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ORIGINAL ARTICLE

Comparison by multilocus variable-number tandem repeat analysis and antimicrobial resistance among atypical enteropathogenic *Escherichia coli* strains isolated from food samples and human and animal faecal specimens

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Abstract

Aim: This study assessed whether multilocus variable-number tandem repeat analysis (MLVA) and antimicrobial susceptibility testing discriminated diarrhoeagenic atypical enteropathogenic *Escherichia coli* (aEPEC) from aEPEC indigenous to domestic animals or healthy people.
Methods and Results: MLVA genotyping of 142 aEPEC strains isolated from foods and faecal samples of domestic animals and humans revealed 126 distinct MLVA profiles that distributed to four clusters, yielding a Simpson's index of diversity (*D*) of 99·8%. Cluster 2 included 87% of cattle isolates and 67% of patient isolates. The plurality (15/34, 44%) of strains from healthy humans mapped to Cluster 1, while half (18/41, 44%) of the swine strains belonged to Cluster 4. Testing for antimicrobial susceptibility revealed that 52 strains (37%) of aEPEC were resistant to one or more agents; only 10 strains (7%) exhibited resistance to more than three agents. Strains isolated from swine or food exhibited a wider variety of resistance phenotypes than bovine or human strains.
Conclusions: MLVA assigned the aEPEC isolates from cattle and patients to Cluster 2, distinct from aEPEC from other sources. Hog yards may be a larger source of drug-resistant strains than are cattle ranches.
Significance and Impact of the Study: MLVA suggests that human diarrhoeagenic aEPEC are derived from cattle and are distinct from strains carried by healthy people and other animals. Cattle appear to be reservoirs of human diarrhoeagenic aEPEC.

Introduction

Enteropathogenic *Escherichia coli* (EPEC), one of the six diarrhoeagenic *E. coli* (DEC) pathotypes, is a major cause of diarrhoeal diseases among young children in developing countries (Scaletsky *et al.* 2002). EPEC can be further classified into typical EPEC (tEPEC) and atypical EPEC (aEPEC), depending on the presence or absence of the *E. coli* adherence factor plasmid; this episome encodes structural and regulatory proteins required for assembly of the bundle-forming pilus (BFP), a type-IV fimbrial adhesin that contributes to localized adherence (LA) to HEp-2 cell monolayers (Girón *et al.* 1991). aEPEC organisms have been reported to be more prevalent than tEPEC in both developing and developed countries (Ochoa *et al.* 2008). Animals can be reservoirs of aEPEC,

whereas humans are considered to be the only reservoir of tEPEC (Trabulsi *et al.* 2002).

Thus, EPEC is a well-recognized DEC; however, neither the origin nor the aetiological role of human aEPEC has been clarified to date (Forestier *et al.* 1996; Nguyen *et al.* 2006). aEPEC has been suggested to have an association with human diarrhoea, although this proposal remains somewhat controversial, in part due to the heterogeneous nature of these strains (Hernandes *et al.* 2009). However, recent epidemiological studies indicate that aEPEC is more prevalent than tEPEC in both developed and developing countries, and that aEPEC is important in both endemic diarrhoea in children and in diarrhoea outbreaks (Ochoa and Contreras 2011). Since the mechanisms and physiopathology of diarrhoea in aEPEC infection are not well elucidated, it is difficult to assess the significance of aEPEC isolates, particularly given that the organisms are isolated only sporadically from patients and foods. Therefore, it would be helpful for inspectors to understand the role of domestic animals as a potential source of aEPEC. Multilocus variable-number tandem repeat analysis (MLVA), the method used for rapid genotyping of aEPEC in the present study, is now used routinely for surveillance and outbreak detection (Lindstedt *et al.* 2007; Løbersli *et al.* 2012). The generic *E. coli* MLVA technique used here was based on known polymorphisms in 10 loci with variable numbers of tandem repeats (VNTR); this method works well as a fast and high-throughput genotyping system. Additionally, we noted that the emergence of antimicrobial resistance among *E. coli* strains of animal origin has important public health implications. EPEC, as a major pathogenic *E. coli*, can acquire, maintain and transmit antimicrobial resistance genes from other organisms in the environment (Zhao *et al.* 2012). Therefore, we tested our collection of aEPEC strains for susceptibility to 12 antibiotics, specifically using the disc-diffusion method on Mueller–Hinton agar plates.

In our previous study, a total of 679 foods or faecal specimens from domestic animals and healthy humans were examined for EPEC using our multiplex real-time PCR method (Hidaka *et al.* 2009) in combination with our newly developed hydrophobic grid-membrane filter (HGMF) colony hybridization method (Wang *et al.* 2011). Hence, we used intimin typing, phylogenetic grouping (Clermont *et al.* 2000) and virulence profiling (Afset *et al.* 2006) to determine whether our aEPEC isolates clustered with those isolated from diarrhoeal patients or with organisms isolated from foods or faecal samples of cattle, swine and healthy humans. In the present study, a total of 142 aEPEC strains were subjected to MLVA typing and antimicrobial susceptibility testing to provide further information for precise discrimination among aEPEC isolates.

