaceous cormorant, black-necked sawn, pigeon, chimango caracara, European sparrow	_____	Unknown	Observational field survey
Broman et al, 2002	Sweden	Black-headed gull	_____	24 months	Longitudinal and molecular epidemiology study
Waldenstrom et al, 2002	Sweden	Sylviidae, Regulidae, Paridae,Passeridae,Fringillidae, Anatidae, Muscicapidae, Sturnidae, Accipitridae,Strigidae, Scolopacidae, Certhidae families	13 migrant families	8 months	Observational field survey
Colles te al, 2003	UK	Starlings	_____	_____	Molecular epidemiology
Wedderkopp et al, 2003	Denmark	Parrots, canaries, hens, peacocks, racing pigeons	Unknown	24 months	Survey of hobby birds summate for PM
Broman et al, 2004	Sweden	Migrating birds	_____	12 months	Field survey, molecular epidemiology
Palmgren et al, 2004	Sweden	Peregrine falcons	_____	_____	
Vlahovic et al, 2004	Croatia	piegons	24 species of birds	Unknown	Observational survey
Mdegela et al, 2006	Tanzania	Crows	_____	Unknown	Observational field survey
Ganapathy et al, 2007	Malasia	Crows	_____	1 month	Observational survey at hunting

Author & Year	Microbiological Method of Isolation	Characterization Method	Data Analysis	Major findings
Smiber et al, 1969	Filtration	Motility, catalase, oxidase, H2S, nitrate, ferment sugars, oxidaze sugars, NaCL, Hydrolisis of deaminase; casein; gelatine; ribonuclease; deoxyribonuclease; phosphatase	Descriptive	*Campylobacter hyointestinalis* isolated from wild birds and poultry had the same biochemical characteristics than strains isolated from healthy and aborted sheep.
Leutchtefeld et al , 1980	BA + amph, ceph, van, trim	Motility, oxidase, catalase, H2S, sensitivity to nalixidic acid	Descriptive, chi - square and McNemar test	High prev. Of *C. jejuni* among migrating waterfowl, differences of carriage among different waterfowl spp. , could be due to different diets. Waterfowl as reservoir and contamination source of *C. jejuni* to water.
Rosef et al, 1982	———	Catalase, oxidase, hippurate, NaCL, H2S, sensitivity to antibiotics	Descriptive	Porcine strains were more diverse in biochemical characteristics than human and avian strains. Avian, human and 2 of the swine groups showed similar biochemical characteristics.
Kapperud et al, 1983	GAB+horse blood+col+nys+ceph	Motility, oxidase, catalase, hippurate, sensitivity to nalixidic acid	Descriptive	Crows, gulls and pigeons around urban areas have the higher *C. jejuni* carriage, differences in diets?. Healthy wild birds as *C. jenuni* reservoir.
Kapperud et al, 1983a	CHA+horse blood+col+nys+ceph	Motility, oxidase, catalase, hippurate, sensitivity to nalixidic acid	Descriptive	Difference in *C. jejuni* prevalence in two populations with different mortalities suggesting a possible c. risk.
Ito et al, 1988	CBA	Biochemical characteristics	Descriptive, chi-square test	Crows, magpies, gray starlings and pigeons have high prev. of *C. jejuni* . Diet seems to have a relationship with C. carriage, not isolated from herbivorous birds but from birds in contact with human sewage as crows.
Pacha et al, 1988	CEB, CBA	Motility, gram, hippurate, sensitivity to nalidixic acid	Descriptive	High carriage of *C. jejuni* found in migrating waterfowl in urban area. More prevalence in ducks than geese. Ducks could be reservoirs for c. jejuni.
Yogasundram et al, 1989	CSM	Colony morphology, hippurate, nalidixic sensitivity, serotyping by passive hemoagglutination test	Descriptive	High prevalence of *Campylobacter jejuni* in waterfowl and chickens suggesting a possible reservoir for this pathogen.
Casanovas et al, 1995	Campysel agar	As described by Morris and Patton (1985), no more refs available about the type of test/s conducted	Descriptive	One in four pigeons were infected with *Campylobacter jejuni* , more incidence in areas with larger pigeons densities , seasonality was not observed.
Oyarzabal et al, 1995	CCSA	Colony morphology, serological latex agglutination test	Descriptive	*Campylobater spp* . in wild birds as possible commensal in their intestines.
Fernandez et al, 1996	SKA	Catalase, oxidase, hippurate, sensitivity to nalidixic acid and ceph	Descriptive	Waterfwol in Chile as possible reservoir of *Campylobacter* . Isolated from spp. of family Falconidae. High prevalence in pigeons and sparrows that live close to human habitats.
Broman et al, 2002	CBA+van+poly+trim	Catalase, oxidase, PCR, PFGE, MRP	Cross-tabulation and paired test	Juvenile gulls show same seasonality as in humans and poultry. Some MRP's identical in human, poultry and gulls. Most genotypes in gulls different to humans, accidental more than reservoir?
Waldenstrom et al, 2002	CBFA+cefo+amph	Catalase, oxidase, hippurate, multiplex PCR	Descriptive, chi-square test	Heterogeneous *C* . prevalence among migrant birds. Diet influences the prevalence, none in granivores and high in opportunistic feeders as raptors. Campylobacter was isolated more in adults than juveniles. Habitat could have an influence in c. prevalence in wild migrant birds.
Colles et al, 2003		MLST	Descriptive	Same ST53 genotype complex was found in calves, sheep, chicken and starling samples collected on farms.
Wedderkopp et al, 2003	CCDCHA+amph	Morphology, motility, hippurate	Descriptive	Hobby birds may act as *C* . reservoir for humans and other birds. Higher prevalence was found in birds kept outdoor than in indoor birds.
Broman et al, 2004		MRP-PFGE	Molecular analysis	Samples from a starling and black bird were very similar to human isolates.
Palmgren et al, 2004	CBA+van+poly+trim	Catalase, oxidase, urease, sentitivity to nalidixc acid, hippurate, PCR-RFLP, MRP-PFGE	Descriptive	MRP form *C. jejuni* isolated from falcons were indistinguishable from human isolates.
Vlahovic et al, 2004	SKA	Gram, Oxidase, catalase, hippurate, sensitivity to malidixic acid and ceph, API	Descriptive	Low prevalence of *Campylobacter spp* . in free wild birds in Croatia. *C. jejuni* found in pigeons
Mdgela et al, 2006	BA	Gram, catalase, oxidase, nitrate, hippurate, PCR	Descriptive, chi-square test	Crows possible reservoir of *C. jejuni* in Tanzania.
Ganapathy et al, 2007	CBFA supplemented by CCDA	Motility, oxidase, catalase, acetate and three-test biochemical system for *Campylobacter* identification	Descriptive	Crows possible reservoir of *C. jejuni* and *C. coli* in Malasia.

1.3 Salmonella

1.3.1 General characteristics

Salmonella was first isolated from pigs by Daniel Elmer Salmon, an American veterinarian, and his colleague Theobald Smith in 1885.

Taxonomy

Salmonella belongs to the proteobacteria, Class III Gammaproteobacteria, Order XIII Enterobacteriales, Family I Enterobacteriaceae, and Genus XXXIII Salmonella (Brenner D.J., 2005). *Salmonella* are Gram negative, facultative anaerobic bacteria generally motile and non-lactose fermenting (Brenner D.J., 2005; OIE, 2004, updated 2007). *Salmonella* can grow within a range of temperatures between 8°C and 45°C but do not survive temperatures higher than 70°C (Acha and Szyfres, 2003).

The classification of *Salmonella* is complex; there are only two species, *S. enterica* and *S. bongoni*. *S. enterica* is subdivided in six subspecies: *enterica*, *arizonae*, *diarizonae*, *houtenae*, *indica* and *salamae*. There are multiple serovars within the two species, approximately 2500, in accordance with the Kauffman-White serotyping scheme for O,Vi and H antigens (Brenner D.J., 2005; Fratamico, 2005). Therefore, *Salmonella* Typhimurium, nomenclature used in this thesis, would also be *Salmonella enterica* sbsp. *enterica* serotype Typhimurium or *Salmonella enterica* serovar Typhimurium. These three ways of referring to *Salmonella* serotypes/ serovars are accepted (Tindall, 2005).

Other characteristics

Salmonella can be found in the intestinal contents of birds and mammals but has also developed strategies to persist and grow in different environments (Acha and Szyfres, 2003).

The host range varies between serovars, and not all *Salmonella* serovars are zoonotic. For example, some of *Salmonella* serovars can be host specific such as *S.* Typhi in humans, *S.* Dublin in cattle and *S.* Pullorum in domestic poultry. Other serovars are able to have

multiple-hosts, such as *S.* Typhimurium. This is important as wide versus narrow host range serovars of *Salmonella* are approached differently in terms of surveillance, animal and public health relevance (Mastroeni, 2006).

1.3.2 Salmonella in human beings

Non-typhoidal salmonellosis is considered the second most commonly reported cause of bacterial gastrointestinal disease in developed countries. Most of the serovars causing human disease belong to *S. enterica* sbsp *enterica.* The average incidence was 39 per 100,000 people in the EU in 2005, the Czech Republic and Slovenia accounted for the highest incidence and Portugal with the lowest. The incidence in the UK was 39.6 in 2005 (Anonymous, 2007b). The most frequently isolated serovars from human cases at a European level were *S.* Enteritidis, *S.* Typhimurium, *S.* Infantis, *S.* Virchow and *S.* Hadar (Anonymous, 2007b).

There were approximately 14,000 reported human cases of salmonellosis in England and Wales in 2006 (DEFRA, 2007a; HPA, 2007). The most commonly isolated serovars isolated were *S.* Typhimurium and *S.* Enteritidis as the two serovars that account for the highest proportion of cases followed by *S.* Virchow, *S.* Infantis and *S.* Newport (VLA, 2006). Furthermore, there was a peak in the number of human cases observed during late summer in temperate countries (Anonymous, 2007b). At present the reasons for this are not completely known but it is thought to be associated with excretion of *Salmonella* in domestic livestock.

In general, the incidence of human cases of salmonellosis in the EU and the UK has decreased compared to previous years (1987-1998) (Figure 1) (Anonymous, 2007b; HPA, 2007), although this pathogen is still highly important for public and animal health. The use of different intervention strategies such as vaccination in egg-laying breeders, improvement in hygienic practices and the introduction of the HACCP system as part of 'from farm to fork' schemes are believed to be some of the factors that have contributed to a decrease in the incidence of human cases in the UK and some other European countries (Anonymous, 2007b; DEFRA, 2007a; HPA, 2007; Smith-Palmer et al., 2003). Despite of this in 2001 there was an

increase in the number of human cases caused by *S.* Enteritidis, this was due to low-cost imported eggs from Spain contaminated with *Salmonella* (Prof. John Threfall, HPA, personal communication).

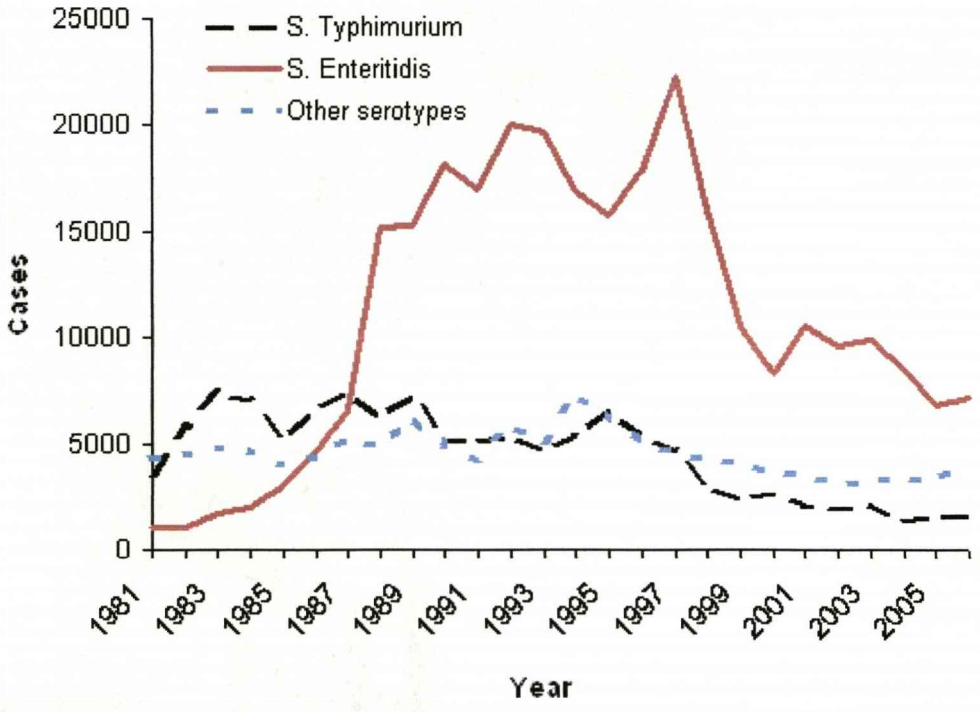


Figure 1. Temporal trends of salmonellosis incidence in the UK (1981-2006) Graph published by HPA/UK 2007 (http://www.hpa.org.uk/infections/topics_az/salmonella/data_human.htm)

Non-typhoidal salmonellosis in humans is characterized by an incubation period of 6 to 72 hours and is normally a self-contained disease. Whereas *Campylobacter* infections tend to be of sporadic nature, outbreaks caused by *Salmonella* serovars that affect high numbers of people are common (Beatty et al., 2008; Franklin et al., 2008).

The main symptoms are diarrhoea, abdominal pain, vomiting and fever. Some cases can present with septicaemia, splenomegaly and even death. This abnormal onset is more common amongst certain groups such as elderly people, infants and immune-compromised

patients (Hugh-Jones et al., 2000; Shakespeare, 2002). Post-infection complications such as reactive arthritis can also occur in a small proportion of cases (Anonymous, 2007b).

Non-typhoidal salmonellosis is a zoonosis associated principally with the consumption of contaminated food of animal origin. Disease cases in humans due to *Salmonella* Enteritidis have been associated principally with the consumption of chicken and eggs while human outbreaks produced by *S.* Typhimurium have been associated with the consumption of a variety of different foods including beef, milk, pork, poultry and salads (DEFRA, 2007a; Hugh-Jones et al., 2000; Smith-Palmer et al., 2003). Poultry meat and eggs are believed to be the most common foods implicated in *Salmonella* outbreaks in humans (Antunes et al., 2003; McNeil et al., 1999; Miller, 1952; Panisello et al., 2000). In Portugal 60% of chicken carcases were found to be contaminated with *Salmonella.* A US survey found between 3 and 84% of carcases and rinse water samples in abattoirs were contaminated with *Salmonella* s serovars (Antunes et al., 2003; Foley et al., 2007). After a 6 year UK survey from 1995 to 2000, 11% of retail chicken was found to be contaminated with *Salmonella* (Antunes et al., 2003; Wilson, 2002). A most recent survey carried out in 2006 showed a lower prevalence of *Salmonella* in retail poultry, 7%, with Northern Ireland accounting for highest proportion of positive samples (30%) (DEFRA-EFSA, 2007b) . A UK survey of imported eggs showed that 3.3% were contaminated with *Salmonella* serovars and *S.* Enteritidis was the main serovar isolated (DEFRA-EFSA, 2007b).

Other factors such as contact with asymptomatic livestock and their environment can also be associated with the disease in humans (Acha and Szyfres, 2003). Moreover, exotic pets such as reptiles can be a source for this disease. It is well documented that reptiles are often asymptomatically infected with *Salmonella* (CDC, 2008; Gugnani, 1999; Hidalgo-Vila et al., 2007; Kaufmann et al., 1967).

1.3.3 Salmonella in domestic animals

Salmonellosis is a well-known disease in the veterinary medicine field. It is capable of producing disease in domestic and companion animals worldwide, causing important economical losses especially in farm animals. Typical syndromes produced in animals are septicaemia, acute/chronic enteritis and abortions in pregnant animals (Anonymous, 1998).

However animals can also be asymptomatic carriers of *Salmonella*. Only 57% of *Salmonella* cases reported in Livestock in Great Britain corresponded to clinical disease cases in 2006 (Anonymous, 1998; DEFRA, 2007a). *Salmonella* is reportable if isolated from livestock in the UK and such reports normally are followed by an epidemiological investigation under the Zoonoses Order 1989 (DEFRA, 2007; DEFRA, 2007a).

Poultry

Birds are an important reservoir for *Salmonella*, especially domestic poultry, due to the high stocking densities and intensive production systems. This facilitates a more rapid spread of *Salmonella* infection throughout the flocks, and, indeed, at slaughter (Antunes et al., 2003; Foley et al., 2007). Chickens and turkeys have two host-specific *Salmonell*a serovars; *S.* Pullorum and *S.* Gallinarum. These two serovars are responsible for pullorum disease and fowl typhoid in poultry populations (Anonymous, 1998). Domestic poultry can also act as a source of other multi-host *Salmonella* serovars of high importance for public health such as *S.* Typhimurium and *S.* Enteritidis and become asymptomatic carriers (Liebana et al., 2001).

Cattle

Salmonella in domestic cattle are capable of producing disease, especially septicaemia in new born calves, sometimes with high mortality, and also abortions in pregnant animals (Anonymous, 1998; Clegg et al., 1983). Cattle can be asymptomatic carriers of different *Salmonella* serovars including *S.* Typhimurium (Clegg et al., 1983) and are considered the main reservoirs for *S.* Dublin (DEFRA, 2007a; Mastroeni, 2006).

Salmonella transmission in cattle is mainly horizontal, but vertical transmission of *S.* Dublin has been suggested (Wray et al., 1989). The environment also seems to play an important role in the persistence and survival of *Salmonella* for long periods of time on farm building, feeding stuffs and pasture contaminated with faecal material (Clegg et al., 1983; Peters et al., 1987; Wray et al., 1989). The existence of 'super-shedders' has been proposed as another explanation for long periods of *Salmonella* persistence in herds (Lanzas et al., 2008; Wray et al., 1989).

Salmonella infections with *S.* Typhimurium and *S.* Dublin tend to follow different temporal patterns of infection in calves. The peak of infection with *S.* Dublin tends to happen later than

the peak of infection with *S.* Typhimurium as *S.* Dublin has a tendency to be more commonly isolated from adult animals (Wray et al., 1987).

Epidemiological risk factors for *Salmonella* infection in cattle are purchasing calves at markets and from dealers (McLaren and Wray, 1991); moving of live animals (Evans and Davies, 1996); poor cleaning and disinfection farm practices (McLaren and Wray, 1991; Vanselow et al., 2007; Wray et al., 1987); absence of isolation facilities (Evans and Davies, 1996); contact with host species such as poultry and poultry manure (Warnick et al., 2001); presence of wild birds and rodents (Warnick et al., 2001) and liver fluke infected animals (Vaessen et al., 1998). Preventive measures such as vaccination against *S.* Dublin and *S.* Typhimurium in herds can help to reduce the levels of infection (DEFRA-EFSA, 2007b) .

As mentioned previously, if *Salmonella* is isolated from cattle in the UK, it has to be officially reported, and in 2006, 90% (n=750) of reported *Salmonella* isolates found in cattle were from clinical cases (DEFRA, 2007a).There is not routine monitoring for cattle herds for *Salmonella* in the UK and in Europe. A UK survey undertaken between 1999-2001 in dairy farms determined an average prevalence of 19% with higher prevalence in late summer, main *S.* serovars isolated were *Salmonella* Dublin, *S.* Agama and *S.* Typhimurium (Davison et al., 2005). A 2003 UK survey in livestock at slaughter found a prevalence of *Salmonella* spp. in cattle of 1.4% and the predominant *Salmonella* serovars isolated were *S.* Dublin and *S.* Typhimurium. This coincides with the serovars most commonly isolated from reported *Salmonella* cases in the UK in 2006 (DEFRA, 2007a; Milnes et al., 2007). These differences in the prevalence of *Salmonella* could be due to a number of factors such as time of sampling, methodology used, type of animals, sample size, diet, etc.

1.3.4 Salmonella in wildlife

Salmonella can be found worldwide in a range variety of environments and animals. It is therefore not unexpected that *Salmonella* has been isolated from a variety of wildlife animals.

Most studies carried out in wildlife have been as part of surveys in domestic animals or have been done on a relatively small scale, so little is known about the epidemiology of wildlife infection.

Birds

There are contradictory statements in the literature about the role of *Salmonella* in wild birds: Some authors have considered their role to be as reservoirs while other just consider wild birds are mere accidental hosts of *Salmonella*. It also seems that the epidemiology and predominant serovars of this bacterium in wild birds is different to serovars domestic poultry. The living habitat of birds e.g. highly *Salmonella* contaminated environment (Cizek et al., 1994) and different bird species' dietary habits, may also have an effect on *Salmonella* carriage by wild birds (Casanovas et al., 1995; Kobayashi et al., 2007; Millan et al., 2004; Robinson and Daniel, 1968) as well as the health status of the bird(Pennycott et al., 2006).

It is not even known if *Salmonella* is part of the normal flora of wild birds. For example, it has been observed that gulls tend to excrete *Salmonella* for 1-4 days in very small quantities, suggesting that this organism is not part of their normal flora and is acquired mainly from the environment (Alley et al., 2002; Palmgren et al., 2006).

The serovar most frequent in wild birds was *S.* Typhimurium, in contrast to *S.* Enteritidis in domestic poultry (Alley et al., 2002; Palmgren et al., 2006). Differences in prevalence have been observed depending on the age, clinical status and bird species. *Salmonella* prevalence in opportunistic feeders such as gulls and pigeons tends to be low, 0.8-38%, with a higher prevalence in younger birds (Cizek et al., 1994). It is also suggested that salmonellosis is more likely to be endemic in those species that have a tendency to be infected asymptomatically (Boqvist et al., 2003; Casanovas et al., 1995; Cizek et al., 1994; Kapperud and Rosef, 1983; Palmgren et al., 2006; Pedersen et al., 2006). In contrast, in different species of migrating birds, only one sample was positive for *Salmonella*, indicating an almost non-

existant prevalence in those species that have reduced contact with livestock, human waste and *Salmonella* contaminated environments (Hernandez et al., 2003).

A higher prevalence of *Salmonella* has been observed in small passerines such as house sparrows (up to 66%) and greenfinches (up to 71%) during winter epidemics amongst these birds in feeding stations and garden feeders in the UK, Norway and several other countries (MacDonald and Brown, 1974; Pennycott et al., 2006; Pennycott et al., 1998; Refsum et al., 2003).

Salmonella isolates from wild birds can be transmitted to domestic animals and humans (Refsum et al., 2002; Tauni and Osterlund, 2000). For example gulls have been identified as possible vectors for *Salmonella* transmission from contaminated environments into cattle (Reilly et al., 1981). On the other hand, certain *S.* Typhimurium strains from passerines collected from gardens and farms in the UK are believed to be host-adapted and pose a very low zoonotic risk for humans, as these strains lacked the *sopE* gene often associated with human salmonellosis (Hughes, 2007). Two cases of disease in humans caused by *S.*Typhimurium have been linked with parallel *Salmonella* epidemics occurring in small passerines.

Rodents

Mice and rats can be reservoirs and excrete high numbers (more than 10^4 organisms) of *Salmonella* serovars in their faeces for long periods of time (Davies and Wray, 1995; Hilton et al., 2002; Khalil, 1938 ; Welch et al., 1941). Transmission of *Salmonella* between domestic poultry and rats and mice is also possible (Liebana et al., 2003) and also between rodents and humans (CDC, 2004). Rodents can become infected with a small dose of organisms (Welch et al., 1941). It has also been demonstrated that one of the main ways of persistence of *Salmonella* in rodent populations is via faeco-oral (Welch et al., 1941). Rodents can have a natural resistance to some *Salmonella* such as *S.* Typhymurium (Hetzer, 1937; Hormaeche,

1979; Wigley, 2004) and have also been associated with outbreaks of *Salmonella* in humans due to close contact with infected pet rodents (Swanson et al., 2007).

High differences in the prevalence of *Salmonella* in rodents have been reported (Guard-Petter et al., 1997; Hilton et al., 2002; Pocock et al., 2001). The presence of *Salmonella* in poultry populations, in the environment (Pocock et al., 2001) and also the infestation densities of rodents can all influence the intra-species transmission and maintenance of *Salmonella* within rodent populations. One study isolated *Salmonella* in house mice only from already infected poultry units but not from rodents caught in "clean" units (Henzler and Opitz, 1992). Many studies on mice have been concentrated on house mice populations around domestic poultry flocks, but not in other livestock such as cattle. The number of studies carried out on the presence of *Salmonella* in rodents on cattle farms is very limited, Warmick *et al* , in a case-control study, determined that the presence of rodents or rodents droppings on US cattle farms could pose a risk for *Salmonella* infection in cattle. A longitudinal study carried out with faecal samples from house mice on livestock farms did not isolate *Salmonella* from 222 mice samples (Pocock et al., 2001).

Information about the dynamics that *Salmonella* spp. have in other wild rodent populations such as wood mice and bank voles around UK cattle farms is almost non-existent (Warnick et al., 2001). A study in 151 wild rodents, field voles and bank voles, did not isolate *Salmonella* from internal organs of any of the rodents in Finland (Soveri et al., 2000). Euden *et al* could not isolate *Salmonella* from a bank vole in Cornwall (Euden, 1990). Currently, there is a lack in the number of studies carried out in wild rodents to understand the role these populations play in the epidemiology of *Salmonella* serovars.

Other wildlife

Salmonella serovars have been isolated from badgers in several studies. This is believed to be associated with their scavenging diet habits, although very few studies have investigated the prevalence and other epidemiological characteristics of *Salmonella* in badgers, and shedding

patterns and the potential transfer of this bacterium to other animal species are not well understood. Variation in the prevalence has also been observed. For example, in a study examining 4881 samples from badgers in Cornwall (UK), *Salmonella* was isolated from 7.2% of the samples(Euden, 1990). A study of badgers in Cheshire determined a social group *Salmonella* prevalence of 72% and a wildlife survey carried out in the Basque Country (Spain) determined a prevalence of 18% (Millan et al., 2004; Wilson et al., 2003). It is not known why such differences in prevalence in badgers from different areas might be seen. Badgers can carry a wide variety of *Salmonella* serovars including *S.* Dublin, *S.*Typhimurium including Definitive Phage Type (DT) 104, *S.* Enteritidis, *S.* Newport, *S.* Lomita, *S.* Ried, *S.* Ajiobo and *S.* Agama, of which is the most often serovar isolated from badger populations (Euden, 1990; VLA, 2005; Wilson et al., 2003; Wray et al., 1977). Some of these serovars rarely produce disease in humans and livestock, although in one an abortion case in cattle due to *Salmonella* Agama badgers were implicated and transmission of *Salmonella* Typhimurium DT42 between badgers and cattle has also been suggested (Euden, 1990; Humphrey and Bygrave, 1988).

Salmonella has been also isolated from a range of other wildlife species including foxes, hedgehogs and even arthropods (Euden, 1990; Handeland et al., 2002; Holt et al., 2007; Millan et al., 2004).

The purpose of this study was the evaluation of the role that wildlife might play in the epidemiology of campylobacteriosis, salmonellosis and VTEC (including *E. coli* O157) infections of domestic cattle on six farms situated in an area of high cattle density in Cheshire (UK). In particular the aims and objectives were:

- To determine the prevalence, risk factors and distribution of these bacteria amongst different wildlife hosts, domestic cattle and farms;

- To determine the virulence and antibiotic resistance genes distribution amongst cattle and wildlife *E. coli* isolates;
- To determine the molecular relatedness of isolates, and thereby investigate the variation in bacteria within and between hosts, and over several spatial scales.

Chapter 2 General Materials and Methods

2.1 Study area and sample collection

2.1.1 Study area

Faecal samples were collected from six cattle farms in Cheshire. The farms had all been part of a previous study of zoonotic bacteria undertaken through the Liverpool Defra Epidemiology Fellowship (Kemp et al., 2005; Leatherbarrow et al., 2004), which included all the farms in a 10x10km area of Cheshire. The area was chosen as being representative of the region, which has one of the highest densities of dairy cattle in Great Britain. For this project, three pairs of neighbouring farms were chosen in order to study bacterial diversity and transmission on several scales: within farm, between farms and over larger distances. The pairs of farms were also chosen in order to include different habitats: two of the farms were on the Sandstone Ridge, the others were on the Cheshire Plain. One farm, MF, was beef, the other five dairy.

Previous studies had shown the area to provide a suitable habitat for a range of wildlife that might come into contact with the domestic livestock. Sampling strategies were as set out below and in individual chapters.

2.1.1 Cattle Sampling

Faecal samples were collected from fresh faecal pats from different husbandry groups of animals within each farm. Approximately 10 grams of faeces per pat, were collected from each group, and placed in a sterile 'universal' tube for transport to the laboratory.

Laboratory processing began within 4 hours of collection.

Sampling of cattle faeces was conducted systematically during the cross-sectional study. The sample size was limited by time and laboratory resources to approximately 50 samples per farm (approximately 10% of the total number of cattle per farm). This sample size was sufficient to be 80% confident that sample prevalences were within 5% of population values, assuming a population prevalence of 10%. Cattle samples were collected in a representative way depending of the different age groups.

2.1.2 Wild birds

Wild bird samples were all collected in collaboration with BTO-licensed ringers from the Merseyside Ringing Group (www.merseysiderg.org.uk), and birds were handled according to strict welfare criteria (Redfern, 2001) Mist nets and Larson traps, specifically for magpies, were placed on the farms at sites judged to enable the sampling of birds representative of those found in each habitat on that farm, but also to sample birds at sites close to cattle in order to assess transmission between cattle and birds. Live birds caught in the mist nets were placed in clean paper bags, and droppings were collected from the bags using sterile swabs (TRANSWAB, Medical Wire&Equipment Co. Ltd., Corsham, Wilts, England). Samples were processed in the laboratory within 24 hours of collection, and often the same day as collection.

Whenever possible, bird samples were collected at approximately the same time as rodent samples. This was not always possible due to adverse weather conditions such as rain or on welfare grounds e.g. nesting season.

2.1.3 Wild Rodents

Wood mice (*Apodemus sylvaticus*), bank voles (*Myodes glareolus*), field voles (*Microtus agrestis*) and house mice (*Mus domesticus*) were live-trapped in sterile Longworth traps (Penlon Ltd.,Oxfordshire, UK). Longworth traps were used as they generally catch only one animal, enabling individuals' faeces to be collected.

Traps were placed at different habitats within each farm, including hedgerows and field margins as well as inside the animal sheds and animal food stores. Trapping sites were chosen in order to be representative of both the habitats available and the areas of the farm where wildlife might be expected to have some contact with cattle.

Each trap was sterilised by autoclaving before use, and was filled with grain and sterile hay prior to trap field placement. Faecal samples were taken from traps where animals were physically trapped. Before using the grain in the traps, this was microbiologically tested to determine that it was free of any of the enteric pathogens being investigated in this study.

Information was recorded for each rodent sampled, including species, weight and sex.

Rodent faecal samples were collected using a sterile cotton swab and scraped into a 5 ml sterile tube before being transported to the laboratory, and laboratory processing was started within four hours of collection.

2.1.4 Other wildlife

Samples from other wild mammals such as rats, badgers , foxes and, occasionally, larger wild birds such as corvids were collected opportunistically from the ground during the field sampling sessions. The source species were identified based on the faecal characteristics, or, in the case of birds, direct observation of defecation. This was recorded together with the date, spatial location and farm.

In addition, rat traps were placed on several farms when farmers reported rat activity or rat droppings were found. Rat traps were mainly located at the cattle barns. Sample collection and processing was as described for other wildlife species.

2.2. *Isolation and Characterisation of Campylobacter* spp

2.2.1 Isolation and culture

The protocol for the isolation of *Campylobacter* spp was as described previously (Kemp et al., 2005): 2 ml of brain heart infusion broth (LabM, Bury, UK) containing 5% glycerol (Sigma, Dorset, UK) were added to 0.5 -2 grams of faeces and thoroughly mixed. Approximately 500 µl of faecal suspension were added to 4.5 ml *Campylobacter* enrichment broth containing 10% lysed horse blood, placed in a microaerophilic variable atmosphere incubator (VAIN) (74% nitrogen, 11% oxygen, 3% hydrogen, 12% carbon dioxide) and incubated at 37°C for 24 hours. One loopful (5µl) of broth was placed on to *Campylobacter* selective agar (CSA) (LabM, Bury, UK) containing cefoperazone and amphotericin and incubated for up to 72 hours. Colonies morphologically characteristic of *Campylobacter* were placed on to Columbia blood agar (CBA) (LabM, Bury, UK) containing 5% defibrinated horse blood (Southern Group Laboratory) and incubated in a VAIN at 37°C for 48 hours. Presumptive *Campylobacter* colonies were allocated a unique culture collection number and frozen in microbank vials at -80°C awaiting further identification.

For identification and confirmation of isolates, campylobacter isolates were resuscitated by placing a microbank (Pro-Lab Diagnostics, UK) bead on a CAB plate and incubating for 48 hours in the VAIN. Each isolate was subjected to a Gram stain and those consisting of Gram negative curved rods were used to prepare cell lysates for PCR confirmation.

Lysate preparation was done by placing a loop (5µl) of pure culture in a sterile 1.5 ml tube containing 100 µl of sterile water and incubated at 100 °C for 20 minutes.

2.2.2 Molecular characterisation of Campylobacter spp

Several PCR methods were used on the isolates to help determine both their genus and species. A hierarchical protocol was developed as outlined in the hierachical flowchart in Figure 1. The sequences of the primers used and product size information are shown in Appendix 1-Chapter2.

Multiplex PCR ('Wang method')

Lysates were tested in a slightly modified protocol adapted from that described by Wang *et al.* (2002)(Wang et al., 2002), which it is claimed can identify *Campylobacter* spp (*C. jejuni*, *C. coli*, *C.lari* and *C. upsaliensis*). This is a multiplex PCR assay in which differences between species level are detected using the 23S rRNA and *hipO* genes for *C. jejuni* and *gly*A gene for *C. coli, C. lari* and *C. upsaliensis*.

Each 25 µl of reaction contained 200 µl of deoxynucleotide triphosphates (dNTPs), 2.5µ l of 10 x reaction buffer (ABgene), 20mM MgCl2 (ABgene), 0.5 µl of *C. jejuni* primers, 1µ l of *C. coli* and *C.lari* primers, 2µl of *C. upsaliensis* primers, 0.2 µl of 23S rRNA primers, 1.25 U of Taq DNA polymerase (ABgene) and 2.5 µl of DNAtemplate. The reaction cycles were as follows: a denaturation cycle at 95°C for 6 minutes followed by 30 cycles of amplification consisting of a first denaturation step at 95°C for 0.5 minutes, then an annealing step at 59°C for 0.5 min and an extension step at 72°C for 0.5 min. This finished after 30 cycles with a final extension at 72°C for 7 minutes. The amplified DNA was analysed by electrophoresis through a 1.5% agarose (Hi-Low EEO agarose, Biogene.com) gel run at 120 v for 90 minutes and stained with ethidium bromide (Sigma, Dorset UK). The PCR products were visualised using a UV light Gel Doc 2000 transilluminator.

Isolates negative in this PCR were considered not to be *Campylobacter* species; isolates giving a positive reaction for *C. jejuni*, *C. coli*, *C. lari* and *C. upsalinesis* were considered

those *Campylobacter spp.* and isolates that gave a positive reaction for the 23 SrRNA gene were examined for determinant genes for *C. hyointestinalis* and *C. fetus* using an multiplex PCR protocol adapted from that developed by Linton *et* al (1996)(Linton et al., 1996).

Multiplex PCR ('Linton method')

This PCR is claimed to detect differences at species level for *C .hyointestinalis* and *C. fetus* based on 16 S ribosomal RNA (rRNA) gene. Each 25 µl PCR reaction contained 0.625 units *Taq*DNA polymerase , 20mM Tris-HCl [pH 8.3]; 50mM KCl (ABgene), 2.5mM $MgCl_2$, 0.2mM dNTP's , 0.4µM each primer and 1µl of DNA template.

This protocol consisted of 25 cycles of a first step of denaturation at 94°C for 1 minute, then an annealing step at 59°C for 1 minute and finally an extension step at 72 °C for 1 minute. The amplified DNA was analysed as described above.

PCR products from isolates positive for *C. hyointestinalis* and *C. fetus* were considered characterised as these species. PCR products negative for *C. hyointestinalis* and *C. fetus* were examined using a multiplex PCR protocol adapted from that described by Gonzalez *et* al (1997)(Gonzalez et al., 1997).

Multiplex PCR ("Gonzalez method")

Isolates were examined for the virulence gene *ceu*E to detect *Campylobacter jejuni* and *C. coli* using the multiplex PCR described by Gonzalez *et* al (1997). Each 25 µl reaction contained 200µl of dNTPs, 50 mM KCL, 3.5 mM MgCl2, 0.5U of Taq DNA polymerase, 1µl of each primer, 1 µl of DNA template. The PCR reactions were as follows: 30 cycles consisting of a denaturate step at 94°C for 30 seconds, then an annealing step at 57°C for 30 seconds followed by an extension step at 72°C for 1 minute. Finally, an additional extension step was performed at 72° C for 5minutes. Negative isolates were tested in a protocol

described by Karenlampi *et al.* (2004) (Karenlampi et al., 2004) to determine *Campylobacter* species based in the conserved *Gro*EL genes.

GroEL PCR ("Karenlampi method")

A single stage PCR was performed to amplify a 592-nucleotide region of the *GroEL* gene of *Campylobacter* and *Arcobacter* spp. Amplification was carried out using M13-H60F and T7-H60R primers in order to avoid cloning (this would be very unpractical in this project due to the volume of samples processed, time and possible contamination post- amplification. Moreover, the H60R primer was slightly modified at the 22-23 nucleotide level following the author's error amendment (highlighted in Appendix 1-Chapter2).

Each 50µl of reaction contained 41 µl of master mix (AB gene) with 2.5 mM MgCl2, 3 µl of each primer and 3µl of DNA template. The PCR reactions consisted of an initial denaturation at 95°C for 2 minutes followed by 40 amplification cycles of a first step of denaturation at 95C for 1 minute, then an annealing step at 50°C for 1 minute and an extension step at 72°C for 3 minutes. After 40 cycles, a final incubation step at 72°C for 5 minutes was conducted. Isolates negative against this PCR were considered not to be *Campylobacter* species Positive isolates were DNA purified and sequenced for identification purposes as follows:

DNA sequencing

Purification of PCR products for nucleotide sequencing was performed using a commercial kit according to the manufacturer's instructions (QIAquick PCR Purification Kit; Qiagen Ltd) to remove excess primer and unincorporated nucleotides.

Purified amplicons were sequenced commercially and the corresponding PCR degenerated reverse primer was also sent with the amplicons in order to obtain the correct sequence (Advanced Biotechnology Centre (ABC), Imperial College London, SW7 2AZ).

2.3. *Isolation and Characterisation of Salmonella Serovars.*

2.3.1 Isolation and culture

Salmonella serovars were isolated using a standard protocol: 500µl from the faecal suspension was placed in 4.5 ml buffered peptone water with vancomycin (selective against cocci and Gram positive bacteria) and incubated at 37°C for 24 hours. Of this suspension, 100 µl were added to 5 ml Rappaport-Vassilladis broth (RVB) (LabM, Bury, UK) broth and incubated at 42°C for 24 hours and 100µl from the resultant broth was placed on the central part of a Rappaport-Vassilladis semi-solid agar (RVA) plate and incubated at 37°C for 24 hours. The highly motile salmonellae move through the semi-solid medium, and positive isolates (those which had swarmed to the outer edge of the plate) were placed on MacConkey agar (LabM, Bury, UK) and incubated at 37°C for 24 hours to determine if they were lactose fermenters.

2.3.2 Characterisation of Salmonella

Non-lactose fermenters (NLF) isolates were tested with somatic polyvalent O antisera and polyvalent flagellar H antisera (Prolab Diagnostics) for agglutination.

Isolates positive for agglutination with poly O and poly H antisera were further confirmed biochemically as *Salmonella* spp. using the API20E biochemical test strip following the manufacturer's instructions (bioMérieux).

Salmonella serovars were identified using specific antisera (VLA Weybridge) against somatic O and flagellar H antigens using the Kauffman and White serotyping scheme.

2.4 Isolation and characterisation of VTEC and E. coli O157

2.4.1 Microbiological isolation of E. coli

Initial isolation of *E. coli* was done following a standard protocol: 500µl from the faeces in brain heart broth with glycerol were added to 4.5 ml of buffer peptone water (LabM, Bury, UK) and incubated at 37°C for 24 hours. A loop (5µl) of the broth was used to inoculate an eosin methylene blue agar (EMBA (LabM, Bury, UK) plate) and incubated at 37°C for 24 hours.

Plates were examined for the presence of blue/purple metallic colonies and 10 individual metallic colonies per plate were used to inoculate a microbank vial, given an individual reference number and frozen to -80°C awaiting further identification.

For resuscitation, a single bead of the 10 pooled colonies from each vial was used to inoculate 3ml of nutrient broth and placed at 37°C for 4 hours, after which 0.5ml was placed in a sterile eppendorf and heated at 100°C for 20 minutes to prepare cell lysates for testing by PCR.

2.4.2 Testing E.coli for vt and eae genes

E. coli cell lysates preparations were examined for *eae*A, *vt*1 and *vt*2 by multiplex PCR. Each 25 µl of reaction contained 200µl dNTPs, 1x reaction buffer, 2.5 MgCl2, 1 M of each primer, 0.5U Taq DNA polymerase, 1µl of DNA template. The primers used were: eaeAF GCTTAGTGCTGGTTAGGATTG, aeaAR CCAGTGAACTACCGTCAAAG (Beebakhee et al., 1992; Yu and Kaper, 1992), VT1F CGCTGTTGTACCTGGAAAGG, VT1R CGCTCTGCAATAGGTACTCC, VT2F GCTTCTGCTGTGACAGTGAC and VT2R TCCATGACAACGGACAGCAG (La Ragione et al., 2002). The reaction conditions were as follows: initially the mix was held for 2 minutes at a temperature of 94°C, followed by a cycle of denaturation for 1 min at 94°C, annealing at 62°C for 1 min 30 seconds and primer

extension at 72 °C for 2 minutes. The cycle was repeated 25 times followed by 5 minutes at 72°C and cooling to 4C. The amplified DNA was analysed as already described. The sizes of expected products were approximately 625 bp for *eae*A, 250 bp for *vt*1, 190 bp for *vt*2.

2.4.3 Isolation of E. coli O157

Samples from domestic cattle, large birds such as corvids, rabbits, foxes and badgers (ie species from which it was possible to collect large enough faeces samples) were examined for *E.coli* O157 by immunomagnetic separation (IMS) (Chapman et al., 1997). For this, 1g faecal sample was added in 9 ml of buffered peptone water and incubated at 37°C for 24 hours, and then 1ml broth was placed in a 1.5 ml sterile tube containing 100µl of IMS beads (Captive O157, Lab M, UK). The tubes were mixed at room temperature for at 20 minutes before being inserted into magnetic separator racks (Dynal MPC-5), shaken, and left for 3 minutes for the beads to be attracted to the magnet.

The supernatant was carefully removed, the magnet was taken away and the beads were washed three times in 1ml phosphate buffered saline (PBS), after which the beads were re-suspended in 100 ml of PBS. Half of this bead suspension was plated onto sorbitol MacConkey agar (SMAC) and the other half onto sorbitol MacConkey agar incorporating cefixime (0.05 mg/L) and tellurite (2.5mg/L) (CT-SMAC). The plates were incubated at 37°C for 24 hours, and colonies with a typical morphology (2-4 mm diameter, translucent glossy, convex with an entire edge) by comparison to control colonies of *E.coli* O157 were selected and plated on to EMBA agar and incubated at 37°C for 24 hours.

Characteristic metallic colonies on EMBA were confirmed as *E. coli* O157 using a dry latex agglutination test for O157 antigen (Dry-spot, Oxoid, UK).

Isolates testing positive on the latex agglutination test for O157 were plated on nutrient agar and incubated at 37C for 24 hours and further confirmed biochemically as *E. coli.* using an API20E biochemical test strip (bioMérieux).

All assays for the isolation of *E. coli* O157 were undertaken under category 3containment.

2.4.4 Molecular characterisation of E. coli O157

PCR

Isolates from 2.4.3 were also examined by PCR for another O157 characteristic virulence determinant, the *rfb* gene which encodes for the O antigen (Paton and Paton, 1998). A loop of the isolates pure culture was prepared for cell lyssates as described in 2.2.1 paragraphs. Each 50 ml of PCR mixture contained 2 ml of DNA extract, 200 mM concentration of dNTP's, 250nM concentration of each primer, 1U of Taq polymerase in 10mM Tris-HCL (PH8.3), 50mM KCL, 2nMMgCl2 0.1% gelatine, 0.1% Tween 20 and 0.1% Nondet P-40. The primer sequences were as follows: F, CGGACTCCATGTGATATGG and R, TTGCCTATGTACAGCTAATCC. The reaction cycle was repeated for 35 times and it consisted of 1 min of denaturation at 65°C, 2 min of annealing at 65°C for the first 10 cycles gradually reduced to 60C by cycle 15 and 1.5 min of elongation at 72°C increasing gradually from cycle 25 to 2.5 min by cycle 35. PCR products were electrophoresed using a 2% agarose gel stained with ethidium bromide and visualised under UV light Gel Doc 2000 transilluminator. The expected product size was 259 bp.

