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Escherichia coli isolates from extraintestinal organs of livestock animals harbour diverse virulence genes and belong to multiple genetic lineages

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Abstract

Escherichia coli, the most common cause of bacteraemia in humans in the UK, can also cause serious diseases in animals. However the population structure, virulence and antimicrobial resistance genes of those from extraintestinal organs of livestock animals are poorly characterised. The aims of this study were to investigate the diversity of these isolates from livestock animals and to understand if there was any correlation between the virulence and antimicrobial resistance genes and the genetic backbone of the bacteria and if these isolates were similar to those isolated from humans. Here 39 *E. coli* isolates from liver (n=31), spleen (n=5) and blood (n=3) of cattle (n=34), sheep (n=3), chicken (n=1) and pig (n=1) were assigned to 19 serogroups with O8 being the most common (n=7), followed by O101, O20 (both n=3) and O153 (n=2). They belong to 29 multi-locus sequence types, 20 clonal complexes with ST23 (n=7), ST10 (n=6), ST117 and ST155 (both n=3) being most common and were distributed among phylogenetic group A (n=16), B1 (n=12), B2 (n=2) and D (n=9). The pattern of a subset of putative virulence genes was different in almost all isolates. No correlation between serogroups, animal hosts, MLST types, virulence and antimicrobial resistance genes was identified. The distributions of clonal complexes and virulence genes were similar to other extraintestinal or commensal *E. coli* from humans and other animals, suggesting a zoonotic potential. The diverse and various combinations of virulence genes implied that the infections were caused by different mechanisms and infection control will be challenging.

1. Introduction

Escherichia coli can be a commensal organism or a causative agent of diarrhoea or extra-intestinal infections-responsible for an estimated 120 million cases of community-acquired urinary tract infections (UTI) diagnosed worldwide annually. It can also cause neonatal meningitis, pneumonia and surgical site infections. The sepsis-associated mortalities due to *E. coli* are estimated at 868,000 per year globally (Russo and Johnson, 2003). In England, Wales and Northern Ireland, *E. coli* has been the most common cause of bacteraemia in humans for most years since 1990 with a year-on-year increases to 27,055 reports in 2010 (HPA). Extra-intestinal pathogenic *E. coli* (ExPEC) strains also cause mastitis, septicaemia, urogenital tract infections and sporadic abortions in cattle, pigs and sheep as well as respiratory infections and colisepticaemia in poultry that can lead to high morbidity and mortality resulting in significant economic losses (Gyles, 1994).

Many lines of evidence suggest links between human and animal extraintestinal pathogenic *E. coli* (ExPEC) (Clermont et al., 2011; Hannah et al., 2009; Johnson et al., 2001b; Johnson et al., 2007; Johnson et al., 2009; Moulin-Schouleur et al., 2007; Warren et al., 2008; Zhao et al., 2009). Although ExPEC from poultry, the avian pathogenic *E. coli* (APEC), have been studied extensively, only 13 ExPEC isolates from livestock animals (excluding poultry) were found in the *E. coli* multi-locus sequence typing (MLST) database (http://mlst.ucc.ie/mlst/dbs/Ecoli). Given this data gap, we aimed to characterize ExPEC strains, mostly from cattle and sheep isolated in England and Wales, in terms of their virulence, antimicrobial resistance profiles, MLST types and serogroups in order to compare with those isolates reported as causing infections in humans. A better understanding of these organisms and their distribution amongst different host species will be an important first step towards the prevention and control of infections in both humans and animals.

2. Materials and methods

2.1. Strain selection

E. coli isolates used in this study are shown in Fig. 1. All were isolated between 1999 and 2008 in England and Wales. They were a subset of those from an enhanced surveillance study. Case selection criteria, bacterial isolation, culture and strain identification methods have been described in detail previously (Hutchinson et al., 2011). Isolates associated with outbreaks where there were no clear alternative diagnoses to *E. coli* infection were included.

2.2. Multilocus sequence typing and data analysis

MLST was performed based on published method (Wirth et al., 2006). However some primers were re-designed, which gave better results for this panel of strains. Locus *adk* was amplified and sequenced with the following primer pairs: adk_vla_F, 5' GCAATGCGTATCATTCTGCTTGG 3' and adk_vla_R, 5' GGCTTGGTGCCGTCAACTTTC 3'. Locus *fumC* was amplified with primer pairs: fumC-P1, 5' TCACAGGTCGCCAGCGCTTC 3'and fumC-P2, 5' GTACGCAGCGAAAAAGATTC 3', but sequenced with fumC_F1, 5' TCCCGGCAGATAAGCTGTGG 3'and fumC_R1, 5' CAAACGGTGCACAGGTAATG 3'. Locus *gyrB* was amplified with gyrB-P1, 5' TCGGCGACACGGATGACGGC 3' and gyrB-P2, 5' ATCAGGCCTTCACGCGCATC 3', but sequenced with gyrB_F1, 5' ATTCCGACCGGTATTCACC 3'and gyrB_R1, 5' AGTACCGCCGTCACGCT 3'. Locus *icd* was amplified and sequenced with icd-P1, 5' ATGGAAAGTAAAGTAGTTGTTCCGGCACA 3' and icd-P2, 5' GGACGCAGCAGGATCTGTT 3'. Locus *mdh* were amplified and sequenced with mdh-P1 5' ATGAAAGTCGCAGTCCTCGGCGCTGCTGGCGG 3'and mdh-P2, 5'