Materials and methods

Strains

aEPEC strains used in the present study were previously isolated via a colony hybridization method using HGMF in combination with multiplex real-time PCR (Wang *et al.* 2011). Briefly, enrichment broth of each food or faecal specimen confirmed to be DEC-positive by PCR was filtered through a HGMF, which then was hybridized with digoxigenin-labelled *eae* DNA probe. Hybridizable strains were isolated from a replicate HGMF membrane, and the presence of the *eae* gene was confirmed by PCR. Each isolate was then screened (by PCR) for the presence of other DEC enterovirulence genes, particularly Shiga toxin (Stx)-encoding genes (*stx1* and *stx2*). Only those strains possessing *eae* (with or without *astA*) were defined as EPEC; these EPEC strains were subjected to further study. Several of these EPEC strains tested positive by PCR with primers for *bfpA*. However, none of these strains tested positive by PCR with primers for the *perA* gene, nor did these strains adhere to HEp-2 cells in a 3-h adhesion assay. Consequently, all of these strains were classified as aEPEC for further analysis, as suggested by Hernandes *et al.* (2009).

Additional aEPEC strains were derived as follows: 15 from food samples, 37 from cattle faeces, 41 from swine faeces and 4 from faecal samples of healthy carriers (Wang *et al.* 2011), along with another 30 from faecal samples of healthy carriers (Fujihara *et al.* 2009) and 15 from faecal samples of diarrhoeal patients (Nishikawa *et al.* 2002). A total of 142 aEPEC strains were tested using MLVA typing and antimicrobial susceptibility testing; the results permitted comparison of the phylogenetic groups and virulence profiles to those reported in our previous study (Wang *et al.* 2013). *Escherichia coli* strain DH5α was used as a nondiarrhoeagenic control.

Extraction of DNA

Suspensions of bacterial cells were boiled for 10 min, then briefly centrifuged at 10 000 × ***g*** for 3 min. The resulting supernatants were used directly in PCR reactions.

MLVA typing

The generic *E. coli* MLVA (GECM10) was performed to indicate the relationship between the aEPEC strains by polymorphisms in nine VNTR loci; primers were constructed so as to amplify the targets in all species where the loci were present, as described by Lindstedt *et al.* (2007) and Løbersli *et al.* (2012). Additionally, locus

CCR001, which is not a VNTR locus but a clustered regularly interspaced short palindromic repeat (CRISPR) locus, was tested in this bacterial typing system. One simplex PCR reaction was run for the CVN002 locus using GoTaqFlexi DNA polymerase and dNTPs (Promega, Madison, WI). The other nine loci were amplified in three multiplex PCR reactions with the KAPA2G^{TM} Fast Multiplex PCR kit (Kapa Biosystems, Boston, MA) according to manufacturer's recommendations, with each reaction consisting of 2 μl of extracted DNA in a total volume of 25 μl. Multiplex-1 (M1) contained 0·2 μmol each of the CVN003 and CVN014 primers; Multiplex-2 (M2) contained 0·2 μmol each of the CVN001, CVN004, CVN007 and CVN015 primers; and Multiplex-3 (M3) contained 0·2 μmol each of the CCR001, CVN016 and CVN017 primers. Each of the multiplex reactions used reagents from the KAPA2G^{TM} Fast Multiplex PCR kit. M1, M2 and M3 were run on a PCR Thermal Cycle Dice Touch TKR-TP350 (Takara, Kusatsu, Japan) under the following conditions: 95°C for 3 min; 30 cycles at 95°C for 15 s, 60°C for 30 s and 72°C for 30 s; followed by a hold at 72°C for 10 min. Primer set CVN002 was run separately using the following temperature profile: 94°C for 5 min; 30 cycles at 94°C for 30 s, 50°C for 30 s and 72°C for 50 s; followed by a hold at 72°C for 7 min (Lindstedt *et al.* 2007; Løbersli *et al.* 2012).

After the PCR amplifications, two samples were prepared for capillary electrophoresis. Sample-1 (S1) contained 1 μl of the Genescan-600LIZ size standard (Applied Biosystems, Foster City, CA), 12 μl of formamide and 1 μl of a 50-μl mixture of the PCR amplification mixtures consisting of a combination of 10 μl of M1, 1·75 μl of M2, 5 μl of the CVN002 amplification and 33·25 μl of water. Sample-2 (S2) was prepared by combining 1 μl of the 1 : 50 diluted M3 PCR reaction with 10 μl formamide and 1 μl of the Genescan-600LIZ size standard. The samples were mixed carefully, denatured for 2 min at 94°C, and cooled to room temperature before being subjected to capillary electrophoresis on an ABI-3130 Genetic Analyzer (Applied Biosystems). Capillary electrophoresis was run at 60°C on POP7 polymer (Applied Biosystems) for 20 min at a run voltage of 15 kV.

The minimum spanning tree (MST) was constructed using BioNumerics ver. 5.10 (Applied Maths, Sint-Martens-Latem, Belgium) (Lindstedt *et al.* 2007; Løbersli *et al.* 2012).

