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Multi-drug resistant *Escherichia coli* in diarrhoeagenic foals: pulsotyping, phylotyping, serotyping, antibiotic resistance and virulence profiling.

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Abstract

Extraintestinal pathogenic *E. coli* (ExPEC) possess the ability to cause extraintestinal infections such as urinary tract infections, neonatal meningitis and sepsis. While information is readily available describing pathogenic *E. coli* populations in food-producing animals, studies in companion/sports animals such as horses are limited. In addition, many antimicrobial agents used in the treatment of equine infections are also utilised in human medicine, potentially contributing to the spread of antibiotic resistance determinants among pathogenic strains. The aim of this study was to phenotypically and genotypically characterise the multidrug resistance and virulence associated with 83 equine *E. coli* isolates recovered from foals with diarrhoeal disease. Serotyping was performed by both PCR and sequencing. Antibiotic resistance was assessed by disc diffusion. Phylogenetic groups, virulence genes, antibiotic resistance genes and integrons were determined by PCR. Thirty-nine (46%) of the isolates were classified as ExPEC and hence considered to be potentially pathogenic to humans and animals. Identified serogroups O1, O19a, O40, O101 and O153 are among previously reported human clinical ExPEC isolates. Over a quarter of the *E. coli* were assigned to pathogenic phylogroups B2 (6%) and D (23%). Class 1 and class 2 integrons were detected in 85% of *E. coli*, revealing their potential to transfer MDR to other pathogenic and non-pathogenic bacteria. With 65% of potentially pathogenic isolates harbouring one or more TEM, SHV and CTX-M-2 group β-lactamases, in addition to the high levels of resistance to fluoroquinolones observed, our findings signal the need for increased attention to companion/sport animal reservoirs as public health threats.

Keywords: *Escherichia coli*, diarrhoeagenic foals, serotyping, antibiotic resistance, adherence and invasion PFGE, ExPEC, ESBL

Introduction

Pathogenic *Escherichia coli* infection is a public health challenge and a continuous source of morbidity/mortality (Croxen et al., 2013). Pathogenic *E. coli* can be split into two categories: intestinal and extraintestinal pathogenic *E. coli*. Intestinal pathogenic *E. coli* cause diarrhoea by expressing virulence genes that produce enterotoxins, facilitate attachment and effacement, and/or invasion of the intestinal mucosa. Extraintestinal pathogenic *E. coli* (ExPEC) possess the ability to cause extraintestinal infections such as urinary tract infections (UTIs), neonatal meningitis and sepsis (Russo and Johnson, 2003). Sources of human infection include direct or indirect contact with animals that carry pathogenic *E. coli*, and from exposure to animal faeces. Recent outbreaks in public settings, such as farms, fairs and petting zoos, have highlighted the public health impact of this route of transmission (Byrne et al., 2015; Murray et al., 2017). While information is readily available describing pathogenic *E. coli* populations in food-producing animals (Lenahan et al., 2007; Lenahan et al., 2009; Kennedy et al., 2017), studies in companion/sports animals such as horses are limited.

One such study, focusing on *E. coli* distribution, reported a greater diversity among pathogenic *E. coli* populations in horses relative to cattle or humans (Anderson *et al.* 2006). In addition, many of the antimicrobial agents used in the treatment of equine infections belong to the same families as used in human medicine (WHO, 2017). These factors, may contribute to the spread of antibiotic resistance determinants to

pathogenic strains; as the *in vitro* and *in vivo* transmission of resistance markers between species has been described previously (Kelly et al., 2009).

Given the high degree of handling and the large number of people (particularly children) who have contact with horses, there is a need to increase awareness of companion/sports animals as potential reservoirs of multi-drug resistant (MDR) and pathogenic *E. coli* (Sequeria et al., 2009; Murray et al., 2017). The aim of this study was to characterise multidrug resistance determinants and virulence genes and to phenotypically characterise those associated with equine *E. coli* isolates recovered from foals with diarrhoeal disease.

Materials and methods

Bacterial isolate collection and antimicrobial resistance profiling

Faecal samples routinely obtained from foals with enteritis and from *post mortem* examinations presenting at the Irish Equine Centre in Kildare, Ireland were cultured onto Columbia blood Agar, Wilkins Chalgren agar (anaerobically) and MacConkey agar and incubated overnight at 37°C. Presumptive *E. coli* were confirmed using API 20E strips (bioMériux, Marcy l'Etoile, France) and antibiotic resistance profiles of all isolates were determined against a panel of 14 compounds using disc diffusion, and where appropriate interpreted according to Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI document VET01-A4, 2013; CLSI supplement VET01S, 2015). Resistance to antimicrobial agents not listed in the CLSI guidelines was determined by the absence of a zone of clearance. The following antimicrobial compounds were included, with their abbreviations and concentrations in parenthesis; ampicillin (A, 10 µg); amikacin (A_K, 30 µg); amoxillin/clavulanic acid (A_M, 20/10 µg); gentamicin (C_N, 10 µg); ciprofloxacin (C_P, 5 µg); enrofloxacin (E, 5 µg),

kanamycin (K, 30 µg), cephalothin (K_F, 30 µg), nalidixic acid (N_A, 30 µg); norfloxacin (N_O, 10 µg); streptomycin (S, 10 µg); sulfonamide (S_3, 300 µg); tetracycline (T, 30 µg) and trimethoprim (W, 5 µg). All antimicrobial-containing discs were supplied by Oxoid (Fannin Healthcare, Dublin, Ireland). Quality control strains, *E. coli* ATCC®25922 and *Pseudomonas aeruginosa* ATCC®27853, were included. Eighty-three isolates were identified that were resistant to 3 or more different antimicrobial classes. These were defined as MDR and were subsequently characterised in greater detail, as described below.

DNA purification

Total DNA was prepared from all isolates using the Promega Wizard Genomic DNA purification kit (Madison, WI) following the manufacturer's instructions. The integrity and concentration of the purified template DNA was assessed by means of conventional agarose gel [1.5%, (w/v)] electrophoresis and by spectrophotometry using a NanoDrop™ ND-1000 (Thermoscientific, Wilmington, DE).