TTAACGAACTCCTGCCCCAGAGCGATATCTTTCTT 3'. Locus *purA* was amplified with purA_F, 5' TCGGTAACGGTGTTGTGCTG 3' and purA_R, 5' CATACGGTAAGCCACGCAGA 3', but sequenced with purA_F1, 5'GCGCTGATGAAAGAGATGAA 3'and purA_R1, 5' GAATTCGTTACCCTGCTTGC 3'. Locus *recA* was amplified with primers: recA_vla-193, 5' GGCCGTATCGTCGAAATCTA 3'and recA-P1, 5' CGCATTCGCTTTACCCTGACC 3' and sequenced with primers: recA_vla_221, 5' AATCTTCCGGTAAAACCACG 3' and recA_val_919, 5' CCTGACCGATCTTCTCACCT 3'. The PCR reactions were carried out for 1 cycle at 95 °C for 2 min, 30 cycles at 94 °C for 1 min, 54-64 °C for 1 min and 72 °C for 2 min, and then 1 cycle at 72 °C for 5 min. The following annealing temperatures were used: *adk, purA and recA* at 56 °C, *fumC* and *icd* at 54 °C, *gyrB* at 60 °C and *mdh* at 62-64 °C. The annealing temperatures were sometimes needed to be adjusted in order for a single band to be observed on the agarose gel before sending the products for sequencing. The sequencing data were imported to Bionumerics (5.1) and allelic numbers and MLST were assigned by submitting the results to the MLST database (http://mlst.ucc.ie/mlst/dbs/Ecoli).

Phylogenetic inferences about ancestral allelic profiles and strain interrelatedness were made using eBURST version 3 http://eburst.nlst.net/ (Feil et al., 2004), SplitsTree4 http://www.splitstree.org/ (Huson and Bryant, 2006) and ClonalFrame version 1.1 http://www.xavierdidelot.xtreemhost.com/clonalframe.htm. Five independent runs of Markov chain were employed and the calculated Gelman-Rubin statistics for all parameters were below 1.20, indicating satisfactory convergence between tree replicates (Didelot and Falush, 2007).

121 2.3. Serotyping, phylotyping, microarrays and PCR analyses of virulence and
122 antimicrobial resistance genes
123 Serotyping was carried out as described earlier (Geue et al., 2010; Hutchinson et
124 al., 2011; Wu et al., 2010b). The details of microarray analyses have been reported
125 (Geue et al., 2010; Monecke et al., 2011) and the layout of the array can be found by
126 following the link (*http://alere-*
127 *technologies.com/fileadmin/Media/Paper/Ecoli/Supplement_Geue__layout_E_coli.xlsx*).
128 PCR virulence typing was performed according to the published method
129 (Johnson and Stell, 2000) and following genes were detected by PCR: *bmaE, cvaC,*
130 *fimH, focG, fyuA, gafD, ibeA, iutA,. kpsMT* II, *kpsMT* III, *kpsMT* K1, *papC, papEF,*
131 *papA, papG I, papG* II, *papG* III, *rfc, sfa/focD, traT*. The genes detected by PCR were
132 analyzed together with microarray data with Bionumerics (5.1).
133 *E. coli* phylotyping was based on the detection of *chuA, yjaA* and TspE4.C2 by
134 PCR (Clermont et al., 2000). However additional primers were used for *chuA,* 5'
135 ATGATCATCGCGGCGTGCTG 3' and 5' AAACGCGCTCGCGCCTAAT-3'; *yjaA,* 5'
136 TGTTCGCGATCTTGAAAGCAAACGT 3' and 5'ACCTGTGACAAACCGCCCTCA
137 3' and TspE4.C2 5' GCGGGTGAGACAGAAACGCG 3' and 5'
138 TTGTCGTGAGTTGCGAACCCG 3'. PCR conditions for above primers were 1 cycle
139 at 94°C for 4 min; 30 cycles of 94°C 30 sec, 65°C 30 sec, 72°C 30 sec; with a final
140 extension at 72°C for 5 min.
141

Results and discussion

3.1 Serogrouping and phylogenetic grouping of ExPEC isolates

All isolates were from internal organs or blood of livestock animals to avoid selecting commensal *E. coli*. Only the results of isolates from different outbreaks were included here with the exception of B2710 (from liver of a calf) and B2711 (from blood of a calf) that were from the same outbreak. Isolates were from different geographic areas in England and Wales. Nineteen different serogroups were identified with O8 being the most common (n=7), followed by O101 and O20 (both n=3) and O153 (n=2), 9 were un-typable and other 15 isolates belonged to 15 different serogroups (Fig. 1).

