Genetic & virulence profiling of ESBL-positive E. coli from nosocomial & veterinary sources

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A B S T R A C T

CTX-M genes are the most prevalent ESBL globally, infiltrating nosocomial, community and environmental settings. Wild and domesticated animals may act as effective vectors for the dissemination of CTX-producing Enterobacteriaceae. This study aimed to contextualise blaCTX-M-14 positive, cephalosporin-resistant Enterobacteriaceae human infections and compared resistance and pathogenicity markers with veterinary isolates.

Epidemiologically related human (n=18) and veterinary (n=4) blaCTX-M-14-positive E. coli were fully characterised. All were typed by Xhol pulsed field gel electrophoresis and ST. Chromosomal/plasmidic locations of blaCTX-M-14 were deduced by S1-nuclease digestion, and association with ISEcp1 was investigated by sequencing. Conjugaation experiments assessed transmissibility of plasmids carrying blaCTX-M-14. Presence of virulence determinants was screened by PCR assay and pathogenicity potential was determined by in vitro Galleria mellonella infection models.

84% of clinical E. coli originated from community patients. blaCTX-M-14 was found ubiquitously downstream of ISEcp1 upon conjugative plasmids (25–150 kb). blaCTX-M-14 was also found upon the chromosome of eight E. coli isolates. CTX-M-14-producing E. coli were found at multiple hospital sites. Clonal commonality between patient, hospitals and livestock microbial populations was found. In vivo model survival rates from clinical isolates (30%) and veterinary isolates (0%) were significantly different (p<0.05). Co-transfer of blaCTX-M-14 and virulence determinants was demonstrated.

There is evidence of clonal spread of blaCTX-M-14-positive E. coli involving community patients and farm livestock. blaCTX-M-14 positive human clinical isolates carry a lower intrinsic pathogenic potential than veterinary E. coli highlighting the need for greater veterinary practices in preventing dissemination of MDR E. coli among livestock.

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1. Introduction

The occurrence of ESBLs (Extended Spectrum β-lactamases) carried by Enterobacteriaceae has increased significantly in the last decade. First identified in the 1980s (Matsumoto et al., 1988), CTX-M-type ESBLs have now established a global presence and in most countries are the most prevalent ESBL (Canton and Coque, 2006; Bonnet, 2004). The CTX-M enzyme family is sub-categorised into five major clusters, group 1, 2, 8, 9 and 25, and now includes 172 distinct gene sequences (at the time of writing) (http://lahey.org/Studies/). blaCTX-M-14 is the precursor of the CTX-M Group 9 cluster, showing 100% homology to the chromosomal β-lactamase, KLUY-1, from Kluvyera georgina (Olson et al., 2005). CTX-M-14 is encoded by two distinct genetic sequences; blaCTX-M-14a (GenBank Accession No. AF252622) and blaCTX-M-14b (GenBank Accession No. D1359215) separated by 2 silent mutations (Navarro et al., 2007). On a global perspective, blaCTX-M-14 is the second most commonly reported CTX-M, after blaCTX-M-15 (Hawkey and Jones, 2009). There are clinical reports worldwide, but in particular CTX-M-14 is the dominant ESBL enzyme in both Asia and the Iberian regions of Europe (Canton and Coque, 2006; Valverde et al., 2009; Hawkey, 2008).

Despite reports of clonal outbreaks (Pitout et al., 2005a), the global foothold of blaCTX-M-14 can be most accurately attributed to the mobility of its immediate genetic context, and its recruitment onto conjugative plasmids. Although there are increasing reports of blaCTX-M-14 linked with ISCR1 elements (Bae et al., 2007),
downstream association with IS\(\text{Ecp1}\) remains \(\text{bla}_{\text{CTX-M-14}}\)’s prevailing genetic context. Additionally, \(\text{bla}_{\text{CTX-M-14}}\) is found upon a diverse range of conjugative plasmid types, including \(\text{IncA/C, IncH2, IncL}\), but favouring \(\text{IncF}\) and \(\text{IncK}\) in Asian and Iberian regions, respectively (Wang et al., 2012; Ho et al., 2012; Valverde et al., 2009).

CTX-M-positive infections are striking in their high rates of community incidence when compared to that of nosocomial settings (Pitout et al., 2005a,b). Outside of the clinical sector, \(\text{bla}_{\text{CTX-M-14}}\) has been reported in bacterial specimens from a range of wild and domesticated animals; birds of prey (Costa et al., 2008), farmed fish (Jiang et al., 2012), gulls (Hernandez et al., 2010), pigs (Brinas et al., 2003), poultry (Smet et al., 2008), rabbits (Blanc et al., 2006) and wolves (Goncalves et al., 2011).