Proposed allele designations

For each locus, the following formulae, modified to suit our strains, yielded the best conversion to actual repeat numbers: CVN001, ((OP(observed PCR product size)+3)−250)/39; CVN002, (OP−272)/18; CVN003, (OP−404)/15; CVN004, (OP−231)/15; CVN007, (OP− 314)/18; CVN014, ((OP + 2)−111)/6; CVN015, (OP−189)/6; CCR001, (OP−131)/59; CVN016, ((OP + 2)−478)/6; and CVN017, ((OP+3)−202)/6. To best fit the data, all VNTR numbers were rounded to the nearest whole number, while the CRISPR numbers were rounded down to the nearest whole number, as described by Lindstedt *et al.* (2007) and Løbersli *et al.* (2012). The absence of PCR product was designated with a negative number (−2), and zero (0) was used to describe a positive PCR product containing no repeats. The results were always reported in the following order: CVN001, CVN002, CVN003, CVN004, CVN007, CVN014, CVN 015, CCR001, CVN016 and CVN017.

Antimicrobial susceptibility testing

A total of 142 aEPEC strains were subjected to antibiotic susceptibility testing for 12 antibiotics using the disc-diffusion method on Mueller–Hinton agar plates. Standard procedure M100-S25 of the Clinical and Laboratory Standards Institute (CLSI) (CLSI 2015) was strictly followed throughout the testing procedure. The discs were purchased from Becton, Dickinson and Company, and the abbreviation of antimicrobial agents and the concentration of the discs that were tested were as follows: ampicillin (AM) 10 μg, amoxicillin–clavulanic acid (AMC) 30 μg, cephalothin (CF) 30 μg, ceftriaxone (CRO) 30 μg, cefoxitin (FOX) 30 μg, aztreonam (ATM) 30 μg, gentamicin (GM) 10 μg, tetracycline (Te) 30 μg, ciprofloxacin (CIP) 5 μg, nalidixic acid (NA) 30 μg, chloramphenicol (C) 30 μg, and sulfamethoxazole–trimethoprim (SXT) 25 μg. The isolates were classified as susceptible (S), intermediate (I) or resistant (R) according to the zone diameter interpretative standards recommendations of CLSI-M100-S25. Confirmation of ESBL production was carried out by the combination disc-diffusion test with clavulanic acid (CLSI 2015), and AmpC-producing aEPEC was confirmed according to the description of Yagi *et al.* (2005).

Subtyping of phylogenetic group and virulence profile

The phylogenetic group and virulence profile results reported in our previous research (Wang *et al.* 2013) were compared with the MLVA and antimicrobial susceptibility testing data in the present study. Briefly, aEPEC strains were classified into four major phylogenetic groups (A, B1, B2 and D), as proposed by Clermont *et al.* (2000). Virulence profiles were based on the scheme of Afset *et al.* (2006), and the aEPEC strains were classified into Ia, Ib, II and N (not detected) groups. Group Ia has the strongest association with diarrhoea, followed by Ib and II.

Statistics

The differences between the aEPEC strains isolated from different sources were analysed by performing a chi-squared test or Fisher's exact probability test by using Prism 5 (GraphPad, San Diego, CA) and SPSS 20.0 (IBM, Armonk, NY). Simpson's index of diversity (*D*) was calculated according to formulae described by Hunter and Gaston (1988).

Results

MLVA typing

The 142 isolates collected for this study showed 126 distinct MLVA profiles, indicating high polymorphism in the tested samples. The resolution for aEPEC isolates was particularly enhanced by the GECM10 assay compared to results obtained previously by the combination of phylogenetic group, O antigen, intimin and virulence group. A Simpson's index of diversity (*D*) was calculated (Table 1). The GECM10 assay presented the highest diversity with a *D* value of 99·8%, followed by 88·5, 70·7, 70·2 and 52·8% for intimin, virulence group, phylogenetic group and O antigens identified using commercially available antisera respectively.

A MST of the total data set is provided in Fig. 1. The majority of strains belong to the central stem (in red); however, other strains form four additional branches (blue, green, pink, yellow). More than a third (15/41, 37%) of the swine strains were particularly easy to distinguish by the GECM10 assay, as these swine strains constituted the majority of the light green and yellow branches in Fig. 1. Four cattle isolates and two patient isolates were assigned to distinct blue and pink branches, respectively, that were clearly separated from other isolates.

We had previously determined the phylogenetic groups and virulence profiles of the aEPEC strains by PCR (Wang *et al.* 2013), and those results were incorporated into Fig. 1a–c, such that the central branch includes a core of phylogenetic Group B1 (closed red squares) in Fig. 1a and virulence Group Ia (closed red squares) or Ib strains (closed blue squares) in Fig. 1b; the representation of the other phylogenetic groups and virulence profiles increases in proportion moving outwards from the core. Combining the results of MLVA, phylogenetic grouping

Table 1 Simpson's index of diversity for groups sorted by phylogenetic group, O antigen, intimin, virulence group and MLVA type

	Phylogenetic group		O antigen		Intimin		Virulence group		MLVA*	
	Type	No.	Type	No.	Type	No.	Type	No.	Type	No.
	A	36	O157	3	UT	12	Ia	18	1	111
	B1	60	O103	4	α1	4	Ib	56	2	14
	B2	32	O115	2	α1,ι1	1	II	47	3	1
	D	14	O119	2	α2	1	None	18		
			O124	1	α2, θ/γ2	1	None/Ib	3		
			O127a	1	β1	31				
			O128	1	γ1	7				
			O145	2	δ/κ/β2O	15				
			O15	2	ε1	6				
			O153	5	ε1,νR/ε2	6				
			O166	1	ζ	7				
			O167	1	η,ε1	2				
			O168	1	θ/γ2	27				
			O20	1						
			O26	4	θ/γ2,γ1	1				
			O27	1	θ/γ2,ι1	1				
			O55	2						
			O63	1	ι1	12				
			O74	9	μB	1				
			O8	1	νR/ε2	1				
			UT	97	ξR/β2B	6				
D	70·2%		52·8%		88·5%		70·7%		99·8%	

UT, untypeable; *D*, Simpson's index of diversity.