Serotyping

Molecular serotyping was performed on all *E. coli* isolates using PCR amplification of the serogroup specific genes, *rfb*$_{O26}$, *rfb*$_{O111}$ and *rfb*$_{O157}$.

A representative group of 25 isolates were then selected at random and sent for conventional serotyping (Public Health England, PHE Colindale, London, UK).

Pulsed-field gel electrophoresis (PFGE)

Molecular subtyping using pulsed-field gel electrophoresis (PFGE) of genomic DNA recovered from equine *E. coli* isolates was performed according to methods described previously (Duffy et al., 2005). Similarity clustering analyses were

performed using an unweighted pair group-matching algorithm and the Dice correlation coefficient with a tolerance and optimization of 1.5% with BioNumerics (Applied Maths, Belgium).

Phylogenetic grouping

Phylogenetic groups were determined for each *E. coli* isolate using an established multiplex PCR targeting *chuA*, *yjaA*, and TSPE4.7 according to the protocol of Clermont et al. (2000). Target amplification was performed using the original primer concentrations (Table 1) and cycling conditions (Supplemental Material Table S1). Amplicons generated were separated by conventional 1.7% (w/v) agarose gel electrophoresis, stained with 0.1 g/ml ethidium bromide (Sigma-Aldrich) in 0.5X Tris-EDTA-boric acid buffer, and subsequently assigned to one of the phylogroups A, B1, B2, or D using the criteria outlined previously by Clermont et al. (2000).

Adherence and invasion assay

Six representative isolates (denoted as Eq23, Eq45, Eq59, Eq67, Eq69 and Eq79) with distinctly different virulence profiles (Supplemental Material Table S2) were selected. These were assessed for their ability to adhere and invade Caco-2 cells as a model for the human small intestinal epithelium. A bacterial adhesion assay was performed with a multiplicity of infection (MOI) of 100 bacteria per epithelial cell according to previously described methods (Simpson et al., 2006). For the invasion assay, Caco-2 monolayers were infected with the same MOI and incubated for 30min at 37°C to allow invasion to occur. The number of intracellular bacteria was determined after the extracellular bacteria were eliminated by incubation of the monolayers with the experimental medium containing gentamicin (100 µg/mL) for a further 30min at 37°C.

All monolayers were subsequently lysed with a 0.5 mL volume of PBS containing 1% (v/v) Triton X-100. Bacteria from each monolayer were collected and plated onto tryptone soy agar (TSA, Sigma) using decimal dilutions. Plate counts from the adhesion assay determined the total number of associated (adhered and invaded) bacteria, and plate counts from the invasion assay determined the number of invaded bacterial cells only. Adherent non-invasive (+-), invasive (++) and non-adherent and non-invasive (--) controls were included in each experiment.

Identification of antimicrobial resistance determinants, integrons, gene cassettes and virulence genes

Detection of antibiotic resistance markers and integron-associated genes was performed by PCR, using the primers listed in Table 1. The following resistance determinants were investigated: *ampC*, *bla*$_{\text{CTX-M-2}}$, *bla*$_{\text{OXA}}$, *bla*$_{\text{SHV}}$, *bla*$_{\text{TEM}}$, *bla*$_{\text{PSE}}$, encoding β-lactamases and β-lactamase groups; *tet*(A) and *tet*(G) tetracycline efflux pumps; and the sulfonamide resistance gene *sul1*.

A PCR amplification of the *gyrA* gene was performed on a subset of 16 ciprofloxacin resistant isolates. Previously published PCR methods were employed to determine the presence of conserved integron-associated genes in all isolates, including *intI1* and *intI2* (coding for integrases of classes 1 and 2, respectively), *qacEΔ1*, *sul1*, the right-sided conserved segments of class 1 integrons, together with their variable regions (Table 1). Gene cassettes and specific *gyrA* amplicons of interest were gel extracted using a Qiagen gel extraction kit (West Sussex, UK). DNA was quantified by spectrophotometry and sequenced commercially (Qiagen, Hilden, Germany). Sequence similarity searches were carried out against sequences deposited in the GenBank database using the BLAST search tool

(https://blast.ncbi.nlm.nih.gov/Blast.cgi). Sequence alignments were performed using the online CLUSTALW2 program available at the European Bioinformatics Institute (https://www.ebi.ac.uk/Tools/msa/clustalw2/).

All isolates were investigated for the presence of the following virulence-associated genes: *stx1* and stx2 which encode Shiga-toxins; *eaeA*, an adherence factor; the alpha-haemolysin, *hlyA*; *fliCh7,* encoding the flagellar antigen H7; *cnf1,* a cytotoxic necrotizing factor from uropathogenic *E. coli*; *iucD*, which encodes the aerobactin operon; *afa/draBC,* a Dr-binding adhesin (F17 fimbriae); *papC,* a P fimbriae; *sfa/focDE,* the S and F1C fimbriae; and *neuC, a* K1 gene implicated in sialic acid synthesis (Table 1).

PCR amplification reaction conditions for all of the investigated genes are presented in supplemental material (Supplemental Material Table S1). All reactions contained 100 ng of purified DNA, 50 pmol/μL of forward and reverse primers (MWG-Biotech AG, Ebersberg, Germany), 10 X amplification buffer containing 2.5 mM $MgCl_2$, 200 μM dNTPs (Promega, Madison, WI) and 0.5 U *Taq*DNA Polymerase (New England Biolabs, Ipswich, MA) or *Pfu* Polymerase (Chimerx, Madison, WI).