The most common ST complexes were ST23 (n=7), ST10 (n=6), ST155 (n=3) and ST117 (n=3) (Fig. 1). Based on the information in the MLST database, clonal complexes ST10, 23, and 155 all contain multiple pathotypes of *E. coli*, including enteropathogenic *E .coli* (EPEC), verotoxigenic *E. coli* (VTEC), diffused adhesive *E. coli* (DAEC), enteroaggregative *E. coli* (EAEC), enterotoxigenic (ETEC) from humans, ExPEC strains from both animals and humans and avian pathogenic *E. coli* (APEC). Clonal complex ST155 also contains enteroinvasive (EIEC) from humans, while ST117 appears to be only associated with ExPEC strains from birds, cats, cattle and humans. Other sequence types or clonal complexes, *i.e.* ST 295, 349, 446, 469, 372 identified in this work have also been found amongst APEC and ExPEC isolates from humans and animals. In addition, the clonal complex ST69 was found only in human ExPEC and ST101 in APEC previously.

There were only 13 ExPEC from livestock animals (excluding APEC) in the database at the start of this work; strains studied here only shared the clonal complexes ST10 and 23 with them. Furthermore, a number of new types were identified in this work; therefore the clonal origins of ExPEC from livestock appeared to be very diverse.

In spite of the diversity, they were not found within the clonal complexes that are specific to diarrhoeic *E. coli*, but rather showed to be similar to ExPEC isolates from humans or animals. This result similar to the observation of the diverse clonal origins of human ExPEC isolates (Jaureguy et al., 2008), although direct comparison between these two studies cannot be made as two different MLST schemes were used.

In January 2012, there were 4245 isolates in the *E. coli* MLST database belonging to 2545 STs. The 29 STs identified in this work were compared with all identified *E. coli* MLST types using the BURST (Based upon Related Sequence Types) algorithm (Feil et al., 2004). it is clear from this analysis that these 39 ExPEC isolates were distributed widely among multiple clonal complexes. However, they were not found among ST95 complex that consists mostly of human and poultry strains nor were they in ST131 and ST73 that consists mostly of strains from humans and their pets (Fig. S1).

The results also showed that the distribution of clonal complexes was not animal specific. For example, ST88 (within the ST23 complex) contained isolates from cattle, a pig and sheep and ST783 contained isolates from both sheep and cattle. This is consistent with other results in the database (Wirth et al., 2006). Furthermore, strains of the same sequence types often belonged to different serogroups and strains of the different sequence types sometimes were of the same serogroup. Similarly, strains of the same sequence types were isolated from different internal organs and those of the different sequence types were often found in same internal organs (Fig. 1).

Phylogenetic typing showed that the isolates were from groups A (n=16), B1 (n=12), B2 (n=2) and D (n=9) (Fig. 2, 3, 4, S2 and S3). As expected ST10 complexes belonged group A; ST 101 belonged to B1; ST 106 belonged to group D, consistent with information in MLST database and published works (Okeke et al., 2010; Wirth et al.,

192 2006). Isolates from ST23 complexes were also assigned to group A due to the lack of
193 amplification products despite of repeated attempts with two different primer pairs for
194 TspE4.C2. Based on the phylogenetic groups determined by multilocus enzyme
195 electrophoresis (MLEE), isolates of the ST23 complex may belong to either group A or
196 group B1 (Okeke et al., 2010). It has been reported that the Clermont method (Clermont
197 et al., 2000) used here for assigning strains to phylogenetic groups, is correct 80-85% of
198 the time, and works best for assigning strains to groups B1 and B2 and worst with group
199 A. This is because the assignment of group A relies on the absence of amplification
200 products (Gordon et al., 2008).

201 Most studies on human ExPEC were concentrated on urinary tract infections and
202 phylogenetic group B2 and D were predominately found (Kanamaru et al., 2006; Zhang
203 et al., 2002). However, a more recent study on bacteraemic *E. coli* isolates from two
204 French hospitals has shown that human ExPEC strains are highly diverse and distribute
205 without bias into five major lineages, corresponding to the classical *E. coli* phylogroups
206 (A+B1, B2, D and E) and group F (which contains strains previously assigned to group
207 D) (Jaureguy et al., 2008). Also, a recent study in Denmark found groups A and B1 are
208 associated with sites of infection other than urinary tract; in that study patients with
209 hepatobiliary septicaemia were relatively numerous (Bukh et al., 2010). As the animal
210 isolates studied here were mainly from animal liver and most of them belonged to group
211 A or B1, it would be interesting to determine if phylogroups A and B1 are associated
212 with infections in the liver of animals and humans.

213 The MLST sequences were further analysed with SplitsTree4 to investigate the
214 influence of recombination on the evolution of each locus. It constructs a split network
215 connection between taxa whenever there is a phylogenetic inconsistency due to
216 homoplasy or recombination. Recombination is generally inferred when competing

splits have equal support. Analysis of sequence data revealed extensive network structures for individual loci and the concatenated sequences (Fig. 2 and Fig. S2), implying extensive sequence exchange between lineages (recombination).