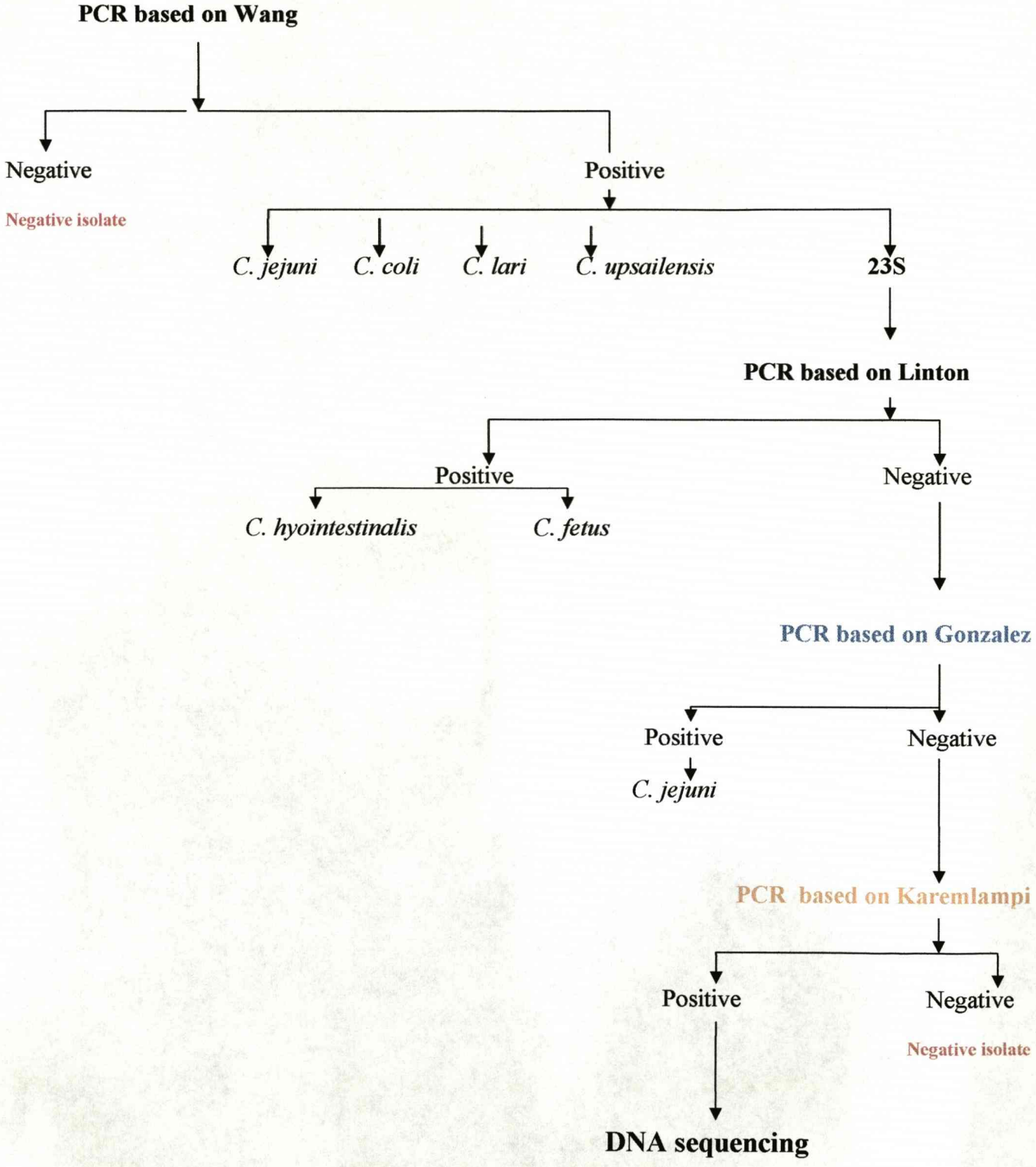
PFGE

Isolates of *E coli* O157 isolates were compared using the rapid *E. coli* PFGE method adapted from the standard protocol developed by PulseNet, Centers for Disease Control and Prevention (CDC), USA. A loopful of culture on nutrient agar was suspended in 2ml fresh-made cell suspension buffer (CSB) (100mM Tris, 100mM EDTA,PH8.0). The optical density at 610nm (OD610) of a 1:10 suspension (100ml suspension in 900 ml of CSB) was measured in a spectophotometer (Secoman, NorthStar Scientific Ltd, UK) and used to calculate the proportion of cell suspension and CSB needed to make a 200 ml suspension with an OD610 of 1.35. Proteinase K (10ml of 20 mgml-1 solution, Sigma, Dorset, UK) was added to each 200 ml suspension, with 200 ml of agarose mixture (1% Bio-Rad (UK) PFGE grade agarose , 1% SDS in 1xTE) and mixed carefully by pipetting. The mixture was transferred in duplicate to moulds and allowed set at 4°C. Later, the solid plugs were placed in 3ml of cell lysis buffer (CLB)(50mM Tris, 50mM EDTA, 1% sacorsyl, PH 8.0) plus 15 µl of proteinase K and incubated while being shaken at 54°C for 2 hours. The buffer was removed and replaced with 3ml pre-heated sterile water and incubated at 54°C for 15 minutes. This procedure was repeated twice. The water was then removed and 3 ml of pre-heated 1x TE was added and incubated for 15 minutes at 54°C. This procedure was repeated four times. One plug was placed in a sterile bijoux in 1ml of CSB and stored at 4°C and the other was placed in a 1.5 ml sterile eppendorf tube containing 200 µl 1x *xba*I restriction buffer (Promega, UK) and incubated at 37°C for 15 minutes.

The buffer was then removed and replaced with a 200µl of xbaI restriction buffer containing 50U of xbaI enzyme (Promega, UK) , and incubated at 37°C for 2 hours. Half of the incubated plug was loaded into 1% PFGE agarose gel (Bio-Rad Laboratories), and the other half kept as back-up. Three ladder plugs (Bio-rad PFG Lambda Ladder) were also loaded to normalise the gel. The gel was run at 14°C for 20 hours at an initial switch time of 2.2s and a

final switch time of 54.2s in a CHEF-DRIII-PFGE machine. The gel was stained in a 0.5 ml/l ethidium bromide solution and visualised under UV light in a Gel Doc 2000 transilluminator.

Figure 1. Flowchart of hierarchical method used to characterise *Campylobacter* isolates to the species level using several PCR's .



Chapter 3 Cross-sectional study of *Salmonella, Campylobacter* and VTEC in domestic cattle and wildlife species from six farms in Cheshire (UK) from July 2004 to May 2005

3.1 Introduction

Approximately 1.3 million cases of food-borne disease were reported in human beings in the UK during 2000. Enteric bacteria, led by *Campylobacter*, *Salmonella* and *E. coli* O157, accounted for a high proportion of those cases (Adak et al., 2002). Domestic animals are known to be a natural reservoir for these bacteria. Furthermore, these bacteria have also been isolated from a wide range of wildlife species, adding to the evidence that wildlife can become infected with these pathogens (Kwan et al., 2008a; Liebana et al., 2003; Nielsen et al., 2004a). The role that wildlife may play in the epidemiology of these bacteria in domestic livestock and human beings is unknown.

Research on the role of wildlife in the epidemiology of these pathogens has so far been undertaken on a small scale, and even fewer studies have been carried out in wildlife species living or having activity in areas close to high concentrations of domestic livestock such as farms or abattoirs. Not even the prevalence of these pathogens in domestic herds is known in the UK. A recent survey carried out in UK cattle, sheep and pigs at slaughter showed that the prevalence of *Campylobacter*, *E. coli* O157 and *Salmonella* in these animal species can be high (Milnes et al., 2007).

The epidemiology of these bacteria in wildlife can be complex and infection with these pathogens may be more prevalent in certain wildlife hosts than in others. Rodents are often blamed for outbreaks of clinical salmonellosis in domestic livestock but studies that test this are difficult to find. A number of studies could not isolate any *Salmonella* from mice on farms (Pocock et al., 2001), but there is increasing evidence that badgers, hedgehogs and reptiles could act as a natural reservoir for *Salmonella* (Bertrand et al., 2008; Handeland et

al., 2002; Wilson et al., 2003). Wild birds are also blamed for carrying *Salmonella* at a high prevalence, but recent studies undertaken in healthy migrant wild birds in Sweden and Norway showed the prevalence of this bacterium to be low (Hernandez et al., 2003; Refsum et al., 2003). In contrast, a high prevalence of *Salmonella* has been reported on sick or dead passerines found around bird feeders in gardens (Pennycott et al., 2005; Pennycott et al., 2006).

Campylobacter is considered to be prevalent in a whole range of domestic animals and the environment. High prevalence of *Campylobacter* infection has been found in wild bird species such as gulls and pigeons (Waldenstrom et al., 2002). *Campylobacter* spp have also been isolated from rodents and rabbits on livestock farms (Kemp, 2005a; Kwan et al., 2008a; Meerburg et al., 2006).

Ruminants, particularly cattle, are considered to be the natural reservoir for *E. coli* O157 and other VTEC. In addition, rabbits are sometimes considered to be a source of *E. coli* O157 (Kemp, 2005a; Leclercq and Mahillon, 2003). Furthermore, *E.coli* O157 has also been isolated from foxes and wild birds such as gulls and pigeons (Dell'Omo et al., 1998; Kemp, 2005a; Pedersen et al., 2006). Wild birds have also been reported as the source of *E. coli* O157 in a human outbreak (Ejidokun et al., 2006). Other non-O157 *E. coli* have been isolated from rodents and wild birds on farms, suggesting that these wildlife species could act as amplifiers of VTEC strains (Nielsen et al., 2004a).

There is a need for epidemiological studies on domestic livestock and wildlife on farms in order to shed some light on the role of wildlife in the epidemiology of these bacterial infections. Recent studies suggest that the transmission of these pathogens and wildlife species can occur (Kemp, 2005a; Liebana et al., 2003; Manning et al., 2003).

The aims of this study were to:

1- Determine the prevalence of *Salmonella*, *Campylobacter* and VTEC in domestic livestock and wildlife species (small rodents, wild birds, larger wildlife mammals) on six cattle farms in Cheshire (UK) ;

2- Determine possible risk factors associated with infection with these three pathogens in domestic animals and wildlife;

3- Determine possible spatial clustering of infection in rodents and wild birds by habitat, within and between farms and at a slightly larger geographic scale;

4- Determine genetic similarities and differences of *E. coli* O157 from domestic cattle and wildlife species from the same farm between 2002 and 2007.

3.2 Materials and Methods

Microbiological isolation and molecular characterisation

General microbiological methods and molecular characterisation methods such as PCR assays, IMS and PFGE have been described in Chapter 2.

Numerical analysis of PFGE-DNA profiles for differentiating different DNA band patterns of O157 *E. coli* was performed using Bionumerics applied maths 1998-2005 software (www.applied-maths.com). Optimal band alignment was conducted using a maximum band position tolerance of 2% to compensate for between- gel variance. A dendrogram based on the Dice coefficient was built using the Underweighted Group Method with Arithmetic Mean (UPGMA).

Due to the small number of animals infected with *E. coli* O157 detected during both studies , all samples plus two strains isolated from wildlife (one fox and one rabbit) and cattle (two calves and one adult) in MF from a previous study on this farm in 2002 were included in the PFGE strains comparison (Kemp, 2005a) an isolate from a fox collected on this farm in

December 2005 was also included. It was hoped that this approach would provide a more accurate picture about the different strains present on the farm, and strain dynamics over time.

Descriptive statistics

The prevalence of *Campylobacter spp* and VTEC virulence determinants together with the binomial 95% Confidence Intervals (C.I.) were calculated using the "exact binomial" command in the Statspages.net free statistical software (www.statpages.org).

A-Epidemiological analysis of risk factors

Variables were classified in different subgroups as follows:

Classification of bovine animals per age and management

Cattle were classified by age and management group in order to explore possible significant differences in the prevalence of *E. coli* and *Campylobacter.* Groups were classified using the following code:

1. *Calves*- young bovine animals that have access to milk, up to five months of age;
2. *Weaned calves*- any young bovine animal not having access to milk but of pre-breeding age – 6 to 10 months of age;
3. *Adult* –animals that have reached sexual maturity- more than 10 months of age. This group includes heifers, steers and finisher beef animals;
4. *Lactating cow*- female animals that after calving are lactating for commercial reasons in dairy farms or have a calf at foot in beef herds;
5. *Dry cow*- adult dairy cows that have been dry of milk prior to calving, or a lactating cow that has previously calved but is not yet producing milk.

Wild bird classification

Wild bird species were grouped for the logistic analysis as follows:

1. *Buzzard-* (*Buteo buteo)* the only bird of prey captured in this study;
2. *Corvids-* magpies (*Pica pica*), jackdaw(*Corvus monedula*), raven (*Corvus corax*), jay (*Garrulus glandarius*) and unidentified corvids;
3. *Other birds associated with farmland-* pigeon (*Columba livia*), meadow pipit(*Anthus pratensis*) swallow (*Hirundo rustica*), wren(*Troglodytes troglodytes*), dunnock (*Prunella modularis*), robin(*Erithacus rubecula*), thrushes (family *Turdidae*), starlings (*Sturnus vulgaris*), finches (family *Fringillidae*) and sparrows(family *Passeridae*);
4. *Birds associated with woodland-* warblers (family *Sylviidae*), tits (family *Paridae*), nuthatch (*Sitta europaea*), great spotted woodpecker(*Dendrocopos major*)

Age conversion in small rodents

Body mass was measured for wood mice and bank voles trapped in this study, and these data were converted into age as described by Telfer *et* al (Telfer, 2002).

1. *Juvenile-* Wood mice (*Apodemus sylvaticus*) captured April-July < 15g.; captured August-March <14 g. Bank voles (*Myodes glareolus*) captured April-July <14g; captured August-March <12;
2. *Sub-juvenile-* Wood mice captured April-July between 15-18g; captured August-March between 14-17g. Bank voles captured April-July between 14-17g; captured August-March between 12-14g;
3. *Adult-* Wood mice captured April-July >18g; captured August-March >17g. Bank voles captured April-July >17g; captured August-March > 14g.

Habitat classification

During the cross-sectional study Longworth traps and mist-nets were set along different habitats within the farms. Five general habitat types were defined as follows:

1. *Hedges*: a combination of closely spaced shrubs intercalated with a small number of trees such as oak (*Quercus spp*), ash (*Fraxinus spp*) and sycamore (*Acer spp*). Shrubs included blackthorn (*Purus spinosa*), berberis (*Berberis thunbergii*) and hawthorn (*Crataegus monogyna*). Other vegetation present could be nettles (*Urtica dioica*) and grasses;

2. *Bank:* areas covered by herbaceous vegetation, shrubs and small trees but more sparse than in hedges. Ivy (*Hedera helix*), brackens (*Pteridium aquilinum*) and nettles can also be present;

3. *Woodland*: areas of the farms where deciduous tress are predominant with some undergrowth vegetation such as brackens;

4. *Water bodies*: areas around ponds with patchy wild vegetation such as reeds (for example *Phragmites communis*) nettles, thistles (*Carduus spp*) and grasses;

5. *Farm buildings*: includes different cattle sheds, hay and silage storage areas and slurry pits.

Months grouped as year season

Sampling months were grouped in 4 three-month climatic seasons typical of temperate countries in the northern hemisphere. The seasons were as follows:

1. *Winter*: December 2204 , January 2005, February 2005;
2. *Spring*; March 2005, April 2005, May 2005;
3. *Summer*: (June), July 2004, August 2004. No sampling was carried out in June 2004
4. *Autumn*: September 2004, (October 2004), November 2004. No sampling was conducted in October 2004.

Geographically close farms

The six participating farms (Chapter 2-Appendix IV and V) were grouped into three geographically close pairs to explore the role of geographical distance in bacterial diversity, and possible risk factors over larger distances. The pairs were as follows

1. *Pair1*: MF-PHF
2. *Pair2*: CLF-BHF
3. *Pair3*: BGF-GF

Statistical analysis

Univariate analysis and multivariate analysis were performed using STATA 8.1 (Statacorp 2003). Univariate analysis was performed using Chi-squared tests in order to explore associations between the outcome and binary and categorical variables as described above.

Multivariate analysis was performed using logistic regression. The models for the multivariate analysis included variables that were p-values equal or less than 0.2 in the

univariate analysis. The models were built including all variables with a backwards stepwise approach. Farm random effects were not included in the models due to the small number of participating farms and the small number of positive samples during the study period. Model selection was based on the likelihood ratio test (lrtest) for inclusion and exclusion of dependent variables into the model with acceptance of a p-value ≤ 0.05; the Akaike Information Criteria (AIC) which aimed to find the simplest model that adequately explains the data. The smaller the AIC the better the model is capable of explaining the data. Furthermore, the Bayesian information criterion (BIC) was taken into consideration as well. The BIC parameter is based on the deviance of the model and the smaller the BIC the better the capacity of the model to explain the data.

Owing to the characteristics of the sampling in this cross-sectional study, every farm was sampled twice during different months and not all with in the same months. Thus, month and season are highly correlated with farm location and pair of farms. These two variables have only been included for the univariate analysis. Therefore, the effect of seasonality and/or sampling month was difficult to explore with this study design.

B-Spatial analysis

The presence or absence of significant clusters of rodents carrying VTEC virulence markers, *Campylobacter* spp. and wild birds carrying VTEC virulence markers was tested using SaTScan ^{TM} software version v7, 0.3 May 2007 (www. Satscam.org). The Poison-model was used as the number of animals that carried those bacteria out of the total number of animals captured or 'population' on the six-participating farms was binomially distributed. The p value of the most likely space cluster was obtained through Monte Carlo hypothesis (10,000 replications). Data from cattle were not included as the exact coordinates for the cattle pat

locations were not recorded, only data about whether the animals were inside barns or grazing were collected. Large wildlife was also not considered for the spatial analysis due to a lack of information about where samples were collected.

The plotting of coordinates of infected and uninfected cases and the cluster buffer was done using ArcGIS 9.2 (ESRI-UK).

3.3Results

A total of 2329 faecal samples from cattle and different wildlife species were collected in this study. The percentage of samples collected from each animal species was 21% cattle, 28% wild birds, 44% small rodents and 8% large wild mammals.

Salmonella

Only nine samples were positive for *Salmonella* serovars; *S.* London was isolated from a badger and a calf with on BHF; *S.* Typhimurium from a house sparrow on BHF; and *S.* Dublin from three calves and one cow from GF and one cow from CLH with. No animals infected with *Salmonella* were detected on the other three farms.

The total prevalence of salmonellosis in cattle was 1% (n=497), in wild birds 0.15% (n=650), and in badgers 1.9% (n=54).

E. coli O157

Prevalence

The results of the prevalence study are shown in Table 1. *E. coli* O157 was isolated only in cattle from MF, the only beef farm participating in the study. A total of 86 faecal samples were collected when this farm was sampled in July 2004 (n=37) and March 2005 (n=49) All *E. coli* O157 isolates carried the *eae, rfb* and *vt1* genes.

The overall prevalence in cattle on MF was 20% (n=86). Different age groups had different prevalence. The prevalence differences between age groups were statistically significant (p= 0.023) with higher prevalence in calves and weaned calves than in adult stock in July 2004.

No significant differences were found in prevalence between different age groups in March 2005 (P= 0.220) within age groups between the two sampling times (p= 0.078).

Table 1. *E. coli* O157 prevalence by age group and sampling dates on MF, the cross-sectional study July04-May05. Proportion of positives out of the total number *n* is in braquets.

	n	Prevalence % July 2004	95% C.I.	n	Prevalence % March 2005	95% C.I.
All age groups	37	19 (7/37)	8-35	49	20 (10/49)	10-34
Calves	3	33 (1/3)	0.8-91	9	0	0-33
Weaned Calves	8	50 (4/8)	16-84	0	0	0
Adult bovines	26	7.7 (2/26)	0.95-25	36	25 (9/36)	12-42
Lactating beef	0	0	0	3	33 (1/3)	0.8-91

PFGE

Eleven unique restriction profiles (RP) or banding patterns were identified among *E. coli* O157 isolates from MF. A RP was unique when it had a banding pattern that differed from all other isolates by at least one band. Isolates that were more than 90% similar in accordance with the Dice index formed five distinct RP groups. There was a large cluster (A) of a predominant E. *coli* O157 strains isolated mainly from adult stock and calves collected in July 2004 and March 2005(Figure 1). A second RP (B) was isolated from a weaned calf and adult stock in July 2004 which was 80% similar to the predominant RP group and a third RP cluster (C) corresponded with strains isolated from wildlife and cattle on MF in 2002 was genetically distant to cluster A with 70% similarity. A further RP strain isolated from a 18 months old animal had low similarity, only 45%, to the main cluster A.

When compared with *E. coli* O157 isolated from cattle in 2002, the predominant *E. coli* strain was 80% similar to the 2002 predominant strain. .

The other two RP clusters contained isolates from wildlife. There were four different *E. coli* O157 isolates from the same faecal sample from a fox (in 2005) that comprised four different RPs, three of which were highly similar, but the fourth RP was only 70% similar to the other three isolates and fell into a different cluster. Cluster (C) had small similarities with cluster A, (60%).

Non O157 *VTEC* and *Campylobacter*

The data for VTEC and *Campylobacter* in small rodents, cattle and wild birds seemed not to fit perfectly the GLM models because of the characteristics of the data including the high correlation between variables and the small frequency of infected animals. However, although not perfect, the attempt to model these data helped to confirm and support what was found in the univariate analysis. Therefore, the GLM model results have been included in order to complete the descriptive analysis.

Non- O157 *'VTEC'*

For the purposes of this study, 'VTEC' was defined as the presence of *vt* on its own or together with other genes such as *eae*. Due to the small number of samples that possessed the *vt1* and or *vt2* genes, the outcome variable in the univariate and logistic regression analysis was presence or absence of the *eae* gene. The eae gene has been associated with APEC and VTEC pathotypes. Therefore, the presence of the *eae* gene did not mean that *E. coli* from the samples were considered VTEC, only samples that carried the *vt1* and/or *vt2* were defined as VTEC strains.

Cattle

The proportion of *E. coli* isolates from cattle that carried at least one of the three VTEC virulence genes (*vt1*, *vt2* and or *eae*) was 7.4% (n=497). Both verotoxin genes (*vt1* and *vt2*) were found separately or together in *E. coli* isolated from cattle (Table 2).

CLF followed by MF had the highest proportion of positives although this was not statistically significant (P= 0.106). There were significant differences ($p<0.0001$) in the proportion of positive isolates by age of host. Young and weaned calves were more likely to be infected than other age groups. Moreover, 20% and 27% of isolates that possessed the *vt2* and *vt1* genes also carried the *eae* gene. (Table 3).

These variables associated with the outcome were supported by the logistic regression model as farm, age group and the *vt1* gene variables were part of the final model. Data with no information about age variable was not considered in the final model (30 data). (Tables 9 and 10).

Wild birds

Isolates from wild birds (n=650) in this study carried the *eae* gene (3.25%) and the *vt1* gene (0.5%). None of the 24 isolates from individual birds carried both genes together. The *vt2* gene was not carried by any of the isolates from wild birds. (Table 2).

PHF and MF (pair 1) had the highest proportion of *E.coli* isolates carrying the *eae* gene. Birds that were associated with farmland and corvids had a higher proportion (13.6%, n=66) of carriers of the *eae* gene compared with birds of prey (0%, n=1) (only a buzzard) and birds associated with woodland (0.7%, n=300) ($p<0.0001$). Also the highest proportion of *eae* gene carrier isolates was in birds trapped in farm buildings (10.6%, n=66) ($p=0.05$). Moreover, no *eae* gene carriers were isolated from birds trapped close to water bodies and hedges. (Table 4).

Only location and bird group variables remained in the final logistic model. Data from BGF was not included in the model as no isolates that carried the *eae* gene were detected. Bird species associated with farmland had a higher probality of being carriers of *eae* positive *E. coli* if trapped in PHF or MF. (Tables 10 and 11).

No significant spatial clusters of wild birds carrying VTEC genes were detected (p=0.73). (Figure 2)

Small rodents

VTEC virulence genes were carried by 4.7% (n=1014) *E.coli* isolates from small rodents. (Table 2).

PHF was the farm with the highest proportion of *E. coli* isolates that carried the *eae* gene. There was not a significant association with any particular species of rodent (p=0.782). Approximately 40% (n=26) of isolates that carried the *vt1* gene also carried the *eae* gene as well. (Table 5).

This was confirmed by the logistic final model in which farm location and the carriage of the *vt1* gene were the significant variables. (Tables 10 and 11).

No significant spatial clusters of small rodents infected by VTEC strains were detected (p=0.60). (Figure 3).

Other wild mammals

This is a very heterogeneous group of hosts. Fifteen isolates possessed the *eae* gene and only one isolate from a fox possessed the *vt1* and *eae* genes altogether.

No carriers were found on two of the six participating farms, PHF and GF. *E. coli* isolates from rats were not found to carry any of the three VTEC virulence determinants.

Only the pair of farms variable was significant in the univariate analysis (p=0.02). CLF and BHF as part of pair 2 had a higher proportion 12.5% (n=64) of *E. coli* isolates that possessed the *eae* gene compared to the other two pair of farms. (Table 6).

Campylobacter spp

Campylobacter spp were isolated from a total of 81 faecal samples (127 isolates) from different animals hosts, producing an overall prevalence of 3.5% (n=2329). The prevalence by host was 8.2% (n=497) in cattle; 3.5% (n=1014) in small rodents; 12.5% (n=16) in rats and birds and 0.46% (n=650) in wild birds (an unidentified corvid, a magpie and a blackbird). (Table 7).

Campylobacter was not isolated from domestic dogs, foxes, rabbits or badgers. No mixed infections with multiple *Campylobacter* spp in the same faecal sample were detected. A small number of isolates (4 from cattle and 3 from rodents) could not be characterised to the species level.

C. jejuni was the only *Campylobacter* spp isolated from wildlife, except for one a *C. coli* isolate from a corvid. In contrast, three different *Campylobacter* spp were isolated from cattle: *C. hyointestinalis*, *C. fetus* and *C. jejuni*, of which *C jejuni* was by far the most common.

Owing to the small number wild birds and 'other mammals' found to carry *Campylobacter* spp in this study, only descriptive statistics have been applied to data from these two host groups: univariable analysis and logistic regression were applied only to small rodents and domestic cattle.

Small rodents

All of the *Campylobacter* isolates from rodents were species identified as *C. jejuni* (91%, n=35), apart from three isolates, the species of which could not be determined by the molecular methods used.

Significant differences in the prevalence of *Campylobacter* between rodent species, and also between farms were observed. The prevalence in bank voles 11.3% (n=194) was significantly higher compared with wood mice 0.9% (n=658) ($p<0.0001$) and no house mice (n=76) were found to be infected with *Campylobacter* spp . *Campylobacter* was also isolated from other rodents such as field voles and shrews. Location and habitat had a significant influence on the probability of finding a rodent infected with *Campylobacter*.

GF was the farm with the highest proportion of infected rodents (12.4%, n=153) ($p<0.0001$). Hedge was the habitat with higher prevalence of *C.* infected rodents (5.4% n=443) ($p=0.03$) compared with other kind of habitats such as water bodies (4%, n=75) and farm buildings (1.6%,n= 184) . No infected rodents were found in MF and on banks or woodland habitats. (Table 7).

The logistic models confirmed what was found on the univariate analysis. Data from MF were excluded from the logistic analysis as no rodents from that particular farm were found to be infected with *Campylobacter*. Only location and species of rodent remained in the final model, confirming that being a bank vole on GF had a higher probability of being infected with *Campylobacter* compared to other rodent species and other farms. (Tables 9 and 10).

A significant spatial cluster of 0.08 km was detected in GF with a central point 53° 08'36 75''N- 2° 43'25 98''E.Spatial map. This cluster was situated on a hedge limiting with a red meat processing abattoir. There were nine rodents (six bank voles, two wood mouse and

two unknown rodents) infected by *C. jejuni*, out of 13 rodents captured, all isolated in December 2004. (Figure 5).

Cattle

There were significant differences in the frequency of cattle infected with *Campylobacter* depending on the location, age and management groups and if the animals were inside the cattle shed or grazing. The proportion of infected animals was higher at PHF than on the rest of the farms (14.5%, n=83) (p=0.10). Weaned calves (14.7%, n=34) and lactating cows (10%, n=171) were the two groups with higher proportion of infected animals (p=0.13). Moreover, the proportion of *Campylobacter* infected animals was higher in animals kept inside sheds than in grazing animals 8.5% (n=424) (p=19). The univariate analysis showed that 20% (n=26) of faecal samples that carried the *vt1* gene were also infected with *Campylobacter* (p= 0.04). The *vt1* gene variable was not kept in the final GLM model (p= 0.28). (Tables 8).

Farm location, animals inside barn or on grazing and age-management groups were kept as significant variables in the final GLM model. Data with no information about age groups and inside barn or grazing variables were excluded from the GLM model (46 observations). (Tables 10 and 11).

Table 2. Proportion (%) of samples from different hosts that carried *E. coli* containing different VTEC associated virulence genes profiles

Animal host	**n**	***eae***	***vt1***	***vt2***	***eae-vt1***	***eae-vt2***	***eae-vt1-vt2***	***vt1-vt2***
Cattle	497	5.4	3.0	1.8	1.4	0.6	0	0.4
Wild birds	650	2.7	0.5	0	0	0	0	0
Small rodents	1014	4.0	0.2	0.3	0.09	0	0.09	0.09
Larger wild mammals	168	10.1	0	0	0.6	0	0	0

Table 3. Results of the univariable analysis exploring the relationship between variables recorded in the cross-sectional study and the isolation of the *eae* gene, carried *E. coli* from domestic cattle.

Variable	**Category**	**n**	**Proportion positive**	**P-value***
Farm location	MF	86	0.105	0.126
	PHF	83	0.048	
	CLF	78	0.090	
	BHF	89	0.124	
	BGF	86	0.035	
	GF	75	0.040	
Pair of farms	Pair1	169	0.080	0.051
	Pair2	167	0.108	
	Pair3	161	0.037	
Month[$]	July 04	75	0.107	0.08
	August 04	50	0.140	
	September04	23	0.130	
	November 04	69	0.015	
	January 05	48	0.125	
	February 05	94	0.064	
	April 05	92	0.044	
	May 05	46	0.044	
Season	Winter	129	0.085	0.07
	Spring	138	0.044	
	Summer	125	0.120	
	Autumn	92	0.044	
Age/management group	Calves	58	0.190	<0.0001
	Weaned calves	34	0.150	
	Adult stock	116	0.070	
	Lactating cows	171	0.041	
	Dry cows	88	0.011	
In shed/outside grazing	Grazing	57	0.090	0.593
	In shed	424	0.068	
vt2 gene carrier	*vt2*-carrier	14	0.214	0.043
	vt2-non carrier	483	0.070	
vt1 gene carrier	*vt1*- carrier	26	0.270	<0.0001
	vt1-non carrier	471	0.064	

$ No sampling was carried out in cattle in October 04, December 04, March 05; *P-value derived from chi-square test

Table 4.Results of the univariable analysis exploring the relationship between variables recorded in the cross-sectional study and the isolation of the *eae* gene, carried by *E. coli* isolated from wild birds.

Variable	**Category**	**n**	**Proportion positive**	**P-value***
Farm location	MF	130	0.085	0.0002
	PHF	61	0.066	
	CLF	119	0.050	
	BHF	179	0.006	
	BGF	99	0	
	GF	62	0.032	
Pair of farms	Pair1	191	0.079	0.001
	Pair2	298	0.024	
	Pair3	161	0.012	
Month[$]	July 04	47	0.064	0.26
	August 04	74	0.081	
	September04	97	0.010	
	November 04	36	0.028	
	December 04	1	0	
	January 05	181	0.037	
	February 05	120	0.058	
	March 05	69	0.015	
	May 05	8	0	
	June 05	17	0.059	
Season	Winter	370	0.032	0.85
	Spring	25	0.040	
	Summer	218	0.046	
	Autumn	37	0.027	
Bird group	Buzzard	1	0	<0.0001
	Corvid	66	0.136	
	Farmland birds	283	0.046	
	Woodland birds	300	0.007	
Habitat	Hedges	21	0	0.05
	Bank	105	0.019	
	Woodland	92	0.044	
	Water bodies	18	0	
	Farm buildings	66	0.106	
vtl gene	*vtl*-carrier	3	0	0.694
	vtl-non carrier	647	0.030	

$ No sampling of wild birds was carried in April 2005; *P-value derived from chi-square test

Table 5 Results of the univariable analysis exploring the relationship between variables recorded in the cross sectional study and the isolation of the [illegible] small rodents.

Variable	Category	n	Proportion positive	P-value*
Farm location	MF	105	0.040	<0.0001
	PHF	187	0.080	
	CLF	205	0.078	
	BHF	162	0.006	
	BGF	202	0.015	
	GF	153	0.026	
Pair of farms	Pair1	292	0.065	0.02
	Pair2	367	0.046	
	Pair3	355	0.020	
Month	July 04	60	0.083	<0.0001
	August 04	155	0.142	
	September04	52	0	
	November 04	165	0.024	
	December 04	61	0.016	
	January 05	105	0.019	
	February 05	135	0.015	
	March 05	152	0.033	
	April 05	74	0.014	
	May 05	55	0.018	
Season	Winter	301	0.017	<0.0001
	Spring	281	0.025	
	Summer	215	0.126	
	Autumn	217	0.018	
Rodent's species	Bank Vole	194	0.041	0.782
	Field Vole	23	0	
	House Mouse	76	0.026	
	Wood Mouse	658	0.047	
	Shrew	17	0.060	
	Unknown Rod.	46	0.022	
Gender	Female	402	0.045	0.624
	Male	480	0.044	
	Juvenile$	13	0	
	Unknown	53	0.076	
Age	Juvenile	95	0.021	0.440
	Sub-juvenile	253	0.040	
	Adult	338	0.050	
Habitat	Hedges	443	0.048	0.380
	Bank	72	0.083	
	Woodland	57	0.035	
	Water bodies	75	0.013	
	Farm buildings	184	0.056	
vt2 gene	*vt2*-carrier	5	0.20	0.08
	vt2-non carrier	1009	0.041	
vt1 gene	*vt1*-carrier	5	0.40	<0.0001
	vt1-non carrier	1009	0.04	

$ with Juvenile animals was not possible to determine the correct gender; *P-value derived from chi-square test

Table 6. Results of the univariable analysis exploring the relationship between variables recorded in the cross-sectional study and the isolation of the *eae* gene, carried *E. coli* from larger wild mammals, domestic dogs and one pony

Variable	**Category**	**n**	**Proportion positive**	**P-value***
Farm location	MF	34	0.147	0.248
	PHF	36	0	
	CLF	28	0.143	
	BHF	36	0.111	
	BGF	26	0.115	
	GF	8	0	
Pair of farms	Pair1	70	0.071	0.566
	Pair2	64	0.125	
	Pair3	34	0.088	
Month	July 04	23	0	0.611
	August 04	26	0.154	
	September04	15	0.133	
	November 04	10	0	
	December 04	1	0	
	January 05	7	0	
	February 05	66	0.121	
	March 05	9	0.111	
	April 05	3	0	
	May 05	5	0	
Season	Winter	74	0.108	0.90
	Spring	17	0.059	
	Summer	49	0.082	
	Autumn	25	0.080	
Animal spp.	Badger	54	0.056	0.56
	Fox	51	0.137	
	Rat	16	0	
	Rabbit	34	0.147	
	Domestic dog	4	0	
	Pony	1	0	
	Unknown host	8	0.125	
vt1 gene[$]	*vt1*-carrier	1	1	-------
	vt1-non carrier	167	0	

*P-value derived from chi-square test ;$ only one sample carried the vt1gene together with the eae gene

Table 7. Results of the univariable analysis exploring the relationship between variables recorded in the cross-sectional study and the isolation of *Campylobacter* spp. from small rodents.

Variable	**Category**	**n**	**Proportion positive**	**P-value***
Farm location	MF	105	0	<0.0001
	PHF	187	0.016	
	CLF	205	0.098	
	BHF	162	0.0123	
	BGF	202	0.0446	
	GF	153	0.1242	
Pair of farms	Pair1	292	0.01	<0.0001
	Pair2	367	0.01	
	Pair3	355	0.079	
Month	July 04	60	0	<0.0001
	August 04	155	0.026	
	September04	52	0	
	November 04	165	0.046	
	December 04	61	0.197	
	January 05	105	0.035	
	February 05	135	0.015	
	March 05	152	0	
	April 05	74	0.054	
	May 05	55	0.073	
Season	Winter	301	0.050	0.25
	Spring	281	0.029	
	Summer	215	0.019	
	Autumn	217	0.037	
Rodent's species	Bank Vole	194	0.113	<0.0001
	Field Vole	23	0.087	
	House Mouse	76	0	
	Wood Mouse	658	0.009	
	Shrew	17	0.059	
	Unknown Rod.	46	0.087	
Gender	Female	402	0.035	0.04
	Male	480	0.030	
	Juvenile$	13	0.154	
	Unknown	53	0	
Age	Juvenile	95	0.042	0.06
	Sub-juvenile	253	0.047	
	Adult	338	0.015	
Habitat	Hedges	443	0.054	0.03
	Bank	72	0	
	Woodland	57	0	
	Water bodies	75	0.040	
	Farm buildings	184	0.016	
vt1, vt2	*Vt* carriers	5	0	0.672
	Vt-non carriers	10009	1	
eae	*eae*-carrier	43	0.023	0.679
	eae-non carrier	971	0.035	

$ with Juvenile animals was not possible to determine the correct gender; *P-value derived from chi-square test

Table 8. Results of the univariable analysis exploring the relationship between variables recorded in the cross-sectional study and the isolation of *Campylobacter* spp. from domestic cattle.

Variable	**Category**	**n**	**Proportion positive**	**P-value***
Farm location	MF	86	0.081	0.106
	PHF	83	0.145	
	CLF	78	0.103	
	BHF	89	0.090	
	BGF	86	0.035	
	GF	75	0.040	
Pair of farms	Pair1	169	0.112	0.03
	Pair2	167	0.096	
	Pair3	161	0.037	
Month[$]	July 04	75	0.080	0.03
	August 04	50	0.160	
	September04	23	0.217	
	November 04	69	0.044	
	January 05	48	0.066	
	February 05	94	0.053	
	April 05	92	0.110	
	May 05	46	0.022	
Season	Winter	129	0.062	0.55
	Spring	138	0.080	
	Summer	125	0.112	
	Autumn	92	0.087	
Age/management group	Calves	58	0.069	0.134
	Weaned calves	34	0.147	
	Adult stock	116	0.078	
	Lactating cows	171	0.100	
	Dry cows	88	0.023	
In shed/outside grazing	Grazing	57	0.035	0.19
	In shed	424	0.085	
eae gene carrier	*eae*-carrier	37	0.135	0.23
	eae-non carrier	460	0.078	
vt1 gene carrier	*vt1*- carrier	26	0.192	0.04
	vt1-non carrier	471	0.076	

$ No sampling was carried out in cattle in October 04, December 04, March 05; *P-value derived from chi-square test

Table 9. The logistic regression model building for risk factors on A- *Campylobacter* infection in small rodents and domestic cattle and B-the *eae* gene carriage by *E. coli* isolated from small rodents, wild birds and domestic cattle in the cross-sectional study from July 2004 to May 2005. Where the following acronyms stand for BIC, Bayesian information criterion; AIC Akaike information criterion; Lrtest :Likelihood ratio test.

A-***CAMPYLOBACTER***

Small Rodents

GLM Model	**BIC**	**AIC**	**Lrtest**
(A)Farm, rodent spp, gender, age, habitat	-2778	0.290	
(B) Farm, rodent spp, age, habitat	-2795	0.287	(B nested in A) 0.80
(C) Farm, rodent spp, habitat	-2805	0.278	(C nested in B) 0.27
(D) ***Farm, rodent spp***	-2845	0.272	(D nested in C) 0.42
(E) Farm	-2825	0.310	(E nested in D) 0.00001

Domestic cattle

GLM Model	**BIC**	**AIC**	**Lrtest**
(A)Farm, age group, inside/outside, *eae* gene, *vt1* gene	-2557	0.540	
(B) Farm, age group, inside/outside, *vt1* gene	-2563	0.569	(B nested in A) 0.42
(C***) Farm, age group, inside/outside***	-2568	0.535	(C nested in B) 0.28
(D) Farm, age group	-2568	0.543	(D nested in C) 0.0136

B-THE *eae* GENE

Wild birds

GLM Model	**BIC**	**AIC**	**Lrtest**
(A) Farm, Bird group, habitat	-1442	0.367	
(B) ***Farm, Bird group***	-1453	0.279	(B nested in A) 0.13
(C) Farm	-3958	0.299	(C nested in B) 0.002

Small rodents

GLM Model	**BIC**	**AIC**	**Lrtest**
(A) Farm, *vt1* gene, *vt2* gene	-6636	0.338	
(B) ***Farm, vt1 gene***	-6643	0.336	(B nested in A) 0.83
(C) Farm	-6642	0.338	(C nested in B) 0.045

Domestic cattle

GLM Model	**BIC**	**AIC**	**Lrtest**
(A) Farm, age group, *vt1* gene, *vt2* gene	-2603	0.464	
(B) ***Farm, age group, vt1 gene***	-2608	0.463	(B nested in A) 0.18
(C) Farm, age group	-2608	0.472	(C nested in B) 0.015

Table 10. Final logistic regression models including coefficient, Wald test p-value and 95% confident intervals (C.I) for A- Campylobacter infection in domestic cattle and small rodents and B- E. coli that carries the eae gene isolated from wild birds, small rodents and domestic cattle in the cross-sectional study from July 2004 to May 2005. Where the following acronyms stand for BV, bank vole; FV, field vole WM, wood mouse; SHW, shrew; UROD, unknown rodent.