Results and Discussion

Serotyping

Initially, PCR was carried out on all 83 equine *E. coli* isolates in order to assign them to one of three known pathogenic *E. coli* serogroups (*rfb*$_{O157}$, *rfb*$_{O26}$ and *rfb*$_{O111}$). One isolate in this study produced a PCR product for serogroup O26, while no amplicons corresponding to the serogroups O157 or O111 were detected (Table 2). However, of the 25 representative isolates sent for conventional serotyping, 10 were typeable

(*E. coli* O1, O101, O153, O19a, O33, O40, O91; Table 2). All but the O19a serogroup have previously been reported in animal sources including: foals, cattle, pigs, sheep, goats, poultry, cats (Krause et al., 2005; Mora et al., 2011). Serogroups O1, O26, and O101 have previously been associated with both healthy and diarrhoeagenic foals (Holland et al., 1996) however, this is the first reported collection of *E. coli* isolates from equine sources to contain serogroups O19a, O33, O40, O91 and O153, though they have been previously assessed for biocide tolerance in a separate study (Sheridan et al., 2012). In human infection, all 8 serogroups are of clinical significance. All but O19a have previously been associated with human clinical shiga toxin-producing *E. coli* (STEC) infection (Werberet. al., 2008; Vally et al., 2012) and serogroups O1, O19a, O40, O101 and O153 have also been identified among human clinical ExPEC isolates (Ciesielczuk et al., 2016). Novel STEC/ETEC hybrid strains have been recently associated with patients with haemolytic uraemic syndrome (HUS), these included isolates of the serogroups O101 and O153 (Nyholm et al., 2015). The German outbreak involving *E. coli* reported in 2011 was linked to a hybrid STEC/EAEC, a feature which highlighted the danger of these combinations and the need to routinely screen for multiple sets of virulence factors (Mora et al., 2011).

Virulence gene characterisation

Of the 83 equine *E. coli* isolates examined, none were found to harbour the *stx 1* or *stx 2* genes; the absence of a single STEC isolate in this collection is unexpected considering the recent increase in outbreaks associated with

domesticated/companion animals in public settings (Byrne et al., 2015, Murray et al., 2017).

Of the 11 virulence genes investigated (Table 1), only 4 were detected among these equine *E. coli* isolates (Table 3). Isolates harbouring two or more of these virulence genes are defined as extraintestinal pathogenic *E. coli* (ExPEC) (Xia et al., 2011). Thirty-nine (46%) of the isolates in this study were classified as ExPEC on this basis, and hence are considered to be potentially pathogenic to humans and animals. The most commonly detected virulence gene in the collection was *iucD* (57 %). This gene codes for a siderophore (an iron chelating compound), which enables *E. coli* to survive in iron-poor environments such as those encountered in UTIs and is related to the virulence of septicemic *E. coli* from non-equine sources (Siqueria et al., 2009). The occurrence of the fimbrae *F17* gene *afa/draBC* (31%) is comparable to the results of one of few studies available on *E. coli* in horses (van Duijkeren et al., 2000). Van Duijkeren and colleagues identified the presence of *afa/draBC* in 30% of *E. coli* recovered from horses with diarrhoeal disease, and none in *E. coli* from healthy horses, suggesting that these fimbrae might have a role in the cause of diarrhoeal disease in horses. *F17* has also been associated with mastitis (Ghanbarpour and Oswald, 2010). The *papC* gene, producing an adhesin associated with both extraintestinal pathogenicity and an increased capacity to colonize the human intestine, was identified in 36% of isolates. In animals, the *papC* gene has also been associated with UTIs, respiratory tract infections, soft tissue infections and diarrhoeagenic infection (Siqueria et al., 2009; Ewers et al., 2014). The presence of the *sfa/focDE* gene was determined in only 2% of isolates. It has been associated with biofilm production in *E. coli* (Naves et al., 2008); biofilm forming properties are considered important for bacteraemia in the urinary tract of humans and animals

(Wiles et al., 2008). All four virulence determinants are frequently documented in ExPEC *E. coli* populations and are of clinical significance (Wiles et al., 2008). In addition, we extended the genetic analysis of ten ExPEC isolates by means of a *E. coli* K12 O157 v2 DNA microarray (Kyle et al. 2010) confirming the presence of these virulence genes and revealing further putative virulence, stress, quorum sensing and antimicrobial resistance (including efflux and porins) genes of interest (Supplemental Material; Word document 1 and Figure S1). These results signal the need for increased attention to be focused on companion/sport animal reservoirs as potential public health risks.

PFGE subtyping and phylogenetic classification

The collection of equine *E. coli* had a diverse range of pulsotypes of which 33 could be grouped into 9 clusters (C1-9) of 3 or more isolates based on a genetic relatedness criterion of 80% (Figure 1). Six pulsotypes (A, B, C, D, E and J) were common to 2 isolates; 4 pulsotypes (F, G, H, I) were common to 3 isolates; 60 isolates had distinct pulsotypes. Pulsotypes A and B were closely related with percentage similarity at 93 % confidence and all 4 isolates belonged to the same phylogenetic group A.

According to Clermont et al. (2000), *E. coli* belonging to phylogenetic groups A and B1 are considered non-pathogenic commensal strains, while strains belonging to groups B2 and D are more likely to be pathogenic. The majority of isolates belonged to phylogenetic group A (57 %); while the remainder of isolates belonged to phylogenetic groups B1 (14 %), B2 (6 %) and D (23 %).

279 ExPEC belonging to both pathogenic and non-pathogenic phylogenetic groups were
280 isolated from foals presenting with diarrhoea. This result is in contrast with other
281 studies reporting on equine and other *E. coli* populations wherein ExPEC isolates
282 predominantly belong to phylogroup B2 (Xia et al., 2011; Ewers et al., 2014).
283 Although typing of human isolates shows good correlation between the phylogenetic
284 group and pathogenicity (Clermont et al., 2000), animal-associated ExPEC can be
285 phylogenetically distinct. Therefore, caution should be exercised when defining such
286 bacteria as commensals (Ghanbarpour and Oswald, 2010). Furthermore,
287 commensal *E. coli* can cause extra-intestinal disease when predisposing factors for
288 infection are present (Russo and Johnson, 2003). Therefore, although the majority of
289 the isolates investigated in this study may be phylogenetically classified as
290 commensal organisms, they may have the potential to be clinically significant.
291 Further refinement of this phylogenetic classification had recently been documented
292 by Clermont et al. (2013).
293
294 **Adherence and invasion**
295 Caco-2 cells, a homolog for enterocytes in the intestinal epithelium, were employed
296 to further assess the pathogenicity of isolates with different virulence gene profiles.
297 Bacterial adherence ranged from 6.31 to 7.73 $\log_{10}$cfu mL^{-1} and bacterial invasion
298 ranged from 1.35 to 5.36 $\log_{10}$cfu mL^{-1} for all 6 isolates (Figure 2). The non-ExPEC
299 *iucD* isolate (Eq23) was more adherent to Caco-2 cells (0.85 $\log_{10}$cfu mL^{-1}, $p \leq$
300 0.001) compared to the adherent control isolate as well as the other isolates
301 examined. However, this did not result in greater invasive ability. The only isolate to
302 be classed as invasive (5.27 $\log_{10}$cfu mL^{-1}, $p \leq 0.05$) was the ExPEC isolate (Eq67)
303 with the *iucD-afa/draBC-papC* virulence profile. Mellor et al. (2009) demonstrated