This sequence based analysis provided addition information regarding the relationship among isolates, for example, two B2 isolates 2723 ST491 and 2774 ST372 that only shared two identical loci, *gyrB* and *icd* were found to be related at the sequence levels at other 5 loci as well. The group D isolates were apparently very diverse and did not form a monophyletic group. For example, group D isolate 2721 (ST753) shared similar *fumC* sequences with ST10 isolates and *recA* was identical. In addition, group D isolates of ST117 were similar to B2 isolates of ST491 and ST372 at *adk, fumC, gyrB* and *purA* (Fig. 2 and 2S). All group D isolates of different MLST types formed distinctive long branches with strong support (bootstrap 100%, Fig. 2.), This kind of diversity was similar to group D isolates previously described for EAEC strains (Okeke et al., 2010). The two fully genome sequenced group D strains are also distinct from each other (Touchon et al., 2009). Most groups A and B1 isolates had shorter branches, suggesting that they were closely related (Fig. 2). This is consistent with previous observations that group A and B1 strains were not distinctly separated and were considered as sister groups (Jaureguy et al., 2008; Lecointre et al., 1998; Okeke et al., 2010). The results suggest that the extensive recombination has obscured the phylogenetic relationships among this group of isolates.

To further explore the impact of recombination on the phylogenetic relationship of these isolates the concatenated 7 gene sequences derived from the MLST analyses were analysed with ClonalFrame, a program designed to infer the clonal relationship of bacteria and the chromosomal position of homologous recombination events that disrupt a clonal pattern of inheritance (Didelot and Falush, 2007). The results of ClonalFrame

242 analysis were similar to those obtained with SplitsTree (Fig. 2 & Fig. S3) and showed
243 strong evidence of recombination. ClonalFrame is able to estimate the relative
244 frequency of recombination compared to point mutation in genetic diversification. For
245 our data set ρ/θ was estimated as 0.72 (95% CI 0.25 to 1.65). This is comparable to the
246 ratio of 0.32-2.14 determined by Wirth ((Wirth et al., 2006) and similar to results for
247 ST10 and triple locus variants (largely ECOR A EAEC strains) described by Okeke et al
248 (Okeke et al., 2010). The relative impact of recombination compared to point mutation
249 (r/m) was 1.9 (95% CI 0.83 to 3.77) which suggests that a basepair is almost twice as
250 likely to change by recombination than by mutation. After analysing the core genes of
251 20 fully sequenced *E. coli*, Touchon et al (Touchon et al., 2009) also found that a gene
252 conversion event is twice as likely as a mutation to occur at a given position. However
253 these r/m ratios are considerably lower than previously found with EAEC strains (2.79-
254 4.52) (Okeke et al., 2010). Touchon et al suggest that due to the short tract length of
255 DNA (at an estimated tract length of 50 bp) involved in gene conversion, "the
256 substantial level of gene conversion in *E. coli* did not blur the phylogenetic signal and a
257 meaningful robust tree topology can be extracted from the sequence (Touchon et al.,
258 2009)". Extensive recombination among isolates of different phylogenetic groups was
259 observed among isolates studied here. This is consistent with the higher recombination
260 rates observed among pathogenic *E. coli* (Wirth et al., 2006).
261
262 3.2. Virulence typing results
263 Thirty-six different combinations of virulence gene patterns were identified
264 among 39 isolates studied here (Fig. 3). At the 50% similarity level based on Jaccard
265 similarity coefficient, isolates could be divided into 16 groups (with numbers per group
266 ranging from 1-8 with a mean of 2.43, a standard deviation of 2.37, and both the mode

and median of 1), indicating remarkable diversity. All isolates harboured *fimH* (a gene for type I fimbriae). Over 60% had *traT* and *iss* (both are serum resistant genes) and *lpfA* (the gene for the long polar fimbriae). About half of isolates contained genes that are related to iron utilisation, *fyuA (*yersiniabactin) and *iutA* (aerobactin). More than 30% of isolates harboured *iroN* (enterobactin siderophore receptor protein), *mchF* and *mcmA* (both related to microcin production) and *prfB* (a fimbriae gene). More than 20% of isolates had *cvaC* (encodes Colicin V), *papC, bmaE* and *f17G* (all associated with fimbriae production). The remaining genes on the arrays were found in less than 20% of isolates. *E. coli* serine protease gene *espP* normally associated with VTEC (Brunder et al., 1997) was found in 6 isolates. This gene has been found in cattle and swine F165-positive strains (Dezfulian et al., 2003). Other genes identified among this group of isolates such as *astA* (heat-stable enterotoxin), *lpfA, pic* (serine protease) were also found in diarrhoeic bacteria especially among EAEC as well as among UPEC strains (Abe et al., 2008). Genes such as *astA, iss, iroN, mchF, pfrB* and *cdtB* were found in *E. coli* strains from both diarrhoea and extra-intestinal infections (Wu et al., 2010b).