A-*CAMPYLOBACTER*

Small rodents (GLM: Farm-rodent species)

Variables	**Coefficient**	**P-value**	**95% C.I.**
BGF *vs* PHF	-0.89	0.269	-2.47 - 0.69
BGF *vs* CLF	-1.67	0.064	-3.43 - 0.09
BGF *vs* BHF	-0.01	0.988	-1.86 - 1.83
BGF *vs* GF	0.86	0.156	-0.33 - 2.05
BV *vs* FV	-15.46	0.992	-2923- 2892
BV *vs* WM	-2.83	0.0001	-3.98 - 1.67
BV *vs* SHW	-1.38	0.206	-3.52 - 0.76
BV *vs* UROD	-0.21	0.744	-1.45 - 1.03

Domestic cattle (GLM: Farm-age group- inside/outside)

Variables	**Coefficient**	**P-value**	**95% C.I.**
MF *vs* PHF	1.98	0.010	0.48 - 3.48
MF *vs* CLF	1.83	0.019	0.30 - 3.36
MF *vs* BHF	1.18	0.098	-0.22 - 2.58
MF *vs* BGF	-0.74	0.394	-2.43 - 0.96
MF *vs* GF	-0.41	0.589	-1.89 - 1.07
Calv *vs* Wcalves	0.94	0.228	-0.59 - 2.48
Calv *vs* Adultstock	1.41	0.073	-0.13 - 2.95
Calv *vs* Lactcows	0.38	0.535	-0.82 - 1.58
Calv *vs* Drycows	-1.72	0.058	-3.50 - 0.06
Outside *vs* Inside	1.69	0.036	0.11 - 3.28

B- THE *eae* GENE

Wild birds (GLM: Farm-bird group)

Variable	**Coefficient**	**P-value**	**95% C.I.**
MF *vs* PHF	0.14	0.832	-1.16 - 1.44
MF *vs* CLF	-0.18	0.744	-1.24 - 0.89
MF *vs* BHF	-2.26	0.033	-4.34 - -0.18
MF *vs* BGF	-15.94	0.987	-1941 - 1909
MF *vs* GF	-0.66	0.414	-2.24 - 0.92
Corvid *vs* Farmland	-0.97	0.053	-1.96 - 0.01
Corvid *vs* Woodland	-2.79	0.001	-4.39 - -1.12

Small rodents (GLM: Farm-the vt1 gene)

Variable	**Coefficient**	**P-value**	**95% C.I.**
MF *vs* PHF	0.74	0.201	-0.39 - 1.87
MF *vs* CLF	0.69	0.229	-0.44 - 1.82
MF *vs* BHF	-1.85	0.100	-4.06 - 0.35
MF *vs* BGF	-0.97	0.212	-2.48 - 0.55
MF *vs* GF	-0.39	0.589	-1.80 - 1.01
No *vt1 vs vt1*	2.11	0.024	0.28 - 3.94

Domestic cattle

(GLM: Farm- age group-the vt1 gene)

Variable	**Coefficient**	**P-value**	**95% C.I.**
MF *vs* PHF	-1.50	0.185	-3.73 - 0.72
MF *vs* CLF	-0.02	0.982	-1.40 - 1.37
MF *vs* BHF	0.68	0.227	-0.42 - 1.78
MF *vs* BGF	-0.65	0.384	-2.11 - 0.81
MF *vs* GF	-1.17	0.114	-2.62 - 0.28
Calv *vs* Wcalves	-0.92	0.191	-2.30 - 0.46
Calv *vs* Adultstock	-1.52	0.009	-2.66 - -0.39
Calv *vs* Lactcows	-1.69	0.002	-2.77 - -0.60
Calv *vs* Drycows	-3.16	0.004	-5.28 - -1.03
No *vt1 vs vt1*	1.54	0.010	0.37 - 2.71

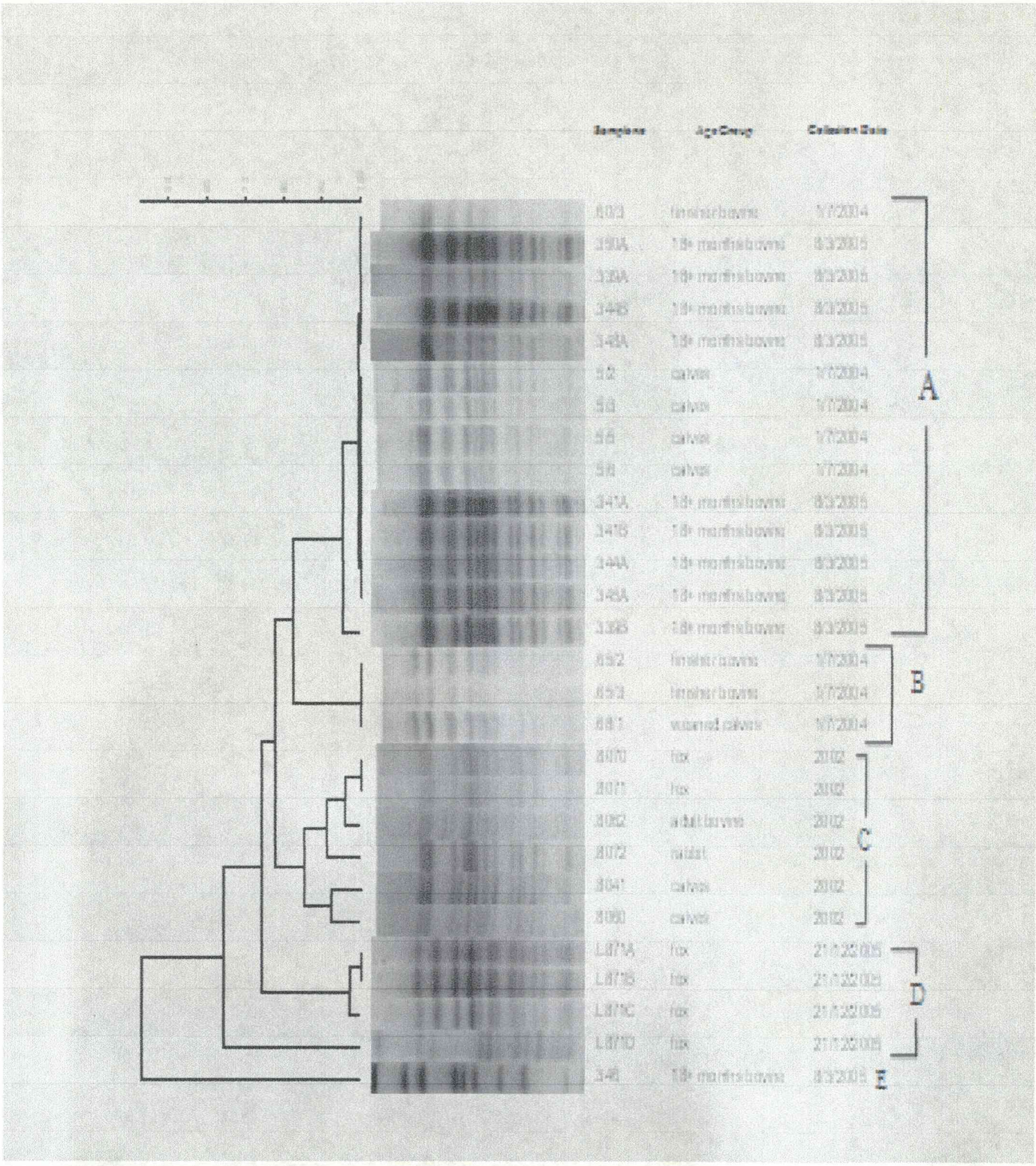


Figure 1. PFGE (*Xba*I digest) of *E. coli* O157 from cattle on MF over time. The axis on the left represents the percentage band similarity between isolates. Information about isolates is shown on the right.

Figure 2. Map with spatial distribution of wild birds infected with *E. coli* carrying VTEC determinants as part of the cross-sectional study carried out from July 2004 to May 2005.

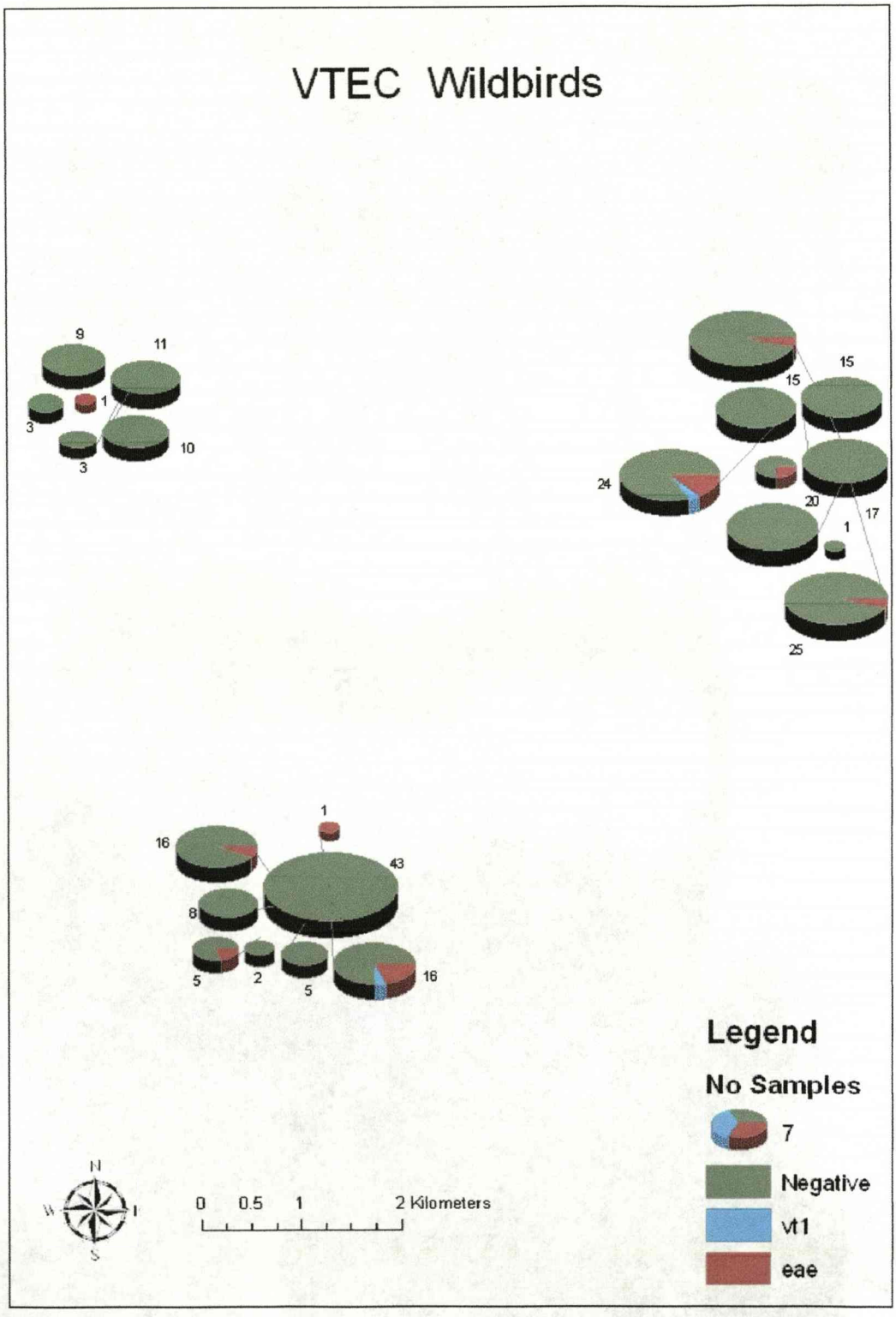


Figure 3. Map with spatial distribution of rodents infected with *E. coli* carrying VTEC determinants as part of the cross-sectional study carried out from July 2004 to May 2005.

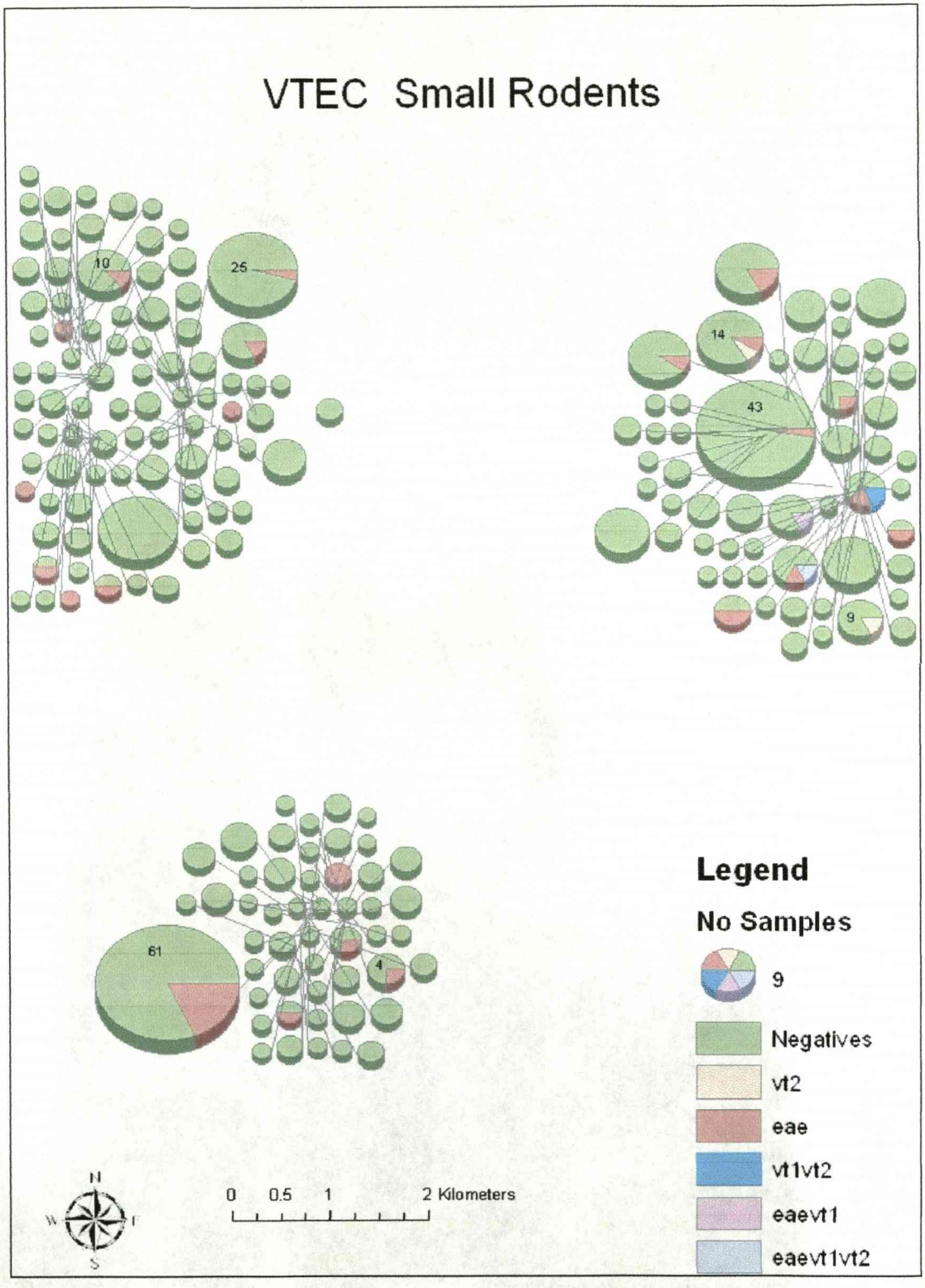


Figure 4. Map with spatial distribution of rodents infected with *Campylobacter jejuni*. The buffer for area for the statistically significant cluster is highlighted in pink as part of the cross-sectional study carried out from July 2004 to May 2005.

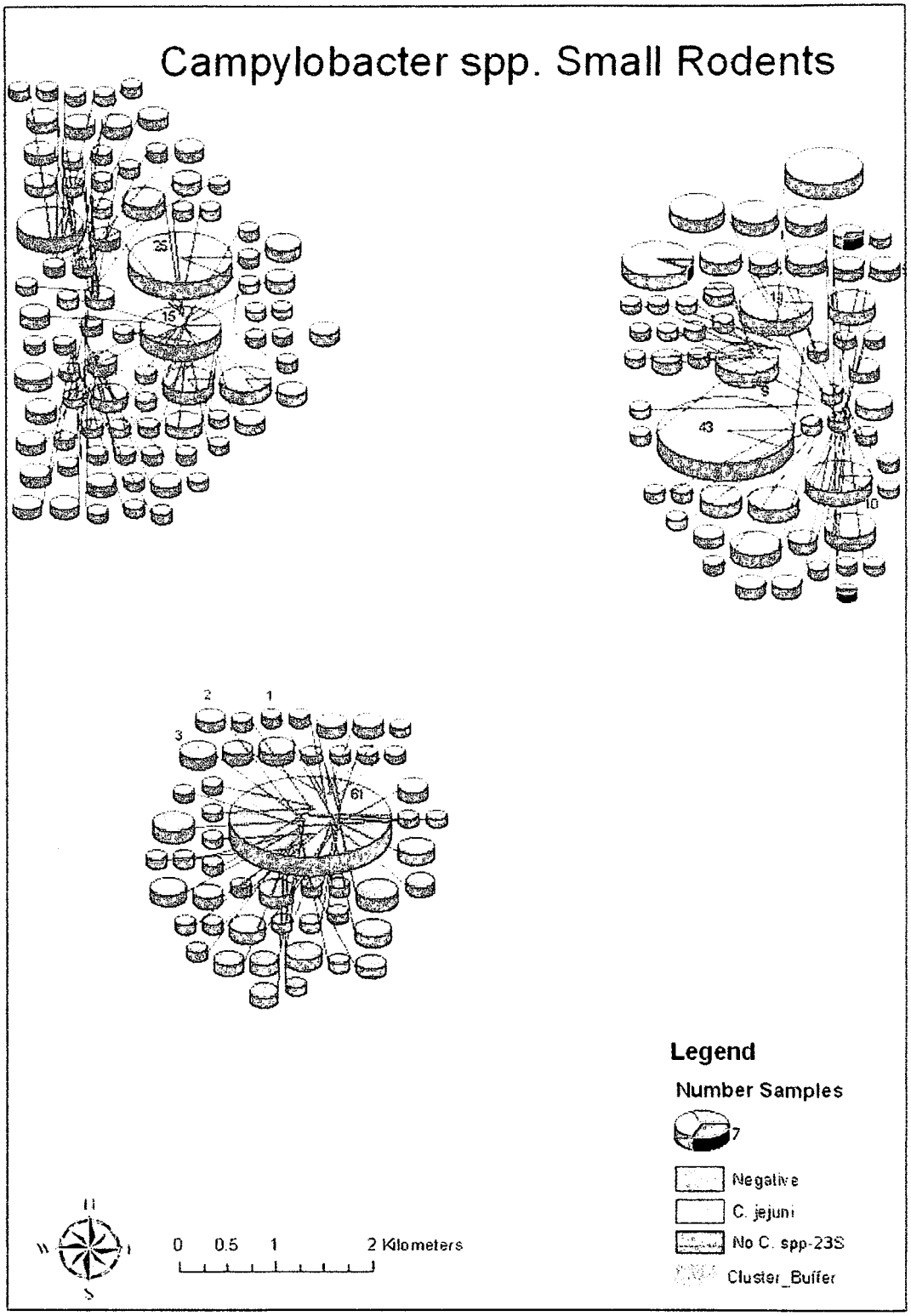


Figure 5. Aerial map that shows the location of the significant C. jejuni spatial cluster in small rodents located at GF in the cross-sectional study from July 2004 to May 2005.

3.4 Discussion

All three enteric bacteria of interest in this cross-sectional study were identified during the study. *Salmonella* infection was rare in both cattle and wildlife; *E. coli* O157 was isolated mainly from beef cattle and only *C. jejuni* and *E. coli* strains that carried VTEC virulence genes were found in a wide range of hosts.

E. coli O157

The prevalence of *E. coli* O157 was 20%. This prevalence is significantly higher than the UK prevalence of *E. coli* O157 in cattle at slaughter 4.7% in 2003 (Milnes et al., 2007). However, these differences may be for a number of reasons such as samples size, age of the animals, time of sampling, diet etc.

It is curious that *E. coli* O157 was only isolated from the only participating beef farm in this cross-sectional study, as it is documented that dairy cattle can also be infected, including other farms in Cheshire (Kemp, 2005a; Robinson et al., 2004). Analysis of PFGE profiles suggested that infection of this farm was dominated by one predominant band pattern carried by cattle, although two other strains were isolated less frequently, suggesting some diversity. *E.coli* O157 tend to be clonal and on some farms the same strain can remain in the animals and the farm environment for long periods of time. Our study shows that a particular strain has remained present on the farm during 2004 and 2005. In another study in 2005, *E. coli* O157 was only isolated from a fox sample in one of the cattle field in December 2005. PFGE patterns in cattle and fox were different suggesting different epidemiological pathways of infection in these two hosts. Moreover, this fox had four *E. coli* O157 isolates presenting three very similar RP (>90%) and a fourth RP quite different to the other 3 RP's (70%). This suggests a possible mixed-infection with multiple *E. coli* O157 strains. *E. coli* O157 had been previously isolated from wildlife (rabbit and foxes) on this farm in a study in 2002 (Kemp, 2005a) and on this occasion cattle and wildlife strains were more similar in PFGE profiles.

The fact that wildlife infected with *E. coli* O157 were present and defecate on the fields where cattle graze could pose a risk of infection to domestic animals with new strains of *E. coli* O157. The number of contaminated wildlife samples could be underestimated as samples from foxes, rabbits and other large wildlife species found on the ground were not actively looked for unless they were located near the small rodent trapping areas.

Comparison between strains collected from animals on this farm in 2002 and 2004-2005 showed that different *E. coli* O157 strains were present during different periods of time. This suggests that the profile of strains of *E. coli* O157 on this farm are dynamic and change over time. The reasons for this are unknown and need further investigation. Some reasons that could contribute to this variability could be domestic animals entering into contact with new strains of this bacterium for the introduction of new infected animals in the herd and contaminated environment with wildlife faeces, mainly terrestrial mammals(Jenkins et al., 2002). *E. coli* O157 was not isolated from samples of badgers, wild birds and rodents. This suggests that these particular hosts pose a limited risk of carrying this bacterium. It is possible that if they came in contact with *E. coli* O157, they could become opportunistically infected.

A previous study did not isolate *E. coli* O157 from 300 rodent faecal samples collected from feed lot farms and the organisim was isolated only from one pooled faecal bird sample (Hancock et al., 1998). However, other study found a high prevalence of *E. coli* O157 from different samples collected from wildlife on beef farms in the Czeck republic (Cizek et al., 1999).

Salmonella

The proportion of samples containing *Salmonella* serovars was low. The most common *Salmonella* serovar was *S.* Dublin and were from cattle. Only three of the six participating farms had infected animals.

Salmonella was isolated only from two wildlife animals and one calf from the same farm (BHF). This farm had just had an outbreak of abortions in cattle caused by *S.* London before this study started. It could be that this *Salmonella* serovar was persistent in the environment or that cattle could be carriers for a period of time after the outbreak. In contrast, the infection could have been transmitted from badgers to cattle. Badgers have been found to carry salmonellae in this study area previously (Wilson et al., 2003). Other wildlife species infected with *S.* Typhimurium was an individual house sparrow from the same location. House sparrows are territorial birds associated with human buildings. However, no *Salmonella* Typhimurium infection was detected on cattle from that farm. Thus, although wild birds could provide a source of Salmonella to non-infected cattle, in this case there was no evidence of transmission.

The low prevalence levels of *Salmonella* in healthy wild birds is consistent with other studies in wild birds. Most *Salmonella* isolates were found in diseased birds (Pennycott et al., 2002; Pennycott et al., 2005).

The majority of the animals that carried *Salmonella* (5/9) were cattle and carried *Salmonella* Dublin.That this serovar has host-specificity for cattle (Anonymous, 1998; Mastroeni, 2006). The lower prevalence of *Salmonella* in cattle (1%) is similar to the prevalence (1.4%) found in recent survey in cattle at slaughter in the UK (Milnes et al., 2007).The "super shedders" or a small number of animals per herd excreting Salmonella in high quantities has been proposed as a possible model to explain this bacterium dynamics within cattle herds. This hypothesis could explain why the number of isolates is low. Further quantification of Salmonella in these samples could be useful.

Salmonella was isolated only from three of the six farms in this study. This could be due to management and biosecurity differences in different premises, it could also have been the time of sampling.

Salmonella was not isolated from rodents, foxes and rabbits in this study. This could mean that wildlife species living in this area may be a low risk to cattle for *Salmonella* infection. This contrasts with the common assumption that often blames rodents for outbreaks of *Salmonella* in domestic cattle and poultry.

The isolation rate of *Salmonella* could be underestimated because of the type of methodology used. A recent study on captive psittacine birds showed a higher prevalence of salmonella applying DNA PCR directly from the faecal sample compared to traditional microbiological isolation methods (Allgayer et al., 2008). It would be therefore appropriate to retest the archive of frozen samples isolated from cattle and wildlife in order to maximize the isolation rate. The results showed that the prevalence of *Salmonella* isolation from wildlife was low suggesting that this bacterium was not endemic on these wildlife and cattle populations. It would help in terms of accuracy to have a precise figure of the rate of infection in cattle and wildlife. As the three enteric bacteria were isolated simultaneously from the same faecal sample, the specificity of the methodology was not perfect. Underestimation of infection can be possibly due to testing methods. Thus the lack of appropriate methods of isolation can have serious consequences for the herd with some pathogens such as *Mycobacterium avium* sbsp. *paratuberculosis*.

An important objective of this study was not so much to determine prevalence as detect opportunities for cross species transmission. Although the isolation methods may not have been perfect, the results do suggest that large amounts of Salmonella are not being shed into the environment by wildlife. So the common assumption that wild rodents and birds are the source of outbreaks in cattle may often be wrong. Furthermore, it's interesting that despite infection being relativly common in badgers in other studies (Wilson et al., 2003)), transmission to cattle is rare – this may also be relevant to the debate about the transmission of TB to cattle from badgers.

Campylobacter

Campylobacter was carried out mainly by rodents, particularly bank voles, and cattle. Only *Campylobacter jejuni* was isolated from rodents. Although wood mice, rats, field voles, shrew and bank voles carried *C. jejuni* there were statistical differences in the prevalence of infection. The prevalence in bank voles was significantly higher, especially compared with wood mice which were the most commonly trapped rodents on these farms. Little has been published about bank voles and *Campylobacter* infection, although, these rodents have been found to carry this bacterium. The prevalence of *C. jejuni* also varied in different farms. The highest prevalence in rodents (12%) was found on a farm (GF), which had one of the lowest prevalence in cattle (4%). The reasons for these differences are not clear. Factors could include farm management factors that favour certain vegetation or suitable areas for the growth of wild rodents, food availability, inherent geographical characteristics of the farm etc. The univariate analysis showed that rodents captured in hedges had higher prevalence of *Campylobacter* infections, whereas housed cattle had a higher prevalence than those grazed. This study also provided evidence of a significant spatial cluster of eight rodents, mainly bank voles, infected with *C. jejuni* in a hedgerow located in the boundaries with a busy red meat abattoir during December 2004. It is not known if these rodents could have become infected by being exposed to *Campylobacter* contaminated by-products such as run-off water or debris from the abattoir. One way of testing this would be to compare the strains of *C jejuni* isolated from rodents in this area with those found elsewhere and with cattle entering the abattoir and 'endemic' to the farm.

No *Campylobacter* spp were isolated from house mice, foxes and badgers in this study. As house mice tend to live close to cattle and farm buildings, this may suggest that transmission between house mice and domestic cattle could be low or the infection by *Campylobacter* spp in mice does not last long or is only intermittently shed in this study. These results also

contrast with a previous epidemiological study that found 8/83 *Campylobacter* infected house mice close to domestic organic farms (Meerburg et al., 2006).

Campylobacter spp were isolated in only a small number of faecal samples from wild birds, from an unidentified corvid, a magpie and a blackbird. Wild birds, unlike rodents, were found to be infected with both *C. coli* and *C. jejuni*. Previous studies have shown that *Campylobacter* infection in wild birds is highly associated with diet habits and species (Waldenstrom et al., 2002). Corvids eat a wide range of foodstuffs, including carrion, whereas blackbirds eat mainly insects and fruits – all three are in part ground feeding, and so might be expected to encounter food contaminated by faeces of, for example, cattle. The prevalence in this study (0.15%), however, is low compared with that found (4.2-89.8%) in wild birds in previous studies (Kapperud and Rosef, 1983; Pacha et al., 1988; Waldenstrom et al., 2002; Yogasundram et al., 1989). The reasons for these differences may be the use of different methods, the species of birds screened, or sample size. Cattle were found to be infected with *C. jejuni*, *C. hyointestinalis* and *C. fetus*. The overall campylobacter prevalence was 8% with variations in the prevalence between the different farms ranging from 4% to 15% compared with the findings of a previous study carried out in the same area in which the prevalence of *Campylobacter* on 61 farms was 55% (Kemp, 2005a), similar to the prevalence of *Campylobacter* in cattle at slaughter (55%) in the UK found in another study (Kemp, 2005a; Milnes et al., 2007). Indeed, other studies across Europe have also found the prevalence of campylobacteriosis in cattle to be up to 47% (Milnes et al., 2007; Stanley and Jones, 2003). Differences in herd size, age of animal, sample size, microbiological methodology used, location of the farms and frequency of sampling could have contributed to these differences. In terms of age group, weaned calves had a higher prevalence than adult animals. This is consistent with previous studies (Stanley and Jones, 2003). The age variable was also part of the final epidemiological GLM model in cattle together with the farm. This

study also showed differences in the prevalence of Campylobacter in cattle on different farms. Management factors probably have an effect on infection with *Campylobacter*.

Non- O157 VTEC

Virulence factors associated with VTEC were identified in *E. coli* isolates from domestic cattle, wild birds, wild rodents and other larger wild mammals. This study shows that the most prevalent virulence gene amongst cattle and wildlife was the *eae* gene on these farms.

E. coli from wild birds were shown to carry only one virulence factor at the time, the *eae* or the *vt1* gene. No carriage of the *vt2* gene was detected by PCR. The *vt1* gene is more conserved genetically in comparison with the *vt2* gene and it was more abundant in cattle on these six farms.

The univariate and multivariate analysis in wild birds showed presence of the *eae* gene was highly associated with particular farms (MF and PHF) geographically close and specific group of birds (corvids and bird species associated with farm land such as pigeons. Although habitat was not kept in the final GLM model, the prevalence of the *eae* gene was higher in birds captured around farm buildings (10% compared with a 3.2% overall, p=0.05). This highlights that species of birds associated with farmland may pose a risk of locally spreading to livestock VTEC virulence genes, or they may be exposed to VTEC genes because of their activity in farms buildings close to cattle.

According to these results no statistical association between the *vt1* and *eae* genes was found in wild birds, unlike in cattle and small rodents, although the prevalence of the *vt1* gene across the sampled wild birds was low. This could also mean that infected wild birds could just be accidental hosts in the carriage of individually acquired VTEC virulence genes. These results contrast with other results found in wild birds in a previous study in this study area in which any of the three VTEC virulence genes were detected in 121 bird samples (Kemp,

2005a). These differences could be due to sample size, sampling time, type of birds, different type of sample such as ground samples, or sample age.

In contrast to wild birds, rodent species and habitat did not seem to have an effect on the probability of a rodent being infected with strains of *E. coli* carrying the *eae* gene. Other risk factors, such as farm, seemed to have a more important effect, especially in two of the farms, PHF and CLF. There was also an association between *E. coli* isolates from rodents that carried the *vt1* gene and carrying the *eae* gene. This should be interpreted cautiously because of the small number of *E. coli* isolates that carried the *vt1* and *vt2* genes. Other studies have shown that rodents can carry VTEC virulence determinants isolated on cattle farms (Nielsen et al., 2004a). Rodent species are independent with the probability of becoming a carrier of VTEC genes in contrast to *Campylobacter* infection where there is strong association of *Campylobacter* infection in bank voles.

Among other wild mammals, the highest prevalence of the *eae* gene was found in rabbits (14.7%, n= 34) and foxes (13.7%, n=51). Only an *E. coli* isolate from a fox carried both the *eae* and *vt1* gene. Farm pair 2 (CLF-BHF) had a higher probability of having a larger wild mammal such as a fox, a rabbit or a badger infected by *E. coli* isolates carrying the *eae* gene (p=0.02). The prevalence of *E. coli* that carried the *eae* gene was found to be 9% (n=11), 4% (n=131) and 0% (n=14) in foxes, rabbits and badgers in a previous study in this study area. Moreover, the *vt1* gene was not carried by any of these three hosts: the *vt2* gene was carried by a small proportion of rabbits and badgers. Other studies have isolated O157 VTEC and non-O157 VTEC in rabbits, and some strains were indistinguishable from cattle strains by PFGE, suggesting cross-species transmission (Kemp, 2005a; Leclercq and Mahillon, 2003).

No VTEC virulence genes were isolated from rats (n=16). This contrasts with other studies that found high prevalence (40%, n=10) of VTEC O157 from rat faecal samples on beef

farms in the Czeck Republic (Cizek et al., 1999) and other VTEC were indistinguishable from bovine isolates (Nielsen et al., 2004a).

Domestic cattle (n=497) on these farms carried the *eae*, *vt1* and *vt2* genes: overall prevalence 5.4% for eae gene, 1.4% vt1-eae genes and 3% for vt2 gene. Although most disease in humans is caused by VTEC O157, it is known that other non-O157 can be responsible for human disease and these could be underestimated (Bettelheim, 2000). Currently there is a lack of surveys to determine the prevalence of non-O157 VTEC in cattle herds in the UK. Most scientific studies and official surveillance activities have been performed to determine the prevalence of O157 VTEC (DEFRA, 2006). Different studies have shown that the prevalence and type of VTEC in cattle can vary considerably between countries and herds (Bettelheim, 2000; Blanco et al., 2003; Jenkins et al., 2002). The prevalence of *eae* positive *E. coli* in domestic cattle in this study was mainly associated with specific farms (BHF, MF and CLF). Age group also had an association with the prevalence of the *eae* gene amongst cattle on particular farms. Hence, a higher proportion of positives were isolated from younger animals than adult stock and lactating cows. These results agree with previous studies (Blanco et al., 2003; Zhao et al., 1995).

No spatial clustering was detected amongst infected wild birds and wild rodents ($p>0.05$). These results should be interpreted with caution given the very fine geographical scale (10 metre separation between individual rodent traps and between 400-800 meters separation between mist nets for birds) of the study. Information about sampling location of a number of samples was not available, these data could therefore not be taken into account for the spatial analysis contributing to an under or overestimation of the results within the analysis. This could also have had an effect on the denominators and captured densities in certain areas of farms. On the wild bird side there could also be bias in the spatial representation of captured places as nets were placed in strategic places on each farm and the place of capture of birds

does not necessarily reflect the place where the birds stopped, nest or carry out most of their behaviour activities. A consensus and standard method for the wild birds captured had to be used even at the risk of introducing location bias.

Moreover, because of the study design *E. coli* isolates from individual samples were frozen as pools of ten *E. coli* colonies. Therefore, it could be the case that when more than one virulence gene was present per sample it was not carried as part of the same colony and could be carried by different colonies present in host's intestine at the same time.

General

In conclusion, *Salmonella*, *Campylobacter*, *E. coli* O157 and VTEC have different host-infection ecologies despite all being enteric bacteria that can be transmitted via the environment. Furthermore, the alternative suggestion that cattle contribute in large scale to high levels of environmental contamination and as a consequence contaminate large numbers of wildlife species with their enteric flora is not true.

Salmonella was rare in cattle and wildlife species isolated from farm habitats. Thus, the assumptions that wildlife act as important sources of *Salmonella* to humans and domestic animals are not necessarily true. The same serovar of *Salmonella* has been found in a badger and a calf on a farm which had a previous outbreak; therefore, environmental transmission or inter-species transmission may be possible.

E. coli O157 was isolated only from beef cattle with no evidence of transmission to or amongst other species. This contrasts with the virulence genes of VTEC that are spread wide amongst cattle and wildlife species; however, the *eae* and *vt* genes rarely appeared together. Prevalence and distribution of these genes varied between farms and host species, suggesting a complex ecology and limited evidence of cross-species transmission.

Campylobacter jejuni is the *Campylobacter* spp with the widest host-range amongst cattle and wildlife but its prevalence in different hosts and farms varied significantly. Infection with this species was mostly found in bank voles and cattle.It is clear that environmental contamination does not lead automatically to transmission amongst different wildlife species and wildlife species and cattle since, house mice sharing a contaminated environment with cattle and wood mice sharing the same environment with bank voles rarely become infected. Hence host factors are obviously important in transmission.

Chapter 4 Molecular characterisation and diversity of Campylobacter spp isolated from domestic cattle and wildlife on six cattle farms in Cheshire (UK)

4.1 Introduction

Campylobacter, and in particular *C. jejuni*, is one of the leading causes of gastroenteric bacterial disease in the UK and many other countries (Anonymous, 2000; Anonymous, 2007b). This bacterium has a zoonotic origin and domestic poultry is considered as one of the major animal reservoirs. The main route of transmission is considered to be via contaminated food of poultry origin(Kapperud et al., 2003; Wilson et al., 2008).

It is well documented that livestock other than poultry can also be important reservoirs for *Campylobacter jejuni* and other *Campylobacter* spp capable of producing disease in humans (Stanley 03). In a recent study 54 % of cattle at slaughter in Great Britain carried *Campylobacter* spp in 2003 (Milnes et al., 2007). Moreover, *Campylobacter* is considered to be common in different environments. Hence, some cases in human beings have been associated with leisure activities that involved countryside water bodies and rural areas with a high density of livestock (Blaser et al., 1983; Sopwith et al., 2006). Molecular evidence has shown that sporadic human cases due to sources other than contaminated food could be underestimated (Colles et al., 2003; Schouls et al., 2003). Although the contribution of these sources is currently unknown, it highlights the complexity and lack of understanding in the epidemiology of *Campylobacter.*

Campylobacter spp have been isolated from different wildlife including wild birds and rodents (Cabrita et al., 1992; Kemp, 2005a; Kwan et al., 2008a; Meerburg et al., 2006; Wahlstrom et al., 2003). There is a paucity of information about the nature of *Campylobacter* isolates from wildlife at a molecular level. Moreover, little is known about the distribution and transmission dynamics of *Campylobacter* spp between healthy cattle and wildlife species

living in a common environment such as farms. This information could be of considerable use when designing surveillance approaches, disease control programmes and developing preventive strategies.

Campylobacter has a small genome compared with other enteric bacteria such as *Salmonella* and *E. coli* (Parkhill et al., 2000). Large genomes allow these two bacteria to become highly resistant in the environment and extremely specialised in terms of pathogenicity within the host. However, it is believed that *Campylobacter* spp are able to overcome the constraints of a small genome through frequent genetic rearrangements in order to adapt successfully to adverse conditions environmentally and within the host (Parkhill et al., 2000; Ridley et al., 2008).

This genetic plasticity makes this bacterium very diverse, difficult to characterise and therefore, challenging to control. However, there is also evidence that *C. jejuni* populations can be weakly clonal and some of them could be host-specific/adapted (Colles et al., 2003; French et al., 2005; Kwan et al., 2008b; Manning et al., 2003; McCarthy et al., 2007; Petersen et al., 2001) . This is believed to happen by genetic rearrangement in the host's intestine that makes *Campylobacter jeuni* adapt in order to cope with the intestinal environment (Kwan et al., 2008b; Rivoal et al., 2005). In addition, it has been observed that there is a predominant *Campylobacter jeuni* strain in the poultry intestinal flora, even if the initial infection was by multiple strains *in vivo* and *in vitro* (Skanseng et al., 2007).

The aims of this study were:

- To determine molecular characteristics and *Campylobacter* spp strain distribution amongst cattle and wildlife species living on farms;

- To investigate the possible transmission dynamics of *Campylobacter* spp due to horizontal transmission isolated from cattle and wildlife living on the same farm or geographically close farms.

4.2 Materials and Methods

Campylobacter isolates were collected from faecal samples, microbiologically processed, molecularly characterised and sequenced as described in Chapter 2 General Materials & Methods. In brief, putative campylobacter colonies were first characterised by the Wang method (multiplex-*Campylobacter* PCR). Isolates that were generic *Campylobacter* spp 23sRNA were further tested by the Lynton and Gonzalez methods for *Campylobacter fetus*, *C. hyointestinalis*, *C. jejuni* and *C. coli*. *Campylobacter* isolates that were unsuccessfully characterised at the species level by the three mentioned methods were then tested by the Karenlampi method for partial amplification of the GroEL gene.

GroEL amplicons were sequenced. Only direct sequencing of each amplicon in both forward and reverse directions was used to produce a consensus sequence for each isolate. Consensus sequences were derived with Chromas Pro. Version 1.42 (technelysium). All primer sites were removed prior to analysis resulting in a final useable sequence of 470 bp. Consensus sequences were compared against the nucleotide BLAST web-based database for identification purposes at http://blast.ncbi.nlm.nih.gov/Blast.cgi. Consensus sequence alignments were performed using CLUSTAL W alignment editor in MEGA version 4 (Tamura et al., 2007). Bootstrapping was used to create a tree with 1000 simulation times based on p-distance to determine the similitude percentage for statistical support.

4.3 Results

In total, 127 isolates of *Campylobacter* spp defined by being positive by the Wang method. These comprised isolates from 81 animals, and included bovine and wildlife isolates (Table 1). The only three *Campylobacter* spp apart from *C. jejuni* were *Campylobacter hyointestinalis* and *C. fetus* isolated from domestic cattle and a *C. coli* isolated from an unknown corvid.

A total of 103 (81%, n=127) isolates were found positive by the Wang method but negative by the Linton and Gonzalez methods. They were however positive by the Karenlampi method for the partial *groEL* gene. The proportion of samples characterisable by the different methods varied slightly according to the host: -For example, the percentage of samples detected by the Karemlampi method was the smallest (75%, n=59) in cattle in comparison with birds (83%, n=6) and rodents (93%, n=57). (Table 1).

Attempted sequencing was carried out on all 103 amplicons detected by the Karenlampi method(Karenlampi et al., 2004). Eleven amplicons could only be characterised one way only; and 39 sequences could not be included in the analysis owing to labelling errors, contamination and poor sequencing results. A further 7 sequences could not be allocated to a particular *Campylobacter spp.* (from 4 cattle isolates and 3 rodent (1 bank vole and 2 wood mice) isolates) based on published sequences.

Thus 47 (46%, n=103) amplicon sequences were used in the CLUSTAL analysis, plus one sequence of an *Arcobacter butzleri* isolate. The validated sequences had between 420 and 470 bp.

The bootstrapping tree (Figure 2) showed that sequences were grouped in two main clusters, one corresponding to *C. jejuni* and another corresponding to *C. hyointestinalis* and *C. fetus-fetus*.

The *C. jejuni* cluster was subdivided into two subclusters, which were95% similar (100 bootstrap value), labelled A and B in Figure 1. One of the subclusters (A) could be further divided into three subgroups that were 98% similar (bootstrap values 100-96-91). Each sub-group comprised sequences of isolates from specific animal hosts, such as rodents, birds and cattle. Only one bank vole isolate had an identical sequence to the cattle sequences. All the sequences in sub group A were 100% identical and were isolated from rodents (bank voles and wood mice).

Rodents, especially bank voles tended to have a greater frequency and diversity of *C. jejuni* strains than wood mice and a rat. A cluster of two wood mice and a rat from BHF and PHF were infected with the same *C. jejuni* strain (bootstrap value 100).

C. hyointestinalis and *C. fetus* were isolated only from cattle in three different farms, which differ by one location (*C. hyointestinalis* in CLF and *C. fetus* in PHF). All *C. fetus* sequences were 100% identical while *C. hyointestinalis* were more diverse in three groups of 99 % similarity.

Figure 2 Phylogenetic tree inferred from comparison of nucleotide sequences of part of the *GroEL* gene of *Campylobacter* isolated from domestic cattle and wildlife in the cross-sectional study between July 2004 and May 2005. Sequneces were aligned,and compared using Chromas pro software, and the tree drawn using MEGA. Colours indicate the animal host: green-domestic cattle; pink-small rodents and rat; blue-wild birds. Numbers at nodes represent bootstraps with a cut off >65.

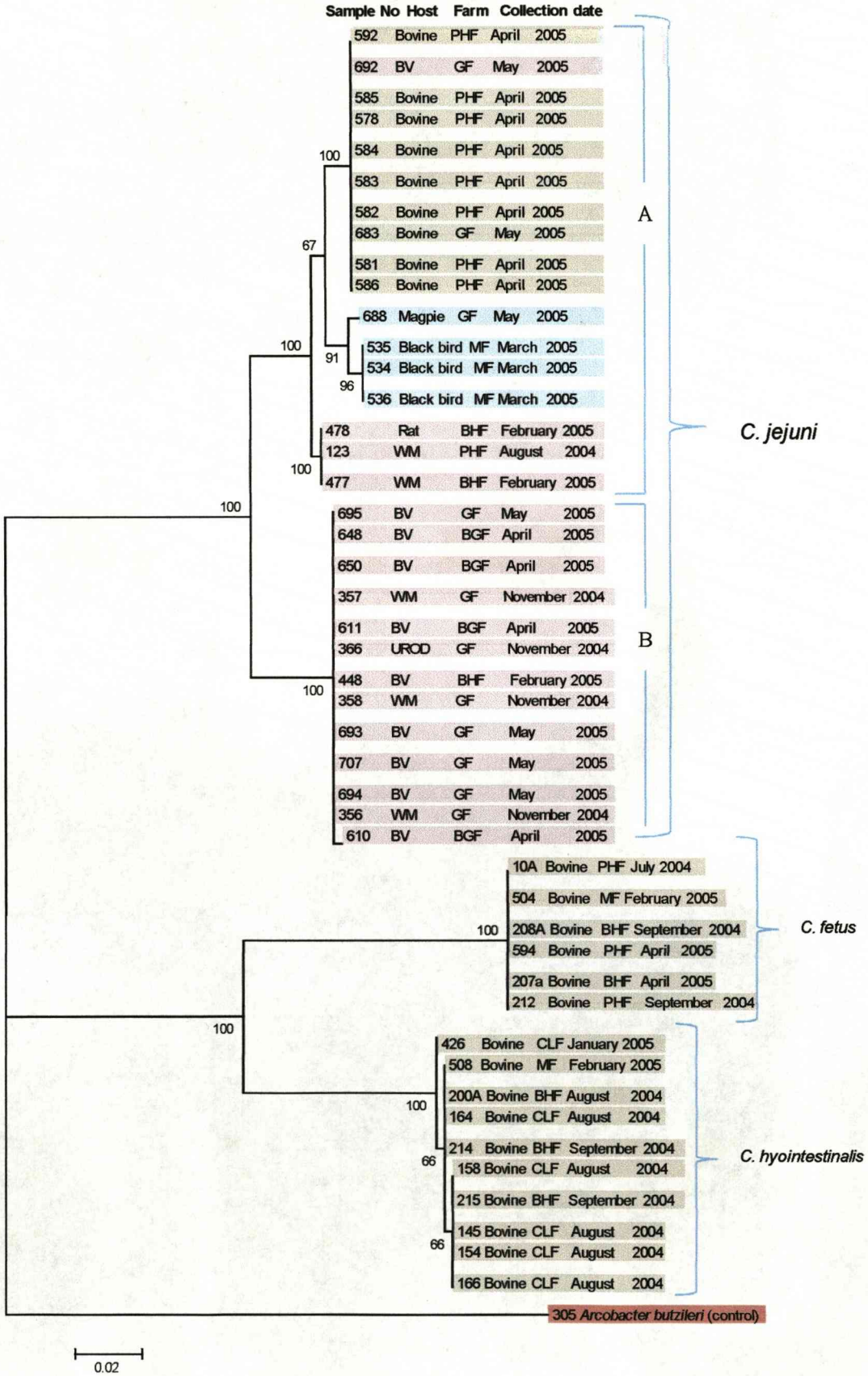


Table 1. Number of *Campylobacter* isolates from the cross-sectional study, by animal host and characterised by different PCR methods

	Wang method			**Lynton method**		**Gonzalez method**		**Karenlampi method**
Animal Host	*C. jejuni*	*C. lari*	23srRNA	*C. hyointestinalis*	*C. fetus*	*C. jejuni*	*C. coli*	*Campylobacter spp*
Cattle	12	0	106	1	2	0	0	44
Wood mouse	1							10
Bank vole	1							43
Unidentified Rodent	1							2
Rat	2							1
Wild Birds	1							3
Total No isolates (%, n=127)	18 (14)	0	106 (83)	1(0.7)	2(1.5)	0	0	103 (81)

4.4 Discussion

This study shows that four different *Campylobacter* spp were isolated on these six farms between July 2004-May 2005. Wildlife carried only *C. jejuni* except for one *C. coli* from an unidentified corvid. In addition, four isolates produced sequences that could not be identified as belonging to a particular species.