Work by Teale et al. (2005) and Liebana et al. (2006) reported the first incident of CTX-M-positive livestock infection in the UK including a \(\text{bla}_{\text{CTX-M-14}}\)-positive \(E.\ coli\). It was unknown whether these findings were isolated incidents, or signs of a new/emerging established antibiotic resistance reservoir. Assumptions on the prevalence of antibiotic resistance gene carriage in livestock specimens would be ill informed and unsubstantiated, and so nationwide surveillance studies were initiated (Snow et al., 2011, 2012). Concurrently, the Specialist Antimicrobial Chemotherapy Unit (SACU) conducted a Wales-wide targeted surveillance of third generation cephalosporin (3GC) resistant Enterobacteriaceae.

Our current work aims to contextualise the discoveries of Teale et al. (2005) and Liebana et al. (2006) with regards to the \(\text{bla}_{\text{CTX-M-14}}\) incidence in 3GC-resistant \(\text{Enterobacteriaceae}\) causing human infection, and compare veterinary and human clinical isolates.

2. Methods

2.1. Bacterial isolates

Cefpodoxime-resistant \(\text{Enterobacteriaceae}\) (\(n = 580\)) were collected and characterised as part of a nation-wide 3GC-resistance surveillance study in Wales, UK. These nosocomial specimens originated from either community-acquired (C) or hospital-acquired (H) infections and were classified as such (Table 1). All ESBL-producers were confirmed phenotypically via susceptibility testing (CLSI) and cephalosporin-clavulanate synergy testing. Screening for \(\text{bla}_{\text{CTX-M}}\) was carried out by multiplex PCR (Woodford et al., 2006) and subsequent sequence analysis.

\(\text{bla}_{\text{CTX-M-14}}\) \(E.\ coli\) collected during the work by Liebana et al. (2006) were included (isolates \#6477, \#6478, \#6479, \#6480) as comparators to the human isolates collected in all further investigation. In short, samples were collected from rectal swabs of scour-suffering calves in a North-Wales farm (location withheld). A history of treatment with amoxicillin-clavulanate, marbofloxacins and cefquinome was noted.

Unless otherwise stated, all bacterial isolates were cultured overnight at 37 °C on CBA plates (Oxoid Ltd., Hampshire, UK) and stored at −80 °C in Microbank Beads (Pro-Lab Diagnostics, Merseyside, UK).

2.2. Identification of \(\text{bla}_{\text{CTX-M-14}}\)

CTX-M Group 9-producing \(E.\ coli\) isolates identified were screened for the presence of \(\text{bla}_{\text{CTX-M-14}}\) using the primers CTX-M-14-F (5’-ccttggacaaaagagttgcaaagcgg-3’) and CTX-M-14-R (5’-cctggagctcgtgatgcgccaagc-3’).
ctg aag cca gca cat cgc-3') with an annealing step of 58 °C for 1 min. Amplicons were visualised by gel electrophoresis. CTX-M-14 DNA was purified and sequenced using suitable reference sequences (Accession numbers: blaCTX-M-14, DQ304479, blaCTX-M-14; FJ668792). Bioinformatics (Applied Maths, USA) and NCBI applications were used for sequence annotation.

2.3. Molecular typing of CTX-M-14-producing isolates

blaCTX-M-14-positive E. coli were classified as one of four phylogenetic groups by the triplex PCR described by Clermont et al. (2000) targeting the chuA, yjaA and tspE4C2 sequences.

Clonal relationships were evaluated by preparation of whole genomic DNA in agarose plugs (Bannerman et al., 1995) and overnight digestion with XbaI restriction enzyme. Digested fragments were separated by PFGE (9 °C, 6 V, 5 to 45s, for 22 h) and digest patterns visualised under UV. blaCTX-M-14 copy number was assessed by hybridisation of the PFGE gel with a [32P] radio-labelled blaCTX-M-14 DNA using a random priming kit (Stratagene). Gene locations were highlighted by incubation with chemi-luminescent autoradiograph films at −80 °C.

All E. coli were sequence typed (ST) using a combination of a two–locus based typing scheme (Weissman et al., 2012) and full MLST (Wirth et al., 2006).