The numbers of virulence related genes in these isolates were from 1-18; this includes genes for microcins and iron utilization that may not be directly associated with the virulence *per se*, but confers competitive advantage for their hosts. Isolates 2774, a member of B2 group, had the highest number of putative virulence genes including those for fimbria, colicin, and iron acquisition. It was the only isolate that harboured *ibeA*, a gene also found in APEC (Germon et al., 2005). The product of *ibeA* is responsible for the invasion of brain endothelium and is associated with human neonatal meningitis (Huang et al., 2001a; Huang et al., 2001b). Isolates 2774 belonged to ST372 complex; this clonal complex has been found among APEC and ExPEC strains from humans, dogs and cats. It was isolated from the liver of a calf that died of septicaemia in

2006 together with 6 other 7 day olds calves. Database record shows that another *E. coli* isolate that was not studied here was from the brain of a calf during the same outbreak. Strain 2778 harboured 17 virulence factors and belonged to ST117. This ST is also found in APEC and human ExPEC. Three ST117 isolates (2762, 2720 and 2778) all isolated from the liver or blood of calves, all harboured *pic* and *vat,* but did not have *ctdB, f17AG, gafD, papG* II *or tsh,*. Isolates 2762 was from a farm in Lincolnshire in 2004, 2720 was from a farm in Dorset in 2005 and 2778 was from a farm in Cumbria in 2007. The *vat* gene has been found among ST117 isolates from human patients with cirrhosis (Bert et al., 2010). Genes *vat, sat* and *pic* are also known to be associated with urinary tract isolates (Restieri et al., 2007).

Isolate 2750 was from the liver of a calf had *kpsMT* II genes belonged to group D and ST69 complex that is usually associated with human UTI or meningitis. It is known that ST69 UTI or bacteraemic isolates consists of 5 serogroups including O17 (Tartof et al., 2005), the serogroup that 2750 belonged to. Two other isolates, 2710 and 2711, were from the same farm and both belonged to ST10 and serogroup O101. Isolate 2710 was from the liver of a calf harboured more virulence and antimicrobial resistance genes than isolate 2711 that was isolated from blood of a calf, suggesting the acquisition or loss of virulence factors either during the infection or after the isolation. Isolate 2839 that was from the liver of a calf contained 15 virulence and 8 antimicrobial resistance genes including a gene encoding extended spectrum β-lactamase ($bla_{\text{CTX-M-group-9}}$) (See later section). No virulence factors (apart from *fimH* that can be found in both commensals as well as pathogens) were detected in some liver isolates, which suggests the presence of unknown or untested virulence factors. This work is consistent with early findings that described the overlapping virulence genes from *E. coli* isolated from diseased calves and pigs and humans (Dezfulian et al., 2003; Girardeau et al., 2003).

Different virulence genes were identified among these ExPEC isolates, which indicated the presence of possible subtypes of ExPEC strains. For *E. coli* causing enteric/diarrhoeal diseases, at least six pathotypes with specific virulence genes have been described (Nataro and Kaper, 1998). The research on the pathogenic mechanisms of ExPEC strains is lagging behind, but nevertheless it has been recognised that different mechanisms must exist among ExPEC to cause diseases (Brzuszkiewicz et al., 2006; Johnson et al., 2001a). Marrs *et al*. have started to define these different 'subtypes' but these authors have pointed out the need for more detailed studies to define specific virulence genes in these organisms (Marrs et al., 2005).

3.3. Antimicrobial resistance gene carriage

The isolates used in this work were selected on the basis of either association with or, probable cause of, diseases and not for antimicrobial resistance. Twenty-one (53%) isolates harboured at least one antimicrobial resistance genes and some have up to 9 antimicrobial resistance genes (Fig. 4). The most common antimicrobial resistance genes were *strB, bla*$_{\text{TEM}}$ and *sul2* that were found in more than 35% of the isolates. The *aadA1, sul1* and *aphA* gene were in more than 10% of isolates. A *bla*$_{\text{CTX-M-group-9}}$ gene was found in isolate 2839 from the liver of cattle. The *intI* 1 gene (for type I integron) was found to be closely associated with *sul1* and *aadA1*, so was *aphA* with *sul2* and *bla*$_{\text{TEM-1}}$ with *strB.*

It has been suggested that there may be a fitness 'trade-off' for organisms between virulence and resistance. For example, B2 strains are considered to be more virulent (contain P fimbriae and are α, β -haemolytic) but less resistant to antimicrobials than B1 (Bukh et al., 2010; Jaureguy et al., 2007; Johnson et al., 1991; Johnson et al., 1994). In Denmark, the prevalence of antibiotic resistance harboured by phylogroup