Most sequences from *C. jejuni* appeared to cluster by animal host. These results suggest that different strains of *C. jejuni* could be contained within the hosts' different niches (Colles et al., 2008a; Manning et al., 2003; Petersen et al., 2001).Some strains of *C. jejuni* can be relatively stable and it is believed some can have genetic re-arengements within the host's intestine in order to become adapted (Hansson et al., 2008; Kwan et al., 2008b; Rivoal et al., 2005) ,which is consistent with our results. This would suggest little cross-species transmission. Alternatively, it could be that the isolates evolve fast within the host.

Wild birds

Wild birds captured in this study were found to carry *C. jejuni* and *C. coli* but at low prevalence.

Three identical *C. jejuni* strains were isolated from a blackbird sample, indicating that there was no mixed-infection, or maybe a dominant strain that kept other *Campylobacter* strains to low levels which were unable to be detected by the methods of isolation used. Infection with *C. jejuni* in domestic poultry is maintained by one or two dominant strains and this has been observed *in vivo* and *in vitro* (de Boer et al., 2002; Skanseng et al., 2007). Moreover, *C. jejuni* isolates from birds appeared to be different from isolates in rodents and cattle suggesting certain host adaption. Host specific *C. jejuni* strains in geese and

starlings have been described before (Colles et al., 2008a). However, the number of positive isolates from birds was low and these results could be biased.

Wild birds are considered a reservoir for *C. lari*. No *C. lari* were isolated from the wild birds captured in this study although *C. lari* have previously been isolated from a wide range of animal hosts including birds and the environment in the same study area in Cheshire (Leatherbarrow et al., 2007). This could be due to a relatively small number of samples collected per farm or to the lack of certain bird species associated with this *Campylobacter* spp.

Rodents

The only *Campylobacter* spp isolated from rodents was *C. jejuni*. There was some genetic diversity amongst such strains. Bank voles carried three different *C. jejuni* strains. The most frequent type seemed to be rodent-adapted as it was carried by wood mice as well, although in small frequencies. Another *C. jejuni* strain carried by a bank vole was identical to strains mainly carried by cattle. Again, this indicates that the probability of transmission between cattle-bank voles and bank vole-wood mice may be possible.

As described in chapter 3, the prevalence of *Campylobacter* in bank voles was higher than in wood mice and isolates were also geographically clustered. *Campylobacter jejuni* has been isolated from rodents before. Although there are a limited number of studies that have compared the genetic profiles of *Campylobacter* strains isolated from domestic animals and wildlife, the possibility of interspecies horizontal transmission has been suggested (Meerburg et al., 2006).

Highly specialised molecular techniques such as Multilocus Sequence Typing (MLST) have been applied to *Campylobacter jejuni* strains from different sources in order to detect strain differences at the species level. This technique has provided evidence that strains from particular hosts could be clonal (Colles et al., 2003; French et al., 2005; Kwan et al., 2008a). We have applied this technique to the *C. jejuni* strains presented in this chapter. Although this work is on-going, preliminary results (data not shown) indicate that there is a novel Sequence Type (ST) not previously identified that seems to be isolated only from rodents, mainly bank voles. A previous survey carried in the study area using MLST identified a considerable number of novel *C. jejuni* strains from wild birds, rabbits and badgers (Kwan et al., 2008a). Once the MLST analysis is finished, it will be important to compare it with the GroEL sequence analaysis described here.

These results in rodents show that some of these *C. jejuni* strains could be rodent-adapted, seemed to be relatively clonal and are stable as highly similar strains have been isolated from different farms. Moreover, different bank voles can be infected with several *C. jejuni* strains genetically very distant and also with other strains predominant in cattle. This shows that bank voles could be a source for strains of *C. jejuni*.

Cattle

This study reiterates that cattle are a reservoir of *Campylobacter* spp. *Campylobacter jejuni* strains from cattle seem to be host adapted and stable over a year period of time and from different locations. This is consistent with other studies carried out in farm animals (Kwan et al., 2008b; Stanley and Jones, 2003). Moreover, previous studies have shown that cattle can carry *C. jejuni*

strains indistinguishable from strains that caused human disease (Nielsen et al., 2000).

Campylobacter hyointestinalis and *C. fetus* were only isolated from cattle. These two *Campylobacter* spp have been isolated from cattle previously (Anonymous, 2008; Milnes, 2007; Anonymous, 1998). *Campylobacter fetus* can produce sporadic abortions and infertility in cattle being of compulsory diagnosis for bulls that are intended to provide semen for artificial insemination in the UK (Anonymous, 2007;Anonymous, 2003a).

Both *C. fetus* and *C. hyointestinalis* are capable of causing gastroenteric disease in human beings although in lower frequency than *C. jejuni* (Gorkiewicz et al., 2002; Krause et al., 2002; Woo et al., 2002).

C. fetus strains seemed to be less diverse than *C. hyointestinalis*. *Campylobacter fetus* has been isolated from reptiles before and *C. fetus* strains of mammal and reptile origin were genetically different, suggesting a possible host adaptation (Tu et al., 2005).

Both species seemed to be quite stable as they were isolated from different farms. *C. hyointestinalis* has been isolated from wild birds captured in the study area before(Brown et al., 2004). A possible explanation could be that cattle would have been exposed to different strains of *C. hyointestinalis* from different sources. One of the reasons that could explain the lack of isolation of *C. hyointestinalis* from wildlife could be the variable sample size in wildlife per farm.

No other *Campylobacter* spp were isolated from cattle. This contrasted with other studies in which *C. coli* has been isolated from domestic cattle and cattle are considered an important reservoir (Milnes et al., 2007).

General

This study shows that *Campylobacter jejuni* strains seem to be host adapted. Bank voles tend to be the host infected with higher diversity of *C. jejuni* strains including an identical sequence shared with cattle suggesting inter-species transmission. Moreover, these *C. jejuni* strains seemed to be genetically stable as identical strains were isolated from the same type of hosts in different farms.

An important question that these results raise is if these *C. jejuni* strains from cattle and wildlife could be zoonotic to humans. All *Campylobacter jejuni* isolates were from healthy animals apparently without any clinical signs of disease detectable by visual inspection. Currently, humans are not considered a reservoir, but an accidental host for *C. jejuni*. Multiple strains from different geographical origins, including new strains, are capable of causing gastroenteric disease in humans(Duim et al., 2003; Quinones et al., 2008). In this study an identical strain was isolated from a bank vole and cattle. Was this bank vole an accidental host of this strain or it could be possible that this "unspecific "strain could infect other animal species including humans if exposed to it? This raises a possible contradiction as host specific strains vs. unspecific diversity as it has been raised before (Manning et al., 2003). This could mean a possible adaptation of a host to different *C. jejuni* strains.

These results should be interpreted cautiously as *groEL* PCR is not considered the most sensitive method, although it has worked satisfactorily in a situation

where the use of conventional PCR did not work for a high proportion of the isolates. Results from the use of MLST provide more complete information about strain genetic similarities as it looks at seven housekeeping genes. Certain ST clonal complexes MLST can be more frequently isolated from cattle while other ST seemed to be more common in wildlife (French et al., 2005; Kwan et al., 2008a).

The results show that a very high proportion of campylobacter isolates from wildlife needed to be sequenced in order to determine *Campylobacter* at the species level as specific PCR assays developed specifically to detect the *ceuE* and *hipO* genes in *C. jejuni* and the 16 S rRNA genes in *C. fetus* and *C. hyointestinalis* failed to detect them.

This could suggest that these *Campylobacter* strains have genetic differences in these genes. It would be useful for future work to sequence the *groEL* gene in the strains that were detected by the other PCRs or even sequence the whole genomes some of "conventional" and "unconventional" strains in order to determine possible differences. Moreover, it was not possible to molecularly characterize seven isolates to the species level with any of the PCR assays used in this study.

This suggests the lack of specific and sensitive methods for the diagnostic of *Campylobacter* spp in wildlife and domestic animals other than poultry.

Molecular methods have been developed in order to detect *C. jejuni* strains in food, domestic poultry and human beings. This could be one of the possible explanations why these methods failed to detect different *Campylobacter jejuni* strains from other sources.

It would not explain why such methods failed to detect other *Campylobacter* spp such as *C. hyointestinalis* and *C. fetus*. One of the reasons could be the lack of appropriate methods to detect these *Campylobacter* spp as they are not considered relevant for public health until very recently.

This could have led to an underestimation and bias in results obtained by the use of conventional PCR on these isolates. There is urgency with regard to further research for more accurate methodology to be developed when working with *Campylobacter* spp from farm animals and wildlife samples. The adoption of standard methodologies to enable to a comparison of different studies should be a "must".

In conclusion, *C. jejuni* strains could be host adapted in rodents, cattle and wild birds from the same farms. There could be differences inherent in the type of wildlife. For example, wild birds may be of less risk in terms of zoonotic spreading than bank voles. Mixed-infection with different *C. jejuni* strains was not very common in the different animal hosts. The possibility of inter-species transmission of *C. jejuni* strains between rodents and cattle was possible. Wildlife might have a limited risk of becoming infected with *C. hyointestinalis* and *C. fetus*.

Chapter 5 Determination of virulence genes carried by *E. coli* strains isolated from multiple healthy animal hosts on six cattle farms in Cheshire (UK) using microarrays

5. 1 Introduction

Escherichia coli is a well adapted and versatile bacterium which is part of the normal intestinal flora of animals. Although most *E. coli* are harmless commensal organisms, there are certain strains that are capable of causing intestinal and extra-intestinal disease in humans and other animals. Such organisms are commonly denominated pathogenic *E. coli.*

Pathogenic *E. coli* are grouped into pathotypes according to the characteristics of the disease produced. Some of the most relevant *E. coli* pathotypes in terms of public health significance are: enterohaemorrhagic *E. coli* (EHEC); shiga toxin or verotoxin producing *E. coli* (VTEC); enteroaggregative *E. coli* (EAEC); enterotoxigenic *E. coli* (ETEC); extraintestinal pathogenic *E. coli* (ExPEC) (which include strains associated with infections of the urinary tract (UPEC)), neonatal meningitis (MAEC), avian pathogenic *E. coli* (APEC) that causes colibacillosis in birds and enteropathogenic *E. coli* (EPEC) (Nataro and Kaper, 1998; Smith et al., 2007; Sousa, 2006).

These pathotypes differ from one another and from commensal strains because they have acquired distinct sets of virulence genes. These genes are mainly carried on plasmids, lysogenic bacteriophages, transposons or in large chromosomal insertions known as pathogenicity islands (Ohnishi et al., 2001; Paiva de Sousa, 2003; Tivendale et al., 2004) . These genes are able to express numerous virulence factors such as adhesins, haemolysins and toxins. Strains classified as part of a pathotype usually carry similar combinations of virulence genes ,(Chapman et al., 2006; Kaper et al., 2004) although sometimes different pathotypes may carry similar virulence genes (Smith et al., 2007). This phenomenon calls

into question whether the current classification system is sufficient to distinguish commensal and pathogenic *E. coli* – pathogenicity is in any case based on the idea of disease-causing potential in humans, and it is therefore not surprising that studies of *E. coli* from healthy non-human animal hosts have shown that such commensal *E. coli* strains can carry virulence genes (Beutin et al., 1995; Chapman et al., 2006; Dixit et al., 2004).

There is currently only limited information available about the virulence genes carried by that *E. coli* from healthy animals (Chapman et al., 2006), and it is not known how much risk for domestic animals, wildlife and humans is posed by virulence genes carried by commensal *E. coli* (Beutin et al., 2003). One obvious example of *E. coli* being commensal in one host and pathogenic in another is *E. coli* O157, in cattle (commensal) and human beings (pathogenic).There is little information on the transmission of 'commensal' *E. coli* between any hosts, and particularly between wildlife and domestic livestock as most studies of *E. coli* in wildlife have concentrated only on VTEC strains (Cizek et al., 1999; Nielsen et al., 2004a; Rice et al., 2003).

Two main factors probably explain this lack of investigation: the difficulty and expense of obtaining isolates from wildlife, and the lack of methodologies available for efficient testing for multiple virulence genes in *E. coli*. The chapters 5 and 6 describe the collection of a panel of wildlife *E. coli*, this chapter focuses on the use of microarrays to test for multiple virulence genes (Anjum et al., 2007).

The main aims of this study were therefore:

1. To determine the presence and distribution of virulence gene combinations (profiles) in *E. coli* amongst faecal samples from sympatric healthy livestock and wildlife.
2. To investigate the usefulness of a recently developed DNA microarray (Anjum et al., 2007) in such studies.

3. To determine whether the gene profiles generated might be useful for characterising 'strains', and therefore investigating possible cross species transmission of *E. coli* between wildlife and livestock.

5.2 Materials and methods

Four hundred individual *E. coli* colonies from faecal samples from domestic cattle and a variety of wild animals were tested using microarray for 45 different *E. coli* virulence genes and 15 23S-rRNA (*rr*_genes). The *gad* gene (glutamate decarboxylase), common to all *E.coli* was used as a control (Chapter 5-Appendix V). The isolates were chosen to be representative (not randomly selected) of those collected during the cross-sectional study on six cattle farms in Cheshire (UK) (Chapter5-Appendix I) as described in Chapter 3. Isolates were selected based on comparing similar numbers of different animal species per farm and area of farm, and also to include isolates already tested for VTEC virulence markers using PCR.

Individual *E. coli* colonies, previously identified morphologically on EMBA as described in chapter 2, were plated onto individual nutrient agar plates and incubated at 37 °C for 24 hours. Following incubation, a loop-full (approx. 10µl) of bacterial growth per plate was mixed with 400µl of lysis buffer (proteinase K and PBS). The mixture was incubated in a water bath at 60°C for 2 hours and boiled at 95°C for 15 minutes. This mix was then centrifuged at 13000 rpm for 5 minutes.

One microgram of supernatant, genomic DNA, was used as a template in a multiplex linear amplification, labelling reaction using the set of primers described by Anjum et *al.* and detailed in Table 1 (Anjum et al, 2007, Balmer et al 2007). The primer amplification was executed using 1µl of primer mix, 1µl of dNTP mix consisting of 1mM dAGCP, 0.65 mMdTTP, 1µl therminator 10x amplification buffer, 0.1 µl therminator DNA polymerase, 0.35 µl biotin-16-dUTP and sterile water up to a volume of 10 µl. PCR reaction conditions

were 5 min at 96°C followed by 40 cycles of 20 seconds at 62 °C, 40 seconds at 72 °C and 60 seconds at 96 °C. Each reaction was held at 4 °C for cooling.

The amplified products were added to array tubes for hybridization performed according to Ballmer *et* al (2007) (Anjum et al., 2007; Ballmer et al., 2007; Monecke and Ehricht, 2005). A total of 500 µl of sterile water was added to each array tube and incubated for 5 minutes (min) at 55 °C using a thermomixing device (550 rpm). The water was removed and 500 µl of hybridisation buffer was added and each tube was incubated 5 min at 55 °C. Then, 100µl of denatured PCR sample (10 µl of PCR labelled product plus 90 µl of hybridisation buffer incubated 5 min at 95 °C and cooled for 1 min in ice) was added to the array tube and incubated for 60 min at 55 °C and 550 rpm. The sample was removed from the tube and washed three times, first by adding 500 µl of a solution containing 2x SSC 0.01% triton incubating for 5 min at 40 °C and shacking at 550rpm; the second wash was done using 500 µl of a solution containing 2xSSC incubating for 5 min at 40 °C and shaking at 550rpm and the third wash was done with 500 µl of 0.2 x SSC incubating for 5 min at 30 °C and shaking at 550rpm. Subsequently, 100 µl of a 2% blocking solution (0.02g of ml powder dissolved in 1ml of 6x sspe-0.005% triton buffer) was added and tubes were incubated for 15 minutes. The solution was removed, 100 µl of poly –horseradish peroxidise (HRP)-streptavidin per tube was added incubated and incubated for 15 min at 30 °C and 550 rpm. This was followed by 3 washes: first with 500 µl/ tube of 2x SSC, 0.01% triton and incubation for 5 minutes at 30°C before centrifugation at 550rpm; a second wash with 500 µl/tube of 2x SSC and incubation for 5 minutes at 20°C and a third wash with 500 µl/tube of 0.2x SSC and incubated for 5 minutes at 20 °C. Finally, 100 µl of peroxidise substrate (True Blue and Seramun Green) was added to each tube and left for 10 min at room temperature. The hybridization signals were visualised and recorded with an ATR01 array tube reader (Clondiag). (Chapter 5-Appendix VI).

The dot signal intensity was obtained by calculating the quantitative staining value with IconoClust®v2 software. The data were normalized using the signal intensity of the *gad* probe, and the normalised signal intensity for genes (which was measured 3 times in order to increase sensitivity, the final intensity being an average of the 3 readings per gene) to differentiate between presence (signal above 0.3) and absence (signal intensity below 0.3).

For each gene i, i=1,2,...,45, a random variable Xi was defined, such that Xi takes the value 1 if the gene i is present (this happens with probability P_i, where Pi is the frequency of *E. coli* isolates that possessed the gene i out of the 374 *E. coli* isolates successfully tested with microarrays) and 0 if not. Therefore, X_i follows the Binomial distribution. Under the assumption that the presence or absence of a gene is independent of the presence of absence of the other genes, the probability P of a given sequence of values for these 45 virulence genes (per isolate), (X_1, X_2, X_3, ..., X_{45}) can be expressed as follows:

$$P = \left(P_1^{X_1} \times (1-P_1)^{(1-X_1)}\right) \times \left(P_2^{X_2} \times (1-P_2)^{(1-X_2)}\right) \times \ldots \times \left(P_{45}^{X_{45}} \times (1-P_{45})^{(1-X_{45})}\right)$$

Thus, for example if gene 1 was carried by the isolate, X_1 will be equal to 1 therefore, the formulae will be $P_i^1 \times (1-pi)^{(1-x_i)}$, equals to p_i; on the contrary, if gene 1 was not carried by the isolate, x_i will be 0 and therefore, $p_i^0 \times (1-p_i)^1$ equals to $(1-p_1)$.

In order to test whether the assumption of randomness holds in the sample of isolates tested (n=374), the expected number of isolates that do not carry any of the 45 genes was compared with the observed number.

To calculate the expected number of isolates that carries the *iss-iroN-mchF* gene profile, the product of the individual expected frequencies was used, for simplicity. In particular, the presence or absence of the other 42 genes was not taken into account in order to explore the possibility of these three genes being carried together as a group for specific isolates.

Statistical analysis was performed using Excel, Stata and R. Uni-variable analysis using Chi squared tests and Kappa agreement was conducted in Stata 8.1 (Statacorp 2003) for isolates that carried the *iss-iroN-mchF* genes profile. Here, the number of significant variables was too low to progress further and apply logistic regression. Frequency of genes graphs were done in Excel (Windows 2007). Cluster analysis for binary data, presence or absence of genes, to show similarities between isolates was carried out using R (http:\\cran.r-project.org/). The distance between isolates was calculated using the Dice index. This is an index in which joint absences are excluded from consideration, and matches are weighted double.

5.3 Results

A total of 400 *E. coli* colonies from different animal hosts were tested for the presence of virulence genes. Of these, 95 did not carry any of the 45 virulence genes in their genome, 11 isolates gave an invalid reading and 15 sample colonies were contaminated with *Proteus* spp. making them invalid to apply the microarray. Those isolates have not been included in analysis. A total of 279 isolates were found to carry virulence genes (75%, n=374).

All 45 genes included in the microarray were detected at least once. Different *E. coli* isolates carried different number of genes, the number varying from 0 to 18. The median (2 genes) and mean (4genes) were very similar amongst these isolates.

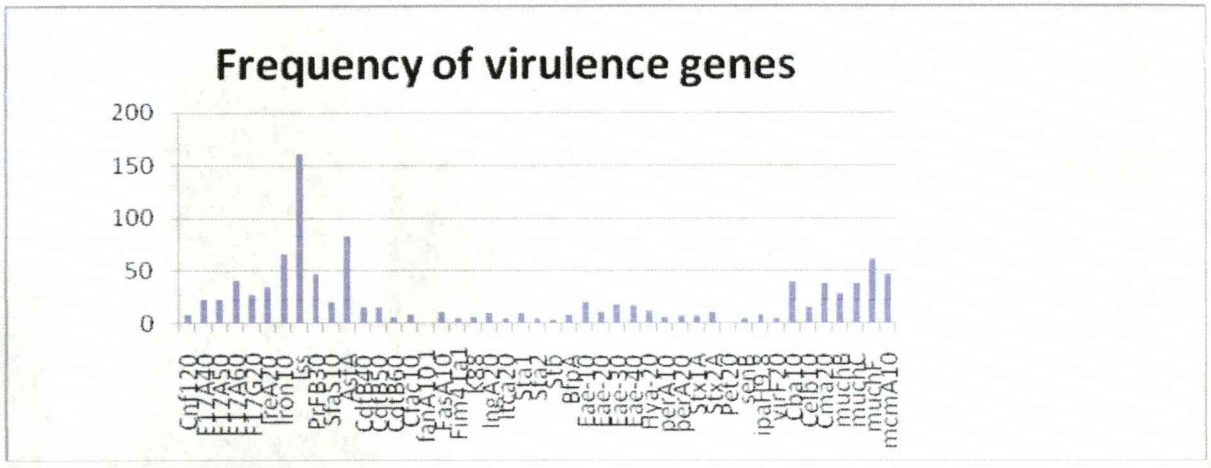
Virulence gene/s general distribution across the isolates

In total, 180 different gene combinations were detected in the 279 isolates that contained one or more virulence genes, And a further 95 isolates contained no genes (other than the control gene). With 45 virulence genes tested, there were 2^{45} combinations theoretically possible per isolate, ie $2^{45} = 3 \times 10^{13}$ combinations in total. This suggests that the distribution of these virulence genes is not random amongst strains. Furthermore, most virulence gene profiles

were encountered only on certain farms (Chapter 5-Appendix II). The most frequently occurring gene was *iss* (42%), followed by *astA* (22%), *iroN* (17%), *mchF* (16%), *mcmA* and *prfB* (12%). The genes with lowest prevalence amongst the isolates were *fanA* (badger MF) and *pet 20* (fox PHF) (0.2%) (Figures 1 and 2). The frequency distribution of individual genes per host is detailed in tables 7 and 8.

Based on equation the expected number of isolates not carrying any of the 45 genes (n=374) was 19. This value is five times smaller than the observed number of 95.

Figure 1 .Frequency of virulence genes in all isolates



Sixteen isolates (4%, n=374) carried 10 or more gene combinations: MF accounts for the higher number of isolates (30%). Wood mice (30%) and calves (18%) were the hosts with a higher proportion of isolates followed by foxes (11.5%) and unidentified wild birds (11.5%). The most prevalent genes across these multi-gene isolates were *iss* (80%) followed by *mchC* (73%) (Figure 2).

Frequency of individual genes and gene combinations per farm

The number of isolates per individual farms was distributed as follows: 66 PHF; 53 MF; 49 CLF; 45 BHF; 42 BGF and 23 GF.

Across farms, *iss* was the most prevalent gene, being present in the 6 participating farms with frequencies that varied between 80% (BHF) and 47% (PHF); *astA* was also carried by high number of isolates, ranging between 48% (GF) and 21% (PHF). The *iroN* gene was found at high frequency (37% to 26%) in 4 out of the 6 farms, and MF (13%) and PFH (9%) accounted for the lowest frequency of this gene.

Each farm's isolates were compared using the "dismatfun" and "hclust" commands in R in order to determine clustering of virulence genes, and to compare these cluster by farm and host. The resultant dendrograms showed that the distribution of genes across samples did not follow particular patterns. The dendrogram for farm GF is shown in figure 3 as an example, and further dendrograms are shown in Chapter 5-Appendix VIII.

Clusters of isolates that carried identical virulence gene profiles

One hundred and twenty two isolates (33%, n=374) had a virulence genes profile identical to at least one other isolate, and these formed into 24 groups of identical profiles. These groups contained different numbers of isolates, and the profiles consisted of different numbers, as well as types of, gene, and were often distributed widely across different hosts and the 6 participating farms (Table 2). The observed prevalence of the various gene profiles encountered was higher than would be expected at random. The *iss* gene cluster was the gene carried by the highest number of isolates (20.8%) followed by *astA* gene (17%), the *iss-astA* genes (9.2%) and the *iroN-iss-mchF* genes (8.33%).

The *iss* gene alone (n=25) was also carried by 10 different wildlife hosts and 1 bovine, 32% of isolates were from rodents, mainly by bank voles and wood mice, from 4 farms, and 44% of isolates were from badgers and foxes and 12% from small passerines.

The *astA* gene alone (n=20) was carried by 11 different wildlife hosts, mainly wood mice (30%) but it was not carried by *E. coli* isolates from domestic cattle.

The *ireA-prfB-mcmA* genes Cluster (n=5) was carried by isolates from cattle (60%) from 3 farms (GF, MF, PHF) and by rodent isolates (40%) from one farm (PHF).

The *cdtB40* and *iss-astA-celb10-mchB-mchC-mchFgene profile* was isolated from bovine animals and rodents sampled from the same farm (PHF and BGF).

Eae, vt1 and vt2 genes

Twenty-nine isolates out the total 279 isolates possessed *eae* and/or *vt1* and/or *vt2* genes. The *eae1* gene was the most frequent (44%). Both *vt1* and *vt2* genes tended to be carried with similar frequency. These two genes were carried for a wide range of different hosts (Table 11), mainly cattle (41%) followed by rodents (28%). One particular farm, MF, had the highest frequency (38%) with the highest proportion of carriers in cattle (54%, n=11), mainly adult animals (67%). The farm with the smallest number of isolates containing any of *eae*, *vt1*or *vt2* genes was GF, where only one calf was positive. These three genes were usually carried together with other virulence genes tested for in the microarray: the most frequent other genes were *astA* (45%) followed by *iss* (41%) and *hylA* (38%) (Table 1).

Table1. Distribution of *E. coli* isolates that carried *eae*, and/or *vt1* and/or *vt2* by hosts and farms

Location	Host	Gene profiles (No of genes)
BGF	Bank vole	*cfa,vt1,vt2,celb* (4)
BGF	Fox	*astA,eae1, mchB,mchC,mchF,mcmA* (6)
BGF	Fox	*eae1,eae3*(2)
BHF	Adult stock	*iss,sfas,eae3,cma*(4)
BHF	Great tit	*f17A60,iss,prfB,astA,cdtb60,fim,bfp,eae1,eae,2,eae3,eae4,hyA,senB,cba,mchC,mchF,mcmA* (17)
BHF	Wood mouse	*ireA,iss,prfB,sfas,cdtb50,fasA,stb,bfp,eae1,vt2,ipaH,mchC,mchF*(13)
CLF	Calf	*astA, eae1,eae2,eae3,eae4,hyA,vt1*(7)
CLF	Chaffinch	*fim41a,eae1,eae2,eae3,eae4*(5)
CLF	Wood mouse	*f17A40,astA f17A50,cdtB40,cfa,k88,bfp,eae1,perA10,vt2,mchC*(11)
CLF	Rabbit	*vt1*(1)
GF	calf	*astA,eae1,eae2,eae3,eae4,hyA,vt1,cba,celb*(9)
MF	Lactating cow	*f17A40,f17A50,f17A60,iss,sfas,astA,cfa,eae1,eae2,eae3,eae4,hyA,vt,2,cba,celb,mchC*(17)
MF	Young stock	*astA,aea1,eae2,eae3,eae4,hylA,vt2*(7)
MF	Calf	*astA,eae2,eae3,eae4,hylA,vt1,cba* (7)
MF	Adult stock	*iss,astA,sta1,hyA,vt2*(5)

MF	Adult stock	*astA,eae1,eae3, hylA,vt2*(5)
MF	Lactating cow	*astA,aea1,2,3,4,hylA,vt2*(7)
MF	Pigeon	*eae1,eae3,eae4,perA10*(4)
MF	Adult stock	*astA,aea1,eae2,eae3,eae4,hylA* (6)
MF	Wood mouse	*f17A40,iron,iss,cfa,ingA,sta1,eae4,vt2,sfas,cba,cma*(11)
MF	Rat	*cnf, f17A40, f17A50, f17G20,iss,cdtB40,cdtB50,fasA,ingA,sta2,eae2,perA20,virF*(13)
MF	Fox	*iss,prfB,cfa,k88,ingA,itcA,bfp,eae1,perA10,perA20,pet, mchB, mchC, mchF* (14)
PHF	Wood mouse	*iss,eae1,eae3*(3)
PHF	Calf	*iss,eae1,eae2eae,3,eae4,vt1,cba*(7)
PHF	Bank vole	*iss,eae1,eae3eae,4*(4)
PHF	Calf	*ireA,prfB,vt1,cba*(4)
PHF	Calf	*f17G20,iss,astA,eae1,eae2,eae3,hylA,cba*(8)
PHF	Wood mouse	*eae1*(1)
PHF	Bank vole	*iss,eae1,eae2,eae3,eae4,cba,cma* (7)

Kappa agreement test in the absence of a "Gold standard" test for eae, vt1 and vt2 results obtained by PCR and microarray techniques

There is no gold standard test method to determine if *E. coli* isolates carry the *eae*, *vt1* and *vt2* genes. Therefore, the results obtained by PCR (Chapter 3) and the microarrays were compared by the use of Kappa agreement for absence of "gold standard" test. The kappa agreement is scaled to be ≤ 0 when the amount of agreement is low, between 0 and 1 when there is some agreement, and 1 when there is perfect agreement.

The *vt1* gene was carried by 13 isolates, detected by a combination of the use of both genomic methods. Kappa agreement for *vt1* gene carriage was -0.723, the actual agreement percentage was 15% and the expected agreement was 51%. Only 15% of the expected 51% of the isolates coincided in their results. Microarray was not able to detect the gene in five of the isolates that were positives by PCR previously, while on six PCR negative isolates microarray detected the *vt1* gene.

Both methods combined were able to detect 13 isolates carrying the *vt2* gene. The agreement for vt2 gene carriage was -0.814, only 8% of the isolates of the expected 49% coincided with the results by both methods.

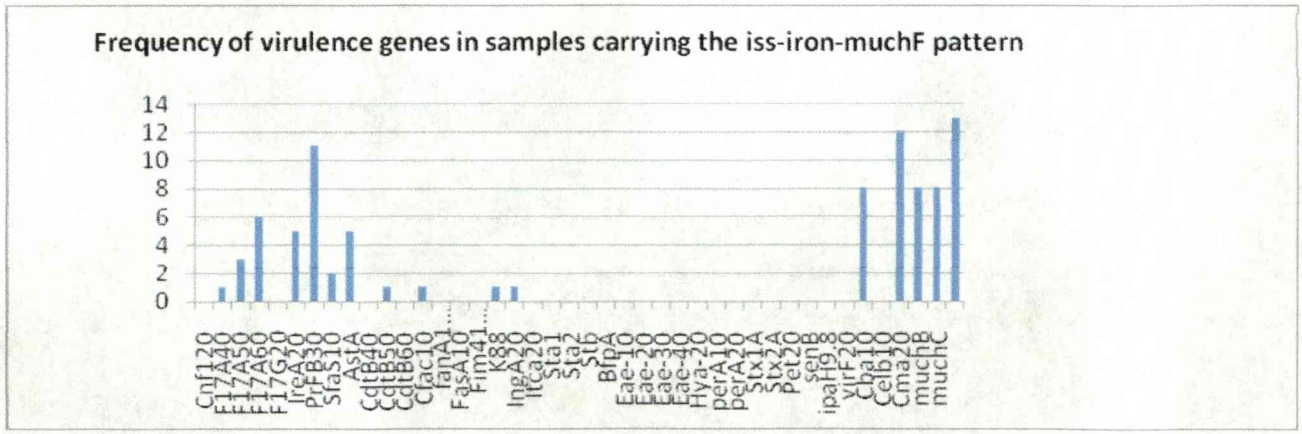
The *eae* gene was detected by both methods combined in 29 isolates. The kappa agreement was -0.07, 17.24% of the isolates coincided with identical results of the 23% agreement expected by both methods. Microarray was able to detect the gene in 23 PCR *eae* negative isolates.

Applying the Kappa agreement under the absence of the gold standard method shows that the agreement for both methods was very low in general, especially with the *vt2* gene that is the most diverse of the three genes.

Iss-iron-mchF genes profile

The *iss-iron-mchF* gene profile was carried by 42 of the 374 isolates (11%).This is ten times more than the expected number of isolates (4) that could carry these three genes together in the 374 isolates. This profile of genes was carried by *E. coli* a variety of different wild and domestic hosts without any other genes (Table 2) and together with other virulence genes (Figure 4). All these genes are associated with the UPEC/APEC pathotype or have an undesignated pathotype. None of these strains carried the *eae, vt1, vt2* or *hlyA* genes, mainly associated with EPEC and VTEC pathotypes.

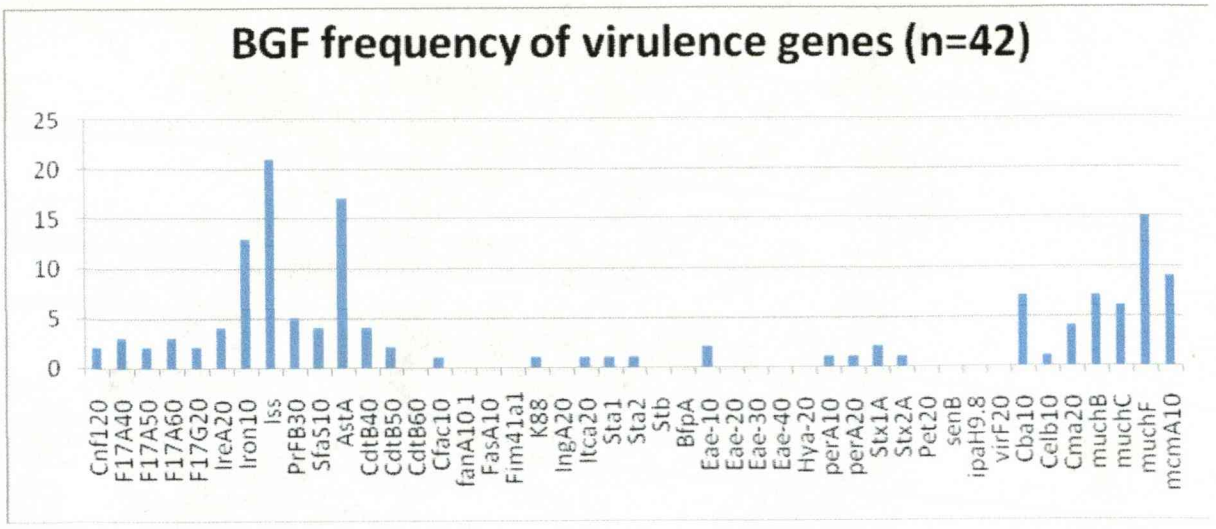
Figure 4. Frequency of genes that were carried together with the *iss-iron-mucF* gene profile

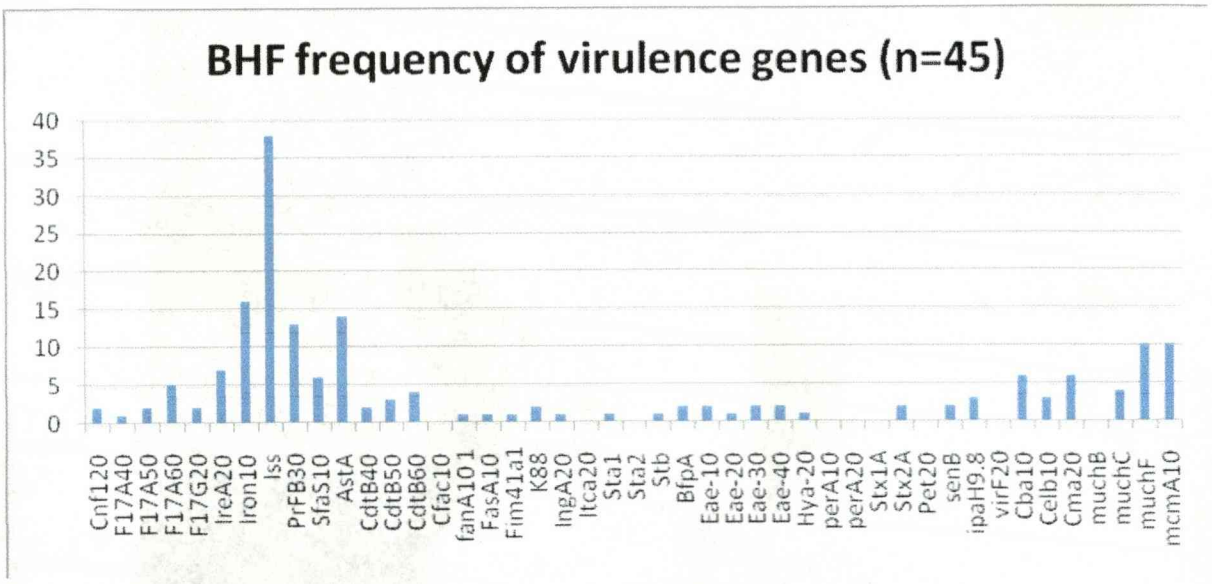


The univariate analysis showed that farm was a significant variable (p=0.05) as 4 farms presented a frequency of isolates carrying this gene pattern of approximately 20-21% compared to two farms (PHF,MF) that both had a much lower frequency of 6 and 7.5%. Animal host was also found significant (p=0.003), the frequency in birds was 36% (n=42) compared with cattle and wild mammals that varied between 7-10%.

This shows that almost 1 in 6 isolates carried these three genes and this profile had a higher prevalence in 4 out of the 6 farms.

Figure 2. The individual gene distribution per participating farm





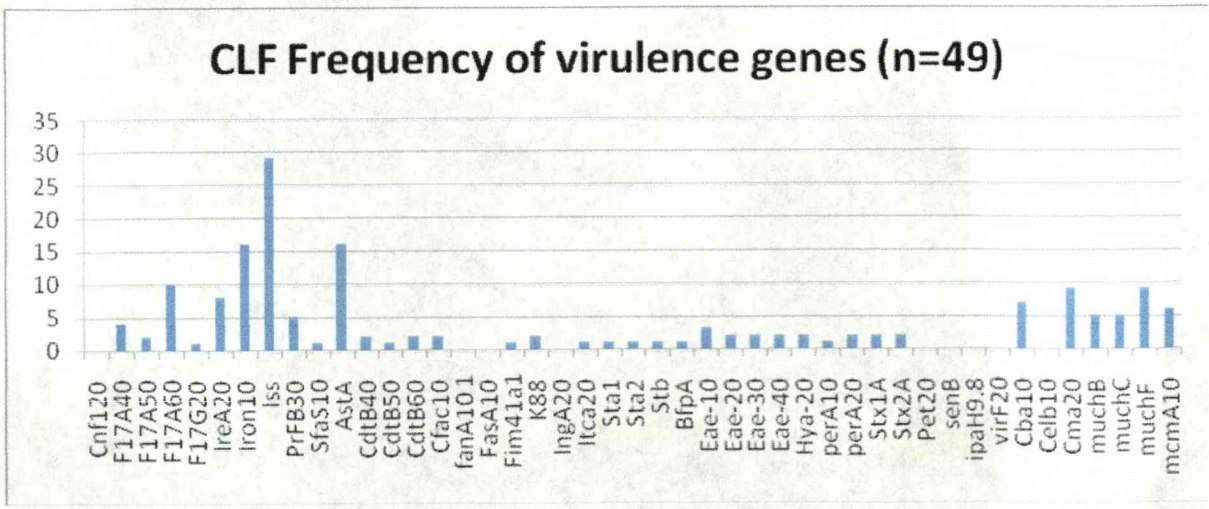


Figure 3. Dendrogram with E. coli gene profiles from GF tested with mycroarrays. The remaining five farms are included in appendix 8-Chapter 5

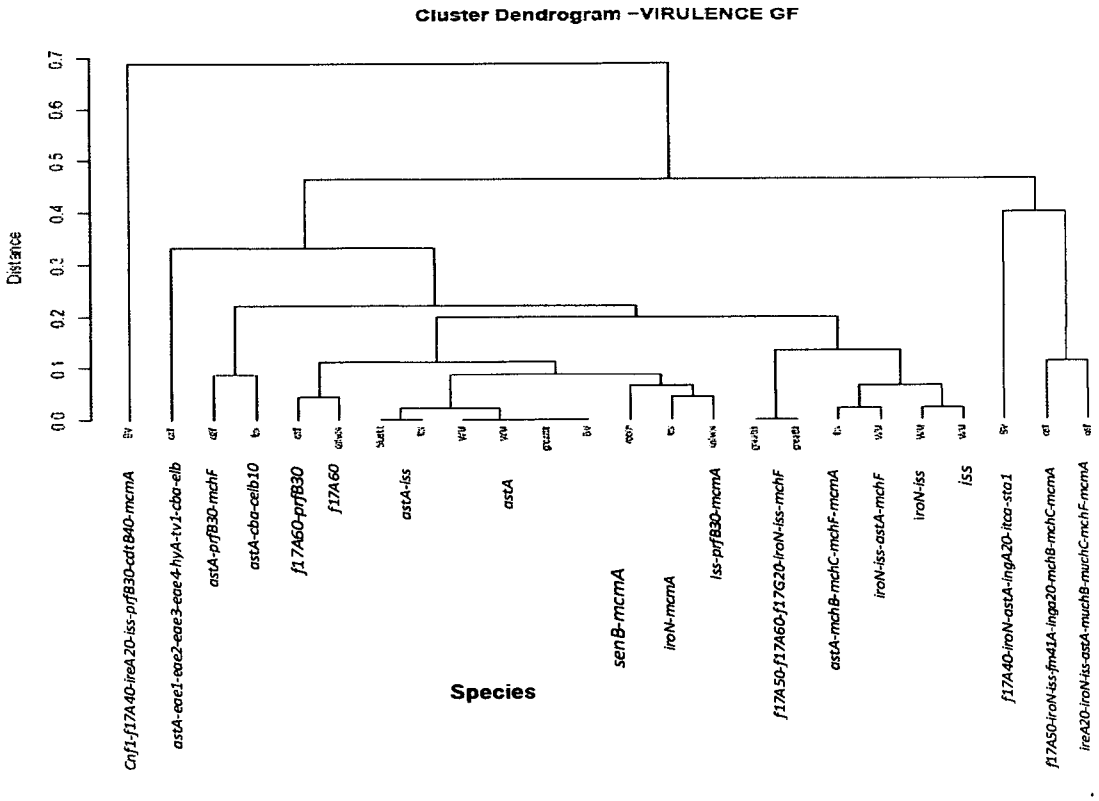


Table 2. Clusters of *E. coli* identical gene/s profiles by animal host isolates per farm Chapter 5. Colours have been allocated by groups of animal hosts as follows: purple-small rodents and rats; green-wild birds; red-larger wild mammals; grey-domestic cattle. The number of Expected isolates with the gene profiles by the binomial distribution is indicated by (E) and the number of actual isolates found to carry the gene profiles is indicated by (O)

Cluster gene/s	**No Isolates (E) (n=374)**	**No isolates (O) (n=374)**	**BGF**	**BHF**	**CLF**	**GF**	**MF**	**PHF**
astA	5	20	3 wood mice, 1 fox	1 wood mouse, 1 fox	1 dunnock, 1 pigeon, 1 corvid, 1 wood mouse	2 wood mice, 1 bank vole, 1 great tit	1 chaffinch, 1 badger, 1 fox	1 bank vole 1 house mo 1 wood mo
iss	11	25	1 badger, 2 foxes	2 foxes, 1 rabbit	1 dunnock, 1 bank vole, 1 rat, 2 badgers	1 wood mouse	1 adult bovine, 1 wood mouse, 1bank vole, 1 fox, 1 badger, 1 wren	2 bank vole 1 wood mo 1 house mo 1 badger, 1 fox, 1 black bird
cdtB40	0.75	2	1 adult bovine, 1 rat					
f17A60	2	7	1 house mouse	1 noID bird	1 house mouse	1 calf	1 noID bird	1 dry cow, 1 house spa
ireA	2	3	1 bank vole		1 bank vole, 1 wood mouse			
iroN	4	2		1 noID rodent	1 pigeon			
f17G20	2	3						1 dry cow, 2 wood mic
Iss-astA	4	11	1 fox	1 black bird, 1 wood mouse	2 bank voles, 1 wood mouse	1 wood mouse	1 bank vole	2 wood mi 1 fox
cdtB40-cdtB50	0.03	2		1 rabbit				1 robin
astA-cdtB50	0.23	2				1 blue tit, 1 fox		
cnf1-20-cdtB40-cdtB50	0.0007	3	1 wood mouse, 1 bank vole				1 adult bovine	

f17A60-iroN-iss	0.33	3	1 calf, 1 bank vole		1 blue tit			
Cluster gene/s	**No Isolates (E)**	**No isolates (O)**	**BGF**	**BHF**	**CLF**	**GF**	**MF**	**PHF**
iroN- iss-cma20	0.31	2		1 dry cow			1 badger	
iroN- iss-mchF	0.6	11	1 robin	2 house mice, 1 robin 1 noID bird, 2 badgers, 1 young bovine	1 noID rodent		1 rat	
ireA-prfB30-mcmA	0.03	5				1 calf	1 adult bovine	1 calf, 1 house mo 1 rat
f17A50-f17G20-iss	0.06	2						2 badgers
iss-astA-cba10-cma20	0.05	3		2 bank voles			1 wood mouse	
F17A60-ireA-prfB30-mcmA	0.004	2						1 wood mo 1 badger
astA-mchB-mchC-mchF-mcmA	0.001	2	1 fox			1 fox		
Iss-astA-mchB-mchC-mchF	0.001	2	1 bank vole					1 lactating
f17A50-f17A60-f17G20-iroN-iss-mchF	0.0002	2				2 great tit		
iron-iss-mchB-mchC-mchF-mcmA	0.0006	2					1 bank vole, 1 rabbit	
iss-astA-celb10-mchB-mchC-mchF	$4x10^{-8}$	3						2 claves, 1 wood mo
astA-eae1-eae2-eae3-eae4-hyA-vt2	$7x10^{-5}$	2					1 young bovine, 1 lactating cow	

5.4 Discussion

The array results presented in this chapter show that *E. coli* isolates from healthy wildlife and domestic cattle carry virulence genes described previously as part of individual *E.coli* pathotypes and /or *E. coli* isolates from diseased humans and domestic animals.