*111 strains formed unique MLVA profiles, with each of 14 pairs of strains sharing a given MLVA profile, and a group of three strains sharing another MLVA profile.

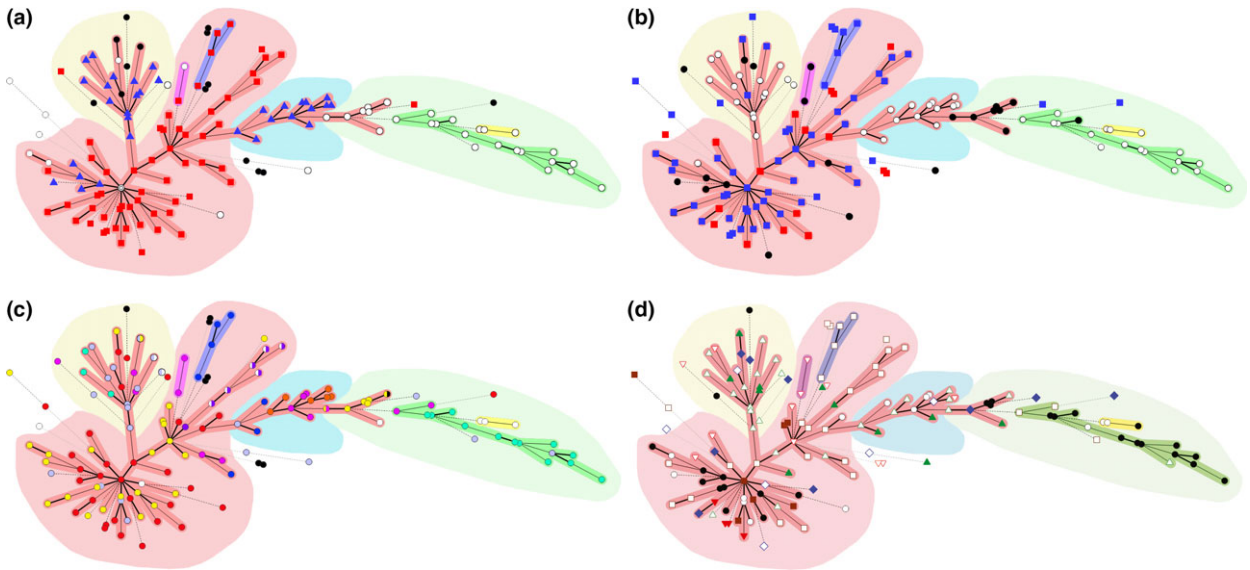

Figure 1 Population modelling using the minimum spanning tree (MST) method on a collection of 142 aEPEC isolates. The MST was constructed using the highest number of single-locus variants as the priority rule with no creation of hypothetical (or missing) types. (a) Relationships between MLVA and phylogenetic groups are shown. Each cluster, including Cluster 1 (yellow), Cluster 2 (red), Cluster 3 (blue) and Cluster 4 (green), was distinguished based on the phylogenetic group; open circles, closed red squares, closed blue triangles and closed black circles indicate strains of Group A, B1, B2 and D respectively. (b) Relationships between MLVA and virulence groups are shown. Closed red squares, closed blue squares, open circles and closed circles indicate strains of virulence Group Ia, Ib, II and the others respectively. (c) Relationships between MLVA and intimin types are shown. Red, yellow, blue, green, pink, orange, purple, white, black and light blue circles indicate β1, θ/γ2, ζ, $\delta/\kappa/\beta$2O, ι1, ξR/β2B, νR/ε2, ε1, γ1, α1/α2/η/μB and untypeable respectively. (d) Relationships between MLVA and host species are shown. Healthy carriers: triangles; patients: inverted triangles; cattle: squares; swine: circles; foods: rhombi. Closed symbols indicate antimicrobial-resistant strains.

and virulence profiling, the 142 strains were arbitrarily classified into four major clusters designated Cluster 1, Cluster 2, Cluster 3 and Cluster 4. Intimin types β1 (closed red circles) and θ/γ2 (closed yellow circles) appeared to accumulate in Cluster 2 (Fig. 1c).