342 decreases in the order of group D>A> B1> B2 (Bukh et al., 2010). In this work, the P-
343 fimbriae positive strains harboured anything from 0 to 9 antimicrobial resistance genes.
344 Resistance genes were found in 6/9 (67%) of group D strains, 12/16 (75%) group A
345 strains, 4/12 (33%) group B1 strains. No resistance genes were identified in two group
346 B2 strains. The average number of antimicrobial resistance genes in group D strains was
347 7.8; group A was 6.8 and group B1 was 3.8.
348
349 3.4. Phylogenetic distribution of virulence genes and antimicrobial resistance genes
350 There was no correlation between the carriage of virulence and antimicrobial
351 resistance genes and the genetic backbone of strains (Fig. 3 and 4). For example, a
352 variety of virulence genes were found in ST10, ST117 and ST23 strains. Occasionally
353 isolates of the same ST from different sources shared similar virulence genes.
354 Furthermore, isolate 2770 from the liver of a sheep and 2839 from the liver of a calf
355 both belonged to ST783 and shared 13 virulence genes; but the sheep isolate harboured
356 *iha* and *bmaE*, while cattle isolate had *ireA* and *papA*. Similarly, isolates that were of
357 the same sequence type contained different antimicrobial resistance genes.
358 Many virulence and antimicrobial resistance genes identified in this set of
359 isolates have disseminated into multiple genetic backgrounds. Previous studies have
360 revealed the parallel evolution of EPEC with multiple acquisitions of virulence genes in
361 different background (Lacher et al., 2007; Reid et al., 2000; Wirth et al., 2006). After
362 studying multiple pathotypes of *E. coli*, Escobar-Paramo et al concluded that a specific
363 genetic background is required for acquisition and expression of virulence factors in *E.*
364 *coli* and the ExPEC associated virulence genes were linked to the phylogenetic group B2
365 in that study (Escobar-Paramo et al., 2004).

Although some sequence types of *E. coli* such as ST10 and ST155 contain multiple pathotypes, others appear to be pathotype specific. For example ST69 is predominately associated with UPEC isolates from human and ST11 contained only EHEC O157:H7 or EPEC O55:H7 strains. Therefore, the interplay between horizontally transferred genetic elements and genetic backbone of bacteria is rather complicated

Two major clonal complexes found among these isolates are ST10 and ST23 and both harboured various combinations of virulence genes. Generally speaking, more virulence genes were associated with ST23 complex than with ST10. As all isolates were from diseased animals, this discrepancy in the number virulence genes was probably due to our limited understanding of those organisms. Many more virulence factors are yet to be discovered; only then we will be able to understand better the association between virulence genes and genetic backbones of *E. coli*. Considerable diversity was observed among strains causing similar infections in animals. Perhaps this is not surprising as even among the isolates of the same serotypes, differences can be substantial due to the constant acquisition and loss of genes (Wu et al., 2010a; Wu et al., 2008). Further work is needed to understand the mechanisms of pathogenicity and their potentials in causing diseases in different hosts.

4. Conclusion

E. coli isolates from extra-intestinal organs of livestock animals belonged to multiple serogroups and phylogenetic groups. The clonal origins and the virulence genes harboured by these strains were similar to ExPEC from humans and other animals, suggesting the zoonotic potential. The many different combinations of virulence factors indicated multiple pathogenic mechanisms. No correlation was found between the genetic backbone, and the virulence and antimicrobial resistance gene content. Because

of this remarkable genetic diversity, it will be challenging to control the infections caused by ExPEC.

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Conflict of interest

There is no conflict of interest.

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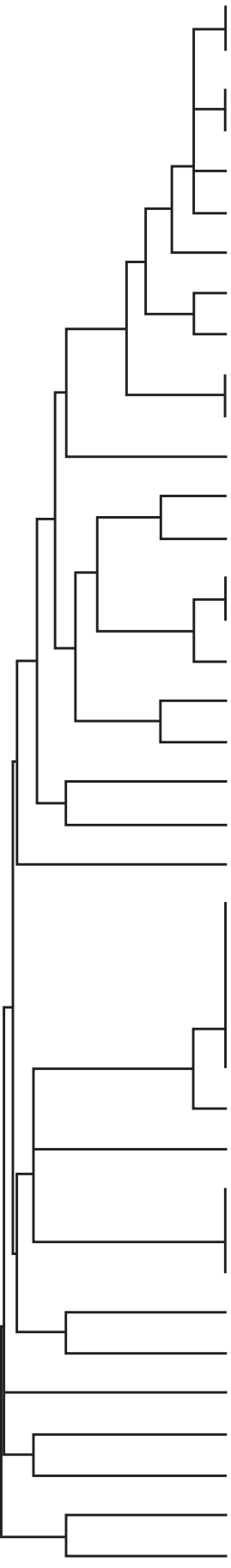
595 Figure legends:

596 Fig. 1. The isolates were clustered based on the allelic numbers of 7 house-keeping
597 genes using categorical coefficient and unweighted pair group method with arithmetic
598 averages (UPGMA, Bionumerics 5.1). Sources and serogroups of the isolates are shown.
599
600 Fig. 2. The concatenated 7 house-keeping gene sequences were analysed with
601 SplitsTree4. Bootstrap scores greater than 90 are given at each node. The scale of the
602 network, MLST types and phylogenetic groups of isolates were indicated.
603
604 Fig. 3. Isolates were clustered based on their virulence gene content using Jaccard
605 coefficient and UPGMA (Bionumerics 5.1). MLST types and phylogenetic groups of
606 isolates are shown.
607
608 Fig. 4. Isolates were clustered based on their antimicrobial resistance genes using
609 Jaccard coefficient and UPGMA (Bionumerics 5.1). MLST types and phylogenetic
610 groups of isolates are shown.
611



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6	4	12	1	20	12	7	2758	88	ST23	Bovine	liver	O8:K+
6	4	12	1	20	12	7	2823	88	ST23	Porcine	blood	O8:K+
6	4	12	1	20	13	7	2755	23	ST23	Bovine	liver	O78:K80
6	4	12	1	20	13	7	2773	23	ST23	Bovine	liver	UT:ND
6	4	12	1	20	8	7	2768	90	ST23	Bovine	spleen	O9:K+
6	4	12	1	20	7	7	2828	360	ST23	Ovine	spleen	O8:K+
6	4	12	1	144	12	7	2771	1088	ST23	Bovine	liver	UT:ND
6	207	12	1	9	2	7	2749	1085	ST295	Bovine	liver	UT:UT
6	4	12	1	9	2	7	2808	295	ST295	Bovine	liver	UT:UT
6	4	33	10	20	12	7	2770	783	ST783	Ovine	liver	O8:K+
6	4	33	10	20	12	7	2839	783	ST783	Bovine	liver	O20:K+
6	4	7	9	7	7	56	2806	1080	None	Bovine	liver	O105:K+
6	19	3	26	11	8	6	2775	446	ST446	Bovine	liver	O88:K+
6	4	3	16	11	8	6	2844	906	ST466	Bovine	liver	O150:K+
6	4	4	16	24	8	14	2709	58	ST155	Bovine	liver	O35:KV79
6	4	4	16	24	8	14	2757	58	ST155	Bovine	spleen	O132:K+
6	4	4	16	24	8	104	2766	767	ST155	Bovine	spleen	O8:K+
6	19	14	16	11	12	2	2776	1097	ST1097	Bovine	spleen	O6:K+
6	29	14	16	11	2	2	2836	1084	ST1097	Ovine	liver	O8:K+
6	6	4	26	9	13	98	2746	1118	None	Bovine	liver	UT:UT
9	65	5	1	9	13	6	2822	162	ST469	chicken	liver	O8:K+
43	41	15	18	11	7	6	2767	101	ST101	Bovine	liver	UT:UT
10	11	4	8	8	8	2	2710	10	ST10	Bovine	liver	O101:K+
10	11	4	8	8	8	2	2711	10	ST10	Bovine	blood	O101:K+
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21	35	27	6	5	8	4	2750	106	ST69	Bovine	liver	O17:K+
20	45	41	43	5	32	2	2720	117	ST117	Bovine	blood	UT:ND
20	45	41	43	5	32	2	2762	117	ST117	Bovine	liver	O85:K+
20	45	41	43	5	32	2	2778	117	ST117	Bovine	liver	UT:ND
12	93	136	30	112	1	2	2721	753	None	Bovine	liver	O153:K+
12	23	136	197	1	2	17	2751	1131	None	Bovine	liver	UT:UT
80	186	54	10	143	35	47	2765	1087	None	Bovine	liver	O98:K+
154	187	22	52	130	129	4	2712	973	None	Bovine	liver	O153:K-
34	36	39	87	67	16	4	2722	349	ST349	Bovine	liver	O166:K+
13	39	19	36	30	14	82	2723	491	None	Bovine	liver	O110:K+
88	103	19	36	23	44	26	2774	372	None	Bovine	liver	O32:K+

Figure

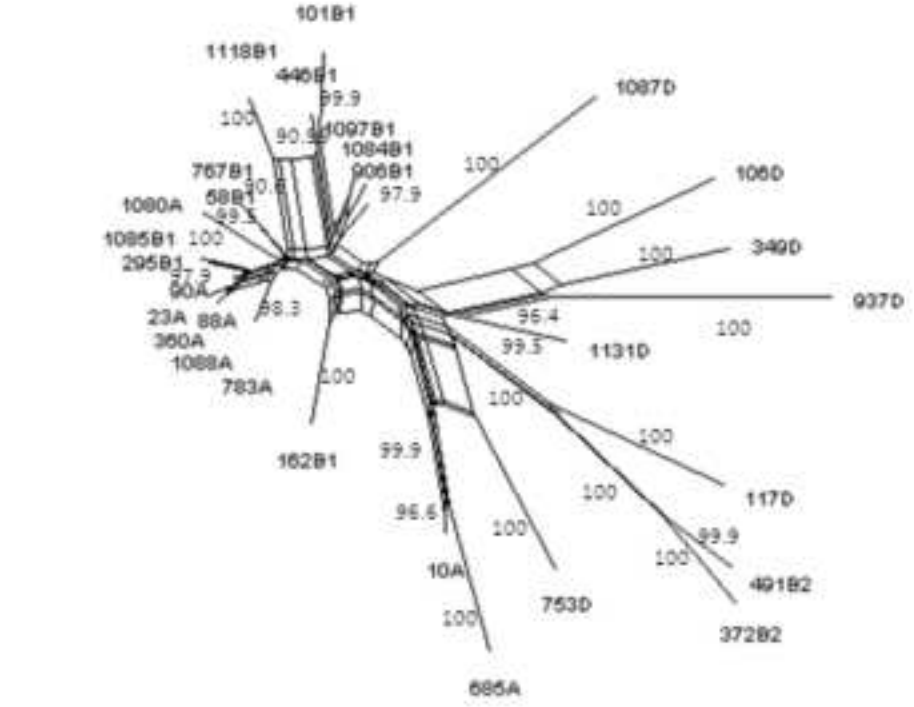
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Figure 4