The possible presence or absence of the 45 virulence genes in the genome of each of the 374 isolates that could carry these genes could have generated as many as 2^{45}different possible combinations. Only 180 different virulence gene combinations have been identified in these isolates which implies that these genes and some of these profiles do not appear at random. This is consistent with the finding that 122 of the isolates contained one of only 24 profiles from a range of different hosts and sites. This suggests the possibility of cross-species transmission or environmental contamination with these *E. coli* strains.

The *iss* gene allows ExPEC strains to survive in serum and increases lethality towards avian embryos. Furthermore, it has been associated with APEC in collibacilosis cases in domestic poultry and it is believed to be implicated in the pathogenesis of APEC in birds (Johnson et al., 2008; Skyberg et al., 2008; Tivendale et al., 2004) . This gene was the most prevalent in five of the six farms, and on the sixth farm the sample size was small. The *iss* gene was also the most prevalent gene amongst all wildlife and domestic hosts. This is consistent with the results obtained by Anjum et *al* for validation of this microarray (Anjum et al., 2007). The reasons for such high frequency are at present unknown. The role of the *iss* gene in the pathogenesis of colibacillosis produced by APEC in birds is not completely understood (Rodriguez-Siek et al., 2005; Someya et al., 2007). It is known that this gene is transmitted by large plasmids denominated colV which are very common in APEC strains (Johnson et al., 2008; Skyberg et al., 2008) and it has been documented that this plasmid has not been found frequently in ExPEC strains producing human disease (Ewers et al., 2007; Johnson et al.,

2008) therefore, it is believed that this gene could imply limited zoonotic potential. In some cases this gene has been present in ExPEC isolates from human disease cases. Moreover, it has been suggested that APEC strains could be a reservoir of virulence genes for human EXPEC (Chapman et al., 2006; Ewers et al., 2007; Rodriguez-Siek et al., 2005; Skyberg et al., 2006).

The *astA* and *iroN* genes were the second most prevalent genes. The *astA* gene encodes for a heat stable enterotoxin (EAST1) and has been associated with different *E. coli* pathotypes including APEC. This gene has been isolated from *E. coli* strains responsible for pre-weaning diarrhoea in pigs and colibacillosis in poultry (Someya et al., 2007; Veilleux and Dubreuil, 2006). Furthermore, the *astA* gene has been identified in *E. coli* strains involved in a case of food poisoning and a waterborne outbreak in Japan (Veilleux and Dubreuil, 2006; Yatsuyanagi et al., 2003). The high frequency of the *astA* gene amongst domestic animals and wildlife could reach human populations via food especially from domestic cattle. This could be consistent with Toshima et *al* (2004) who found the EAST1 present in food of animal origin and has been implicated in human outbreaks transmitted by food (Toshima et al., 2004).

The *iroN* gene encodes for a enterobactin siderophore receptor associated with ExPEC (UPEC and APEC) and allows the bacteria to retain the necessary iron for their metabolism specially in presence of antibiotics. This receptor acts as a virulence factor during infections of the urinary tract (Skyberg et al., 2006; Skyberg et al., 2008). Moreover, this gene is transmitted by the colV plasmid as the *iss* gene (Johnson et al., 2006). Both genes, *astA* and *iroN* had the lowest prevalence at PHF. It is not clear why this particular farm (PHF) had a significantly lower prevalence amongst its hosts of both genes (*iroN*, *astA*) than the other 5 farms. This farm had a higher number of isolates than the other five farms but there could be other unknown differences between these farms that could contribute to such prevalence

differences or, indeed, the selection of samples or sample size per farm may have had an effect.

The above discussion is relevant also to finding the *iss-iroN-mucF* gene pattern so frequently amongst the isolates. This pattern was carried by 15% of the total number of isolates either on its own or with other virulence genes. The virulence genes carried together with this profile of genes are mainly associated with ExPEC/UPEC strains (*prfB*, mchC and *f17A60*) or without a specific pathotype (*cma, cba,mcmA* and *mchF*). None of the isolates that carried this gene pattern also carried the *eae, vt1, vt2 and hlyA* genes. The reason is unknown but it is possible that groups of specific genes are incompatible, or that this profile and the VTEC profiles are selected for in different environments.The *iss-iroN-mucF* profile was significantly associated with wild birds with 36% prevalence compared to cattle and terrestrial wildlife (7-10%). As most of these genes are associated with APEC strains, this could simply represent host affinity. Four of the six farms had a prevalence of this pattern of approximately 20% compared to PHF and MF which had a much smaller prevalence (6-7%). These two farms are geographically close, and were only separated by a road. One farm (MF) had a high prevalence of VTEC strains amongst its hosts, including birds that could move freely across both farms. This result is surprising as one field on MF has boundaries with a poultry broiler farm house and material, such as running water and chicken by-products, were found on the farm embankment. A possible explanation for this may be that specific strains and or virulence genes are predominant over other or may establish competitive exclusion if they are of higher prevalence than others. Scott et *al* (2007) observed that *Campylobacter jejuni* carrying bacteriophages become antibiotic-resistant but could not then carry the virulence markers that enable them to colonise the chicken's intestine, making them immobile(Scott et al., 2007). Similarly, Soto et *al* observed that the gaining of quinolone resistance required the loss of virulence genes from pathogenicity islands in UPEC strains

(Soto et al., 2006). The *iss-iroN* genes are usually carried by a large plasmid, colV, and the genome weight of this plasmid combined with vt1-2 bacteriophages and/or the LEE could be incompatible.

Although *iss-iroN-mucF* gene profile seemed to be wild bird associated, these genes were also carried by other animal hosts, albeit less frequently. Thus this profile is not totally host specific. Most dairy or beef cattle farms in the UK have extensive production system, the animals spend long periods of time grazing in the field and have contact with wild birds and or their droppings in the fields or around ponds. Moreover, wildlife rodents and large mammals live around cattle fields and carry out a large variety of activities such as feeding, drinking, nesting and defecating, which increase the probability of contact with wild bird droppings or cattle manure. At present no data are available about the prevalence of APEC strains or colibacillosis outbreaks in domestic poultry in the UK, although it is considered to be common (Dr. Paul Wigley personal communication). Therefore, it is not strange to find a number of gene/s associated with APEC strains spread widely in wild birds, especially if they could have been in contact with domestic poultry. It is not known if APEC strains that produce colibacillosis in domestic poultry could produce disease in wild birds or if wild birds have a natural resistance to disease but could act as a reservoir.

Ten percent (n=374) of the isolates carried the *eae* and/or *vt* genes. Cattle *E. coli* were the main carriers (30%) but these genes were also carried by wildlife hosts including birds and mammals. This is consistent with other research showing that cattle are the main reservoir of VTEC but that wildlife can carry VTEC markers such as *eae* and *vt* genes, probably transmitted by direct contact with cattle faeces or other faecal contaminated environment (Nielsen et al., 2004a). MF was one of the farms with the highest prevalence as this was observed previously in other chapters (Chapter 3). In addition, a pigeon sample from MF carried the four *eae* genes. This result is not surprising as high number of pigeons were seen

around the cattle barns and also fed from the cattle silage inside the barn. *E. coli* from a rabbit was also found to carry the *vt1* gene at CLF. VTEC genes were not detected in rabbits using IMS and PCR techniques during the cross-sectional study (Chapter 3), although a previous study carried in this area isolated *E. coli* O157 from rabbits (Kemp, 2005a). Most of the 39 VTEC isolates carried multiple genes, the most frequently carried genes being *iss*, *astA* and *hlyA*, but only one sample carried the *iroN* gene. The *astA* gene has been isolated before from VTEC isolates from bovine animals together with *bfp*, *hlyA* and *eae* genes (Blanco et al., 2005) although only 3% (n=39) samples carried the *bfp* gene within our study. These results are consistent with two other studies that detected the *eae*, *astA* and *hlyA* genes carried by *E. coli* strains in pigs and sheep (Cookson et al., 2002; Zweifel et al., 2006).

The detection of *eae*, *vt1* and *vt2* genes previously by the use of PCR was compared with the results obtained by this microarray technique. The agreement between both methods was low for all three genes, indicating that in some cases PCR failed to detect the genes in isolates that were detected by the use of microarray and/or vice-versa. The lowest kappa agreement was with *vt2* which is considered very diverse gene (Mainil, 1999; Nataro and Kaper, 1998) followed by the *vt1* and *eae* genes that tend to be more conserved genes.

The detection of the *eae* gene by microarray was not as specific as almost 100% specificity showed when this microarray was developed. Anjum et *al* only considered 5 out of 45 genes at random to determine the method's specificity (Anjum et al., 2007), and it might be expected that a greater diversity of genes, leading to lower sensitivity and specificity of diagnostic assays, might be found in isolates from a more diverse range of hosts. Meanwhile, the use of both the PCR and microarray methods should probably be used in future studies. It might also be interesting to sequence the *eae* and *vt* genes from wildlife isolates to investigate this diversity further.

Data from this study showed that 43% of isolates were clustered in 24 identical gene profile groups amongst different hosts and farms. Some gene profiles were specific to certain farms but many others appeared in every farm. The *iss* and *astA* genes, for example, were widely dispersed across farms and hosts. Other combinations appeared to be more associated with a particular host, such as the *astA-mchB-mchC-mchF-mcmA* found in 2 foxes in BGF and GF. These two farms were geographically very close and could be the same animal that defecated in both. Other genes combinations such as the *iroN-iss-mchF* (as described before), the *iss-astA-celb10-mchB-mchC-mchF* genes, *f17A60-iroN-iss* and the *irA-prfB30-mcmA* were carried by both cattle and wildlife on the same farm. This could indicate that *E. coli* virulence genes may be transmitted between cattle and wildlife via direct contact or contaminated environment although, the direction of transmission is not known. It remains unknown whether these genes are endemic in *E. coli* amongst these animal populations or will disappear over time because of the *E. coli* strain dynamics within the farm. More research into the temporal and dynamic ecology of these virulence genes would be beneficial to our understanding of the dynamics of transmission and persistence in hosts and on farms.

One of the most important issues that the results highlight about these *E. coli* strains from healthy animal hosts is: Are these *E. coli* strains commensal or pathogenic? As already discussed, the genes most frequently carried by these isolates are associated with ExPEC strains, and it is well documented that such strains can behave as non-pathogenic strains in one host's intestine and as pathogenic elsewhere (Welch et al., 2002). Other studies state that the difference between ExPEC and commensal strains is that ExPEC do not establish long term relationships with their hosts (Chapman et al., 2006). It should also be noted that the presence of these virulence genes in *E. coli* isolates does not mean that these are expressed phenotypically.Other studies have also observed that commensal enteric bacteria can carry virulence genes and that the difference between pathogenic and commensal strains is not at

all clear (Chapman et al., 2006; Dixit et al., 2004; Dobrindt et al., 2003; Gilmore and Ferretti, 2003; Rodriguez-Siek et al., 2005).

The number of isolates with no virulence genes detected by the microarray was 95 (25%) compared with the 279 (75%) that carried at least one of the 45 virulence genes out of 374 isolates successfully tested. This means that 3 in 4 isolates did possess one or more virulence genes. This suggests that there are strong selection factors in favour of the acquisition of these virulence genes, and probably means that these virulence genes have functions, and advantages, beyond the disease-causing functions.

This study is the first to compare *E. coli* isolates from healthy wildlife and domestic cattle by using microarray. Our findings suggest that a wide range of virulence factors circulate in *E. coli* that are part of the normal intestinal flora of healthy wild and domestic animals. Further research is needed to understand the dynamics and selection pressures that apply to these genes, their transmission amongst bacteria and the transmission of those bacteria amongst various animal hosts and the environment. Until this work is done it is difficult to estimate the zoonotic potential of what is a potentially sustantive reservoir of pathogens.

Chapter 6 Microarray analysis of virulence and antibiotic resistance genes in *E. coli* isolated on six cattle farms in Cheshire (UK).

6.1 Introduction

Antibiotic resistance in bacteria can occur for a number of reasons, as a natural or innate property of the bacteria and as an adaptation process following exposure to the antibiotics. The rapid spread of resistance through a bacterial population is mediated by horizontal transfer via plasmids, transposons or bacteriophages (Arber, 2000; Paiva de Sousa, 2003)

There is a growing concern about an increase of enteric bacteria resistant to antimicrobials commonly used for veterinary and human disease therapy and prevention (Aarestrup, 1999). It is believed that the use of antibiotics for animal prophylaxis and growth promotion in animal food has been one of the reasons for the rapid spread of resistance amongst bacteria in farm animals (Blanco et al., 1997; Boerlin et al., 2005; Depaola et al., 1995; Sawant et al., 2007) and persistence long after the drugs have been used (Depaola et al., 1995; Maynard et al., 2004). Some of resistant enteric bacteria such as *Salmonella* and *E. coli* are zoonotic and this raises the possibility of infecting humans via food of animal origin or contaminated water (Pathak and Gopal, 2008; van den Bogaard et al., 2001).

E. coli is a commensal bacterium of the intestinal flora of humans and animals but it can also be an important pathogen that produces a very diverse type of clinical disease from diarrhoea to septicaemia, meningitis and infections in the urinary tract in humans (Nataro and Kaper, 1998; Smith et al., 2007). The difference between commensal and pathogenic *E. coli* is based on the acquisition of virulence genes (Sousa, 2006). Like antibiotic resistance genes, virulence genes can be acquired via plasmids, bacteriophages and transposons, and chromosal pathogenicity islands reflect past acquisition of collections of virulence genes via these routes or transduction (Donnenberg and Whittam, 2001). The products of these genes

can harm the host animal(Skyberg et al., 2006), although it is not clear that pathogenicity is the primary or selected function of all these genes.

As virulence and antibiotic resistance genes use similar vehicles of transference between bacteria, both type of genes can be transmitted together, on the same mobile elements (Barza, 2002; Boerlin et al., 2005; Chapman et al., 2006; Johnson et al., 2003; Travis et al., 2006).

Little is known about the occurrence, distribution and spread of *E.coli* carrying antibiotic resistance and virulence genes in wildlife populations, although pathogenic *E. coli* that carry antibiotic resistance can exist in domestic animals and the environment (da Costa et al., 2008; Hamelin et al., 2007; Sayah et al., 2005), and wildlife commensal bacteria, including *E. coli*, are often resistant to a range of antibiotics. (Costa et al., 2008; Gilliver M, 1999)Indeed, resistant isolates have been detected from certain wildlife hosts and not others sharing the same habitat, suggesting possible host association (Hughes, 2007; Lemus et al., 2008; Mallon et al., 2002). Such host association might be due to dietary habits, with some wild animals coming into contact with resistant bacterial strains via food (Dolejska et al., 2007; Lemus et al., 2008). Furthermore, some studies have shown that wildlife can carry a higher prevalence of antibiotic resistance in areas close to human populations than in more man isolated areas (Osterblad et al., 2001; Rolland et al., 1985; Routman et al., 1985).

Most research has compared clinical and commensal isolates using techniques such as multiplex PCR that can only detect a small number of genes. Novel techniques such as microarrays allow detection of a high number of virulence and antibiotic resistance genes that could be carried by individual *E. coli* strains. This technique is easy to perform and provides quick results (Anjum et al., 2007; Batchelor et al., 2008).

This chapter describes the application of a microarray to *E. coli* from healthy catlle, wild mammals and birds on six cattle farms in order to determine and compare their virulence and antibiotic resistance genes.

The aims of this study were to determine if the samples carried antibiotic-resistance and virulence genes, if so, which genes and how frequently they were present in these samples. These data were then used to investigate possible associations between virulence and antibiotic genes, host and site associations, or possible shared gene profiles that might indicate cross species transmission.

6.2 Materials and methods

Two hundred individual *E. coli* colonies from faecal samples of domestic cattle and a variety of wildlife animals as part of the cross-sectional study (Chapter 3), were tested both the microarray for 45 different *E. coli* virulence genes (Chapter 5) and a further microarray for 47 antibiotic resistance genes (Batchelor et al., 2008). Appendix V-Chapter 5 and Appendix I-Chapter 6 describe the primers, probes and control genes also included in the arrays.

Individual *E. coli* colonies, previously identified morphologically on EMBA as described in Chapter 2, were plated onto individual nutrient agar plates and incubated at 37 °C for 24 hours. Afterwards, one loopfull (approx. 10µl) of bacterial growth per plate was mixed with 400µl of lysis buffer (proteinaseK and PBS). The lysate was incubated in a water bath at 60°C for 2 hours and boiled at 95°C for 15 minutes, and finally centrifuged at 13000 rpm for 5 minutes.

Both array methods followed the method described previously by Ballmer *et* al, Anjum *et* al and Batchelor *et* al and have been described in detail in Chapter 5.

Due to the high complexity and the amount of information per sample, only results for ten most prevalent antibiotic resistance genes and one associated gene intl1 were included in the

comparison with virulence genes. These ten antibiotic resistance and associated with resistance genes were: *sul1*, *sul3*, *tetA*, *tetB*, *intl1*, *catA1*, *dfr12*, *drfA14*, *aadA1* and *blaTEM1 (tem1)*. The *intl1* gene is a conserved region of an integron and encodes for an integrase. Integrons can integrate and express antimicrobial resistance genes. (Appendix I-Chapter 6).

Statistical analysis was performed using Excel, Stata and R. Uni-variable analysis using 2×2 tables and Chi^2 tests was conducted in Stata 8.1 and SPSS. Clustering analysis was as described in Chapter 5.

6.3 Results

Two hundred *E. coli* isolates were tested to detect virulence and antibiotic resistance genes. Of those samples, 41 (20.5%) were considered invalid owing to contamination, and were not included in the analysis. Of the remaining 149 isolates, cluster O* four isolates that did not contain any virulence nor antibiotic resistance genes detected by the microarrays (Figure 1), these isolates were from three unidentified birds from BHF and a song thrush from MF. A further 35 (22%) isolates did not contain any of the 45 virulence genes but carried antibiotic resistance genes, and 120 (75%) isolates contained both virulence and antibiotic resistance genes.

Isolates that only carried antibiotic resistance

Isolates (n=35) that contained only antibiotic resistance genes were mainly from cattle (48.5%), badgers (14%) and wood mice (8.5%). At a farm level, 31% of the isolates were from BHF, 20% BGF followed by 17% CLF, PHF and 14% MF. No such isolates were isolated from GF (Table 1).

A dendrogram to identify possible clusters of these profiles was plotted (Figure 1). Four main clusters were identified. Cluster A comprised 12 isolates (34%) that carried the *tem1 and tetB* genes. Cluster A isolates were from six cattle (50%, n=12) (two calves MF, one calf BGF,

one adult BHF, one adult BGF and one dry cow BGF) and from a house sparrow, a badger MF, a wood mouse BHF and two foxes, the animals being sampled from five of the six farms. Cluster B comprised three isolates with the gene profile *aadA1-catA1-tetB-sul3*. All three isolates were from one farm (CLF), from two dry cows and an unidentified corvid. Cluster C comprised just two isolates with the profile *tem1-tetA-tetB*, and Cluster D also comprised just two isolates containing *tem1*, from a wood mouse and a calf from different farms.

The *tem1* gene was the most prevalent gene, being carried by 30 of the samples (85.7%). This was followed by *tetB* gene carried by 23 samples (65.7%) and *aadA1* gene carried by 10 samples (28.6%).

BGF, MF and PHF were the farms with the least number of antibiotic resistance genes amongst their animal hosts (2-3 genes), except for five genes carried by a wood mouse on MF and four genes carried by a house mouse at PHF. A domestic dog carried a very similar antibiotic resistance gene pattern (*tem1-tetB-intl1-sul1-dfr12*) to a lactating cow (*tem1-tetB-intl1-sul1-catA1* genes) at BHF.

Figure 1. Dendrogram comparing the antibiotic resistance gene profiles of isolates that did not contain any of the 45 virulence genes tested.

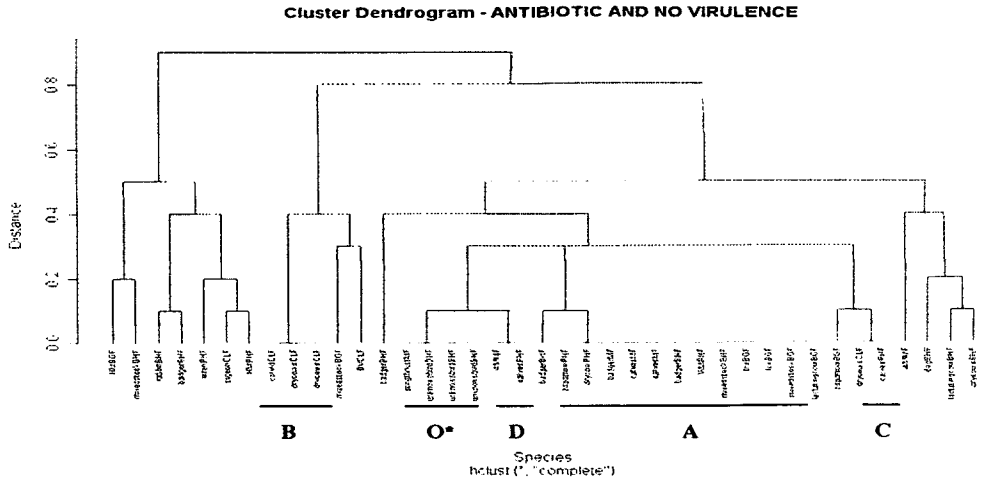

Cluster Dendrogram - ANTIBIOTIC AND NO VIRULENCE
Distance
B
O*
D
A
C
Species
hclust (*, "complete")

Table 1. Antibiotic resistance and associated gene profiles of the 35 isolates without virulence genes by host and location.

Location	Host	*teml*	*aadA*	*tetA*	*tetB*	*dfrA*	*Intl1*	*sul1*	*catA1*	*dfr12*	*sul3*
BGF	House mouse	+	+	+				+			
BGF	House sparrow	+		+							
BGF	Fox	+			+						
BGF	Fox	+			+						
BGF	Lactating cow	+			+						
BGF	Adultstock	+			+						
BGF	Adultstock		+	+			+				+
BHF	Rabbit	+	+	+		+	+	+			
BHF	Badger	+	+	+		+	+	+		+	
BHF	Adultstock	+		+				+	+		
BHF	Badger	+			+						
BHF	Adultstock	+			+						
BHF	Wood mouse	+			+						
BHF	Dry cow	+			+		+	+			
BHF	Lactating cow	+			+		+	+	+		
BHF	Dog	+			+		+	+		+	
BHF	Badger	+			+				+		
BHF	Badger	+					+			+	
CLF	Pigeon	+	+			+		+	+		
CLF	Dry cow	+		+	+						
CLF	Corvid		+		+				+		+
CLF	Dry cow		+		+				+		+
CLF	Dry cow		+		+				+		+
CLF	Bank vole			+					+		+
MF	Wood mouse	+									
MF	Wood mouse	+	+		+	+	+				
MF	Badger	+			+						
MF	Calf	+			+						
MF	Calf	+			+						
PHF	Calf	+									
PHF	House mouse	+	+			+		+			
PHF	Calf	+		+	+						
PHF	Dry cow	+			+						
PHF	House sparrow	+			+						
PHF	Wren	+			+			+			

Isolates that carried virulence and antibiotic resistance genes

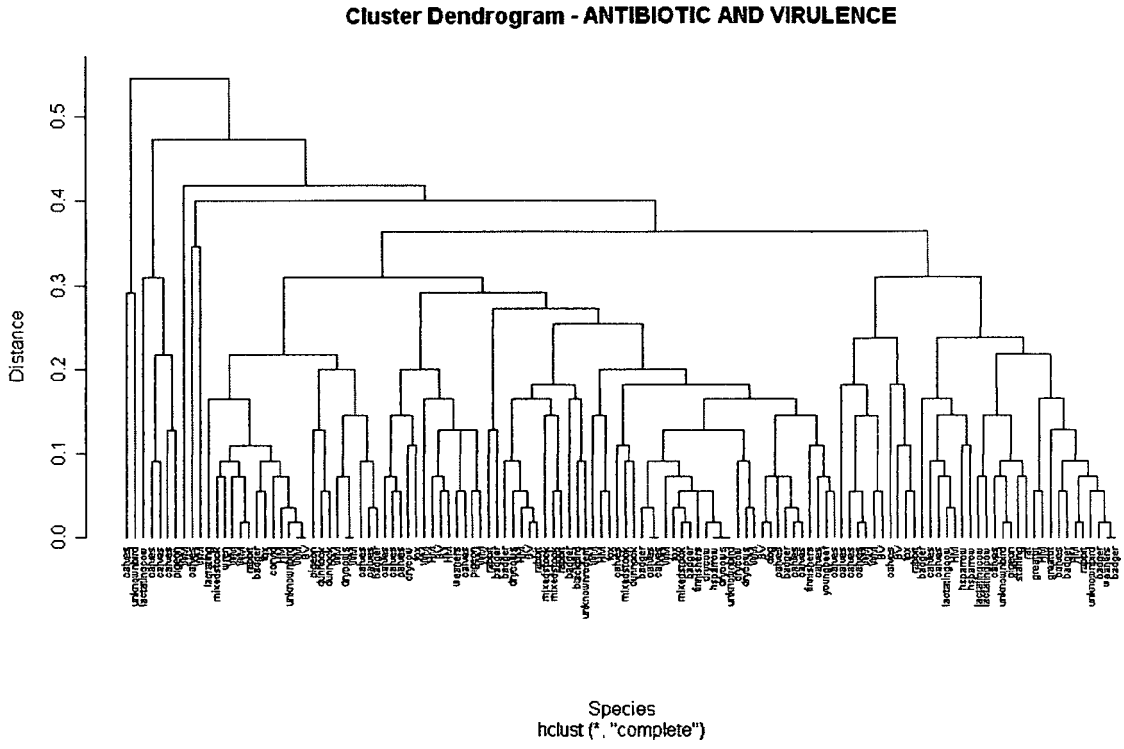
In total, 120 *E. coli* isolates contained both virulence and antibiotic resistance genes. These were found in samples from domestic cattle (39%), large wild mammals (20%), wild rodents (18%), wild birds (15.8 %) and a farm dog (0.8%) (Table 2). BHF accounted for the highest proportion of such isolates (23.3%) and BGF for the lowest number (5.8%) (Table 2).

Table 2. Isolates that carried virulence and antibiotic resistance genes by host and individual farm.

Farm	No isolates (%)	Hosts
BHF	28 (23.3%)	7 house mice, 6 badgers, 1 rabbit, 1 dog, 1 no ID bird, 12 cattle (3 calves, 2 young stock, 1 dry cow, 2 lactating cows, 3 adult stock)
PHF	27 (22.5%)	8 wood mice, 1 bank vole, 3 badgers,1 fox,1 rabbit, 2 dunnocks, 1 house sparrow, 1 starling, 9 cattle (4 calves, 2 lactating cows, 2 dry cows, 1 adult stock)
MF	25 (20.8%)	3 badgers, 2 rabbits, 1 rat, 2wood mice, 2pigeons, 3 noID bird, 1wren, 11 cattle (7 calves, 1 young stock, 1 lactating cow, 1 adult stock)
CLF	22 (18.3%)	1 corvid, 1 dunnock, 2 pigeons, 1 black bird, 2 rabbits, 3 wood mice, 4 bank voles, 1 noID rodent,7 cattle (6 calves, 1 dry cow)
GF	11 (9.2%)	3 foxes, 2 great tit, 6 calves
BGF	7 (5.8%)	2 foxes, 1 house mouse, 1 bank vole,1 house sparrow, 2 cattle (adult stock, 1calf)

The gene profiles of these isolates were again analysed for clustering, and the resultant dendrogram is shown in Figure 2. There were four identical profiles found in more than one isolate. A wood mouse from PHF and bank vole from CLF both provided isolates with the profile *iss-astA-tem1-aadA1-tetB-dfrA-Intl1-sul1-ctaA1* genes; two calf isolates from PHF had the profile *iss-astA-celb10-mchB-mchC-mchF-tetB*; a house sparrow, a dry cow from PHF and an unidentified bird from BGF provided isolates with the profile *f17A6-tem1-aadA-tetA* genes; and a badger and a calf isolates from BHF with the profile *iron-iss-mchF-tem1-aadA1-tetB-dfrA-Intl1*. Otherwise, each isolate had a unique profile.

Figure 2. Dendrogram of profiles of virulence, antibiotic and associated genes in *E. coli* isolates from a variety of cattle and wildlife on six farms.



The number of antibiotic resistance genes found in these isolates varied between one and seven, with the following frequency (n=120); 1 gene (9%), 2 genes (29%), 3 genes (10%); 4 genes (13%); 5 genes (17%), 6 genes (12%) and 7 genes (10%). The mean was three genes, although 52 % of the isolates carried four genes or more. The most frequent antibiotic gene found was *tem1* carried by 98 isolates (82.5%), followed by *aadA* 61 isolates (51%), and *tetA* 54 isolates (45%). These proportions were different from samples that only carried antibiotic resistance as *tem1* and *tet B* were the most frequently carried genes. The frequency in which different antibiotic genes appeared together is shown in Table 3. Some genes, such as *tem 1*, *aadA1* and *dfrA1* were seldom carried alone, whereas others, such as *tetA* and *tetB* were seldom found in the same isolate as other resistance genes.

Table 3. Frequency in which the 10 different antibiotic resistance genes are carried together

Gene(n)*	*teml*	*aadA1*	*tetA*	*tetB*	*Intl1*	*sul1*	*catA1*	*dfr12*	*sul3*	*dfrA*
***teml*(99)**		**49**	**46**	**26**	**38**	**28**	22	15	9	**37**
***aadA*(61)**			**31**	16	**34**	**30**	23	7	10	**38**
***tetA* (54)**				1	25	19	19	7	5	26
***tetB* (36)**					12	7	7	5	6	13
***Intl1*(42)**						23	15	11	4	27
***sul1*(34)**							20	8	0	21
***catA1*(28)**								0	5	17
***Drf12*(20)**									0	7
***sul3* (14)**										2
***drfA14*(43)**										

*n= number of samples that carried that particular antibiotic resistance gene

These isolates contained different numbers of virulence genes with the following frequencies; 1 gene (15%), 2 genes (14%), 3 genes (21.6%), 4 genes (16%), 5 genes (10%), 6 genes (8.3%), 7 genes (6%), 8 genes (1.7%), more than 8 genes (0.8%). The median number of genes found was four genes and 75% of isolates carried between one and five genes. The *iss* gene was the most prevalent gene (63.3%) followed by *iroN* (33%), *f17A60* and *astA* (25.8%), *mchF* and *mcmA* (20.8%). The *fasA*, *stb* and *pet20* genes were not found in these samples (Figure 3).

Figure 3. Virulence gene carriage amongst the 120 isolates also containing antibiotic resistance genes

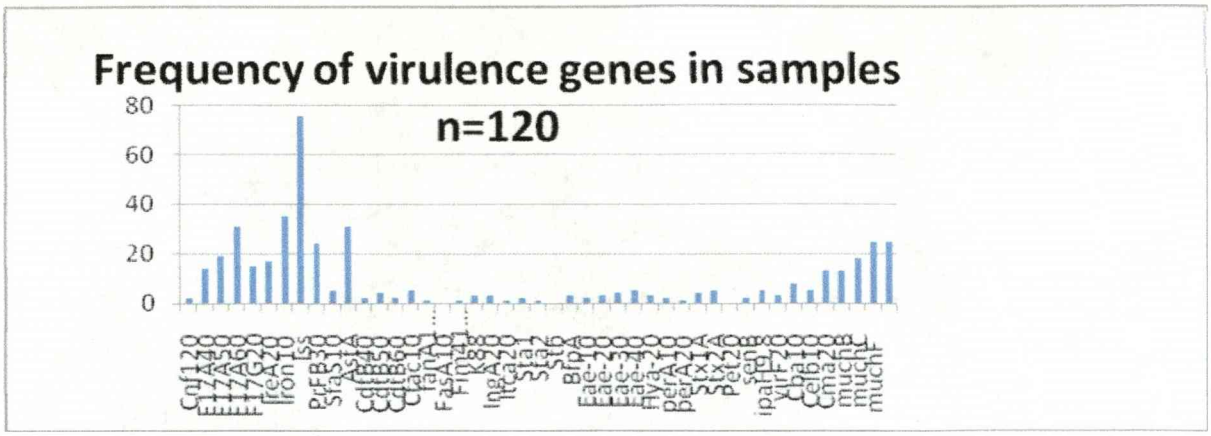


Table 4. The frequencies which different combinations of virulence and antibiotic resistance genes were found in 120 isolates.

Genes	*tem1*	*aadA1*	*tetA*	*tetB*	*Intl1*	*sul1*	*catA1*	*dfr12*	*sul3*	*dfrA*
cnf1-2	1	1	1	1	1	2	1	1	0	1
f17A40	10	5	7	3	3	4	1	5	1	1
f17A50	**15**	**6**	**10**	**7**	**8**	**5**	**5**	**4**	**2**	**7**
f17A60	**25**	**13**	**18**	**8**	**10**	**6**	**7**	**3**	**3**	**8**
f17G20	**12**	**9**	**8**	**5**	**10**	**5**	**4**	**8**	**3**	**10**
ireA20	**15**	**9**	**7**	**4**	**4**	**6**	**6**	**2**	**0**	**7**
Iron10	**38**	**23**	**22**	**8**	**17**	**16**	**10**	**7**	**2**	**22**
iss	**67**	**41**	**33**	**27**	**32**	**28**	**21**	**16**	**6**	**32**
prfB30	**17**	**10**	**6**	**8**	**8**	**7**	**5**	**6**	**1**	**6**
Sfas10	4	2	3	2	0	2	1	0	0	1
astA	**29**	**10**	**8**	**14**	**7**	**9**	**8**	**7**	**3**	**4**
cdtB40	2	0	3	0	1	1	2	0	0	1
cdtB50	3	0	4	0	0	1	1	1	0	2
cdtB60	2	1	2	0	1	1	0	1	0	0
Cfa-c10	5	2	4	1	2	2	1	0	0	1
fanA	1	0	1	0	0	0	0	0	0	0
fim41a	1	0	0	1	0	0	0	0	0	0
K88	3	0	3	0	1	1	1	0	0	0
ingA20	4	2	2	3	1	0	0	1	0	2
itcA20	1	1	0	1	1	0	0	0	1	0
Sta1	2	1	2	0	1	0	0	0	0	1
Sta2	1	0	0	0	0	0	0	1	0	0
bfpA	3	0	1	2	1	0	1	0	0	0
eae10	2	1	2	0	1	1	1	0	1	0
eae20	3	1	2	1	1	1	0	0	1	0
eae30	4	1	2	2	2	2	1	0	0	0
eae40	5	1	3	2	2	1	1	0	1	1
hlyA-20	3	1	2	1	1	1	1	0	0	0
perA-10	2	0	1	1	1	1	1	0	0	0
perA-20	1	0	1	0	0	0	0	0	0	0
vt1	4	2	3	1	2	2	1	1	1	1

vt2	5	2	5	0	2	2	0	0	0	1
senB	2	1	2	0	1	0	0	0	0	1
ipaH9.8	3	0	1	4	0	0	0	0	0	0
virF-20	2	0	0	2	0	0	1	0	1	0
cba-10	8	3	5	3	2	2	1	0	0	1
celb-10	6	3	3	4	2	0	2	3	3	2
cma-10	**13**	**7**	**12**	**2**	**6**	**3**	**1**	**3**	**1**	**6**
mchB	8	1	1	7	1	0	1	2	2	2
mchC	11	2	5	7	2	2	2	1	2	2
mchF	**24**	**16**	**14**	**12**	**10**	**9**	**9**	**6**	**3**	**15**
mcmA	**23**	**14**	**7**	**8**	**9**	**10**	**8**	**5**	**2**	**10**

Antibiotic resistance and virulence genes carriage at the farm level

There was a significant association between antibiotic resistance genes carried and farms, indicating that particular combination/s or carriage of particular genes were related to particular farms ($p < 0.0001$). In addition, the carriage of particular virulence genes was significantly associated with farms ($p < 0.0001$), hosts ($p=0.021$) and antibiotic resistance genes carriage ($p<0.0001$).

However, when virulence and antibiotic resistance gene carriage was considered as a combined profile, farm ($p=0.181$) and animal host ($p=0.145$) were not significantly associated. This suggests that virulence and antibiotic resistance genes act independently as variables, although selection of isolates and size may have been a problem with this analysis.

The carriage of antibiotic resistance and virulence genes by farm was as follows.

BGF

Every sample (n=7) from BGF carried the *tem1* gene and five isolates carried the common profile of *tem1-aadA1-tetA-dfrA14-intl1*genes (Table 5). These five *E. coli* isolates were from a calf, fox, house sparrow and a house mouse. Four of those five isolates also carried *sul1*, *catA1* and *sul3* genes. (Table 5).

Table 5. Antibiotic resistance and virulence genes carried by *E. coli* isolates from BGF (n=7)

Host	**Virulence genes**	**Antibiotic resistance genes**
Bank vole	*f17A6,iroN,iss,astA,mchB,mchC,mchF*	*tem1*
Adult cattle	*cdtB40*	*tem1,aadA1*
Calf	*iroN,iss ,mcmA*	***tem1,aadA1,tetA,dfrA,intl1***
House sparrow	*f17A5,f17G2,ireA2,iroN,cba10,cma2, mchF ,mcmA*	***tem1,aadA1,tetA,dfrA,intl1****,sul1,catA1*
House sparrow	*f17A4,f17G2,iroN,iss,prfB3,vt1,mchF ,mcmA*	***tem1,aad,tetA,dfrA,intl1****,sul1,drf12*
House mouse	*f17A5,f17G2,iroN,iss,prfB3,mchF ,mcmA*	***tem1,aadA1,tetA,drfA,intl1****,sul1,catA1*
Fox	*celb10*	***tem1,aadA1,tetA,dfrA14,intl1****,sul3*

GF

Every isolate containing an antibiotic resistance and virulence gene from GF (n=11) carried the *tem1* gene. The frequency for other antibiotic resistance genes was *tetB* (45%) followed by *tetA*, *drfA* and *drf12* (36%). There were five profiles of genes carried amongst these isolates. There was a cluster of 5 calf isolates that carried the *tem1-tetB* genes and two of those calves also carried almost identical virulence genes (Table 6)

The number of samples from different hosts in GF seemed to be very limited (only 3 different animal hosts) and the antibiotic resistance genes in wildlife and domestic cattle isolates seemed to be independent from each other. Wildlife carried a higher number of antibiotic resistance genes than domestic cattle.

Table 6. Antibiotic resistance and virulence genes carried by *E. coli* isolates from GF (n=11)

Host	Virulence genes	Antibiotic resistance genes
Fox	*astA,mchB,mchC,mchF ,mcmA*	*tem1*
Fox	*astA,cdtB50*	*tem1,tetA,dfrA,dfr12*
Fox	*celb10*	*tem1,tetA,intl1,dfr12*
Great tit	*f17A5,f17A6,f17G2,iroN,iss,mchF*	*tem1,tetA,dfrA,dfr12*
Great tit	*f17A5,f17A6,f17G2,iroN,iss,mchF*	*tem1,tetA,dfrA,intl1,dfr12*
Calf	*f17A5,f17A6,iroN,iss,fim41a,inga20,bfpA,ipaH9.8, virF,cba10,mchB.mchC,mcmA*	*tem1,tetB*
Calf	*f17A6,iroN,iss,inga20,bfpA,ipaH9.8,virF, cba10,mchB.mchC,mcmA*	*tem1,tetB*
Calf	*ireA,prfB30,mcmA*	*tem1,tetB*
Calf	*ireA,iroN,iss,astA,cfac10,mchB.mchC,mchF,mcmA*	*tem1,tetB*
Calf	*iss,prfB30,mcmA*	*tem1,tetB*
Calf	*f17A6*	*tem1,aadA1*

BHF

Each of the isolates (n=28) from BHF carried the *tem1* gene, followed by *aadA1* (86%), *dfrA*, *catA1* and *intl1* (46%), *tetA* and *sul1* (36%), and *tetB* (32%). Twenty four isolates (86%) carried *tem1-aadA1-* these two genes being the most prevalent amongst samples from BHF. There was a cluster of isolates from a badger and a bovine animal that carried *iron-iss-mchF-tem1-aadA1-tetB-dfrA-intl1* (Table 7).

Table 7. Antibiotic resistance and virulence genes carried by *E. coli* isolates from BHF (n=28)

Host	Virulence genes	Antibiotic resistance genes
House mouse	*cnf12,iss,astA*	*tem1,aadA1,tetB,dfrA,intl1,sul1,dfr12*
Dog	*ireA,iss,prfB30,,mcmA*	*tem1,aadA1,catA1*
Adult cattle	*k88,celb10*	*tem1,aadA1,catA1*
House mouse	*ireA,iroN,iss,prfB30,astA*	*tem1,aad,A1,dfrA,sul1,catA1*
Young acattle	*ireA,iroN,iss,prfB30,astA,mcmA*	*tem1,aadA1,dfrA,sul1,catA1*
Lactating cow	*ireA,iroN,iss,prfB30,mchF,mcmA*	*tem1,aad,A1,dfrA,sul1,catA1*
Calf	*iss,prfB30,sfas10,mcmA*	*tem1,aad,A1,dfrA,sul1,catA1*
House mouse	*iroN,iss,prfB30,mcmA*	*tem1,aadA1,dfrA,intl1,sul1*

Badger	*iroN,iss,prfB30,mchF,mcmA*	*tem1,aadA1,dfrA,intl1,sul1,catA1*
Calf	*ireA,iss,prfB30,astA,mcmA*	*tem1,aadA1,intl1,sul1,dfr12*
noIDbird	*f17A6*	*tem1,aadA1,tetA*
House mouse	*iroN,iss ,mcF*	*tem1,aadA1,tetA,dfrA,catA1*
Rabbit	*iroN,iss*	*tem1,aadA1,tetA,dfrA,intl1,sul1,catA1*
Dry cow	*iroN,iss ,cma20*	*tem1,aadA1,tetA,dfrA,intl1,sul1,dfr12*
House mouse	*iroN,iss ,mcF*	*tem1,aadA1,tetA,intl1,sul3*
Badger	*iroN,iss ,mcF*	*tem1,aadA1,tetA,sul1*
Young cattle	*iroN,iss ,mcF*	*tem1,aadA1,tetB,dfrA,intl1*
Badger	*iroN,iss ,mcF*	*tem1,aadA1,tetB,dfrA ,intl1*
House mouse	*iss,astA*	*tem1,aadA1,tetB,dfrA,intl1,sul1,catA1*
Badger	*f17A6,iss*	*tem1,aadA1,tetB,intl1,catA1,sul3*
Dry cow	*f17A6,iss,astA,mcmA*	*tem1,aadA1,tetB,intl1,catA1,sul3*
Adult cattle	*iss,Sas10,eae3,cma2*	*tem1,aadA1,tetB,intl1,sul3*
House mouse	*prfB30,itcA,mcmA*	*tem1,aadA1,tetB,intl1,sul3*
Badger	*iss,cma20*	*tem1,aadA1,tetB,intl1,sul1,dfr12*
Calf	*f17A4,f17A5,f17A6,iss,prfB30,sfas, astA,cdtb40, cdtb 50,cdtb60, k88,inga20,senB,ipaH9.8,cba10, mchC*	*tem1,tetA*
Badger	*fanA,eae4,ipaH9.8,mchC*	*tem1,tetA*
Adult cattle	*iss,vt2,cba10,cma2*	*tem1,tetA*

CLF

Every isolate from CLF (n=22) carried the *tem1* gene (95%), and the frequency of carriage of other antibiotic resistance genes by those isolates was *tetA* (86%), *dfrA* (36%), *aadA1* and *sul1* (32%), *catIA1* (27%). There were even clusters in terms of antibiotic resistance gene carriage; 9 isolates (41%) carried *tem1-tetA* (Table 8).

Isolates from a house mouse, a young bovine and a lactating cow possessed very similar antibiotic and virulence gene profiles, *ireA-iroN-iss-prfB30-(astA,mchF,mcmA)-tem1-aadA1-dfrA14-sul1-catA1*.

Table 8. Antibiotic resistance and virulence genes carried by *E. coli* isolates from CLF (n=22).