304 that *E. coli* isolates considered pathogenic to humans do not display a greater ability
305 to attach to the Caco-2 cell line than those that are not. Perhaps cell lines modelling
306 other common ExPEC sites of infection, such as the human J-82 bladder homolog,
307 might reveal more about the potential these equine ExPEC have to cause infection.
308
309 **Antimicrobial resistance profiles**
310 All isolates demonstrated an MDR phenotype to critically important antimicrobial
311 agents for human medicine (WHO, 2017). Resistance was demonstrated to between
312 4 and 13 of the antimicrobial agents tested with some 46 different resistance profiles
313 recognised (Supplemental Material Table S2). The majority of isolates (74 %) were
314 resistant to 10 or more antimicrobial compounds. A summary of the frequency of
315 antimicrobial resistance is presented in Table 3.The most common MDR profile
316 among isolates (16 %) had resistance to 12 different compounds, including
317 ampicillin, trimethoprim, ciprofloxacin, streptomycin and tetracycline. Recently,
318 increasing numbers of MDR *E. coli* have been isolated from animal and human
319 sources (ECDC, 2017; Kennedy et al., 2017) and the acquisition of resistance genes
320 may convey a certain competitive advantage and ultimately lead to increased levels
321 of MDR *E. coli* in microbial populations (Webber et al., 2017).
322
323 **Occurrence of resistance determinants**
324 Antimicrobial agents frequently used in veterinary hospitals include broad-spectrum-
325 activity drugs, such as β-lactams and fluoroquinolones. Thus, hospitalized animals
326 may constitute an important reservoir of antimicrobial resistance (Karczmarczyk et
327 al., 2011a; WHO, 2017). Twenty-one percent of isolates possessed the commonly
328 reported *tet*(A) gene, 61% possessed the *tet*(G) gene and 15% of isolates carried

329 both, accounting for 53 of the 81 tetracycline resistant isolates. Sixty-five percent of
330 the MDR equine *E. coli* isolates harboured one or more TEM, SHV and CTX-M-2
331 group β-lactamases (Table 3), as determined by PCR. The specific variants of these
332 β-lactamases were not identified. AmpC β-lactamases are cephalosporinases that
333 confer resistance to a wide variety of β-lactam drugs and give rise to serious
334 therapeutic challenges in veterinary and human medicine. β-Lactam resistance in *E.*
335 *coli* generally occurs as a result of deregulation of the putative *ampC* gene or the
336 acquisition of a mobile genetic element containing an *ampC* gene (Li et al., 2007).
337 The presence of plasmid-mediated *ampC* genes such as the *bla*$_{CMY}$ variants were
338 not investigated here. In this study, the majority of isolates (79%) were positive for
339 *ampC* and *bla*$_{TEM}$ (55%). Isolates with the endogenous *ampC* gene and the narrow-
340 spectrum *bla*$_{TEM-1}$ gene are common in animals (Li et al., 2007). Consequently, the
341 high rates of ampicillin-resistant *bla*$_{TEM}$-positive isolates among the equine collection,
342 was to be expected (Li et al., 2007).
343 Both TEM and SHV enzymes belong to the class A family of β-lactamases and are
344 widely disseminated among the Enterobacteriaceae from veterinary sources (Li et
345 al., 2007). In this study *bla*$_{TEM}$ was identified in over half the equine isolates however
346 *bla*$_{SHV}$ was detected in only 4% of isolates. All were negative for *bla*$_{PSE}$ or *bla*$_{OXA}$.
347
348 TEM enzymes often co-exist with CTX-M enzymes in bacteria of animal origin (Li et
349 al., 2007). CTX-M genes are currently regarded as the predominant extended-
350 spectrum β-lactam (ESBL) type of animal origin, while they have also been
351 associated with human isolates in Europe since the late 1990s (Bevan et al., 2017).
352 In Ireland, *bla*$_{CTX-M}$-mediated ESBL resistance is widespread (Burke et al., 2016,
353 Morris et al., 2016) however the number of *bla*$_{CTX-M-2}$ group-positive isolates in this

study was low (n=5). To our knowledge, only one instance of *bla*$_{\text{CTX-M-2}}$ has previously been reported in animals in Ireland, as well as from bacteria of equine origin (Karczmarczyk et al., 2011a). These results are of interest considering the current epidemiology of these genes (Bevan et al., 2017).

Eighty-six percent of isolates were resistant to nalidixic acid, 79% were resistant to norfloxacin, 77% resistant to enrofloxacin and 73% resistant to ciprofloxacin. The high levels of resistance to quinolones and fluoroquinolones observed in this study are of medical concern, since ciprofloxacin is considered a very valuable antimicrobial agent and is the most effective drug in the treatment of Gram-negative bacterial infections, such as those caused by *E. coli* and *Salmonella* species (WHO, 2017). Ciprofloxacin resistance is generally conferred by mutations in target genes coding for DNA topoisomerases (*gyrA*, *gyrB*, *parC*, and *parE*) (Webber et al., 2017). Mutations in the DNA gyrase *gyrA* have been commonly reported in ciprofloxacin-resistant *E. coli* and *Salmonella* isolates (Karczmarczyk et al., 2011b). All isolates in this study were found to have two amino acid substitutions in their GyrA subunit (D87Y and S83F).