The majority (15/34, 44%) of the strains from healthy carriers (triangles in Fig. 1d) fell within Cluster 1 (Table 2); 80% (12/15) of these isolates belonged to B2-II (phylogroup-virotype), with the remaining 20% belonging to D-Ib (Fig. 1a,b). Healthy carrier- and food-derived strains were prevalent in Cluster 1 than cattle- and swine-derived strains ($P < 0{\cdot}05$). Another 21% of the strains from healthy carriers showed the same phylogroup-virotype profile (B2-II) but fell within Cluster 3, in which

Table 2 Distribution of aEPEC strains in each cluster based on the MST*

	N (%) of strains					
	Total (n = 142)	Cattle (n = 37)	Swine (n = 41)	Food (n = 15)	Healthy carriers (n = 34)	Patients (n = 15)
Cluster 1	23 (16)	0^{b}†	2 (4·9)^{b}	4 (27)^{a}	15 (44)^{a}	2 (13)^{ab}
Cluster 2	71 (50)	32 (87)^{a}	17 (42)^{bc}	5 (33)^{bc}	7 (21)^{b}	10 (67)^{ac}
Cluster 3	13 (9·2)	0^{b}	4 (9·8)^{ab}	1 (6·7)^{ab}	7 (21)^{a}	1 (6·7)^{ab}
Cluster 4	28 (20)	3 (8·1)^{b}	18 (44)^{a}	3 (20)^{ab}	4 (12)^{b}	0^{b}
Outside main clusters	7 (4·9)	2 (5·4)	0	2 (13)	1 (2·9)	2 (13)
P value‡		0·000	0·000	0·504	0·003	0·000

*The Pearson's chi-squared test was used to compare the overall distribution of aEPEC strains in the four clusters, and the *P* value is $0{\cdot}000 < 0{\cdot}05$.

†Cells in each row with different lowercase letters 'a–d' are significantly different ($P < 0{\cdot}05$) from each other.

‡*P* values were calculated by single sample chi-squared test.

healthy carrier-derived strains were prevalent than cattle-derived strains ($P < 0{\cdot}05$). Half (71/142) of the strains investigated in this study mapped to Cluster 2, including the majority of the cattle- (32/37, 87%) and patient (10/15, 67%) derived strains. Cattle-derived strains were prevalent at higher frequencies in Cluster 2 than were swine-, food- and healthy carrier-derived strains ($P < 0{\cdot}05$), and patient-derived strains also were prevalent at higher frequencies in Cluster 2 than were healthy carrier-derived strains ($P < 0{\cdot}05$). Phylogenetic Group B1 was the most prevalent group in Cluster 2 (58/71, 82%), especially among patients (7/10, 70%) and cattle (30/32, 94%); the exceptions were three patient strains (one each belonging to phylogenetic Groups A, B2 and D), and two cattle strains (belonging to phylogenetic Group D) that fell far from the centre of Cluster 2. In contrast, most of the swine strains (35/41, 85%) were distributed to Clusters 2 and 4, in proportions of 42% (17/41) and 44% (18/41) respectively. Notably, swine-derived strains were prevalent at higher frequencies in Cluster 4 than were other strains ($P < 0{\cdot}05$), with the exception of food-derived strains. All of the strains in Clusters 3 and 4 belonged to phylogroup-virotype B2-II and A-II/N, respectively, except one fish-borne strain of B1 and one chicken-borne strain of D that had distant phylogenetic relationships. The results of single sample chi-squared tests showed that aEPEC strains from cattle, swine, healthy carriers and patients exhibited significant P values ($P < 0{\cdot}05$) when classified by the MLVA-based clustering (Table 2). The Pearson's chi-squared test also showed that the clustering was sufficient to provide significant distinction of the aEPEC strains from different sources ($P < 0{\cdot}05$).

Antimicrobial resistance of aEPEC isolates

The 142 aEPEC characterized in this study were isolated between 1997 and 2009. All were examined for the profiles of antimicrobial resistance. In terms of drug resistance patterns, aEPEC strains isolated from 2005 to 2009 exhibited resistance at a higher frequency than strains isolated from 1997 to 1999. No drug-resistant strains were detected in 1999. Only one strain (1/15, 6·7%) from 1998 exhibited duplex drug resistance, and one strain each from 1997 and 1998 showed resistance to a single drug (data not shown). In contrast, 21 strains (21/127, 17%) isolated from 2005 to 2009 were resistant to more than two antimicrobial agents. No multidrug-resistant strains were found from 1997 to 1999, or in 2005, whereas four strains resistant to three drugs, three strains resistant to four drugs and three strains resistant to more than five drugs were detected in 2006, 2007 and 2009, representing total resistance frequencies of 3·2, 2·4 and 2·4% respectively. No significant differences in aEPEC drug resistance frequencies were found via year-to-year comparison over the 7 years of the study, although several years had nominally higher resistance frequencies than others. However, resistance profiles of the aEPEC isolates differed significantly based on the sources from which the strains were isolated.

Table 3 shows the antimicrobial susceptibility of aEPEC isolates ($n = 142$) to 12 antimicrobial agents. Resistance patterns of these isolates are shown in Table 4. Among 142 aEPEC strains, 52 (37%) were resistant to one or more antimicrobial agents; the remaining 90 isolates (63%) were sensitive to all 12 of the antimicrobials tested in this study. Resistance to Te (26%) was observed at significantly ($P < 0{\cdot}0003$) higher frequencies than resistance to other antimicrobials (Table 3). Resistance to AM (9·2%) and CF (9·2%) occurred at significantly ($P < 0{\cdot}01$) higher frequencies than resistance to AMC (1·4%), CRO (1·4%) or FOX (1·4%) (Table 3). All isolates were susceptible to ATM, GM and CIP. The isolates derived from swine exhibited the highest frequency of resistance to Te (51%); this frequency was nominally higher than that observed in strains derived from food (40%), and significantly higher than those observed in cattle (16%; $P < 0{\cdot}01$), healthy carrier (8·8%; $P < 0{\cdot}001$) and patient (6·7%, $P < 0{\cdot}01$) isolates.