Host	Virulence genes	Antibiotic resistance genes
Bank vole	*ireA*	*tem1,aadA1,tetA,dfrA*
Wood mouse	*ireA*	*tem1,aadA1,tetA,dfrA,intl1*
noID rodent	*iroN,iss ,mcF*	*tem1,aadA1,tetA,dfrA,cat1A*
Calf	*f17A6,iroN,iss,prfB30*	*tem1,aadA1,tetA,dfrA,intl1*
Bank vole	*f17A6,iroN,iss*	*tem1,aadA1,tetA,dfrA,intl1,sul1,*
Claf	*f17A6,iroN,iss*	*tem1,aadA1,tetA,intl1,sul3*
Bank vole	*iss,astA*	*tem1,aad,tetB,dfrA,intl1,sul1,cat1A*
Calf	*f17A6,iroN,iss*	*tem1,tetA*
Dry cow	*f17A6,astA*	*tem1,tetA*
Rabbitt	*f17A6,ireA*	*tem1,tetA*
Wood mouse	*f17A6,iss,astA,cma2*	*tem1,tetA*
Calf	*ireA,iron,iss*	*tem1,tetA*
Black bird	*iroN,iss,cba10,cma2,mchF*	*tem1,tetA*
Calf	*iroN,iss,prfB30,astA,cma2,mchF,mcmA*	*tem1,tetA*
Rabbit	*iss,cba10,cma2*	*tem1,tetA*
Corvid	*astA*	*tem1,tetA*
Pigeon	*F17A6,,iron,iss,muchF*	*tem1,tetA,dfrA,cat1A*
Bank vole	*astAeae1,eae2,eae 3, eae4,hlyA,vt1*	*tem1,tetA,intl1,sul3,cat1A*
Wood mouse	*f17A4,f17A5,astA,cdtb40,cfac10,k88,bfpA, eae1,perA10,vt2,mchC*	*tem1,tetA,intl1,sul1,cat1A*
Bank vole	*f17A6,ireA,iss,prfB30*	*tem1,tetA,intl1,sul1,cat1A*
Pigeon	*AstA*	*tem1,aadA1,dfrA,sul1,catA1*
Dunnock	*AstA*	*tetB,catA1,sul3*

MF

The frequency of antibiotic resistance genes and associated with antimicrobial resistance genes carried by isolates positive for antibiotic and virulence genes from MF was: *tem1* (100%), *tetB* (36%), *dfrv* (28%), *intl1*, *tetA* and *aadA* (24%). The *catA1* gene was not carried by any of the isolates. Genes were carried by these samples in 12 different profiles. The most frequent profile was *tem1-tetB-(dfrA)* carried by nine isolates (36%, n=25) from cattle, wild birds, small rodents and a rabbit (Table 9).

Table 9. Antibiotic resistance and virulence genes carried by *E. coli* isolates from MF (n=25)

Host	Virulence genes	Antibiotic resistance genes
Badger	*astA*	*em1*
Wren	*iss*	*em1,aadA1*
Wood mouse	*f17A40,iroN,iss,cfac10,ingA,sta1,eae4,vt2 ,senB,cba10,cma2*	*em1,aadA1,tetA,dfrA,intl1*
Calf	*iss,cba10,cma2,mchB.mchC,mchF,mcmA*	*em1,aadA1,tetA,dfrA,intl1*
Badger	*roN,iss ,cma2*	*em1,aadA1,tetA,drfA,intl,sul1,df1 2*
Lactating cow	*f17A40,f17A5,f17A6,iss,sfas10,astA,cfac10 eae1, eae 2, eae3 eae,4,hlyA,vt1,vt2, cba10,celb10,mchC*	*em1,aadA1,tetA,sul1*
Rat	*roN,iss ,mucF*	*em1,aadA1,tetA,sul1*
Young cattle	*f17A5,f17G2*	*em1,dfrA,sul3*
Adult cattle	*f17G2,,iss,prfB30,astA,fim41a,mcmA*	*em1,dfr12*
Badger	*roN,iss, astA,celb10,mchB*	*em1,dfr12*
Pigeon	*f17A40,f17A5,f17A6,sta2*	*em1,dfr12*
Young cattle	*f17A5,f17G2,iss,prfB30,mchF*	*em1,intl1,dfr12*
Adult cattle	*f17A40,f17G2*	*em1,intl1,dfr12*
Calf	*f17A40,f17G2 ,iss,prfB30*	*em1,intl1,dfr12*
Rabbit	*roN,iss,mchB.mchC,mchF,mcmA*	*em1,sul3*
hoID bird	*f17A40,f17A5,F17A6,LiroN,iss,prfB30, sfas10,astA,cdtB50,cdtB60,cfa-c10 k88,sta1,perA20,cdba10,celb10, cma-2,mchC*	*em1,tetA*
Calf	*astA,eae2 eae,3, eae 4,hlyA,vt1,cbA10*	*em1,tetB*
Pigeon	*eae1, eae 3, eae 4,perA10*	*em1,tetB*
Wood mouse	*f17A5,f17G2,iss, astA*	*em1,tetB*
Calf	*f17A5,f17A6,iss,prfB30*	*em1,tetB*
hoID bird	*f17A6, iss, astA*	*em1,tetB*
hoID bird	*f17A6,iroN,iss,mchF*	*em1,tetB*
Calf	*reA,iss,prfB30*	*em1,tetB*
Rabbit	*roN,iss,cdtB5,mchF*	*em1,tetB*
Calf	*f17A5,f17G2*	*em1,tetB,dfrA*

PHF

The antibiotic resistance and associated gene frequency amongst the *E. coli* isolates from PHF was *aadA1* (63%), *tetB* (48%), *dfrA* (44%), *tetA* (41%), and *catA1*(33%). The proportion of

samples from PHF that carried the *tem1* gene was smaller (22%) than on the other five farms (100%). In addition, isolates from PHF carried a more heterogeneous combination of gene, (17 profiles) compared to the other farms. There were two profiles found in more than one animal: isolates from a dry cow and a house sparrow that carried *f17A6-tem1-aadA-tetA* and two calves that carried *iss-asta-celb10-mch-mchC-mchF-tetB* (Table 10).

Virulence genes found most frequently were *iss* (44%), *mchF* (33%), *f17A60* and *f17G20* (26%). The frequency of the *iroN* gene (7%) was lower compared to on the other five farms (32%-71%) (Table 10).

Table 10. Antibiotic resistance and virulence genes carried by *E. coli* isolates from PHF (n=27)

Host	Virulence genes	Antibiotic resistance genes
Wood mouse	*f17A5,iss,astA,celb10,mchB.mchC, mchF*	*tetB,dfr12*
Wood mouse	*f17A4,f17A6,f17G20, iss*	*aadA1,intl1,sul1,dfr12*
Dunnock	*f17A5,f17A6,*	*aadA1,tetA,dfrA,intl1,sul1,cat1A*
Wood mouse	*f17G20*	*aadA1,tetA,dfrA,intl1,sul1,cat1A*
Wood mouse	*f17G20*	*aadA1,tetA,dfrA,intl1,sul1*
Badger	*f17A5,F17A6, iss*	*aadA1,tetA,intl1,cat1A*
Rabbit	*sfas,mchC*	*aadA1,tetA,intl1,sul3*
Fox	*prfB30,mcmA*	*aadA1,tetA,sul1,cat1A*
Dry cow	*f17G20*	*aadA1,tetA,tetB,sul3*
Starling	*f17G2,iroN,iss,cma2,mchF*	*aadA1,tetB,dfrA*
Calf	*ireA,prfB30*	*aadA1,tetB,dfrA*
Badger	*iroN, iss,prfB3,ingA,cma20,mchF,mcmA*	*aadA1,tetB,dfrA,dfr12*
Dunnock	*f17A4,f17A5,f17A6,f17G20, sfas10*	*aadA1,tetB,dfrA,sul3*
Adult cattle	*virF2*	*cat1A,sul3*
Bank vole	*iss,celb10,mchB.mchC,mchF*	*dfrA*
Lactating cow	*K88,celb10*	*tem1,aadA1,sul3*
Dry cow	*f17A6*	*tem1,aadA1,tetA*
House sparrow	*f17A6*	*tem1,aadA1,tetA*
Wood mouse	*iss,astA*	*tem1,aadA1,tetB,dfrA,intl1,sul1,cat1A*
Wood mouse	*iss,astA*	*tem1,aadA1,tetB,dfrA,intl1,sul1,dfr12*
Calf	*ireA,prfB3,mcmA*	*tem1,tetA,drf12*
Lactating cow	*cnf1,f17A6,f17G2,iss,cbtB4,cbtB5*	*tetA,dfrA,sul1,cat1A*
Calf	*iss,asta,celb10,mchB.mchC,mchF*	*tetB*
Calf	*iss,asta,celb10,mchB.mchC,mchF*	*tetB*
Badger	*f17A4,prfB30,sfas10,ipaH9.8,mchF*	*tetB*
Wood mouse	*f17A4,prfB3,ipaH9.8,mchF*	*tetB*
Wood mouse	*iss,asta,celb10,mchB.mchC,mchF*	*tetB,cat1A,sul3*

6.4 Discussion

The relationship between virulence and antibiotic resistance at a genetic level in *E. coli* from diverse domestic and wildlife hosts in close geographical proximity is not known. This study shows many *E. coli* isolates, 75% (n=210) from domestic cattle and a variety of wildlife hosts carried multiple antibiotic resistance genes (four genes median) together with multiple virulence genes (four genes median). There was statistical evidence that patterns of virulence and antibiotic genes tended to be more similar within farms and to differ between hosts. Occasional clusters of identical isolates carrying the same antibiotic resistance and virulence genes were observed in different animal species, for example a house sparrow and a cow on the same farm carried the same virulence and antibiotic resistance patterns. Clusters of isolates with the same antibiotic resistance, but without virulence genes, were also detected. This suggests that inter-species transmission may be possible, although the frequency seems low. This could be associated with certain *E. coli* isolates present in a close geographical area. Alternatively, the same profiles could appear by chance.

Only four samples did not carry virulence or antibiotic resistance genes, and all four samples came from wild birds (one song thrush and three unidentified birds), however 19 (11.9%, n=159) samples from wild bird carried antibiotic and virulence genes and 5 (14%, n=35) carried antibiotic resistance and no virulence genes. Most of the identified birds were pigeons, corvids, or small passerines. Antibiotic resistance carried by enteric bacteria isolated from wild birds has been described before and it has been suggested that may be associated with hosts with specific feeding habits (Dolejska et al., 2007; Lemus et al., 2008). If we assume that wild birds from this study had never received treatment with antibiotics, these birds could have been in contact with antibiotic resistance genes via contaminated environment, contaminated food (including bird feeders in gardens) or by direct contact with contaminated cattle, as could be the case of house sparrow and a domestic cow at PHF.

Wildlife hosts and domestic cattle presented similar patterns of antibiotic resistance and sometimes shared similar virulence genes on certain farms such as PHF, MF,CLF, BHF and BGF, suggesting cross-transmission of genes (if not isolates) between cattle and wildlife. However, on farm GF, each domestic and wild animal carried a completely different antibiotic resistance gene profile, suggesting that cross-species transmission of *E. coli* on this farm may be low and/or that gene transmission is rapid and dynamic leading to constantly changing gene profiles. A longitudinal study would be useful in order to study this further.

Amongst samples that only carried antibiotic resistance genes, similar pattern of genes were carried in cattle and house mice at PHF. These results are not surprising, as this farm had a house mouse infestation around the cattle and food storage buildings and a high number of these rodents would have been in contact with bovine carriers and/or faecal contaminated areas. It has previously been shown that wild rodents can carry enteric bacteria resistance to antibiotics (Mallon et al., 2002).

Isolates from a domestic dog and a lactating cow had a very similar antibiotic resistance gene pattern at BHF. This could represent a zoonotic risk for the farmer and farmer's family who tend to have a more close interaction with the pet than with the livestock. Pets can pose a risk for transmission of antibiotic resistance to humans as antibiotic resistant *E. coli* strains have been isolated previously from healthy pets and also have indistinguishable PFGE patterns with strains that produced urinary infections in humans (Costa et al., 2008; Johnson et al., 2008).

The antibiotic resistance genes most commonly detected in the *E. coli* isolates were *tem1* (beta lactams) and *aadA1* (aminoglycoside) followed by *tetA* (tetracycline), *tetB* (tetarcycline) and *dfrA* (trimethoprim). These antibiotics are commonly used for treatment of domestic livestock (OIE, 2007; VMD, 2007) and have been previously associated with

bacterial resistance in animals even a long time after use (Maynard et al., 2004).These results on antibiotic resistance genes amongst isolates were similar to those of Batchelor et *al.* (2008) when this microarray was validated (Batchelor et al., 2008). Maynard et *al.* (2004) observed that beta-lactam resistance was the most frequent drug resistance in ExPEC strains from humans and animals in Canada, this was followed by sulphonamide resistance by *sul1* and *sul3* which is not consistent with our results, and that could explain how specific antibiotic resistance patterns are driven by the antibiotics used or dispensed in geographical areas, within countries, specific treatments for different livestock or even different antibiotic used in different farms.

Previous studies have shown that wildlife can carry antibiotic resistance in areas where there was human use of antibiotics. Furthermore, the use of antibiotic contributes to the development of resistance in bacteria. This is a dynamic process driven through a selection determined by the type of antibiotic used within the host (Maynard et al., 2004; Rolland et al., 1985; Routman et al., 1985).

It is logical to think that the transmission of specific antibiotic resistance genes was from the domestic animals or contaminated environment with faecal contents e.g. manure spread on the farm pasture land as fertilizer to wildlife (never previously treated with antibiotics). Curiously on some of the farms such as GF, we observed that patterns of antibiotic resistance genes carried by wildlife were completely different to those carried by cattle, and also carried a higher number of resistance genes. This suggests that both groups have different pathways of exposure or contact with different antibiotics, or at least are subject to different selection pressures. The wildlife isolates from this farm were mainly foxes and great tits. Foxes can move long distances looking for food, they can eat all kinds of different things from a variety of sources, from human by-products to earthworms. Foxes could have been in contact with other *E. coli* strains carrying a complete different pattern of antibiotic resistance from other

sources. A similar situation could have happened to great tits, although they tend to move around the same area, they can move along close farms and urban areas (Chester is just 2-3 miles away). Great tits eat mainly insects, seeds and nuts (Mr Peter Coffey personal communication), but rarely feed from the ground – the most likely source of *E. coli* might be flying insects contaminated by faeces (Hume, 2007). There were two clusters of samples that carried an identical combination of virulence and antibiotic resistance genes in cattle and wildlife hosts. One cluster involved a bovine and a house sparrow from PHF farm and a non-identified bird from CLF farm: all three isolates had the profile *f17A6-tem1-aadA1-tetA*. House sparrows tend to be very territorial birds and do not move great distances. They are also largely found around buildings and are ground feeders. Dolejska *et* al (2008) in the Czech Republic, however, found that cattle and house sparrows on two farms carried different antibiotic resistance genes (Dolejska et al., 2008). The second cluster comprised an isolate from a badger and from a calf from BHF with the profile *iron-iss-mchF-tem1-tetB-dfrA-intI1*. Badgers tend to be very territorial animals, but can have large territories, and they eat a wide range of foodstuffs including small mammals, birds, earthworms and roots, and also cattle feed. They often have their latrines in close proximity to cattle. Thus if transmission between cattle and badger occurred it could have been in either direction.

The most common virulence genes among the isolates were *iss, iroN, astA, f17A60, mchF and mcmA,* and isolates that carried these genes also carried different antibiotic resistance gene profiles. There were differences in percentages of these genes by farm, for example PHF had a very low frequency of *iroN* compared to the other five farms with only one sample out of 27 carrying *iroN*. This farm also had a very low frequency of the *tem1* gene. It is difficult to know why this should be, except that specific management practices and the environment did vary between farms.

Iss, *iroN* and *f17A60* are genes considered part of UPEC (ExpEC), APEC pathotypes, while the *astA* gene is associated with EAEC, ETEC, EHEC, APEC pathotypes. Infections produced by such *E. coli* have therefore been widely treated with antimicrobials and might be expected to have been under strong selection pressure for resistance (Smith et al., 2007) (Hamelin et al., 2007; Johnson et al., 2003). This could be why the frequency of antibiotic resistance to wide spectrum antibiotics in these isolates samples was high.

Antibiotic resistance has been found in different wildlife animals (Costa et al., 2008; Dolejska et al., 2007; Gilliver M, 1999) , but there is limited information about *E. coli* antibiotic gene carriage in wildlife that also carried virulence genes. Previous studies to determine virulence in *E. coli* strains isolated from wildlife have concentrated mainly on detection of a small number of specific virulence genes associated with VTEC (Kobayashi et al., 2002; Nielsen et al., 2004a). Our study shows that wildlife animals found on farms can carry a wide variety of different virulence and antibiotic resistance genes.

Based on the results obtained we can not be certain that clusters of animals carrying an *E. coli* with the same virulence and antibiotic resistance genes are genetically identical. The use of another more discriminative method used in parallel such as PGFE could provide more precise information about identical strain's genomes. Even if the genomes of clustered strains of *E. coli* are different, our results still show that domestic animals and wildlife on farms shared virulence and antibiotic genes that could have been transmitted by plasmids or other mobile DNA vehicles via contaminated environment or direct contact.

In addition, molecular studies to determine the presence of plasmids and other transmissible elements would be very beneficial in order to have a better understanding of the virulence and antibiotic resistance genes dynamics amongst *E. coli* strains in these animal populations.

Sample collection from farmers would provide clear information about possible *E. coli* zoonotic strains persistent on these farms. Two similar studies have been carried out previously on meat industry workers and poultry farmers and showed that these workers carried VTEC and antibiotic resistant *E. coli* strains of zoonotic origin (Stephan et al., 2000; van den Bogaard et al., 2001). Equally beneficial would be the testing of environmental samples such as soil and water from pond and cattle troughs to determine the role of the abiotic environment in the ecology of virulence and antibiotic resistance genes.

It would be appropriate to compare the results with the microbiological phenotypic profile of antibiotic resistance in order to determine if the genes are expressed and the possible inconsistencies of both methods. Some *in vitro* techniques could be applied to determine which virulence genes were expressed and under which environmental pressures could be expressed if they were not initially expressed. This was one of the limitations of this microarray: it did not provide information about genes expressed by the bacteria. A further limitation of the use of this microarray is that it does not provide information about other possible genes that could be carried. Thus, it would be interesting to include other virulence genes that are associated with ExPEC pathotypes, as these are the most frequent genes carried among these samples. However, the data collected suggest that this, or a modified microarray, is a feasible means of undertaking this kind of research.

In conclusion, this study shows that wildlife and cattle carried a wide number of virulence and antibiotic resistance genes. Clusters of identical patterns of carriage between domestic cattle and wildlife were observed, implying that inter-species transmission may be possible – but such occurrences were unusual. It is not known, as discussed in Chapter 5, if these *E. coli* strains that seemed to inhabit the intestine of different healthy hosts are commensal or pathogenic and their zoonotic potential.

The virulence patterns amongst these samples do not seem to be animal/species host specific and show a high grade of diversity even between the same species of animals suggesting that this could be a dynamic process and genetic exchange between *E. coli* strains could be an active process in the host intestine. Antibiotic resistance patterns appeared to be closely linked to individual farms, which might indicate the importance of management or other environmental factors in the ecology of resistance. A further investigation of the use of antibiotics and medicine management on cattle present on these farms would be interesting. The collection of environmental samples would also help to understand the possible persistence of carriers *E. coli* strains and provide information about possible environmental transmission.

Chapter 7 General Discussion

Introduction

The major findings of this thesis were:

- A low prevalence of salmonellosis in wildlife, suggesting that the risk posed to cattle of infection with *Salmonella* from wildlife is low.
- VTEC O157 was isolated only from beef cattle, and not dairy cattle in this study.
- A survey for the genes associated with VTEC and the *eae* gene assoiacted with VTEC/EPEC found that these genes were found in both domestic cattle and a range of wildlife. Among birds, the *eae* gene was particularly associated with farmland birds and those captured in farm buildings.
- *Campylobacter* was isolated mainly in bank voles and cattle. There was only a low prevalence in birds. The main *Campylobacter* species from wildlife was *C. jejuni*. DNA sequencing suggested that strains are host-specific and that transmission between species, although possible, was rare.
- Using microarrays, a high proportion of *E. coli* isolates from healthy cattle and wildlife were found to contain genes associated with virulence and antibiotic resistance.
- There was not clear association between virulence and antibiotic resistance genes. Pattern of antibiotic resistance genes were associated with individual farms.
- Analysis of the resultant gene profiles provided little evidence for cross species transmission, but did suggest that the ecology of these genes is dynamic and influenced by the environment.

The overall aim of this study was the evaluation of the role that wildlife might play in the epidemiology of campylobacteriosis, salmonellosis and VTEC (including *E. coli* O157) infections of domestic cattle, through a cross sectional survey of six farms situated in an area of high cattle density in Cheshire (UK). In particular, the aims were:

- To determine the prevalence, risk factors and distribution of these bacteria amongst different wildlife hosts, domestic cattle and farms.
- To determine the molecular relatedness of isolates, and thereby investigate the variation in bacteria within and between hosts, and over several spatial scales.

Study design and collection of samples

Observational epidemiological studies are important in order to determine frequency of disease or infection in animal populations, and to examine its relationship with different risk factors of exposure. A cross-sectional study is a very valuable approach when the disease or infectious status is unknown in a population. In this case, the cross-sectional study met expectations as it enabled a preliminary understanding of how the frequency and risk factors for *Campylobacter spp. Salmonella* serovars, *E. coli* O157 and VTEC virulence determinants were distributed amongst wildlife and cattle populations in the six farms.

There were fundamental differences in terms of host populations that required a different sampling approach depending on the type of host. The cattle sampled were a well known, well characterised and well delimited population. In contrast, little was known about the rodents, wild birds and other wild mammals. Although rodents were clipped on the left side of the back leg and wild birds were ringed after a first sample was collected, the short time frame of a cross-sectional study did not allow for the making of accurate assumptions about denominators in populations.

Wild birds were largely captured in areas selected on the basis of bird activity, the likelihood of the nets or traps working, and nearness to cattle, rodents were trapped mainly in hedgerows and alongside walls in buildings, cattle and larger wild mammals were sampled by the collection of faeces deposited on the ground. Each of these approaches, although pragmatic, may have introduced biases that should be taken into account when interpreting the results from the study. Furthermore, the frequency of sampling for wild birds was not as regular as it was with the rodents. At certain times of the year (May-July) sampling could not be conducted as this is the nesting time for a large number of species. In addition, sampling was conducted in collaboration with the local BTO group and it was subject to finding a convenient time for both groups from the university and the BTO ringers.

Samples from larger wild mammals such as rabbits, foxes, badgers and larger wild birds such as corvids were collected from the ground when found. Sampling was not active for these species, and was rather *ad hoc* in its nature. Therefore, this could result in an underestimation of those populations. Furthermore, every sample was assumed to come from a different individual, but samples could have been repeatedly deposited by the same animal/s as the collection method did not involve physical capture and identification of individual animals. Likewise, the age of the sample was not known and this could have had an effect on the successful isolation of the bacteria: it is known that sample age is a risk factor for the viability of some bacteria and in particular *Campylobacter* (Stanley and Jones, 2003). These are largely unavoidable limitations of working with wildlife populations.

There may also be some differences in the results owing to the collection of samples in cattle, which were taken from fresh pats on the ground and not directly from the rectum of individual animals (Stanley and Jones, 2003). On the other hand, we considered that the difficulty involved in rectal sampling in terms of animal stress, handling and time

consumption would have made the process complex and difficult, especially for the farmers whose cooperation was essential to the project.

Cross-sectional studies are not ideal to determine seasonality and trends of change in the disease/infection over time. For these three bacteria, it is documented that a higher prevalence in animals together with an increase in the number of disease cases in human beings can be associated with particular times of the year in temperate countries (Meldrum et al., 2005; Paiba et al., 2002; Wray et al., 1987). We attempted to explore the effect of seasonality on these farms by sampling each farm twice a year. However, not all the farms were sampled at the same time. This makes 'season' a factor difficult to consider when comparing differences between farms, as it is difficult to deduce whether prevalence differences are due to the farm or to the time of sampling. Indeed, the sequencing of farm sampling makes time and farm variables highly correlated. Data on the month/season of sampling were therefore not taken into account in the epidemiological analysis, though they were included in the univariate analysis to complement the information shown.

At the geographical level, basic spatial analysis was undertaken to determine if clusters or aggregations of infected animals tended to be concentrated in particular areas of the farm or around particular habitats. The relationship between habitat and spatial dependency has been well studied in wildlife populations (Aspinall, 1993).In this study the spatial frame used was to a very small scale, making results difficult to interpret as it did not provide information about possible clusters of infection across the whole region.

Laboratory methodology

There is a lack of standard methods of isolation and molecular characterisation for these bacteria. The methods used for the microbiological isolation of the three bacteria were chosen

based on experience within the group a wide consultation at the beginning of the study (French et al., 2005; Kemp, 2005a).

In order to process samples during field work periods, faecal samples underwent a common preliminary incubation for 24 hours in buffer peptone water could reduce sensitivity. Indeed recent papers suggest that PCR might be a better approach than any culture method (Allgayer et al., 2008; Persson and Olsen, 2005).

E. coli colonies were archived as frozen pools of 10 colonies per faecal sample. This made it difficult to be sure that when more than one VTEC gene was found in an isolate, this reflected the wild type organisim or a more laboratory phenomenon. On the other hand, the correspondence of *eae* and either *vt* gene was rarely detected outside of *E. coli* O157. This has made any attempt for further characterization of pooled colonies by the use of PFGE in samples impossible as the colony used from the microarray test might not be exactly the same one selected to carry out the microarrays technique in Chapters 5 and 6.

Epidemiological approach to data analysis

Different variables were collected from each different animal host – not least because some variables (for example age, or even identity) were difficult or impossible to know for many host animals. This made the creation of a single epidemiological model that could integrate all existent information in order to provide an explanation for the dynamics of these bacteria in different hosts on the farm almost impossible.

Due to the high number of negative samples and isolates, the fitting of conventional epidemiological multivariate models was difficult. The attempt to use this approach appears to have worked well enough in that the results from the multivariate models support the results observed in the univariate analysis. More continuous data over time may fit better in an epidemiological model and even predict the dynamics of these bacteria on the farms under

the observed conditions through time. This would be beneficial for evaluating the effect on prevalence/incidence of different interventions such as biosecurity measures or vaccination.

A possible introduction of bias in the data analysis was the lack of precise information about the spatial position of some traps and the type of habitat. This resulted in the exclusion of incomplete data from the spatial, uni/multivariate analyses.

The scope of the geographical area was limited to only six farms. It is impossible to say how well the results can be generalized to the rest of Great Britain. On the one hand, this study shows that the role of wildlife in the epidemiology of these three enteric bacteria may be very limited even though it was carried out in an area with one of the highest dairy cattle densities in the country. The results also showed that these bacteria in wildlife and cattle populations are dynamic and can be associated with multiple risk factors besides environmental contamination leading to transmission between cattle and wildlife and vice-versa. Moreover, these bacteria in animal populations could be spread because of other factors, for example other disease or animal health and production policies. For example bovine tuberculosis (bTB) in the UK, is a multi-host pathogen that can be transmitted by multiple routes including via wildlife. The prevalence of this disease has been concentrated mainly in the Southwest of England, which has the highest dairy livestock density in the UK. As a result of the Foot and Mouth Disease (FMD) epidemic in 2001, many infected premises around the country bought cattle from bTB infected farms for re-stocking after the killing of infected herds. This has contributed enormously to the spread of this disease to previously bTB- free areas of the UK and this indirectly may include some of the wildlife reservoirs such as badgers and deer. To carry out a similar study on a larger geographical scale would be very intense in terms of collection of samples, personnel involved, microbiological processing and molecular characterisation of samples. A study of these characteristics could have a high number of confounders or "noise" in terms of species populations that could make accurate

interpretation of the results difficult. If reliable results could be obtained at a higher geographical scale, it would be very beneficial in terms of interventions and policy-making.

Conclusions

Veterinary public health is facing complex and challenging times. There is an overlap of veterinary and human health in terms of zoonotic and emerging diseases. The concept of animal health has changed over the last ten years and so has the public perception of zoonoses that can be transmitted via contaminated food and water. Diseases such as BSE and the new variant Creutfeldt-Jakob disease (vCJD), salmonellosis, campylobacteriosis and VTEC O157 infections have contributed enormously to the creation of strict policies in livestock production and food safety in Europe (http://europa.eu/scadplus/leg/en/s84000.htm).

In addition, factors such as intensive farming, increased movement of domestic stock, the use of veterinary medicines in food-production animals, and changes in people's lifestyle in terms of an increase in the consumption of ready meals have all had an important impact on the epidemiology of zoonotic bacteria responsible for gastroenteric disease in humans.

Wildlife species are part of our identity, culture and heritage. There is a real contradiction in public health and biodiversity policies with regard to wildlife animals in many countries. On the one hand, nowadays there is a tendency to introduce wildlife conservation and protection policies amongst most European countries. On the other hand, the high level of man-made modifications to natural ecosystems in favour of certain domestic animal species and specific plant crops has made the wildlife ecosystem completely unbalanced. The increase in human population and the use of massive amounts of resources to sustain this species in terms of food and sheltering has contributed to the use of natural wildlife habitats to develop urban or farming areas, making a fragile separation between urban and countryside areas and therefore

a decrease in the levels of natural biodiversity. The detrimental effect on wildlife is combined with increased probability of interactions between wildlife, domestic animals and human beings. These interactions are high risk in terms of the transmission and spread of disease as it has been documented that wildlife can be the carriers of many zoonotic diseases and other diseases transmissible to domestic livestock (Acha and Szyfres, 2003). Zoonotic diseases can also be spilled from human and domestic livestock populations into wildlife thus, increasing the sources of infection. Inevitably, this creates conflict in terms of biodiversity, animal and human health. It also brings welfare, ethical and even moral issues into consideration.

There is a lack of research aimed at understanding and quantifying the domestic-wildlife-human interface in terms of zoonotic disease. Answers to such research could provide information necessary in order to develop appropriate disease surveillance and intervention programmes. Such research would also indirectly contribute to the creation of adequate biodiversity conservation programmes that would help to preserve our native wildlife and minimise this conflict.

This project has attempted to understand some of the factors mentioned above. It was undertaken in the belief that zoonotic research and the domestic-wildlife-human interface require a multidisciplinary approach.

The results suggested that the epidemiology of *Campylobacter*, *Salmonella* and VTEC in domestic and wildlife populations on farms largely involved within species transmission. The prevalence of *Salmonella* was low in both domestic cattle and wildlife. *Salmonella* Dublin was isolated only from cattle, and cattle are usually regarded as the natural reservoir for this serovar. *S.* London was isolated from a badger and a calf on a farm that previously had an outbreak, suggesting possible environmental spillage and badgers as a possible natural reservoir for *Salmonella* serovars. In addition, among birds, *Salmonella* was isolated only

from one house sparrow. This agrees with the hypothesis that the prevalence of *Salmonella* in healthy wild birds is low or that shedding is at undetectable levels with the methodology used in contrast to diseased wild birds in which the prevalence can be high (Pennycott et al., 2005).

Campylobacter jejuni was the predominant *Campylobacter* species from wildlife, and was mainly found in bank voles. This rodent species might be useful as a sentinel indicator for *Campylobacter* at the farm level. A spatial cluster was detected in rodents in the boundaries of a busy red meat abattoir on one of the farms in which the prevalence of *Campylobacter* in cattle was low suggesting different infection sources in both hosts. *Campylobacter* infection in rodents and cattle was farm-associated, suggesting that currently unknown management factors have an effect on the frequency of infection. The prevalence of *Campylobacter* in wild birds was low, in contrast to domestic poultry flocks, suggesting that this bacterium may have different epidemiology in wild and domestic birds.

Campylobacter DNA sequences for the partial *gro*EL gene revealed that *Campylobacter jejuni* seems to be host adapted. A higher diversity of *C. jejuni* was observed in bank voles, including a bank vole that carried a *C. jejuni* strain identical to the one isolated mainly from domestic cattle. This suggests that although most isolates tend to be host associated, cross species transmission of *C. jejuni* is possible. The zoonotic potential of the *C. jejuni* isolates from wildlife and cattle in was unknown.

E. coli was isolated from all types of wildlife samples and domestic cattle. A high proportion of wildlife species and cattle *E. coli* isolates carried virulence and antibiotic resistance genes. The high prevalence of antibiotic resistance genes in wildlife animals may be due to environmental exposure, however cattle and wildlife rarely had the same resistance profiles. The *iss* and *iroN* genes were the most prevalent virulence genes and the *tem1*, *tetA* and *tetB*

genes were the most prevalent antibiotic resistance genes amongst *E. coli* isolates. The carriage of antibiotic resistance genes seemed to be associated with farms. Microarrays would be a useful method to use for molecular characterization of individual *E. coli* strains producing outbreaks.

VTEC O157 was found only on the beef farm and only in cattle in the cross sectional study. There was a dominant strain, but also evidence of some diversity in strains, and changes in the dominant strain over time, either through evolution or competition between strains.

The *eae* gene was the most predominant VTEC virulence determinant isolated from cattle, rodents, badgers, foxes, rabbits and wild birds in this study. Wild bird species associated with farmland and corvids had a higher probability of carrying the *eae* gene if isolated from two particular farms, MF and PHF. Moreover, there was a higher prevalence of infection in birds captured from farm buildings. There were significant variations in prevalence in rodents and cattle on different farms. After the *eae* gene, the *vt1* gene was the most commonly detected gene and it tended to be carried together with the *eae* gene in *E. coli* pooled isolates from cattle and rodents. Wildlife species in this study could contribute to the amplification of VTEC virulence genes within the farm or other close by surroundings.

Interventions

This study has shown that cattle are the main reservoir for VTEC O157, other VTEC, *Salmonella* Dublin, *Campylobacter jejuni* and other *Campylobacter* spp such as *C. fetus* and *C. hyointestinalis*. This study has also found that wildlife animals on farms are capable of carrying *C. jejuni*, *Salmonella* serovars and also VTEC virulence determinants. Thus, the dynamics of transmission and infection of these pathogens in wildlife and cattle appears complex and multifactorial, and this suggests that interspecies transmission of strains or genes could be possible. In addition, GLM models have shown that farms have a high

influence in the frequency of *Campylobacter* and VTEC infections in both wildlife and cattle. This has an important consequence for strategic interventions. The evidence that farms may be the nucleus where transmission occurs suggests that intervention should be addressed principally at farm level. In this study we were not able to determine the direction of transmission of these pathogens between hosts. Due to the ubiquitous nature of these three pathogens, a total eradication from the animals could be difficult to achieve. Hence, the most indicated intervention could be based on a preventive approach taking appropriate biosecurity measures to maintain the low or undetectable levels of these bacteria in animals.

For instance, the measures could be applied to four different levels;

1. *At the cattle level*; Breaking the transmission routes between animals should be a priority. As the GLM models in this study showed, the frequency of infection for VTEC and *Campylobacter* was age related. Good quarantine procedures such as isolated areas for animals with diarrhoea in order to avoid infection of healthy animals should be implemented. Moreover, animals of different ages should not be mixed.

Another possible intervention in cattle is vaccination. Vaccination against abortions caused by *Salmonella* Dublin and *S.* Typhimurium has been carried out as part of routine herd health plans with success (Anonymous, 2007) . Moreover, *Salmonella* Enteriditis and *S.* Typhimurium have been controlled in hen layers by the use of an inactivated vaccine in the UK and other EU countries with success (EFSA, 2004). Currently, research is being conducted in order to develop vaccines against VTEC O157 and *Campylobacter*. Consideration should be given before opting for vaccination. Factors such as the cost-benefit of the vaccine treatment and the possible introduction of new strain of these bacteria once a particular strain is "under control" should be considered carefully.

Other control methods might include, minimising the number of new animals introduced in the herd and avoiding common grazing with cattle from other herds. In addition, an early detection and elimination of "super-shedders" could be carried out especially on farms where a high prevalence of any of these three pathogens is confirmed.

2. *At the wildlife level*; Actions to avoid contact between wildlife and cattle should be implemented on farms in order to reduce transmission in both directions. Good pest and insect controls should be in place around farm buildings and cattle barns. Measures should particularly be taken to prevent the access of wildlife to animal feeding stuffs, and open barns should have a way of avoiding the entrance of birds when animals are there.

3. *At the farm environment*; The environment may play an important role in the indirect transmission between cattle and wildlife and vice versa. Simple measures to decrease the amount of faecal contamination should be adopted. Slurry could be treated as proposed by Bucjoczek to avoid the viability of these pathogens, before spreading on grazing field as fertilizer (Bujoczek et al., 2001).

Another possible measure could be to maintain good cleaning practices around cattle barns and other buildings, for example, the routine cleaning of pens especially in young and weaned calves. Changing water from the animals' troughs regularly to avoid contamination and cross-infection is also recommended.

Nevertheless, some of the possible environmental sources of infection may be difficult to control. The presence of abattoirs close to the farms could be the source of environmental contamination due to debris, run-off water and the possible increase in the number of rodents due to food availability. The approach to this could be the creation of policies that do not allow the building of abattoirs in agricultural land.

4. *Other*; The implementation of Hazard Analysis and Critical Control Points (HACCP) systems on farms that take into account wildlife as a possible critical control point could be adopted by farmers as part of the herd health and welfare plans.

Targeting farmers in terms of education and consciousness about food production and safety would also be beneficial. The willingness of farmers to adopt preventive measures such as the ones mentioned above may increase if they understand the impact on human health behind the preventive measures applied on their farms.

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Appendices

Appendices

Chapter 2

Appendix I. PCR primers used to characterise *Campylobacter* isolates.

Multiplex PCR for *hipO*, 23*S rRNA*, *gly*A genes ('Wang method')

Assay	Target gene	Oligonucleotide Sequence	Amplicon size (bp)
C. jejuni	*hip*O	F: ACTTCTTTATTGCTTGCTGC R: GCCACAACAAGTAAAGAAGC	323
C. spp.	*23S rRNA*	F: TATACCGGTAAGGAGTGCTGGAG R: ATCAATTAACCTTCGAGCACCG	650
C. coli	*gly*A	F: GTAAAACCAAAGCTTATCGTG R: TCCAGCAATGTGTGCAATG	126
C. lari	*gly*A	F: TAGAGAGATAGCAAAAGAGA R: TACACATAATAATCCCACCC	251
C. upsaliensis	*gly*A	F: AATTGAAACTCTTGCTATCC R: TCATACATTTTACCCGAGCT	204

Multiplex PCR for 16S rRNA gene ('Linton method')

Assay	Target gene	Oligonucleotide Sequence	Amplicon size (bp)
C. hyointestinalis	16SrRNA	F:GCAAGTCGAACGGAGTATTA R:GCGATTCCGGCTTCATGCTC	1287
C. fetus	16SrRNA	F:GCAAGTCGAACGGAGTATTA R:GCAGCACCTGTCTCAACT	997

Multiplex PCR for *ceuE* gene ('Gonzalez method')

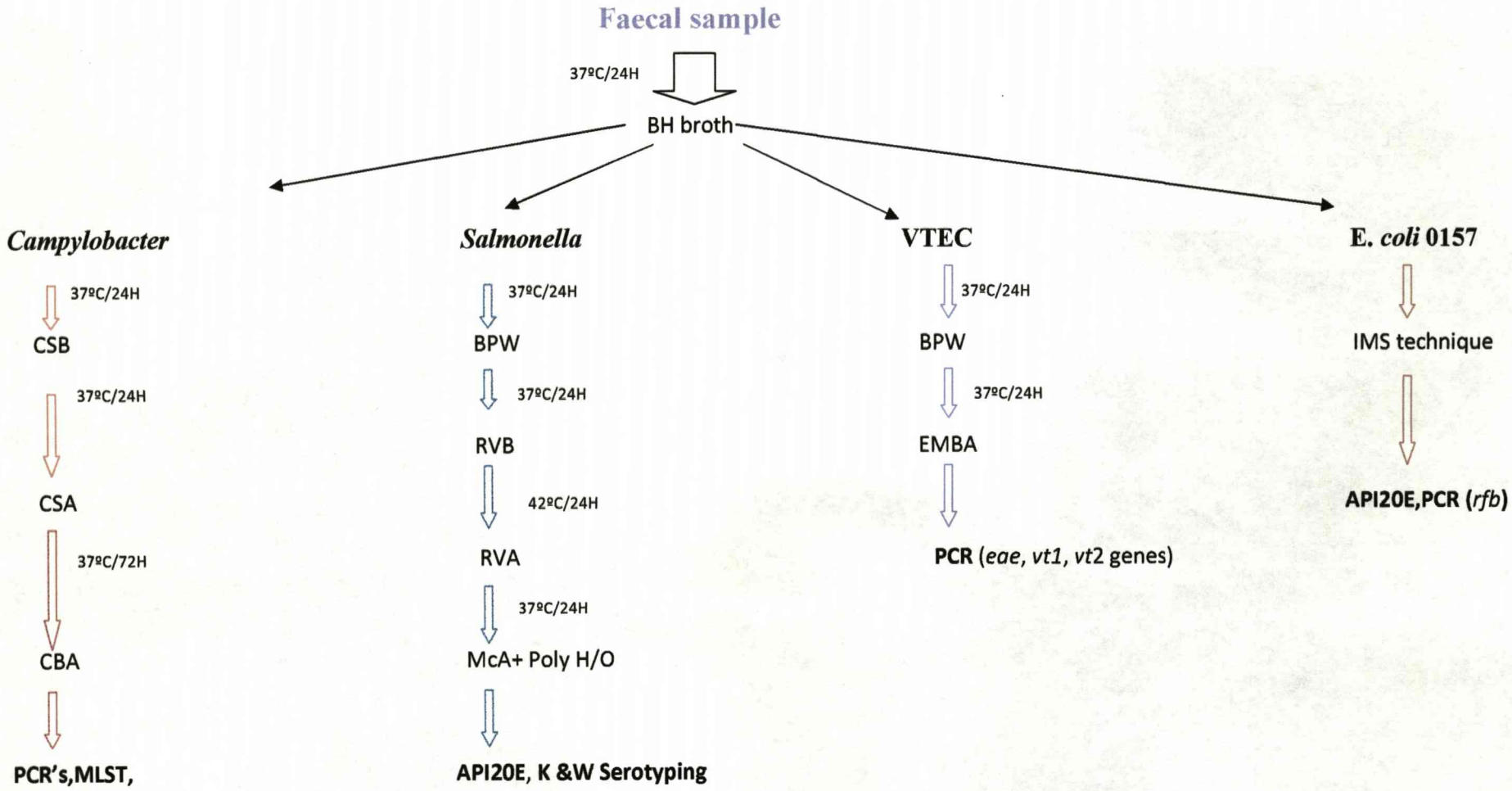
Assay	Target gene	Oligonucleotide Sequence	Amplicon size(bp)
C. jejuni	*ceuE*	F: CCTGCTACGGTGAAAGTTTTGC R: GATCTTTTTGTTTTGTGCTGC	793
C. coli	ceuE	F: ATGAAAAAATATTTAGTTTTTGCA R: ATTTTATTATTTGTAGCAGCG	894

PCR for *GoEL* gene ("Karenlampi method")

Assay	Target gene	Oligonucleotide Sequence	Amplicon size(bp)
C. spp	*groEL*	F:GAGCGGACAATTTCACACAGG(AGCT)GA(CT)GG(AGCT)AC(AGCT)AC(AGCT)AC(AGCT)GC (AGCT)AC (AGCT) R: TAATACGACTCACTATAGGGTC(AGCT)CC(AG)AA(AGCT)CC(AGCT)GG(AGCT)GC (CT)TT(AGCT)AC(AGCT)GC	592

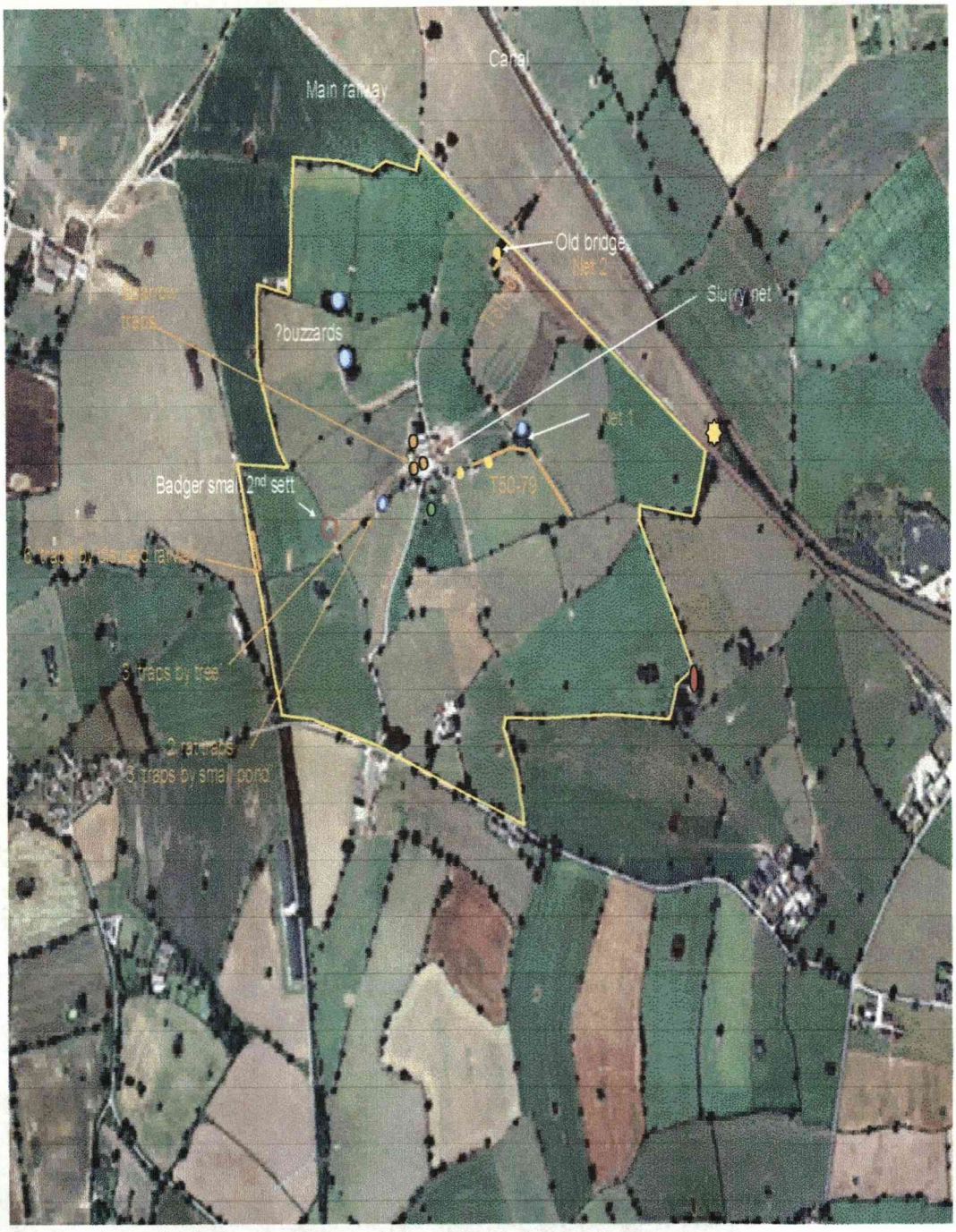
-Universal sequences primers (M13 and T7), **AC** nucleotides modified in comparison with original primers

Appendix II. Summary of the microbiological processing of samples for isolation of *Campylobacter*, VTEC and *Salmonella*.