Distribution of integrons and gene cassettes

Class 1 and class 2 integrons were detected among 85 % of MDR *E. coli* isolated from diarrhoeagenic foals. Seventy-one percent of isolates were determined to possess the class 1 integrase gene (*intI1*), and just over half of these (54%) also carried both the *qacEΔ1* and *sul1* genes. Class 1 integrons may be one of the mechanisms responsible for the rise in MDR *E. coli* in animal production environments (Kelly et al., 2009; Kennedy et al., 2017). Their contribution to

379 resistance appears to be directed against antimicrobial compounds, including
380 streptomycin, trimethoprim and sulfonamides. The presence of class 1 integrons
381 associated with resistance to trimethoprim, streptomycin, and sulfonamide, is in
382 agreement with previous investigations (Kelly et al., 2009; Karczmarczyk et al.,
383 2011a; Kennedy et al., 2017).
384 PCR amplification with consensus primers targeting the regions flanking the gene
385 cassettes yielded 6 different amplicons, ranging from 0.5- to 2.5-kbp in size.
386 Integrase I (*intI1*)-positive isolates possessed none (5%), 1 (31%), 2 (21 %), 3 (5 %),
387 4 (7 %) or 5 (2%) gene cassettes (Supplemental Material Table S2). A
388 representative of each of these 6 gene cassettes was sequenced and annotated. A
389 schematic representation of the gene cassettes is shown in Figure 3. These include
390 the *aadA1* and *aadA2* genes conferring aminoglycoside resistance, *dfrA1* and *dfrA2*
391 conferring trimethoprim resistance and *sat1* conferring streptothricin resistance.
392 Fourteen percent of isolates carried class 2 integrons, as determined by amplification
393 of the integrase gene (*intI2*). These isolates possessed none (2%), 1 (6%), 2 (4%), 3
394 (1%) or 5 (1%) gene cassettes (Supplemental Material Table S2). The most common
395 cassettes carried by the *intI2*-positive isolates were the 0.5-kbp (67 %) and the 1.0-
396 kbp cassettes (42 %). The variable gene cassette regions from within the integron
397 structures identified were typical of cassettes present in class 1 and class 2 integron
398 structures in *E. coli* and are widely disseminated among the Enterobacteriaceae
399 (Kadlec et al. 2008). These results reveal the potential to transfer their MDR to other
400 pathogenic and non-pathogenic bacteria.
401
402 The development of MDR in any zoonotic bacterial species gives rise to the potential
403 for it to be transmitted from animals to humans and this could lead to major health

issues such as the transfer of MDR to other human intestinal mircoflora as well as other human pathogens that are traditionally susceptible to these agents (Kelly et al., 2009). MDR infections are associated with poorer clinical outcomes and higher cost of treatment than other infections, and there are already reports of pan-resistant strains in Gram-negative bacteria leading to treatment failure (Xiong et al., 2017). Colonization of diarrhoeagenic foals with MDR *E. coli* is particularly challenging, given the importance of these drugs. The ongoing usage of antimicrobial compounds in the treatment of animals increases the selective pressure for emergence of MDR organisms and dissemination of resistance (Webber et al., 2017).

Conclusion

The detection of potentially pathogenic MDR equine ExPEC in this study suggests a need for heightened attention to be focused on companion/sport animals as possible sources for human acquisition of disease-causing *E. coli.* Close monitoring of the virulence and antimicrobial resistance of these bacterial populations, in order to better understand their potential public health risk, is of great importance.

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Tables

Table 1: PCR primer characteristics

Table 2: Typable equine *E. coli* strains as determined by Public Health England

Table 3: The percentage of antibiotic resistance, resistance markers and virulence genes among equine *E. coli* isolates.

Figure captions

Figure 1: Dendrogram showing genotypic similarities between the equine *E. coli* isolates (n=83) based on pulsotypes.

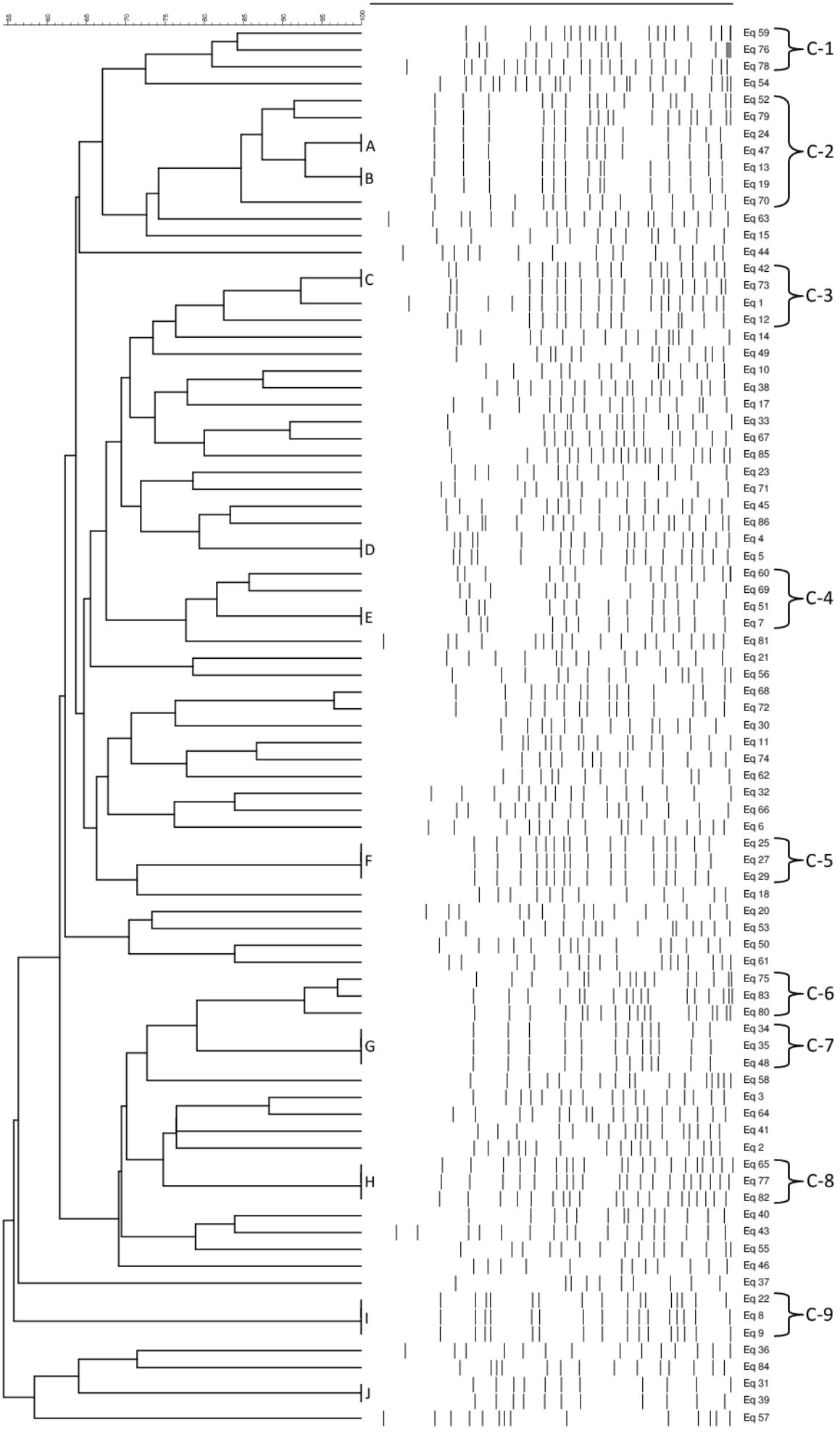
Figure 2: Adherence (A) and invasion (B) of Caco-2 cells by equine *E. coli* isolates positive or negative for virulence genes.