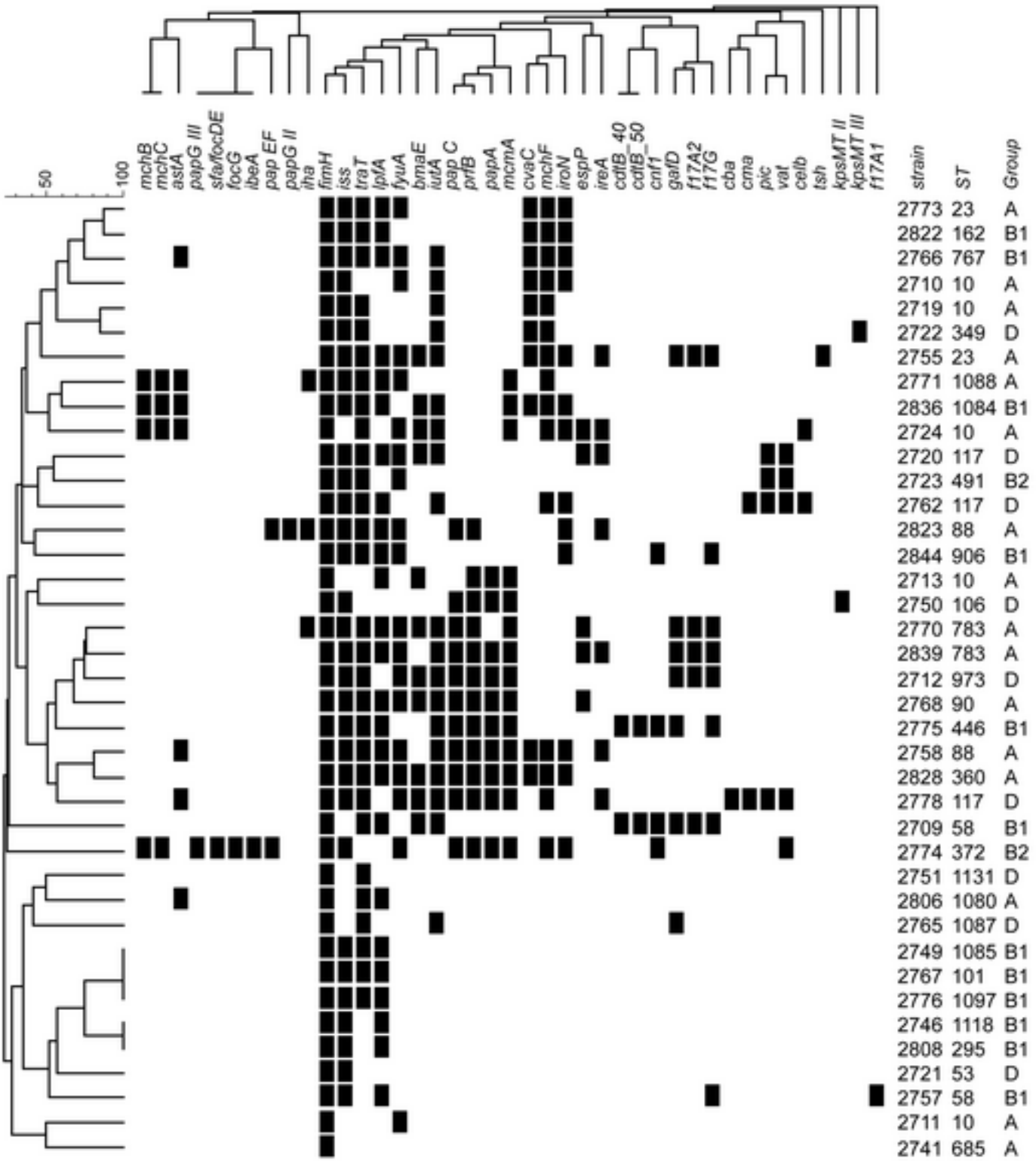

Figure 5