** BH broth (brain heart broth); BPW (buffer peptone water); CSB (Campylobacter selective broth) ; CSA(Campylobacter selective agar); CBA(Columbia blood agar); RVB (Rappaport-Vassiladis broth); RVA (Rappaport Vassilladis agar), McA(McConkey agar); EMBA (Eosin methylene blue agar).

Appendix IV. Aerial maps of small rodents traps located in two of the six participating farms (as an example) in the cross-sectional study from July 2004 to may 2005.


Canal
Main railway
Old bridge
Net 2
Slurry net
?buzzards
Net 1
T50-79
Badger small 2nd sett
3 traps by tree
2 ramps
3 traps by small pond

Appendices

Chapter 5

Appendix I. Distribution of samples and *E. coli* isolates that were tested with microarrays per host /location .

DOMESTIC CATTLE

Host	N0 colonies	N0 faecal samples	Location
Dry cows	14	14	3BHF,7CLF,4PHF
Lactating cows	9	8	3PHF, 2MF,1BGF,3BHF
Young stock	28	25	13MF, 5BGF, 9BHF,1PHF
Calves	35	30	6MF, 9PHF,7GF,6CLF, 3BHF,2BGF

TERRESTRIAL WILDLIFE

RODENTS

Host	N0 colonies	N0 faecal samples	Location
Bank vole	44	43	11CLF,7BGF,9PHF,6GF,6MF,5BHF
Wood mouse	58	57	7BGF, 8BHF, 10CLF, 6GF,12MF,15PHF
House mouse	17	13	2BGF, 8BHF,2 CLF,4PHF,1BGF
Field vole	1	1	BGF
Rat	16	11	2BGF,1BHF,3CLF,3MF,8PHF
noID rodent (unidentified)	2	2	1BHF,1CLF

OTHER WILDLIFE

Host	N0 colonies	N0 faecal samples	Location
Fox	31	27	12BGF,4BHF,1CLF,5GF,4MF,5PHF
Badger	30	25	2BGF,12BHF,3CLF,5MF,8PHF
Rabbit	16	15	1BGF,4BHF,5CLF,3MF,3PHF

WILDLIFE BIRDS

SMALL PASSERINES

Host	N0 colonies	N0 faecal samples	Location
Blackbird	5	5	1BGF,1CLF,1MF, 1BHF,1PHF
Blue tit	4	4	1BGF,1CLF,1GF,1BHF
Chaffinch	5	5	1BGF,1BHF,1MF,1PHF,1CLF
Dunnock	7	6	2CLF,1GF,1MF,3PHF
Great tit	5	5	1BGF,1BHF, 3GF
House sparrow	8	5	4BGF, 3PHF,1BHF
Redwing	1	1	BHF
Robin	6	5	1BGF, 1BHF,1CLF,1GF,1MF,1PHF
Song thrush	1	1	MF
Starling	3	3	PHF
Wren	4	4	1BGF,1CLF,1MF,1PHF
Long-tailed tit	2	2	1CLF, 1BHF
Raven	1	1	CLF

OTHER BIRDS

Host	N0 colonies	N0 faecal samples	Location
Pigeon	7	5	4 CLF, 2MF,1BGF
Pheasant	2	2	CLF
noID bird (unidentified)	17	10	6CLF, 5BHF, 6MF

OTHER ANIMALS-PETS

Host	N0 colonies	N0 faecal samples	Location
Dog	3	3	2BHF, 1BGF
Pony	1	1	PHF

Appendix II. Different profiles of virulence genes present in *E. coli* isolates per location as part of the test with microarrays.

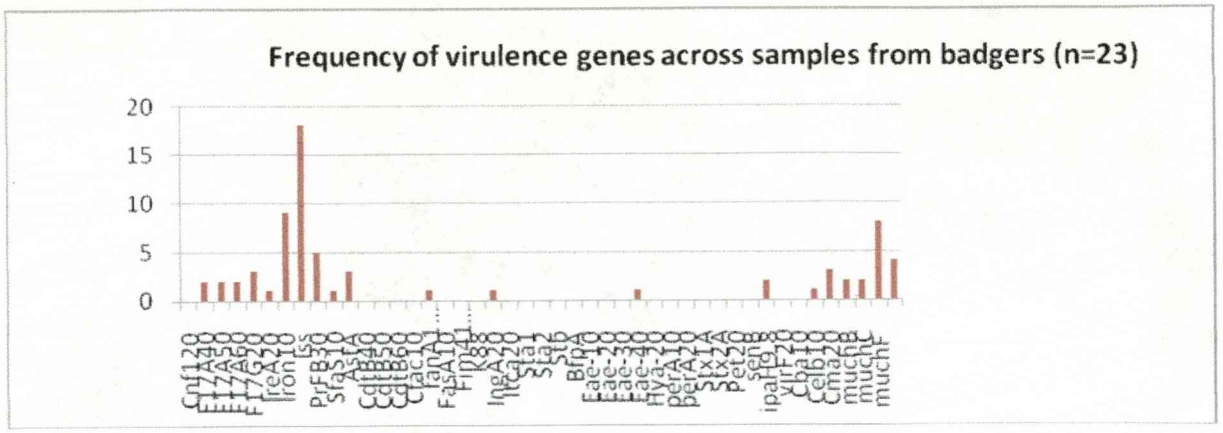
Code number	Combination of Genes (No of genes)	Location
1	*f17A40,f17A60,iss,sfas,astA,perA10,mchB,mchC,mchF,mcmA* (10)	BGF
2	*astA,eae1, mchB,mchC,mchF,mcmA* (6)	BGF
3	*astA, mchB,mchC,mchF,mcmA* (5)	BGF,GF
4	*iss, astA, mchB,mchC,mchF* (5)	BGF,PHF
5	*ireA, iss, astA,perA20, mchB,mchC,mcmA* (7)	BGF
6	*f17A60,iroN, iss, astA, mchB,mchC,mchF* (7)	BGF
7	*astA*(1)	BGF,BHF,CLF,GF,MF,PHF
8	*iss, astA* (2)	BGF,GF,MF,BHF,CLF,PHF
9	*iss, astA,cba* (3)	BGF
10	*iss* (1)	BGF, BHF,CLF,GF, MF,PHF
11	*iss, perA-10* (2)	BGF
12	*iss, mchF* (2)	BGF
13	*cdtB-40* (1)	BGF,BHF
14	*f17A60*(1)	BGF,BHF,GF,MF,PHF,CLF
15	*ireA* (1)	BGF, CLF
16	*Celb* (1)	BGF
17	*itcA*(1)	BGF
18	*eae-10,eae-30*(2)	BGF
19	*iss,sta2*(2)	BGF,CLF
20	*f17A40,f17G20,astA* (3)	BGF
21	*Cnf,cdtb40,cdtb50*(3)	BGF,MF
22	*f17A60,iron,iss* (3)	BGF,CLF
23	*iroN,iss,mcmA*(3)	BGF
24	*ireA, iroN,iss,prfB30, mcmA* (5)	BGF
25	*iroN,iss,k88,muchF* (4)	BGF
26	*cfa,vt1,vt2,celb* (4)	BGF
27	*iroN,iss,astA,cma,cba,mchF* (6)	BGF
28	*iroN,iss,astA cba,mchF*(5)	BGF
29	*iroN,iss,prfB,sfas,astA,cba, cma, mchF* (8)	BGF
30	*iroN, sfas , cba, cma, mchF* (5)	BGF
31	*iroN, sfas,astA,sta1,cba,mchB*(6)	BGF
32	*f17A40,f17G20,iroN, iss,prfB,vt1,mchF,mcmA*(8)	BGF
33	*f17A50f17G20, iron,iss,prfB, mchF,mcmA* (7)	BGF
34	*f17A50,f17G20,ireA,iss,cba,cma, mchF,mcmA* (8)	BGF
35	*iroN,iss* (2)	BHF
36	*iroN,iss,sfas,mchF*(4)	BHF
37	*iroN,iss,cma* (3)	BHF,MF
38	*iroN,iss,muchF* (3)	BHF,BGF,CLF,GF,MF
39	*iss, astA,cma*(3)	BHF
40	*cnf, iss, astA* (3)	BHF
41	*iss,cma*(2)	BHF
42	*iss, astA,cba,cma*(4)	BHF,MF

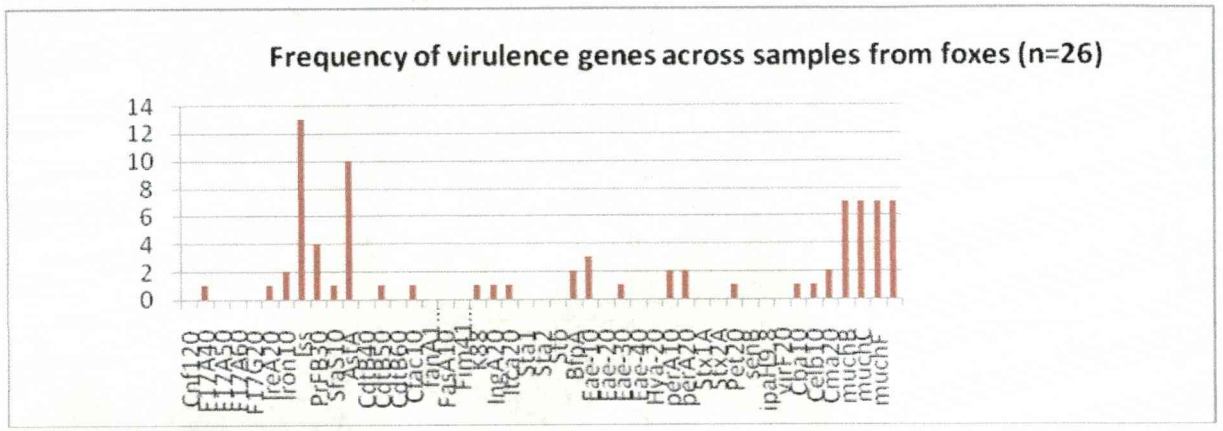
43	*f17A60,iss* (2)	BHF
44	*f17A60, iss, astA,mcmA*(4)	BHF
45	*iss,sfas,eae3,cma*(4)	BHF
46	*iss,sfas,sta1*(3)	BHF
47	*iroN*(1)	BHF, CLF
48	*prfB,itcA, mcmA* (3)	BHF
49	*iroN,iss, prfB, mcmA* (4)	BHF
50	*iroN,iss, prfB,mchF, mcmA* (5)	BHF
51	*ireA, iroN,iss, prfB,mchF, mcmA* (6)	BHF
52	*iss, prfB, sfas, mcmA* (4)	BHF
53	*ireA, iroN,iss, prfB,astA* (5)	BHF
54	*ireA, iroN,iss, prfB,astA , mcmA* (6)	BHF
55	*ireA,iss, prfB,astA , mcmA* (5)	BHF
56	*ireA, iroN,iss, prfB,* (4)	BHF
57	*ireA,iss, prfB , mcmA* (4)	BHF
58	*iss, prfB,astA,cdtb60,k88,cba,celb*(6)	BHF
59	*k88,celb* (2)	BHF
60	*iss,vt2,cba,cma*(4)	BHF
61	*Cdtb40,cdtb50*(2)	BHF,PHF
62	*cnf,f17G20,iss, Cdtb40,cdtb50, celb* (5)	BHF
63	*fanA,eae4,ipaH,mchC*(4)	BHF
64	*ireA,iss,prfB,sfas,cdtb50,fasA,stb,bfp,eae1,vt2,ipaH,mchC,mchF*(13)	BHF
65	*f17A60,iss,prfB,astA,cdtb60,fim,bfp,eae1,2,3,4,hyA,senB,cba,mchC,mchF,mcmA*(17)	BHF
66	*f17A40,f17A50,f17A60,iss,prfB,sfas,astA,cdtb40,cdtb50,cdtb60,k88, ingA,senB,ipaH,cba,mchC*(16)	BHF
67	*iroN,iss, prfB,cba,cma, mchB, mchC, mchF, mcmA*(9)	CLF
68	*iroN,iss, prfB , mchB, mchC, mchF, mcmA* (7)	CLF
69	*iroN,iss, prfB,astA,cma, mchF, mcmA* (7)	CLF
70	*f17A60, iroN,iss, prfB, cma, mchF, mcmA* (7)	CLF
71	*iss,cba,cma*(3)	CLF
72	*iroN,iss, cba,cma, mcmA* (5)	CLF
73	*ireA, iroN,iss, cba,cma, mcmA* (6)	CLF
74	*F17A60, ireA, iroN,iss,cba,cma, mchB, mchC, mchF, mcmA* (10)	CLF
75	*f17A60, ireA,iroN,iss,cba,cma, mchB, mchC, mchF* (9)	CLF
76	*iroN,iss ,astA*(3)	CLF
77	*astA,cma*(2)	CLF
78	*vt1*(1)	CLF
79	*cfa*(1)	CLF
80	*f17A60,f17G20*(2)	CLF
81	*f17A60,astA*(2)	CLF
82	*f17A40, f17A60* (2)	CLF
83	*f17A60,iss,astA,cma*(4)	CLF
84	*prfB, cma* (2)	CLF
85	*f17A40, iss,astA* (3)	CLF
86	*ireA,iron,iss*(3)	CLF
87	*f17A60,iron,iss,mchF*(4)	CLF
88	*iss,itcA,mcmA*(3)	CLF
89	*f17A60,ireA,iss,prfB*(4)	CLF
90	*sfas,cdtB60,sta1*(3)	CLF
91	*f17A40,iss, sfas,astA,perA20*(5)	CLF

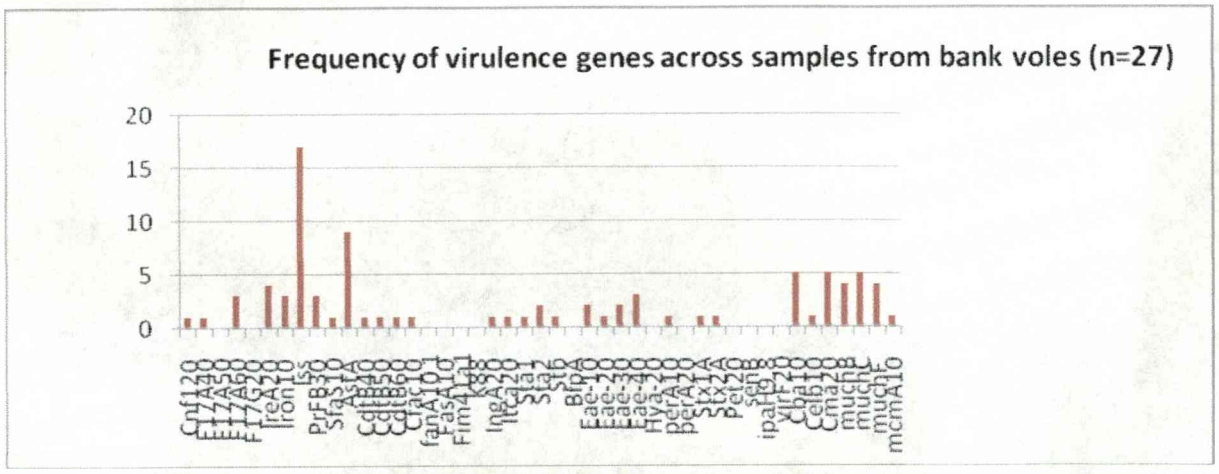
92	*fim,eae1,2,3,4*(5)	CLF
93	*astA, eae1,2,3,4,hylA,vt1*(7)	CLF
94	*f17A40,astA f17A50,cdtB40,cfa,k88,bfp,eae1,perA10,vt2,mchC*(11)	CLF
95	*f17A40,f17A50,f17A60,iss,satA,cdtB40,cdtB50,cdtB60,k88,stb,hyA, perA20,cba,cma,mchB*(15)	CLF
96	*iroN,iss,astA,mchF*(4)	GF
97	*f17A50,f17A60,f17G20, iroN,iss,mchF*(6)	GF
98	*astA,cdtB50*(2)	GF
99	*celb*(1)	GF
100	*senB, celb* (2)	GF
101	*ireA,prfB,mcmA*(3)	GF,MF, PHF
102	*iss, prfB,mcmA* (3)	GF
103	*ireA, iroN,iss,astA,cfa, mchB, mchC, mchF, mcmA* (9)	GF
104	*f17A50,f17A60,iroN,iss,ingA,bfp,ipaH,virF,cba, mchB, mchC , mcmA* (12)	GF
105	*astA,eae1,2,3,4,hylA,vt1,cba,celb*(9)	GF
106	*f17A50,f17A60,iron,iss,fim,ingA,bfp,ipaH,virF,cba, mchB, mchC,mcmA*(13)	GF
107	*cnf,F17A40,iroN,prfB,astA,ingA,itcA,sta1,sta2,stb,eae4,perA1 , cba, cma,mchC*(15)	GF
108	*astA,aea1,2,3,4,hlyA,vt2*(7)	MF
109	*astA,aea1,2,3,4,hylA* (6)	MF
110	*astA,eae1,3, hylA,vt2*(5)	MF
111	*astA,eae2,3,4,hylA,vt1,cba* (7)	MF
112	*eae1,3,4,perA10*(4)	MF
113	*cnf,f17A40,f17G20,ireA,iss,astA,cdtB40,cdtB50*(8)	MF
114	*bfp*(1)	MF
115	*cnf,f17A60,iss,astA*(3)	MF
116	*iss,sta1*(2)	MF
117	*f17A60, mchC* (2)	MF
118	*iron,cma*(2)	MF
119	*iroN,iss,cdtB50,mchF* (4)	MF
120	*f17A60,iroN,iss, mchF* (4)	MF
121	*ireA,iss,prfB*(3)	MF
122	*f17A50, f17A60, iss,prfB* (4)	MF
123	*f17A50, f17G20,iss,astA*(4)	MF
124	*f17A50, f17G20* (2)	MF
125	*f17A50, f17G20,iss,prfB,AmcmA*(5)	MF
126	*f17A40, f17G20*(2)	MF
127	*f17A40, f17G20, iss,prfB* (4)	MF
128	*f17A60,astA,cma,mcmA*(4)	MF
129	*f17A40, f17A50, f17A60,sta2*(4)	MF
130	*iss,astA,sta1,hylA,vt2*(5)	MF
131	*f17G20, iss,prfB, astA,fim,mcmA*(6)	MF
132	*iroN,iss,astA,celb,mchB*(5)	MF
133	*f17G20, iss,prfB,sfas,cdtB60,cba*(6)	MF

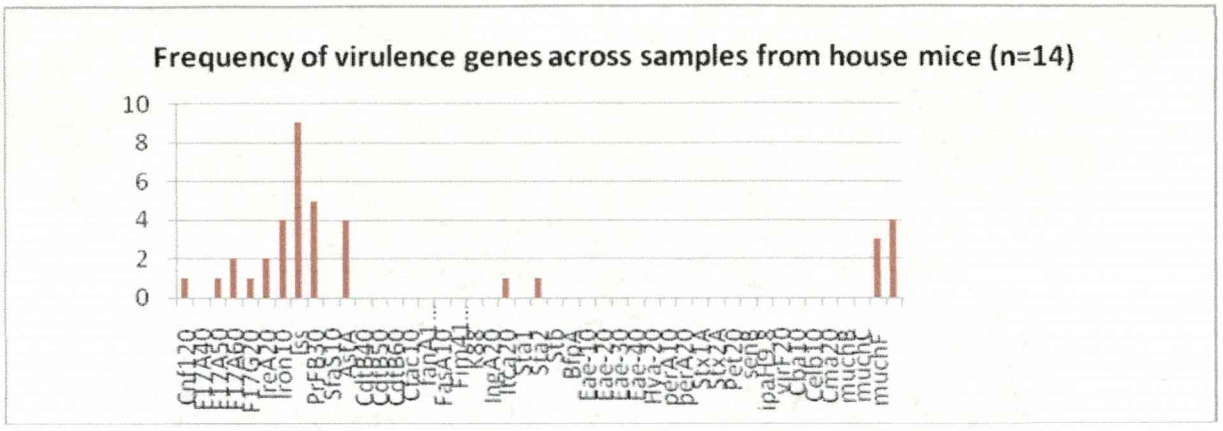
134	*iroN,iss, mchB, mchC, mchF, mcmA* (6)	MF
135	*iss,cba,cma, mchB, mchC, mchF, mcmA*(7)	MF
136	*f17A40,ireA,astA,ingA,cba,cma,mchC*(7)	MF
137	*f17A40,iroN,iss,cfa,ingA,sta1,eae4,vt2,sfas,cba,cma*(11)	MF
138	*cnf, f17A40, f17A50, f17G20,iss,cdtB40,cdtB50,fasA,ingA,sta2,eae2 ,perA20,virF*(13)	MF
139	*iss,prfB,cfa,k88,ingA,itcA,bfp,eae1,perA10,perA20,pet, mchB, mchC, mchF* (14)	MF
140	*f17A40, f17A50, f17A60,iss,sfas,astA,cfa,eae1,2,3,4,hylA,vt1,2,cba,celb,mchC*(17)	MF
141	*f17A40, f17A50, f17A60,iron,iss,prfB,sfas,astAcdtB50,cdtB60,cfa,k88,sta1,perA20, cba,celb,cma,mchC*(18)	MF
142	*f17A40* (1)	PHF
143	*eae1*(1)	PHF
144	*f17G20* (1)	PHF
145	*f17A50, f17A60* (2)	PHF
146	*f17A50, f17G20,iss*(3)	PHF
147	*iss,eae1,3*(3)	PHF
148	*iroN,iss,cba*(3)	PHF
149	*iroN,iss,fasA*(3)	PHF
150	*f17G20,iroN,perA20*(3)	PHF
151	*sfas,cdtB50,cdtB60* (3)	PHF
152	*sfas,bfp,mchC*(3)	PHF
153	*ingA,ipaH,mcmA*(3)	PHF
154	*ireA,prfB,vt2,cba*(4)	PHF
155	*ireA,prfB,cba,cma*(4)	PHF
156	*prfB ,mcmA*(2)	PHF
157	*ireA,prfB*(2)	PHF
158	*ireA,iss,prfB,mcmA*(4)	PHF
159	*f17A60, ireA,prfB,mcmA* (4)	PHF
160	*f17A60, prfB,sta1,mcmA*(4)	PHF
161	*prfB,cdtB40,celb,mcmA*(4)	PHF
162	*astA, cdtB40,cba,cma*(4)	PHF
163	*iroN,iss,cma,mchF*(4)	PHF
164	*f17G20,IroN,iss,cma, mchF* (5)	PHF
165	*iroN,iss, prfB ,ingA, cma, mchF,mcmA*(7)	PHF
166	*f17A40, f17A50, f17A60,f17G20,sfas*(5)	PHF
167	*f17A40, f17A60,f17G20G,iss*(4)	PHF
168	*cnf, f17A60,f17G20G,iss ,cdtB40,cdtB50*(6)	PHF
169	*f17A40,prfB,sfas,ipaH,mchF*(5)	PHF
170	*iss,astA,celb, mchB, mchC, mchF* (6)	PHF
171	*f17A60, iss,astA,celb, mchB, mchC, mchF*(7)	PHF
172	*iss, celb, mchB, mchC, mchF* (5)	PHF
173	*iss, mchB, mchC, mchF* (4)	PHF
174	*cba,celb,cma, mchB, mchC, mchF,mcmA*(7)	PHF
175	*iss,eae1,3,4*(4)	PHF
176	*iss,eae1,2,3,4,vt1,cba*(7)	PHF
177	*iss,eae1,2,3,4,cba,cma* (7)	PHF
178	*f17G20,iss,astA,eae1,2,3,hylA,cba*(8)	PHF
179	*f17A40,iss,prfB,sfas,ingA,itcA,sta1,senB,ipaH,cma,mchC* (11)	PHF
180	*virF*(1)	PHF

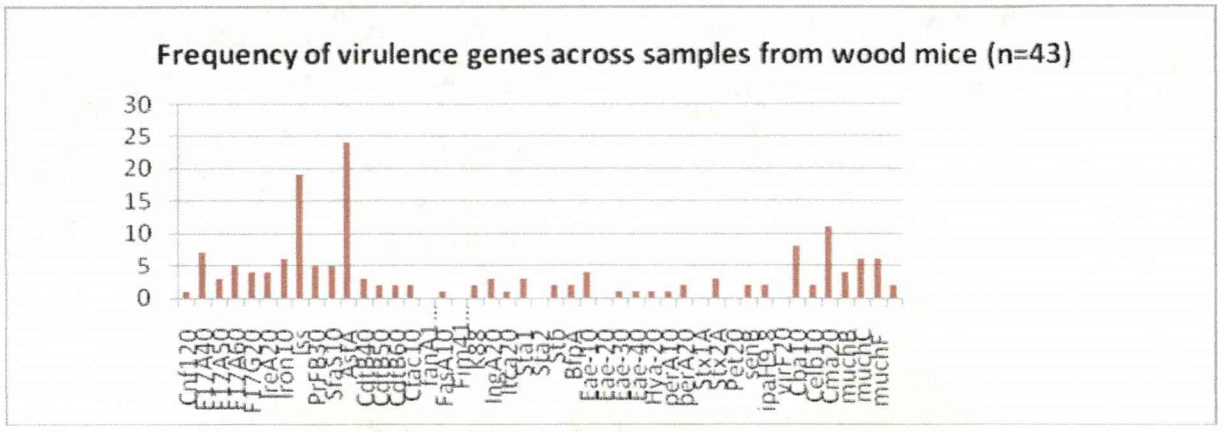
Appendix III .Frequency of virulence genes carried by *E. coli* isolates from different animal hosts tested with microarrays.

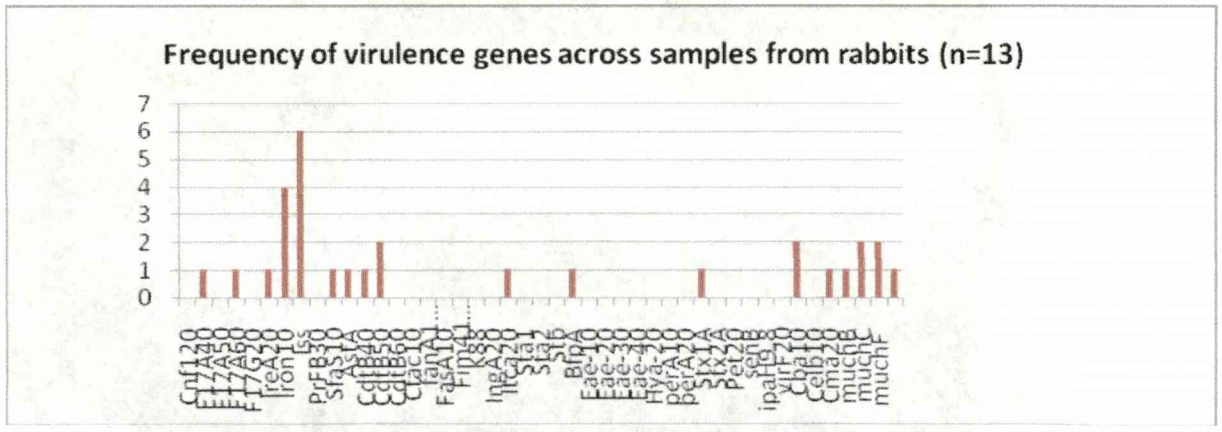


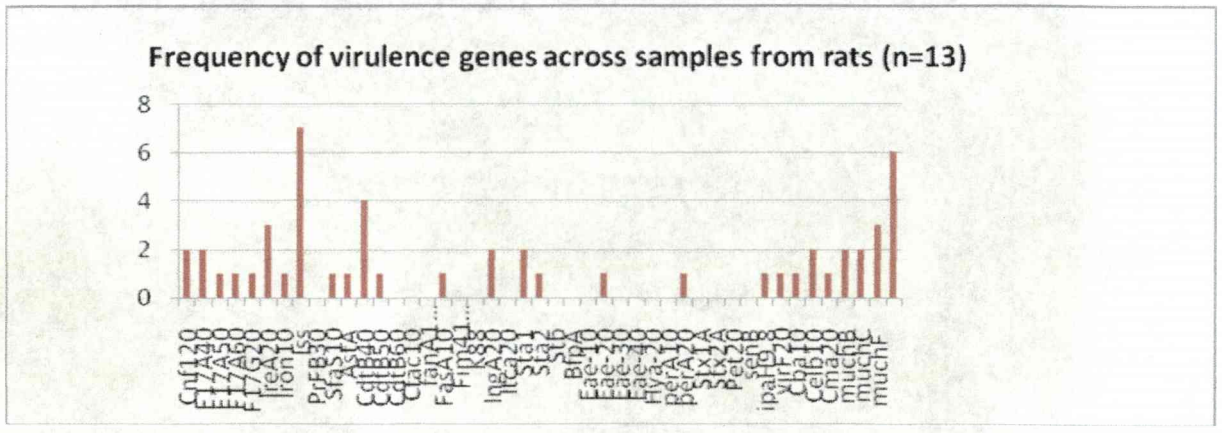


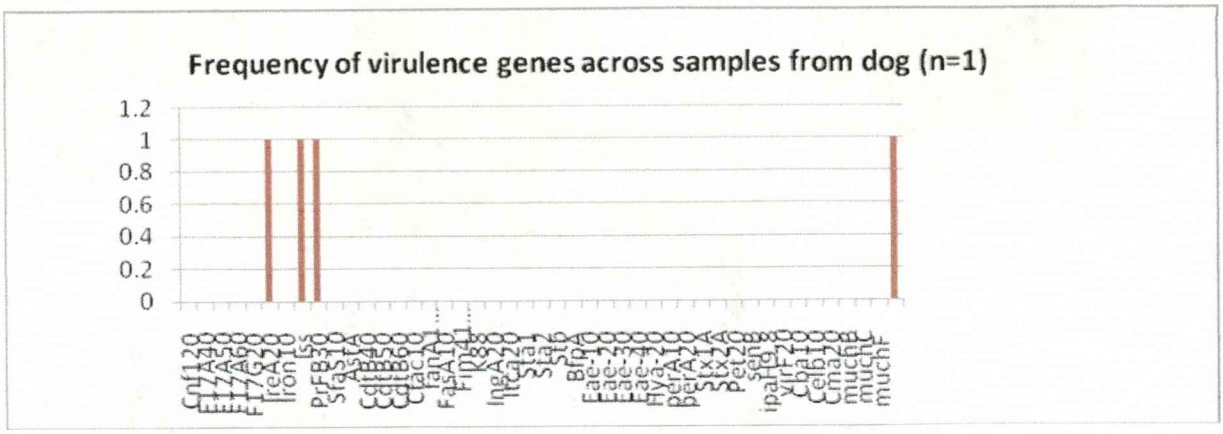


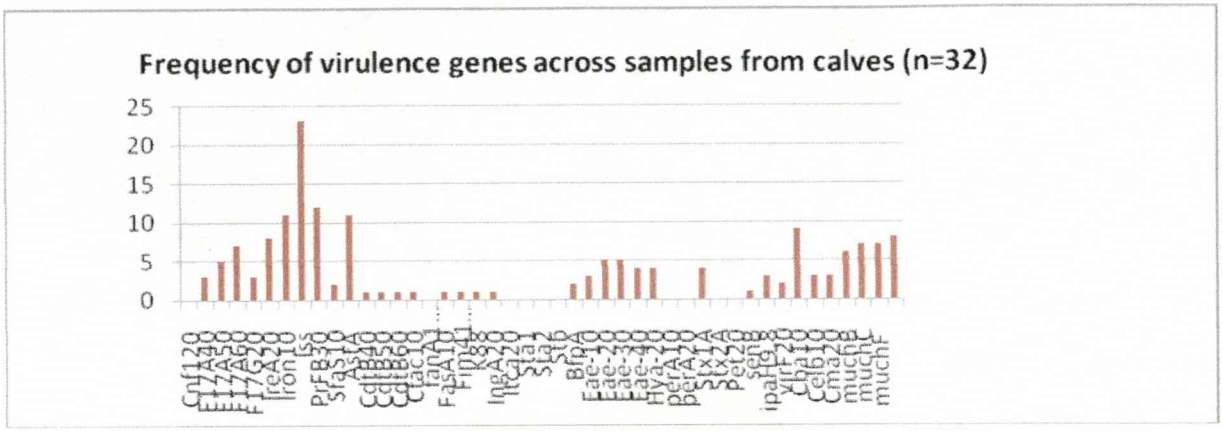

Frequency of virulence genes across samples from house mice (n=14)
10
8
6
4
2
0
Cnf1 20
EtxA 20
EtxA 50
EtxA 60
F17G 20
IreA 20
Iron 10
fss
PrfB 30
SfaS 10
AfaA
CdtB 40
CdtB 50
CdtB 60
Clac 10
fanA 1
FasA 10
FimA 21
FimG
IngA 20
IrgA 20
Sta 1
Sta 2
Stb
BfpA
Eae-10
Eae-20
Eae-30
Eae-40
Hya-10
BfrA 20
StxA
StxB
Pet 20
sen
ipaH9.8
VirF 20
Cba 10
Celb 10
CmaA 20
muchB
muchC
muchF

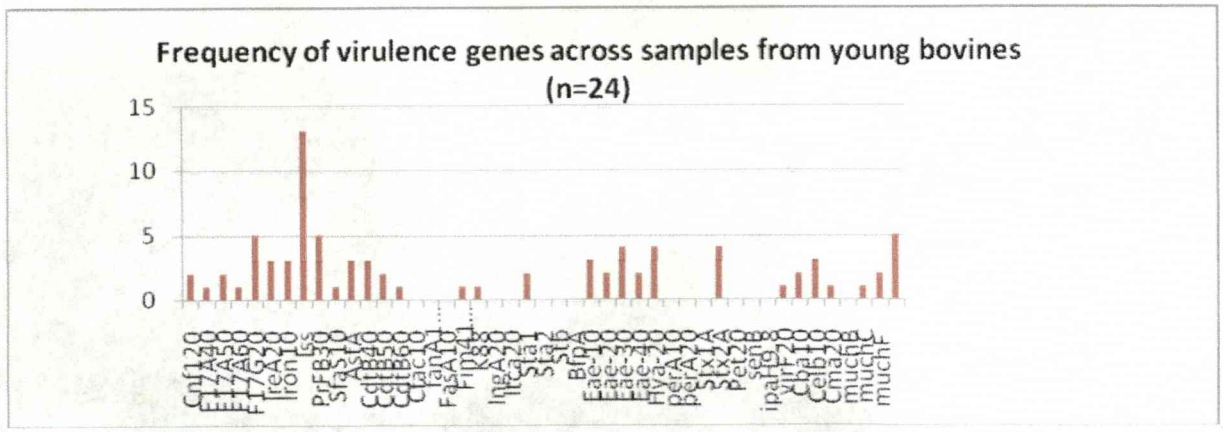

Frequency of virulence genes across samples from wood mice (n=43)
30
25
20
15
10
5
0
Cnf1 20
EtxA 20
EtxA 50
EtxA 60
F17G 20
IreA 20
Iron 10
fss
PrfB 30
SfaS 10
AfaA
CdtB 40
CdtB 50
CdtB 60
Clac 10
fanA 1
FasA 10
FimA 21
FimG
IngA 20
IrgA 20
Sta 1
Sta 2
Stb
BfpA
Eae-10
Eae-20
Eae-30
Eae-40
Hya-10
BfrA 20
StxA
StxB
Pet 20
sen
ipaH9.8
VirF 20
Cba 10
Celb 10
CmaA 20
muchB
muchC
muchF

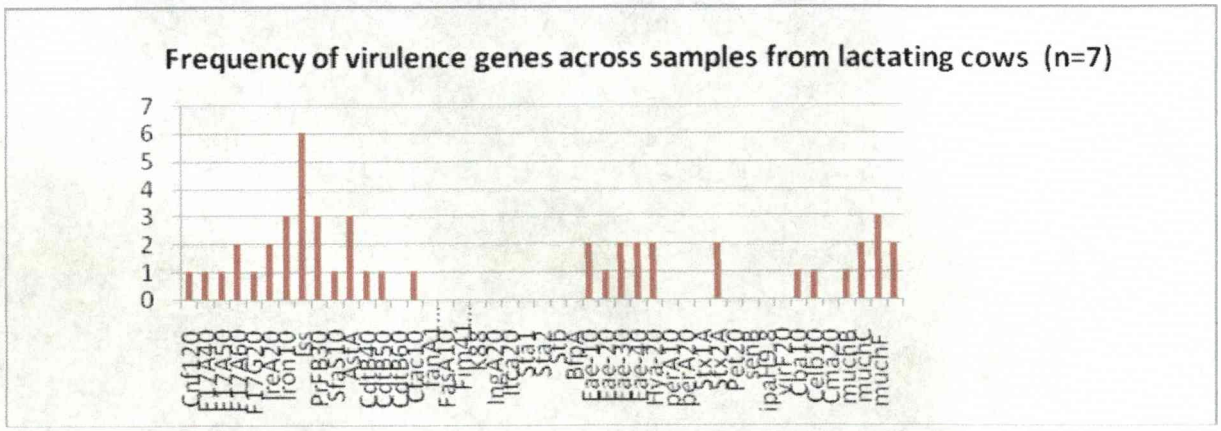

Frequency of virulence genes across samples from rabbits (n=13)
7
6
5
4
3
2
1
0
Cnf1 20
EtxA 20
EtxA 50
EtxA 60
F17G 20
IreA 20
Iron 10
fss
PrfB 30
SfaS 10
AfaA
CdtB 40
CdtB 50
CdtB 60
Clac 10
fanA 1
FasA 10
FimA 21
FimG
IngA 20
IrgA 20
Sta 1
Sta 2
Stb
BfpA
Eae-10
Eae-20
Eae-30
Eae-40
Hya-10
BfrA 20
StxA
StxB
Pet 20
sen
ipaH9.8
VirF 20
Cba 10
Celb 10
CmaA 20
muchB
muchC
muchF

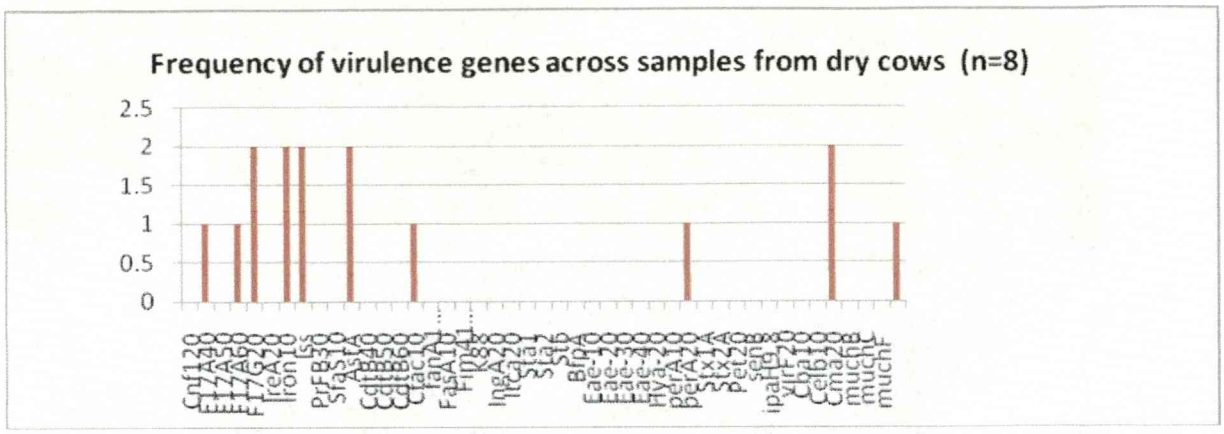

Frequency of virulence genes across samples from rats (n=13)
8
6
4
2
0
Cnf1 20
EtxA 20
EtxA 50
EtxA 60
F17G 20
IreA 20
Iron 10
fss
PrfB 30
SfaS 10
AfaA
CdtB 40
CdtB 50
CdtB 60
Clac 10
fanA 1
FasA 10
FimA 21
FimG
IngA 20
IrgA 20
Sta 1
Sta 2
Stb
BfpA
Eae-10
Eae-20
Eae-30
Eae-40
Hya-10
BfrA 20
StxA
StxB
Pet 20
sen
ipaH9.8
VirF 20
Cba 10
Celb 10
CmaA 20
muchB
muchC
muchF

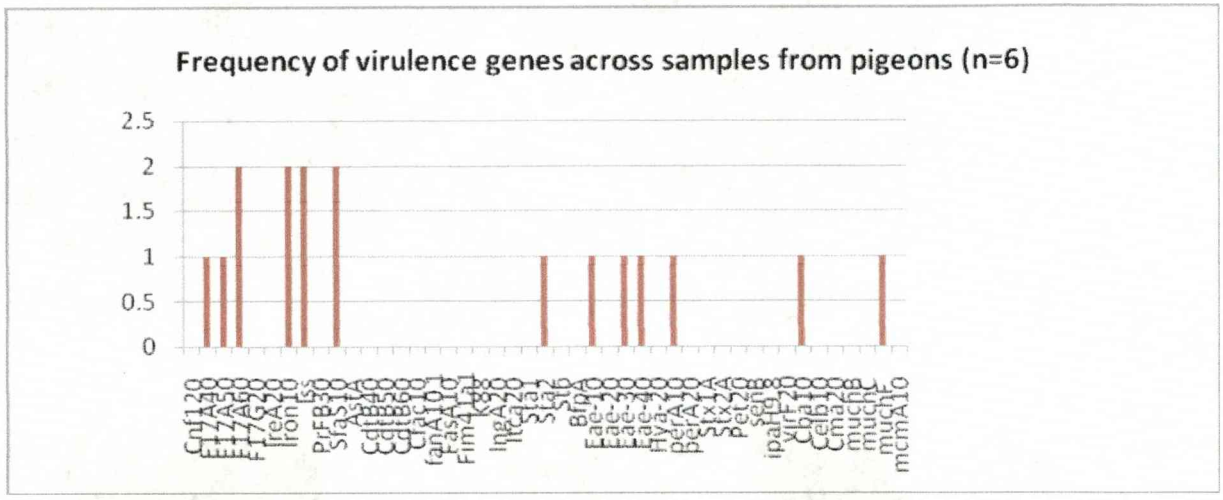

Frequency of virulence genes across samples from dog (n=1)
1.2
1
0.8
0.6
0.4
0.2
0
Cnf1 2a0
EtxA 3a0
EtxA 5a8
EtxA 6a8
F17G 2a0
IreA 2a0
Iron 1ss
PrfB 3a0
SfaS 1a0
AslA
CdtB 4a0
CdtB 5a8
CdtB 6a8
Clac 1a0
IanA 1
FasA 1a0
Fim 41
K88
IngA 2a0
IteA 2a1
Sta 1
Sta 2
Stb
BfpA
Eae-1a0
Eae-2a0
Eae-3a0
Eae-4a0
Hya-1a0
perA 1a0
perA 2a0
Stx 1A
Stx 2A
Pet 2a0
sen
ipaH 8
VirF 2a0
CbaI 1a0
CelB 1a0
Cma 2a0
muchB
muchC
muchF