Figure 3: Schematic representation of the organization of sequenced gene cassettes of class 1 integrons.

Figure legends

Figure 2 legend: Adhesion (A) and invasion (B) of equine *E. coli* to human Caco-2 cells. Values indicate means of three separate experiments ± S.E. The results of statistical analysis are indicated by the letters a, b and c. Strains with different letters are significantly different from each other ($p < 0.05$).

Figure 3 legend: *aad* - aminoglycoside adenyltransferase; *attI1* - Integron associated recombination site; *dfr* - dihydrofolate reductase; *sat1* - streptothricin acetyl transferase; 59bp - 59 base pair element recombination site; hp - hypothetical protein; CS - conserved segment. Arrowheads represent the direction of the transcription.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Eq 59
Eq 76
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Eq 13
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Eq 15
Eq 44
Eq 42
Eq 73
Eq 1
Eq 12
Eq 14
Eq 49
Eq 10
Eq 38
Eq 17
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Eq 22
Eq 8
Eq 9
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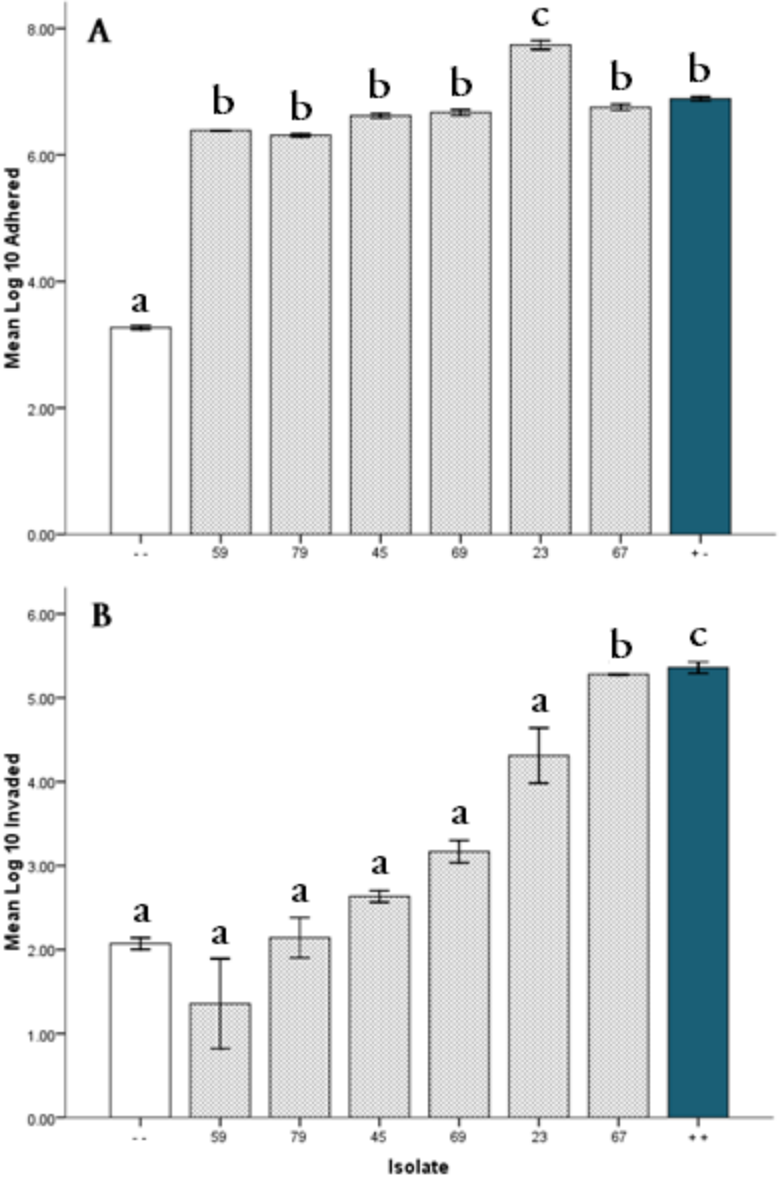
A
Mean Log 10 Adhered
8.00
6.00
4.00
2.00
0.00
a
b
b
b
b
c
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- -
59
79
45
69
23
67
+ -
B
Mean Log 10 Invaded
6.00
5.00
4.00
3.00
2.00
1.00
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a
a
a
a
a
b
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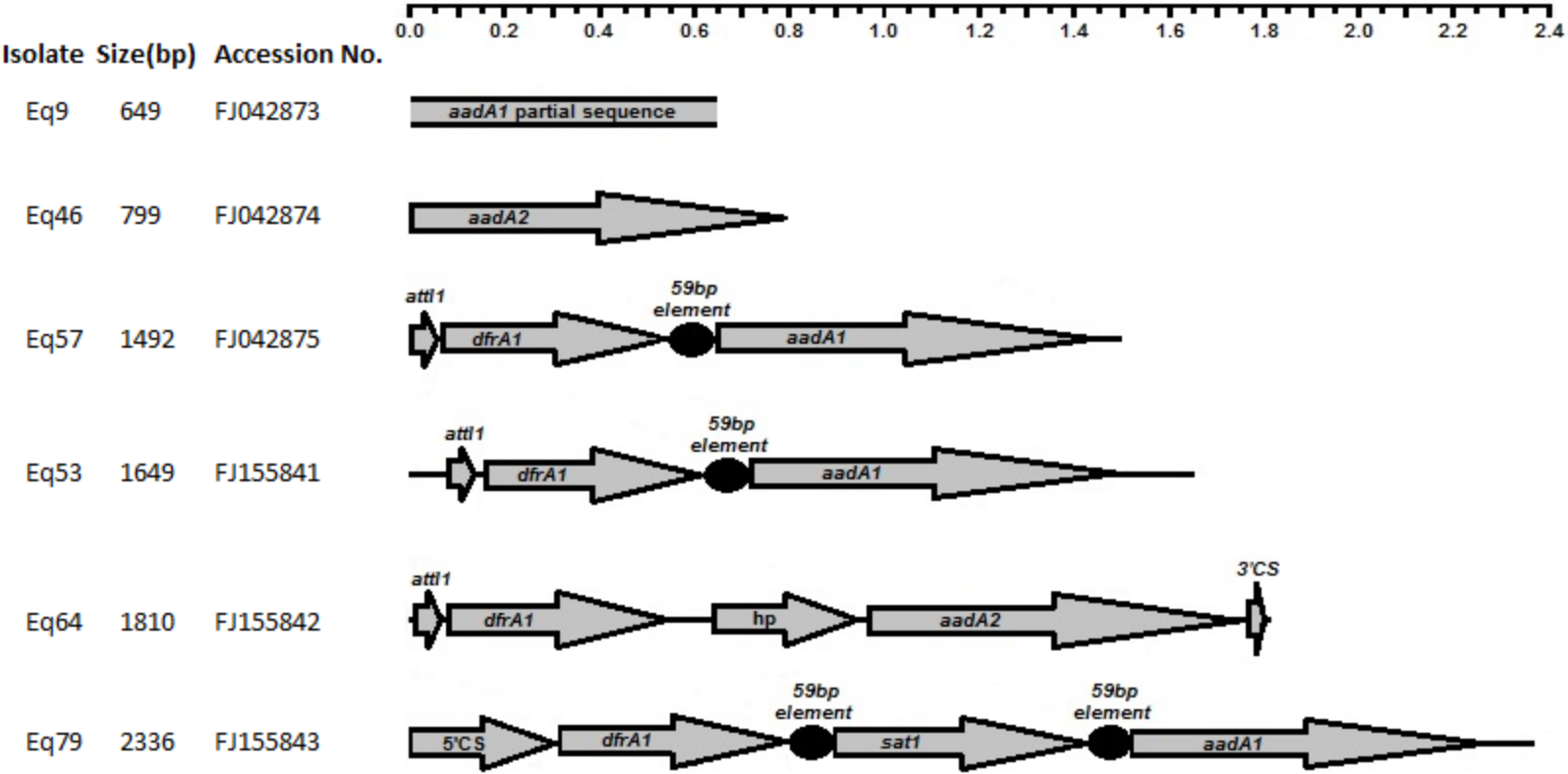
- -
59
79
45
69
23
67
+ +
Isolate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0.6
0.8
1.0
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Isolate
Size(bp)
Accession No.
Eq9
649
FJ042873
aadA1 partial sequence
Eq46
799
FJ042874
aadA2
Eq57
1492
FJ042875
attI1
dfrA1
59bp element
aadA1
Eq53
1649
FJ155841
attI1
dfrA1
59bp element
aadA1
Eq64
1810
FJ155842
attI1
dfrA1
hp
aadA2
3'CS
Eq79
2336
FJ155843
5'CS
dfrA1
59bp element
sat1
59bp element
aadA1