The majority (30/52, 58%) of the strains were resistant to only one antimicrobial agent (Table 4). A total of 10 strains (10/52, 19%) showed resistance to more than three antimicrobial agents, including two isolates from healthy carriers that exhibited resistance to seven antimicrobial agents (AM, AMC, CF, CRO, FOX, Te and NA) or to five antimicrobial agents (AM, AMC, CF, CRO and FOX); both of these last two strains were negative for ESBL but positive for AmpC. Additionally, one isolate from swine also exhibited resistance to five antimicrobial agents (AM, CF, Te, C and SXT). In conclusion, strains isolated from swine (56%) and food (60%) displayed resistance more frequently than did isolates from other sources. The prevalence of resistance in porcine isolates was significantly higher than those in cattle (19%; $P < 0{\cdot}01$), healthy carrier (29%; $P < 0{\cdot}05$) and patient (20%, $P < 0{\cdot}05$) isolates. The frequency of resistance in food isolates was higher than that in bovine strains ($P < 0{\cdot}01$), but not significantly higher than that in human isolates.

Compared with the MLVA data in Fig. 1c, 14% of cattle and 33% of patient isolates mapped outside of Cluster 2 and were sensitive to all antimicrobial agents; in contrast, all drug-resistant isolates derived from cattle and patients belonged to Cluster 2, with the exception of one cattle strain that was resistant to Te and C and mapped far from the centre of Cluster 2. On the other hand, most

Table 3 Antimicrobial resistance frequencies of aEPEC isolates

	N (%) of resistant strains					
Antimicrobial resistance*	Total (*n* = 142)	Cattle (*n* = 37)	Swine (*n* = 41)	Food (*n* = 15)	Healthy carriers (*n* = 34)	Patients (*n* = 15)
AM	13 (9·2)*	2 (5·4)	3 (7·3)	3 (20)	4 (12)	1 (6·7)
AMC	2 (1·4)				2 (5·9)	
CF	13 (9·2)*	1 (2·7)	2 (4·9)	3 (20)	5 (15)	2 (13)
CRO	2 (1·4)				2 (5·9)	
FOX	2 (1·4)				2 (5·9)	
ATM	0					
GM	0					
Te	37 (26)†	6 (16)^{bc}‡	21 (51)^{a}	6 (40)^{ab}	3 (8·8)^{c}	1 (6·7)^{bc}
CIP	0					
NA	8 (5·6)		3 (7·3)	3 (20)	2 (5·9)	
C	7 (4·9)	3 (8·1)	4 (9·8)			
SXT	11 (7·7)	2 (5·4)	5 (12)	2 (13)	2 (5·9)	

AM, ampicillin; AMC, amoxicillin–clavulanic acid; CF, cephalothin; CRO, ceftriaxone; FOX, cefoxitin; ATM, aztreonam; GM, gentamicin; Te, tetracycline; CIP, ciprofloxacin; NA, nalidixic acid; C, chloramphenicol; SXT, sulfamethoxazole–trimethoprim.

*The frequencies of resistance to AM or CF were significant higher than those to AMC, CRO or FOX ($P < 0·01$).

†The frequency of resistance to Te was significantly higher than those to other antimicrobials ($P < 0·0003$).

‡Cells in the Te row with different lowercase letters (a–d) are significantly different ($P < 0·05$) from each other.

Table 4 Antimicrobial resistance patterns of aEPEC isolates

	N (%) of resistant strains					
Resistance pattern (18 profiles)	Total (*n* = 142)	Cattle (*n* = 37)	Swine (*n* = 41)	Food (*n* = 15)	Healthy carriers (*n* = 34)	Patients (*n* = 15)
AM	3 (2·1)		1 (2·4, A-N)	1 (6·7, B1-Ib)	1 (2·9, B1-Ib)	
CF	6 (4·2)	1 (2·7, B1-Ib)		1 (6·7, B1-Ib)	3 (8·8, B2-II, 2; D-Ib, 1)	1 (6·7, B1-Ia)
Te	17 (12)	3 (8·1, B1-Ia, 1; B1-Ib, 2)*	12 (29)†	1 (6·7, B1-Ib)		1 (6·7, B1-Ib)
NA	1 (0·7)		1 (2·4, D-Ib)			
SXT	3 (2·1)			1 (6·7, B1-Ib)	2 (5·9, D-Ia; B1-Ib)	
AM-CF	1 (0·7)					1 (6·7, B1-Ia)
AM-Te	3 (2·1)		1 (2·4, B2-N)	1 (6·7, A-N)	1 (2·9, B2-II)	
CF-Te	1 (0·7)		1 (2·4, B1-Ib)			
Te-NA	5 (3·5)		2 (4·9, A-II; A-N)	2 (13, A-II; D-Ib)	1 (2·9, A-N)	
Te-C	1 (0·7)	1 (2·7, A-Ib)				
Te-SXT	1 (0·7)		1 (2·4, A-Ib)			
CF-Te-NA	1 (0·7)			1 (6·7, A-N)		
Te-C-SXT	3 (2·1)		3 (7·3, A-II)			
AM-CF-Te-SXT	1 (0·7)			1 (6·7, D-N)		
AM-Te-C-SXT	2 (1·4)	2 (5·4, B1-Ib)				
AM-CF-Te-C-SXT	1 (0·7)		1 (2·4, B2-N)			
AM-AMC-CF-CRO-FOX	1 (0·7)				1 (2·9, B2-N)	
AM-AMC-CF-CRO-FOX-Te-NA	1 (0·7)				1 (2·9, A-N)	
Total	52 (37)	7 (19)^{c}‡	23 (56)^{a}	9 (60)^{ab}	10 (29)^{bc}	3 (20)^{bc}