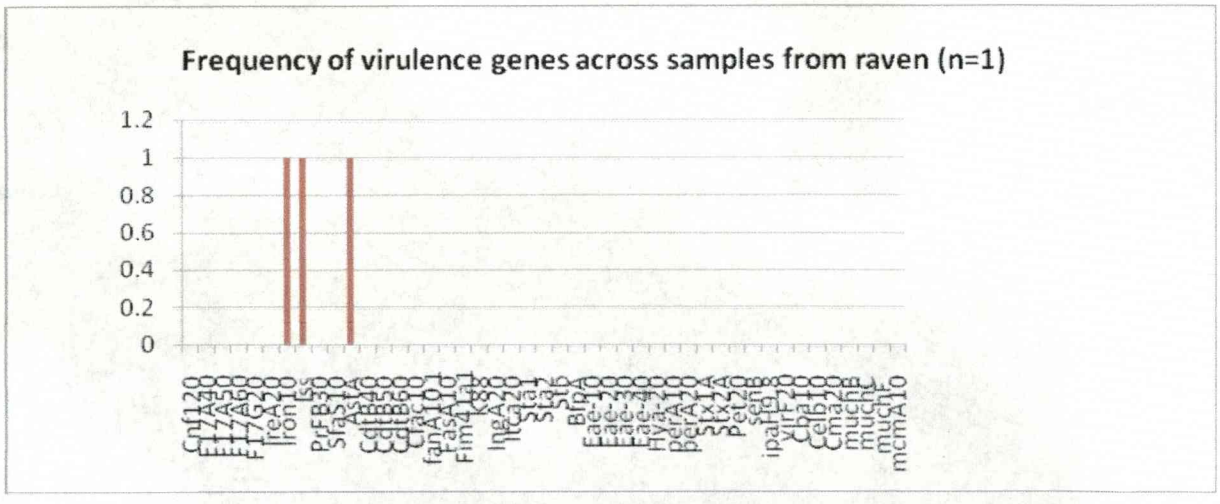

Frequency of virulence genes across samples from calves (n=32)
25
20
15
10
5
0
Cnf1 2a0
EtxA 3a0
EtxA 5a8
EtxA 6a8
F17G 2a0
IreA 2a0
Iron 1ss
PrfB 3a0
SfaS 1a0
AslA
CdtB 4a0
CdtB 5a8
CdtB 6a8
Clac 1a0
IanA 1
FasA 1a0
Fim 41
K88
IngA 2a0
IteA 2a1
Sta 1
Sta 2
Stb
BfpA
Eae-1a0
Eae-2a0
Eae-3a0
Eae-4a0
Hya-1a0
perA 1a0
perA 2a0
Stx 1A
Stx 2A
Pet 2a0
sen
ipaH 8
VirF 2a0
CbaI 1a0
CelB 1a0
Cma 2a0
muchB
muchC
muchF

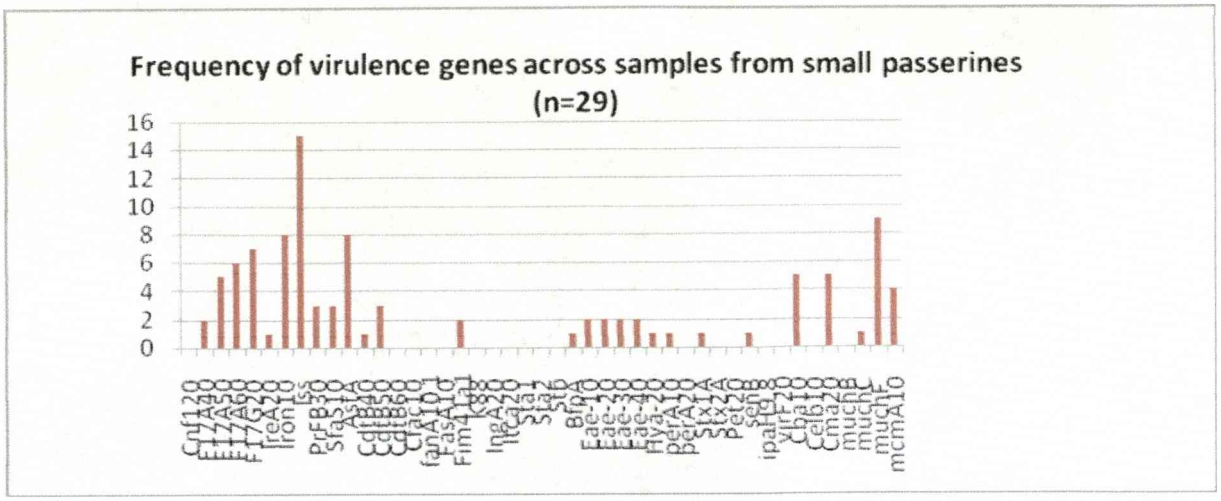

Frequency of virulence genes across samples from young bovines (n=24)
15
10
5
0
Cnf1 2a0
EtxA 3a0
EtxA 5a8
EtxA 6a8
F17G 2a0
IreA 2a0
Iron 1ss
PrfB 3a0
SfaS 1a0
AslA
CdtB 4a0
CdtB 5a8
CdtB 6a8
Clac 1a0
IanA 1
FasA 1a0
Fim 41
K88
IngA 2a0
IteA 2a1
Sta 1
Sta 2
Stb
BfpA
Eae-1a0
Eae-2a0
Eae-3a0
Eae-4a0
Hya-1a0
perA 1a0
perA 2a0
Stx 1A
Stx 2A
Pet 2a0
sen
ipaH 8
VirF 2a0
CbaI 1a0
CelB 1a0
Cma 2a0
muchB
muchC
muchF

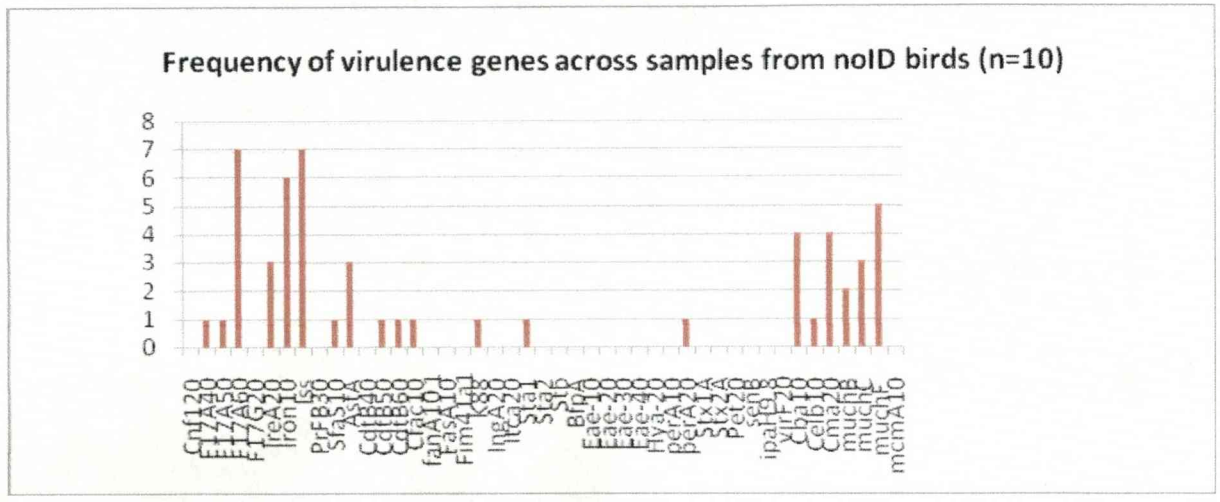

Frequency of virulence genes across samples from lactating cows (n=7)
7
6
5
4
3
2
1
0
Cnf1 2a0
EtxA 3a0
EtxA 5a8
EtxA 6a8
F17G 2a0
IreA 2a0
Iron 1ss
PrfB 3a0
SfaS 1a0
AslA
CdtB 4a0
CdtB 5a8
CdtB 6a8
Clac 1a0
IanA 1
FasA 1a0
Fim 41
K88
IngA 2a0
IteA 2a1
Sta 1
Sta 2
Stb
BfpA
Eae-1a0
Eae-2a0
Eae-3a0
Eae-4a0
Hya-1a0
perA 1a0
perA 2a0
Stx 1A
Stx 2A
Pet 2a0
sen
ipaH 8
VirF 2a0
CbaI 1a0
CelB 1a0
Cma 2a0
muchB
muchC
muchF


Frequency of virulence genes across samples from dry cows (n=8)
2.5
2
1.5
1
0.5
0
Cnf1 20
EtxA 30
EtxA 50
F17G 60
F17G 20
IreA 20
Iron 10
PrfB 30
SfaS 10
AslA
CdtB 40
CdtB 50
CdtB 60
Cfac 10
fanA 10
FasA 10
Fim4 10
K88
InaA 20
Intg 20
Sta1
Sta2
Stb
BfpA
Eae-10
Eae-30
Eae-40
Hya-20
PerA 10
PerA 20
Stx1A
Stx2A
Pet20
sen8
ipaH9
VirF20
Cba10
Ceb10
Cma20
mucB
mucC
mucF


Frequency of virulence genes across samples from pigeons (n=6)
2.5
2
1.5
1
0.5
0
Cnf1 20
EtxA 30
EtxA 50
F17G 60
F17G 20
IreA 20
Iron 10
PrfB 30
SfaS 10
AslA
CdtB 40
CdtB 50
CdtB 60
Cfac 10
fanA 10
FasA 10
Fim4 10
K88
InaA 20
Intg 20
Sta1
Sta2
Stb
BfpA
Eae-10
Eae-30
Eae-40
Hya-20
PerA 10
PerA 20
Stx1A
Stx2A
Pet20
sen8
ipaH9
VirF20
Cba10
Ceb10
Cma20
mucB
mucC
mucF
mcmA10


Frequency of virulence genes across samples from raven (n=1)
1.2
1
0.8
0.6
0.4
0.2
0
Cnf1 20
EtxA 30
EtxA 50
F17G 60
F17G 20
IreA 20
Iron 10
PrfB 30
SfaS 10
AslA
CdtB 40
CdtB 50
CdtB 60
Cfac 10
fanA 10
FasA 10
Fim4 10
K88
InaA 20
Intg 20
Sta1
Sta2
Stb
BfpA
Eae-10
Eae-30
Eae-40
Hya-20
PerA 10
PerA 20
Stx1A
Stx2A
Pet20
sen8
ipaH9
VirF20
Cba10
Ceb10
Cma20
mucB
mucC
mucF
mcmA10


Frequency of virulence genes across samples from small passerines (n=29)
16
14
12
10
8
6
4
2
0
Cnf120
EtxA30
EtxA60
F17G20
IreA20
Iron Fss
PrfB30
SfaS10
AslA
CdtB40
CdtB50
CdtB68
CtaE
fanA10
FasA10
Fim41a1
K88
IngA20
IroA21
Sta1
Stb
Stx2
BfpA
Eae-10
Eae-30
Eae-4
HlyA20
perA10
perA20
Stx1A
Stx2A
Pet20
sen
ipaH9.8
YirF20
Cba10
Celb10
Cma20
mucNB
mucNC
mucNE
mcmA10


Frequency of virulence genes across samples from noID birds (n=10)
8
7
6
5
4
3
2
1
0
Cnf120
EtxA30
EtxA60
F17G20
IreA20
Iron Fss
PrfB30
SfaS10
AslA
CdtB40
CdtB50
CdtB68
CtaE
fanA10
FasA10
Fim41a1
K88
IngA20
IroA21
Sta1
Stb
Stx2
BfpA
Eae-10
Eae-30
Eae-4
HlyA20
perA10
perA20
Stx1A
Stx2A
Pet20
sen
ipaH9.8
YirF20
Cba10
Celb10
Cma20
mucNB
mucNC
mucNE
mcmA10

Appendix IV. Description, genetic location and *E. coli* pathotypes association of the different virulence genes targeted by microarrays.

Gene symbol	**Location**	**Description**	***E. coli* pathotype**
cnf1	chromosome, pathogenicity island	Cytotoxic necrotizing factor 1	UPEC
f17A(4,5,6)	Chromosome, Vir plasmid	Subunit A of major fimbrial protein	ETEC,UPEC
f17G (2)	Chromosome, Vir plasmid	Adhesin subunit of fimbrial protein	ETEC,UPEC
ireA	pathogenicity island?	Siderophore receptor	UPEC
iroN	plasmid	Enterobactin siderophore receptor protein	UPEC, APEC
iss	chromosome ,plasmid	Increased serum survival	UPEC,APEC
prfB	chromosome	P-related fimbriae regulatory gene	UPEC
sfaS	pathogenicity island	Adhesion, minor Shigella fimbriae subunit	UPEC
astA	plasmid	Heat stable enterotoxin	EAEC,ETEC,EHEC, APEC
cdtB(4,5,6)	chromosome?	Cytolethal distending toxinB	EPEC,STEC,ETEC,EXPEC
cfa	plasmid	Colonisation factor antigen I	ETEC
fanA	plasmid	Involved in biogenesis of k99 fimbriae	ETEC
fasA	chromosome	Fimbriae 987P subunit	ETEC
fim41a	unknown	Mature Fim41a protein	ETEC
k88	plasmid?	K88 protein subunit gene	ETEC
ingA	plasmid	Longus pili gene	ETEC
itcA	plasmid	Heat labile enterotoxin subunit A	ETEC
sta (1,2)	plasmid	Heat stable enterotoxinI	ETEC
stab	plasmid	Heat stable enetrotoxin II	ETEC
bfpA	plasmid	Major subunit of bundle forming pili	EPEC
eae (1,2,3,4)	Pathogenicity island	intimin	EPEC, EHEC
hylA	plasmid	Haemolysim A	EPEC,EHEC,UPEC
perA (1,2)	plasmid	EPEC adherence factor	EPEC
vt1A	bacteriophage	Verotoxin 1 A subunit	VTEC
vt2A	bacteriophage	Verotoxin 2 A subunit	VTEC
pet	plasmid	Autotransporter enterotoxin	EAEC

Gene symbol	**Location**	**Description**	***E. coli* pathotype**
senB	plasmid	Plasmid encoded enterotoxin	EIEC
ipaH9.8	plasmid	Invasion plasmid	*Shigella*
virF	pathogenicity island	VirF transcriptional activator , ipaBCD positive regualtor	*Shieglla flexneri*
cba	plasmid	Colicin B-pore forming	Undisgnated
celb	plasmid	Endonuclease colicin E2	Undesignated
cma	plasmid	Colicin-M resembles B-lactam	Undesignated
mchB	plasmid	microcitinH47 part of colicin H	Undesignated
mchC	plasmid	MchC protein	UPEC,Undesignated
mchF	plasmid	ABC transporter protein MchF	Undesignated
mcmA	plasmid	Microcitin M part of colicin H	Undesignated

AppendixV. Probes and primers sequences used in the microarrays for detection of virulence genes associated with *E. coli*.

Probes/genes	Target Accession no	Control strain/origin or reference	Probe sequence (5'-3')	Primer sequence (5'-3')
astA-11 *astA*-21	AE005345.1	Abbotstown	TCgTgCATATGGTGCgCAACAG	GACGGCTTTGTAgTCCTTCC TACGGCTTTGTAtTCCTTCC
bfpA	AB024946.1	E2348/69	GGTGTGATGTTTTACTACCAGTCTGC	CGTCATTACTTCTGAAATaGCA
cba	M16816.1	E2334/03	GGATGGTCTGTCAGTGTGCATAGC	GCGGAAACTTTCTCGTTTCC
*cdtB*_40 *cdtB*_50	AJ508930.1	EC934/04	GCTGTTGATGCCtTtGGTGGAAG GCTGTTGATGCCcTtGGTGGAAG	GCTAACCAGAGCAAGATTGAC
celb	X03632.1	EC2334/03	GGACCGTATCTCCGTCATCAACAG	GCGTTGCTAATCCGGTCAC
cfaC	M55661.1	IMI100	GGAATAGCGCGCTGGGTATTACAGA	TCATCCACCAATTTAAGACAGC
cma	M16754.1	EC2334/03	TGTAACGCCACCGAAATCTGGT	TCATAAACGCTTATTCCAGGGT
cnf	AF483828.1	S5	CTTCCAGTATGGGGATCAGTTTTGATCA	CGACGTTCTTCATAAGTATCACC
*eae*_10 *eae*_20 *eae*_30 *eae*_40	AJ579371.1	E2348/69	GTTACAaCaTTATGGAACGGCAGAGGT GTTACAaCgTTATGGAACGGCAGAGG TGGTgAtAATACCCGtTTAGGtATtATtGGt TGGTgAtAATACCCGcTTAGGtATtATtGG	CgTCAAAGTTATtACCaCTCTGC AGTcTCGCCAgTATTCgC
*f17A*_40 *f17A*_50 *f17A*_60	AF022140.1	CK210, S5	ggTAcTAtGCaACgGgtcaGGC CagTAcTAcGCaACgGgtgtGG aCaaTAtTAtGCcACaGcgccGG	TGATAAgCGATGGTGTAATTcACaG TGATAAgCGATGGTGTAATTaACtG CTGATAAaCGATGGTGTAATTtACtG
f17G	AF022140.1	CK210	TGCAATGGATAACCTGCCATTTGTCT	CCAGACATTTGCATTCTGATATCC
fanA	X05797.1	ETEC562	AGCAAGGTGCTTCCAATTATTAGTGGA	CGTAAATACCCCTAGAACTACGT
fasA	M35257.1	HM1535	GCCAAGTGGATACTTCTAATCTGTCGC	GAGCAGAAGTAGACAACTCTCC
fim41a	X14354.1	ETEC562	GGCTTGTTAATCCAGGTCGATTTACTG	GAGAGTCCATTCCATTTATAGGCT
gad	M84025.1	all	GATATCGTCTGGGACTTCCGCCT	TGAAGCACTGATCGATTTCACA
ehx(hlyA)	AB011549.2	EDL933	TGTAGGATTAACTGAACGTGGTGTTGC	GCAGAAGTTTGTCAAGTTGTGG
ipaH9.8	AF047365.1	NCTC8192	TCGCGCTCACATGGAACAATCT	GCCTGATGGACCAGGAGG
ireA	AF320691.1	CFT073	CCACAAATGACTTCTATCTGTCAGGC	CTCCATATAGCTGAAGACCAAGT
iroN	AF449498.1	CFT073	GCCTGTCGAGTAACATGATCAATGCT	GAGGCTTTGCGAAGTGAGC
iss	AF042279.1	CFT073	CCGCTCTGGCAATGCTATTACAGG	gGTTTGTTTccAACAGTAAACGT
K88	V00292.1	Abbotstown	GCCTGGATGACTGGTGATTTCAATGG	GTGATACTACCACCGATATCGAC

lngA	AF004308.1	B1308	CGTCTGGTTCATATGCATGACAGC	CCACAGACATATCTACACCAGT
lthA	AB011677.1	ETEC21d	GGTTTCTGCGTTAGGTGGAATACCA	ACCAAAATTAACACGATACCATCC
mchB	AJ515252.1	CFT073	GGTTGTAGTTGGAGCCGTATCTGC	GGTCGAGCCAATTGCTGT
mchC	AJ515252.1	CFT073	CTGTCGGGTTAGATCTGTGATCCAC	CCGGTGGTACAGGTAGATATCC
mcmA	AJ515252.1	CFT073	CCTCCATGTCTCCCTCAGGTATAGG	GGCACTTGATGTACCTCTGC
*perA*_10 *perA*_20	AF255772.1	E2348/69	TGTTTGGTTGGGTTTAATTCCACATCA GCTTGGTTGGTTTTAATTCCACGTC	TTGGTGTTGTGTTGTAATATTCCT
pet	AF056581.1	NZ1470-95	GCTGACAAGGATAATTCTGCCACAAGA	GCATCGCGAGAGCAAACT
prfB/papB	X76613.1	CFT073	GGGAGACTTATACGGCTGAATGCTC	TCATCTGTATAATAAGGTGGTGCAAGC
senB	Z54195.1	NCTC9774	GCTCTATATCGGACACACCCAGTCAG	GGTGTCAAACATACTGATACGC
sfaS	X16664.4	E536	CAATGCAGGAAGTGGATCTCCATGG	TCCGGTGAGAGACAGATCA
*staIA*_111 *staIA*_121	AJ555214.1	ETEC562	ACACATTTTACTGCTGTGAACTTTGTTG	AACATggAGCACAGGCAG AACATccAGCACAGGCAG
staIB	STAY342058	IMI 100	AGCAATTACTGCTGAATTGTGTTGT	AGCACCCGGTACAAGCAG
stb	AJ555214.1	Abbotstown	GAGATGGTACTGCTGGAGCATGCT	TTGCTGCAACCATTATTTGGG
vt1	AB035142.1	EDL933	GTGACAGTAGCTATACCACGTTACAGC	TCTGCATCCCCGTACGAC
vt2	AB035143.1	EDL933	GCAGTTATTACCACTCTGCAACGTGTC	CtgAttTGCATtCCgGaACG
virF	AF386526.1	NCTC8192	GCCTTTTATCAGCTGTTTCTGATGAGGA	GAGAAGAAGCTATCGATATCGAAGT
rrl_0101_0177_10	M25458.1	E2348/69	GTGTGTTTCGACACACTATCATTAACTGA	GGTTCGCCTCATTAACCTATGG
rrl_0101_0177_20	M25458.1	E2348/69	GTGTGATTCGTCACACTATCATTAACTGA	
rrl_0260_0330_10	M25458.1	E2348/69	CAGAGCCTGAATCAGTATGTGTGTTAGT	GCCTTTCCAGACGCTTCC
rrl_0260_0330_20	M25458.1	E2348/69	GAGCCTGAATCAGTGTGTGTGTTAGT	
rrl_0260_0330_30	M25458.1	E2348/69	AGAGCCTGAATCAGTTTGTGTGTTAGT	
rrl_0520_0580_10	M25458.1	E2348/69	GCAGTGGGAGCACGCTTAGG	AAGGTACGCAGTCACACG
rrl_0520_0580_20	M25458.1	E2348/69	AAGCAGTGGGAGCATGCTTAGG	
rrl_1480_1560_coli_10	M25458.1	E2348/69	CCGGAAAATCAAGGATGAGGCGTG	CACCGTAGTGCCTCGTCA
rrl_1480_1560_coli_20	M25458.1	E2348/69	CGGAAAATCAAGGCTGAGGCGTG	
rrl_1480_1560_coli_30	M25458.1	E2348/69	GGAAAACCAAGGCTGAGGCGTG	
rrl_1480_1560_shig_40	M25458.1	E2348/69	GGAAAATCAAGGCCGAGGCGTG	
rrl_1690_1770_coli_10	M25458.1	E2348/69	GCTGATATGTATGTAGGTGAAGCGACTTGC	CGACTGATTTCAGCTCCACG
rrl_1690_1770_freu_30	M25458.1	E2348/69	CGCTGATATGTAGGTGAAGTGGTTTACT	
rrl_1690_1770_shig_20	M25458.1	E2348/69	GCTGATACGTAGGTGAAGCGATTG	

Appendix VI- Chemical buffers and reagents used in the microarrays technique.

Reagent	Volume	pH	Commercial Origin
3DNA/SDS Buffer	**100 ml**	**7.2**	
1M NaPOi	25ml		
20%SDS	22.5 ml		
0.5M EDTA	200µl		Sigma
20xSSC	5ml		
Sterile water	47ml		
1M NaPOi	**1L**	**7.2**	
Sodium phosphate dibasic (anhydrous)	141.96g		Sigma
20 x SSC	**100 ml**	**7.0**	
Sodium chloride	17.53g		Sigma
Sodium citrate	8.82g		Sigma
Sterile water	100 ml		
2 x SSC+0.01% Triton	**100ml**		
20 x SSC	10ml		
Triton x 100	10µl		Sigma
Sterile water	90ml		
2 x SSC	**100ml**		
20 x SSC	10ml		
Sterile water	90ml		
0.2 x SSC	**100ml**		
20 x SSC	1 ml		
Sterile water	99ml		
10 x SSPE	**200 ml**	**7.4**	
Sodium chloride	17.53g		
Sodium dihydrogen phosphate (Na H_2PO_4)	2.76g		Sigma
EDTA	0.74g		
Sterile water	200ml		
6 x SSPE 0.005% Triton	**100ml**		
10 x SSPE	60ml		
Triton x 100	5µl		
Sterile water	40ml		
Poly-HRP-streptavidin	**1mg/ml**		Pierce
True blue			Insight biotechnology

Reagent	**Commercial origin**
Seramun green	Seramun diagnostic GmbH
Therminator buffer polymerase	NEB
Primer mix	Clondiag
dNTPs	Clondiag
Biotin-16-dUTP	Roche

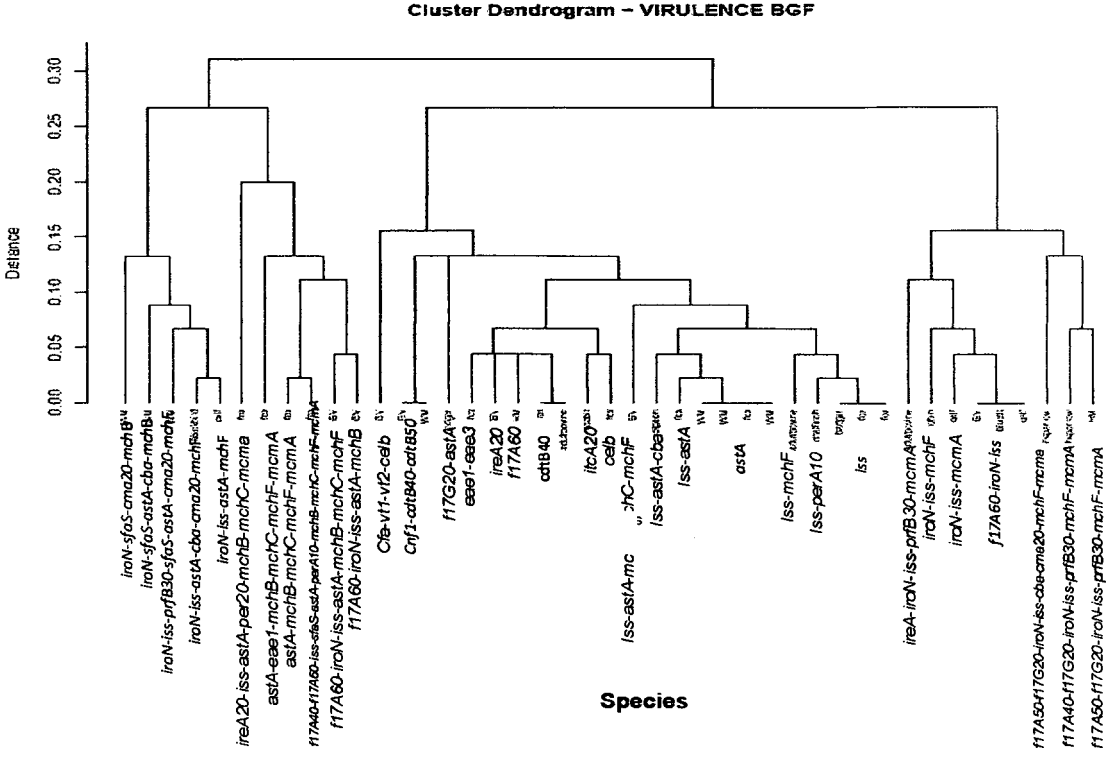
Appendix VII(b). Virulence gene frequency in *E. coli* isolates from wild bird species tested with microarrays.

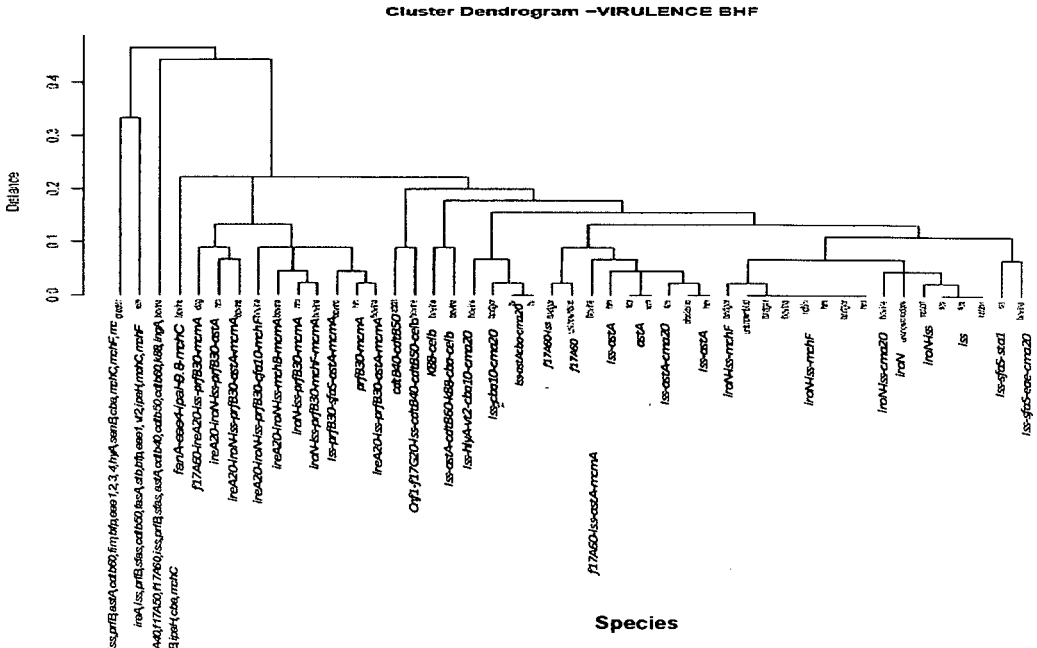
Gene/Host	Pigeons(6)	Raven(1)	Passerines(29)	Unknownbird(10)
Cnf120	0	0	0	0
F17A40	1	0	2	1
F17A50	1	0	5	1
F17A60	2	0	6	7
F17G20	0	0	7	0
IreA20	0	0	1	3
Iron10	2	1	8	6
Iss	2	1	15	7
PrFB30	0	0	3	0
SfaS10	2	0	3	1
AstA	0	1	8	3
CdtB40	0	0	1	0
CdtB50	0	0	3	1
CdtB60	0	0	0	1
Cfac10	0	0	0	1
fanA10	0	0	0	0
FasA10	0	0	0	0
Fim41a10	0	0	2	0
K88	0	0	0	1
IngA20	0	0	0	0
Itca20	0	0	0	0
Sta1	0	0	0	1
Sta2	1	0	0	0
Stb	0	0	0	0
BfpA	0	0	1	0
Eae-10	1	0	2	0
Eae-20	0	0	2	0
Eae-30	1	0	2	0
Eae-40	1	0	2	0
Hya-20	0	0	1	0
perA10	1	0	1	0
perA20	0	0	0	1
Stx1A	0	0	1	0
Stx2A	0	0	0	0
Pet20	0	0	0	0
senB	0	0	1	0
ipaH9.8	0	0	0	0
virF20	0	0	0	0
Cba10	1	0	5	4
Celb10	0	0	0	1
Cma20	0	0	5	4
muchB	0	0	0	2
muchC	0	0	1	3
muchF	1	0	9	5
mcmA10	0	0	4	0

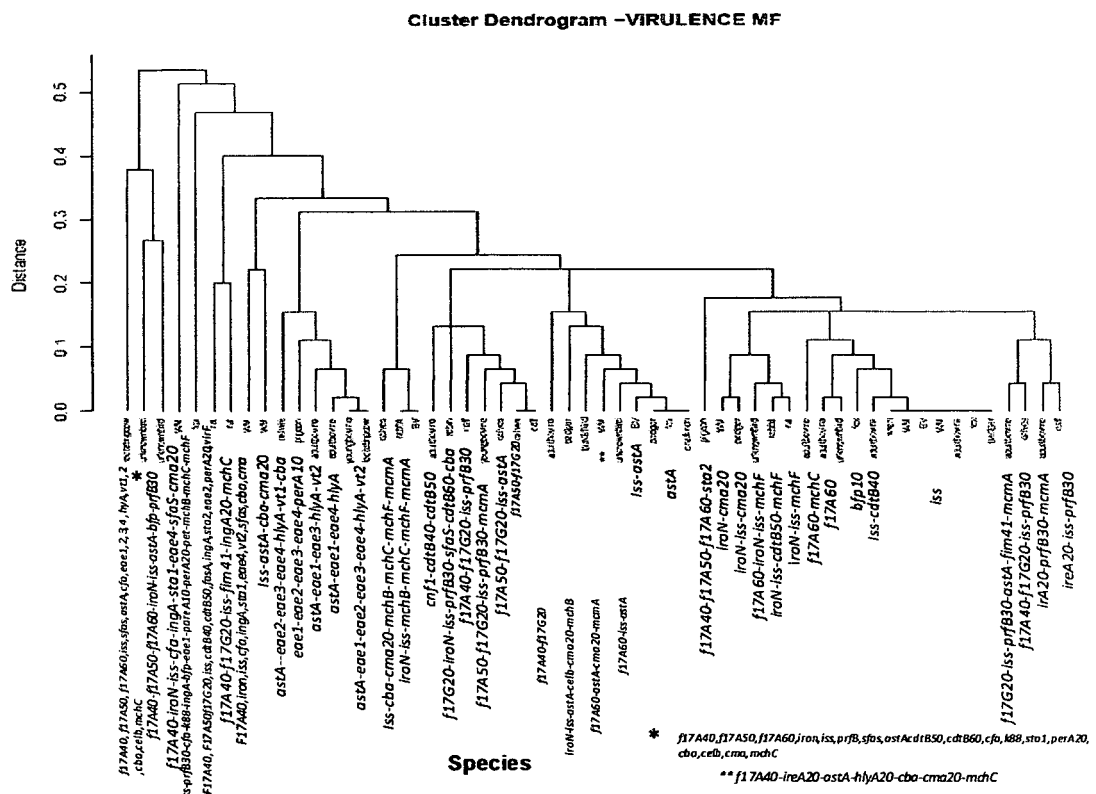
AppendixVII (a). Virulence gene frequency in *E. coli* isolates from different terrestrial animal hosts tested with microarrays.

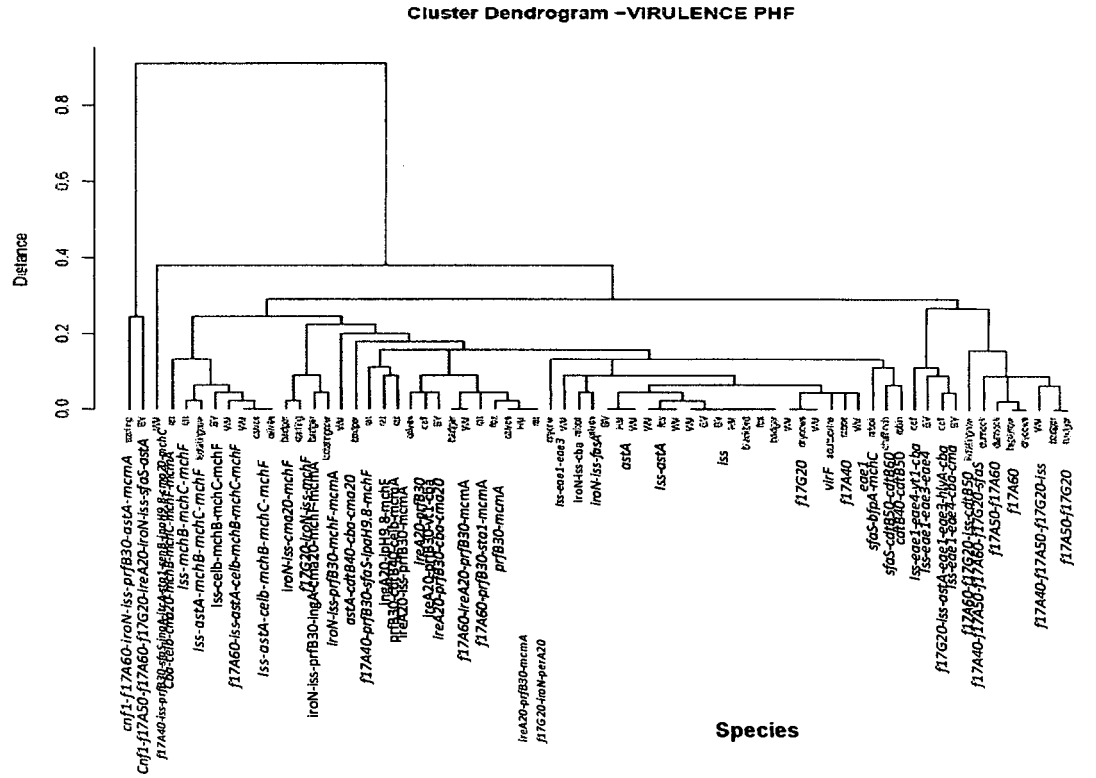
Gene/Host(n)	Calves(32)	Youngstock(24)	Lactating(7)	Dry(8)	Badger(23)	Fox(26)	Rabbit(13)	Bankvole(27)	Hmouse(14)	Wmouse(43)	Rat(13)	noIDrodent(2)	Dog(1)
Cnf120	0	2	1	0	0	0	0	1	1	1	2	0	0
F17A40	3	1	1	1	2	1	1	1	0	7	2	0	0
F17A50	5	2	1	0	2	0	0	0	1	3	1	0	0
F17A60	7	1	2	1	2	0	1	3	2	5	1	0	0
F17G20	3	5	1	2	3	0	0	0	1	4	1	0	0
IreA20	8	3	2	0	1	1	1	4	2	4	3	0	1
Iron10	11	3	3	2	9	2	4	3	4	6	1	2	0
Iss	23	13	6	2	18	13	6	17	9	19	7	1	1
PrFB30	12	5	3	0	5	4	0	3	5	5	0	0	1
SfaS10	2	1	1	0	1	1	1	1	0	5	1	0	0
AstA	11	3	3	2	3	10	1	9	4	24	1	0	0
CdtB40	1	3	1	0	0	0	1	1	0	3	4	0	0
CdtB50	1	2	1	0	0	1	2	1	0	2	1	0	0
CdtB60	1	1	0	0	0	0	0	1	0	2	0	0	0
Cfac10	1	0	1	1	0	1	0	1	0	2	0	0	0
fanA10	0	0	0	0	1	0	0	0	0	0	0	0	0
FasA10	1	0	0	0	0	0	0	0	0	1	1	0	0
Fim41a10	1	1	0	0	0	0	0	0	0	0	0	0	0
K88	1	1	0	0	0	1	0	0	0	2	0	0	0
IngA20	1	0	0	0	1	1	0	1	0	3	2	0	0
Itca20	0	0	0	0	0	1	1	1	1	1	0	0	0
Sta1	0	2	0	0	0	0	0	1	0	3	2	0	0
Sta2	0	0	0	0	0	0	0	2	1	0	1	0	0
Stb	0	0	0	0	0	0	0	1	0	2	0	0	0
BfpA	2	0	0	0	0	2	1	0	0	2	0	0	0
Eae-10	3	3	2	0	0	3	0	2	0	4	0	0	0
Eae-20	5	2	1	0	0	0	0	1	0	0	1	0	0
Eae-30	5	4	2	0	0	1	0	2	0	1	0	0	0
Eae-40	4	2	2	0	1	0	0	3	0	1	0	0	0
Hya-20	4	4	2	0	0	0	0	0	0	1	0	0	0
perA10	0	0	0	0	0	2	0	1	0	1	0	0	0
perA20	0	0	0	1	0	2	0	0	0	2	1	0	0
Stx1A	4	0	0	0	0	0	1	1	0	0	0	0	0
Stx2A	0	4	2	0	0	0	0	1	0	3	0	0	0
Pet20	0	0	0	0	0	1	0	0	0	0	0	0	0
senB	1	0	0	0	0	0	0	0	0	2	0	0	0
ipaH9.8	3	0	0	0	2	0	0	0	0	2	1	0	0
virF20	2	1	0	0	0	0	0	0	0	0	1	0	0
Cba10	9	2	1	0	0	1	2	5	0	8	1	0	0
Celb10	3	3	1	0	1	1	0	1	0	2	2	0	0
Cma20	3	1	0	2	3	2	1	5	0	11	1	0	0
muchB	6	0	1	0	2	7	1	4	0	4	2	0	0
muchC	7	1	2	0	2	7	2	5	0	6	2	0	0
muchF	7	2	3	0	8	7	2	4	3	6	3	1	0

Appendix VIII. Dendrogram of *E. coli* isolates with their virulence gene profiles tested with microarrays in the five remaining farms-Chapter 5.






Cluster Dendrogram –VIRULENCE MF
Distance
0.0
0.1
0.2
0.3
0.4
0.5
Species
* f17A40,f17A50,f17A60,iron,iss,prfB,sfas,ostAcdtB50,cdtB60,cfa,k88,sta1,perA20, cba,celb,cma, mchC
** f17A40-ireA20-ostA-hlyA20-cba-cma20-mchC


Cluster Dendrogram –VIRULENCE PHF
Distance
0.0
0.2
0.4
0.6
0.8
Species

Appendices

Chapter 6

Appendix I. Selection of antimicrobial resistance genes for inclusion on the microarrays. The probes and primer sequences of genes are listed together with the target gene accession number, gene description and genotypic characteristics also included.

Target	Resistance phenotype or gene description	Target gene accession no.	Control strain	Location	Probe sequence (5'-3')	Primer sequence (5'-3')
teml	β-lactamase	AF309824	Co34	plasmid	CGAACTACTTACTCTAGCTTCCCGGCAA	TATCCGCCTCCATCCAGT
aadA1	aminoglocoside	AY125351	99-2175	integron (cassettes)	AGATTCTCCGCGCTGTAGAAGTACC	TTATGTCGTCGTGCACA
Tet-A	tetracycline	X75761	NCTC50269	plasmid/transposon	CTCATGCTCGGAATGATTGCCGACG	AGCAGGATGTAGCCTGTG
Tet-B	tetracycline	AF223162	NCTC50269	plasmid/transposon	CGTTTGCTTTCAGGGATCACAGGAGC	GGTATCGGCAATGACCGA
Intl1	Class1 integrase	AY260546	P3170700	plasmid	CCATTCCGACGTCTCTACGACGATGA	CTTTCAGCACATGCGTGT
sul1	sulphonamide	X12869	00-419	plasmid	CCTTCCTGTAAAGGATCTGGTCCAGC	CGATCGCGTGAAGTTCC
catA1	Chloramphenicol acetyltransferase	AP000342	99-2175	integron	CGTCTCAGCCAATCCCTGGGTGAG	GTTGTCCATATTGGCCACG
drf12	trimethropin	Z21672	P5061800	plasmid	CAGTACGCATTTATCTCGTTGCTGCGA	TTGCCAATAACCCGATTGG
sul3	sulphonamide	AJ459418	01-2571	plasmid	GCTCTGCATTTGGTTGAAGATGGAGCA	CGGCTCCCAAATCAATCAC
drfA1	trimethroprim	AY146989	NCTC50535	plasmid	CAATAGACATCGAGCCGGAAGGTGATG	ACTGGCCTAAAATTGCTGG

Appendices

General

Appendix I. the common and scientific name of the species of wild birds of which faecal samples were collected as part of the cross-sectional study.

Common Name	Scientific Name
Blackbird	*Turdus merula*
Blue Tit	*Cyanistes caeruleus*
Bullfinch	*Pyrrhula pyrrhula*
Buzzard	*Buteo buteo*
Chaffinch	*Fringilla coelebs*
Chiffchaff	*Phylloscopus collybita*
Coal Tit	*Periparus ater*
Dunnock	*Prunella modularis*
Feral Pigeon	*Columba livia*
Goldcrest	*Regulus regulus*
Goldfinch	*Carduelis carduelis*
Great Tit	*Parus major*
Great Spotted Woodpecker	*Dendrocopos major*
Greenfinch	*Carduelis chloris*
House Sparrow	*Passer domesticus*
Jay	*Garrulus glandarius*
Jackdaw	*Corvus monedula*
Long-tailed Tit	*Aegithalos caudatus*
Magpie	*Pica pica*
Meadow Pipit	*Anthus pratensis*
Nuthatch	*Sitta europaea*
Pied Wagtail	*Motacilla alba*

Raven	*Corvus corax*
Redwing	*Turdus iliacus*
Robin	*Erithacus rubecula*
Song Thrush	*Turdus philomelos*
Starling	*Sturnus vulgaris*
Swallow	*Hirundo rustica*
Treecreeper	*Certhia familiaris*
Tree Sparrow	*Passer montanus*
Willow Warbler	*Phylloscopus trochilus*
Woodpigeon	*Columba palumbus*
Wren	*Troglodytes troglodytes*

Appendix II. Common and scientific names of wild mammals of which faecal samples were collected as part of the cross-sectional study.

Common Name	Scientific Name
Badger	*Meles meles*
Bank Vole	*Myodes glareolus*
Brown Rat	*Ratus norvergicus*
Field Vole	*Microtus agrestis*
Fox	*Vulpes vulpes*
House Mouse	*Mus musculus*
Pygmy Shrew	*Sorex minutus*
Rabbit	*Oryctolagus cuniculus*
Wood Mouse	*Apodemus sylvaticus*

List of abbreviations

BTO - British trust of ornithology

CDC - Centers for disease control and prevention

CFU - Colony forming unit

DEFRA - Department of environment, food and rural affairs

DNA -Deoxyribonucleic acid

EFSA - European food safety authority

EU - European Union

HPA - Health protection agency

MLST - Multi locus sequence typing

OIE - Office international des epizooties

PCR - Polymerase chain reaction

PFGE - Pulsed field gel electrophoresis

RNA - Ribonucleic acid

23S rRNA - 23S ribosomal nucleic acid

UK - United Kingdom

VLA - Veterinary laboratories agency

VMD - Veterinary medicine directorate

WHO - World health organisation