Table 1: PCR primer characteristics

		Sense[a]	Primer sequence (5'-to-3')	Reference
Serogroup	*rfb*$_{O157}$	F	CGG ACA TCC ATG TGA TAT GG	Paton and Paton, 1998
		R	TTG CCT ATG TAC AGC TAA TCC	
	rfb$_{O111}$	F	TAG AGA AAT TAT CAA GTT AGT TCC	Paton and Paton, 1998
		R	ATA GTT ATG AAC ATC TTG TTT AGC	
	rfb$_{O26}$	F	GCG CTG CAA TTG CTT ATG TA	Debroy et al., 2004
		R	TTT CCC CGC AAT TTA TTC AG	
Phylogenetic Group	*chuA*	F	GACGAACCAACGGTCAGGAT	Clermont et al., 2000
		R	TGCCGCCAGTACCAAAGACA	
	yjaA	F	TGAAGTGTCAGGAGACGCTG	Clermont et al., 2000
		R	ATGGAGAATGCGTTCCTCAAC	
	TSPE4C2	F	GAGTAATGTCGGGGCATTCA	Clermont et al., 2000
		R	CGCGCCAACAAAGTATTGCG	
Virulence Gene	*stx1*	F	ACA CTG GAT GAT CTC AGT GG	Gannon et al., 1992
		R	CTG AAT CCC CCT CCA TTA TG	
	stx2	F	CCA TGA CAG ACG GAC AGC AGT T	Gannon et al., 1992
		R	CCT GTC AAG CTG AGC ACT TTG	
	eaeA	F	GAC CCG GCA CAA GCA TAA GC	Lenahan et al., 2007
		R	CCA CCT GCA GCA ACA AGA GG	
	hlyA	F	GCA TCA TCA AGC GTA CGT TCC	Lenahan et al., 2007
		R	AAT GAG CCA AGC TGG TTA AGC T	
	fliCh7	F	GCGCTGTCGAGTTCTATCGAGC	Lenahan et al., 2007
		R	CAACGGTGACTTTATCGCCATTCC	
	cnf1	F	GAA CTT ATT AAG GAT AGT	Siqueira et al., 2009
		R	CAT TAT TTA TAA CGC TG	
	iucD (aerobactin operon)	F	TAC CGG ATT GTC ATA TGC AGA CCG T	Siqueira et al., 2009
		R	AAT ATC TTC CTC CAG TCC GGA GAA G	
	papC	F	GAC GGC TGT ACT GCA GGG TGT GGC G	Siqueira et al., 2009
		R	ATA TCC TTT CTG CAG GGA TGC AAT A	
	sfa/focDE	F	CTC CGG AGA ACT GGG TGC ATC TTA C	Siqueira et al., 2009
		R	CGG AGG AGT AAT TAC AAA CCT GGC A	
	afa/draBC	F	GCT GGG CAG CAA ACT GAT AAC TCT C	Siqueira et al., 2009
		R	CAT CAA GCT GTT TGT TCG TCC GCC G	
	neuC(K1)	F	AGG TGA AAA GCC TGG TAG TGT G	Johnson & Stell, 2000
		R	GGT GGT ACA TCC CGG GAT GTC	
A^R Gene	*bla*$_{TEM}$	F	GTA TGG ATC CTC AAC ATT TCC GTG TCG	Zhou et al., 1994
		R	ACC AAA GCT TAA TCA GTG AGG CA	
	bla$_{PSE}$	F	CGC TTC CCG TTA ACA ACT AC	Winokur et al., 2000
		R	CTG GTT CAT TTC AGA TAG CG	
	bla$_{SHV}$	F	TCA GCG AAA AAC ACC TTG	Kennedy et al., 2017
		R	TCC CGC AGA TAA ATC ACC A	
	bla$_{OXA}$	F	TAT CTA CAG CAG CGC CAG TG	Kennedy et al., 2017
		R	CGC ATC AAA TGC CAT AAG TG	
	ampC	F	CCC CGC TTA TAG AGC AAC AA	Kennedy et al., 2017
		R	TCA ATG GTC GAC TTC ACA CC	
	tet(A)	F	GCT ACA TCC TGC TTG CCT TC	Kennedy et al., 2017
		R	CAT AGA TCG CCG TGA AGA GG	
	tet(G)	F	GCT CGG TGG TAT CTC TGC TC	Kennedy et al., 2017
		R	AGC AAC AGA ATC GGG AAC AC	
	bla$_{CTX-M-2}$ group	F	TGA TAC CAC CAC GCC GCT C	Xu et al., 2005
		R	TAT TGC ATC AGA AAC CGT GGG	
	gyrA	F	TGT CCG AGA TGG CCT GAA GC	Baucheron et al., 2002
		R	CGT TGA TGA CTT CCG TCA G	
Integron Components	gene cassette	F	GGC ATC CAA GCA GCA AGC	Sandvang et al., 1998
		R	AAG CAG ACT TGA CCT GAT	
	intI1	F	GTG GAT GGC GGC CTG AAG CC	Sandvang et al., 1998
		R	ATT GCC CAG TCG GCA GCG	
	intI2	F	CAC GGA TAT GCG ACA AAA AGG T	Kennedy et al., 2017
		R	GTA GCA AAC GAG TGA CGA AAT G	
	sul1	F	CTT CGA TGA GAG CCG GCG GC	Sandvang et al., 1998
		R	GCA AGG CGG AAA CCC GCG CC	
	qacEΔ1	F	ATC GCA ATA GTT GGC GAA GT	Sandvang et al., 1998
		R	CAA GCT TTT GCC CAT GAA GC	

[a]F, forward; R, reverse; A^R, antibiotic resistance target

Table 2: Typeable *E. coli* strains as determined by conventional serotyping of 25 representative *E. coli* isolates and in-house PCR recovered from diarrhoeagenic foals by the Irish Equine Centre

	Isolate Code	Serotype
Conventional Serotyping	Eq4	*E. coli* O1
	Eq48	*E. coli* O1
	Eq74	*E. coli* O101
	Eq50	*E. coli* O153
	Eq77	*E. coli* O153
	Eq44	*E. coli* O19a
	Eq54	*E. coli* O33
	Eq45	*E. coli* O40
	Eq75	*E. coli* O91
In house PCR serotyping	Eq64	*E. coli* O26

Serotyping results for the remaining 16 isolates were not available, as the isolates were 'unidentifiable' using traditional serotyping techniques.

Table 3: Antibiotic resistance, associated resistance markers, and virulence genes among equine *E. coli* isolates

Antimicrobial Agents	**% Resistance**
Ampicillin*	93
Amikacin*	6
Amoxillin/clavulanic acid*	30
Gentamicin*	68
Ciprofloxacin*	73
Enrofloxacin*	77
Kanamycin*	70
Cefalothin	67
Naladixic Acid*	86
Norfloxacin*	79
Streptomycin*	98
Sulfonamide	98
Tetracycline	96
Trimethoprim	98
Integron Components	**% Present**
intI1	71
intI2	14
qacEΔ1	71
sul1	57
Gene Cassettes (kb)	**% Present**
0.5	64
1.0	40
1.5	35
1.7	10
2.0	4
2.5	6
Antibiotic Resistance Determinants	**% Present**
ampC	80
bla_{SHV}	4
tet(A)	21
tet(G)	61
bla_{TEM}	55
$bla_{CTX\text{-}M\text{-}2}$ group	6
Virulence Genes	**% Present**
iucD	57
afa/draBC	31
papC	36
sfa/focDE	2

*Critically important antimicrobial agents (WHO, 2017)