*Percentage, phylogenetic group – virulence group, number of strains.

†A-II, 5; B1-Ib, 5; D-Ib, 1; B2-N, 1.

‡Cells in the total row with different lowercase letters (a–d) are significantly different ($P < 0·05$) from each other.

(21/23, 91%) of the swine-derived, antimicrobial-resistant strains were located in Clusters 2 and 4, which correlated with the proportion of swine-borne strains in the collection. Nearly 80% (11/15) of the swine strains in the light green and yellow branches of Cluster 4 exhibited resistance to Te (six strains), Te-NA (two strains) or Te-C-SXT (three strains). However, except for the strains resistant only to Te, the swine-derived resistant strains in Cluster 2 showed distinct resistance patterns (such as CF-Te, AM-Te, Te-SXT and AM-CF-Te-C-SXT) compared to those in Cluster 4. Resistant strains isolated from healthy carriers were found in each of the four clusters, including four strains in Cluster 1, three in Cluster 2 and one each in Clusters 3 and 4. Although more than half of the strains in Cluster 3 were isolates from healthy carriers, only one of these strains was resistant to AM and Te. The five antimicrobial-resistant strains from a healthy carrier mapped to Cluster 1, and the seven drug-resistant strains mapped far away from Cluster 2. Of the three food-derived resistant strains, one each was assigned to Clusters 1, 2 and 4.

Discussion

aEPEC was commonly present in all five varieties of samples examined, with especially high recovery rates observed among domestic animals. However, it is not clear whether all of the aEPEC strains described here are enteropathogenic in humans. Nonetheless, our results demonstrate that genotyping could provide a powerful and useful tool for epidemiological discrimination of these isolates. Using the GECM10 assay of Lindstedt *et al.*, we obtained a *D* value of 99·8%. In a separate study that also used the GECM10 assay, Staples *et al.* (2013) obtained a total of 59 different MLVA profiles among 61 EPEC isolates derived from Australian patients, with no more than two isolates sharing the same profile ($D = 99{\cdot}9\%$). Similarly, a high diversity index value of 97% was observed in a set of 72 non-O157 : H7 VTEC isolates analysed in Argentina using the GECM10 assay (González *et al.* 2014). The GECM10 assay is very useful for genotyping all of the known serogroups of *E. coli*, and the high *D*-values obtained in the present study confirmed the results of previous studies (Staples *et al.* 2013; González *et al.* 2014). The GECM10 assay appears to provide sufficient discriminating power in epidemiological investigations of aEPEC; although Izumiya *et al.* (2010) reported that an alternative 18-locus MLVA system was required to distinguish a set of 641 EHEC strains belonging to the limited O serogroups such as O26, O111 and O157. However, the MST assigned most of the aEPEC strains onto the red arm and did not by itself show the relationship between the strains and their sources very well.

In the arbitrary trial where the MST was combined with conventional typing, MLVA permitted us to assign our collection of strains to four major clusters (1, 2, 3 and 4), as shown in Fig. 1. A total of 87% of cattle and 67% of patient isolates were mapped to Cluster 2. Phylogenetic Groups B1 and D, and virulence Group Ia were specific among patients and cattle strains in Cluster 2, which confirmed the results of our previous study (Wang *et al.* 2013). Bok *et al.* (2015) also observed that the phylogenetic Group B1 was predominant among isolates from beef cattle; similarly, an Australian survey (Staples *et al.* 2013) reported that the majority of EPEC strains fell into Group B1. Together, these findings suggested that cattle are a major source of strains that are diarrhoeagenic in humans, particularly for strains of Group B1. Our results also indicated that patient and Group-Ia strains were commonly found on the peripheral branches of the MST. Thus, diarrhoeagenic aEPEC strains may have evolved from common strains following the introduction of virulence genes. However, the present clustering may be somewhat arbitrary unlike MST. The robustness of present results will need to be validated by incorporating additional strains, and reappraisal of the clustering also will need to be performed in the next stage by introducing suitable statistical methods such as self-organizing maps and so on.

In contrast to the case with cattle, our results indicated that swine is a less frequent source of human aEPEC diarrhoeal strains. A majority (22/34, 65%) of the isolates derived from healthy carriers mapped to Clusters 1 and 3 of the MST, with 80% of the healthy carrier-derived strains in Cluster 1 and all of the strains in Cluster 3 belonging to the prevalent phylogenetic group and virulence profiles of B2-II. Furthermore, more than half (22/41, 54%) of the swine strains mapped to Clusters 3 and 4 with the profiles of B2-II and A-II/N respectively. In contrast, most (10/17, 59%) of the swine strains belonging to B1-Ib, B2-N or A-Ib/N were assigned to Cluster 2. These Cluster-2 strains differed widely from the swine strains in Clusters 3 and 4; notably, the antimicrobial resistance profiles were distinct between the drug-resistant swine strains of Clusters 2 and 4. These results showed that the strains from healthy carriers and swine mapped to Clusters 1, 3 and 4, and were different from cattle or patient strains that mapped to Cluster 2. Similar results were reported by Baldy-Chudzik *et al.* (2008), who observed that Phylogroup B1 was prevalent in herbivorous animals, while Group A was prevalent in carnivorous and omnivorous animals. In separate work, Afset *et al.* (2008) reported that strains of phylogenetic Group B2 were observed significantly more frequently among healthy controls. The present MLVA study yielded results consistent with those of our previous study (Wang *et al.*

2013), suggesting that aEPEC from cattle are diarrhoeagenic in humans. Thus, MLVA classification could be considered as a screening method to estimate the source of the diarrhoeagenic isolates and their etiological potential.

The use of antimicrobials in food animals and the role of antibacterials in promoting resistance in bacterial pathogens is an important public health issue. Although our results did not reveal statistically significant differences in aEPEC drug resistance frequencies by year, resistance profiles were significantly different depending on the sources from which the aEPEC organisms were isolated. In this context, we note that Boulianne *et al.* (2016) reported that the use of ceftiofur in hatcheries was significantly associated with the proportion of ceftiofur-resistant *E. coli* in chicken flocks. Similarly, Herrero-Fresno *et al.* (2016) demonstrated that apramycin-resistant *E. coli* strain 912 spread readily among pigs in the same pen; the apramycin treatment of these animals resulted in significantly higher counts compared to those in a nontreated group.

In the present work, aEPEC isolates were more frequently resistant to Te than to other antimicrobials; similarly, resistance to AM or CF was observed significantly more frequently than resistance to AMC, CRO or FOX. Similar findings of common resistance to Te and AM among *E. coli* isolates from food animals and meats also have been reported by other investigators (Sáenz *et al.* 2001; Schroeder *et al.* 2003; Zhao *et al.* 2012). For the strains characterized here, the level of resistance to different antimicrobials varied according to the source of the isolates. Te resistance was detected significantly more frequently among porcine strains than among isolates derived from cattle, healthy carriers, and patients. Food isolates (five from chicken and one from pork; data not shown) also exhibited a high frequency of resistance (40%) to Te. Similarly, *E. coli* isolates from poultry meats have been reported to exhibit resistance more frequently than isolates from beef and pork, with isolates derived from turkey meat exhibiting resistance to Te at frequencies significantly ($P \leq 0{\cdot}05$) higher than those observed in other meat isolates (Zhao *et al.* 2012). These findings could reflect the selective pressures imposed by antimicrobial use in different food animal production facilities.

All but one of the drug-resistant strains from cattle and patients mapped to Cluster 2 and belonged to Group B1-Ia or B1-Ib; the sole exception was a cattle-derived Group A-Ib strain that mapped well outside of the centre of Cluster 2 and was resistant to Te and C. These findings were in agreement with the MLVA results of the present study and those of our previous study (Wang *et al.* 2013), which found that strains belonging to phylogenetic Group B1 and virulence Group Ia were specific among cattle and patients. These results support the hypothesis that aEPEC strains from cattle and patients are distinct from those isolated from swine or healthy carriers (Wang *et al.* 2013). Indeed, our results suggested that cattle are a major source of strains diarrhoeagenic in humans. Additionally, we noted that ESBL/AmpC production was detected in only two of the 142 (1·4%) *E. coli* characterized in the present study. This frequency was much lower than those recently reported for isolates from pig faeces (20%) in Korea and for isolates from broiler chicken (82%) at slaughterhouses in Germany and the Netherlands (Pacholewicz *et al.* 2015; Han *et al.* 2016).

In conclusion, MLVA was sufficient to distinguish aEPEC strains from a variety of sources. The results of our present study suggested that cattle are potential reservoirs for aEPEC strains that are diarrhoeagenic in human. Healthy carriers and pigs are unlikely to be sources of human diarrhoeagenic aEPEC. High prevalence of aEPEC was demonstrated in cattle faecal specimens, indicating that faecal contamination at slaughter and processing may increase the spread of pathogenic strains. However, antimicrobial properties of strains isolated from cattle and patients revealed single or duplex resistance. In contrast, strains derived from swine, food and healthy carriers displayed various and complicated resistance patterns; swine and healthy carriers may act as reservoirs for multiresistant bacteria. The association between food animals and aEPEC impacts food safety and has implications for public health. Additional surveillance for aEPEC in food animals, particularly in poultry, is recommended, given that poultry increasingly is being recognized as a source of *E. coli* infections (Vincent *et al.* 2010; Overdevest *et al.* 2011; Bergeron *et al.* 2012).

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Conflict of Interest

No conflict of interest declared.

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