2.4. Genetic context of blaCTX-M-14

Presence of ISEcp1 upstream of blaCTX-M-14 was confirmed using primers ISEcp1-U1 (5’-aaa aat gta aag aag gtt gtt-3’) (Lefon-Guibout et al., 2004) and ISEcp1-U2 (5’-gca ata ct acc tga ttt ct-3’) (Ho et al., 2005) coupled with the CTX-M-14 R primer. This PCR assay used an annealing temperature of 58 °C for 1.5 min. Positive amplicons were sequenced and annotated as previously described.

Genomic DNA prepared in agarose plugs was digested with S1-nuclease, separated by PFGE, and again hybridised with [32P] radio-labelled blaCTX-M-14 DNA. Subsequent analysis allowed identification of chromosomal and/or plasmidic locations of the blaCTX-M-14 genes. Replicon types of plasmids carrying blaCTX-M-14 were deduced by multiplex PCR assay (Carattoli et al., 2005).

2.5. Transmissibility of resistance

Conjugative matings was carried out in LB broth (Fisher Scientific Ltd.) between blaCTX-M-14–positive E. coli and the recipient strain GFP-E. coli HB101 (UA6190) (resistant to gentamicin, kanamycin and rifampicin) (Mata et al., 2010) in a 1:1 ratio. Transconjugants were positively selected for blaCTX-M-14 gene and associated phenotype upon LB agar (Fisher Scientific Ltd., Loughborough, UK) containing 50 μg/ml rifampicin and 2 μg/ml cefotaxime. Gene transfer was further confirmed by PCR. S1-nuclease digestion was used for plasmid characterisation in positive transconjugants, as described.

2.6. Evaluation of pathogenic potential

E. coli isolates were screened for a selection of virulence factors, as described previously by Johnson and Stell (2000); Multiplex 1- sofS, focC, fimH, papA, papGII. Multiplex 2– papC, papEF, bmaE, fyuA, papGII & III. Multiplex 3– kpsMT K1, iutA, nfsA, hlyA. Multiplex 4– kpsMT K5, kpsMT II, cnfI, cvc, PAI. Multiplex 5- traT, kpsMT III, afa/draBC, gfdD.

In vivo infection models used larvae of the wax moth Galleria mellonella and were infected with bacterial loads of approximately 1.2 × 10^6, 10^5 and 10^4 CFU/ larva. 10 μl of bacterial suspension was injected into the larval haemocoel through the rear-left proleg. Larvae were incubated in sterile petri plates at 37 °C for 72 h. Death was accepted when the larvae no longer responded to touch.

Table 2
Characteristics relating to virulence & pathogenic potential of blaCTX-M-14-positive E. coli.

<table>
<thead>
<tr>
<th>XbaI Type</th>
<th>Isolate #</th>
<th>Source</th>
<th>ST Type</th>
<th>Phylo Group</th>
<th>Larvae Survival vs Infection Challenge</th>
<th>VFs</th>
</tr>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>10^3 CFU/larva</td>
<td>10^4 CFU/larva</td>
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<tr>
<td>I</td>
<td>#5714</td>
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<td>B2</td>
<td>10%</td>
<td>0%</td>
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<td></td>
<td>#5722</td>
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<td>538</td>
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<td>0%</td>
<td>0%</td>
</tr>
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<td>80%</td>
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<tr>
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<td>#3021</td>
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<td>70%</td>
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<td></td>
<td>#6478</td>
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<td>A</td>
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<td>0%</td>
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<td></td>
<td>#6479</td>
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<td>30%</td>
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<tr>
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<td>#5737</td>
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<td>D</td>
<td>0%</td>
<td>0%</td>
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<tr>
<td></td>
<td>#3341</td>
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<td>38</td>
<td>D</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
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<td>#5720</td>
<td>Bangor (C)</td>
<td>38</td>
<td>D</td>
<td>100%</td>
<td>100%</td>
</tr>
</tbody>
</table>

a Indicates collection site and origin of specimen (C-community, H-hospital, V-veterinary).
b Phylogenetic grouping of E. coli.
c Virulence factors present.
d XbaI/PFGE types. E. coli shown to be clonal were grouped I, II, III and IV.
with larvae being checked every 24h up to the experiment endpoint.

3. Results

3.1. Summary of targeted surveillance of 3GC-resistance

Of 580 cefpodoxime-resistant Enterobacteriaceae received from participating hospitals across Wales as part of the targeted surveillance of 3GC-resistance 26 were found to harbour CTX-M Group 9 enzymes and 19 of these were confirmed for the presence of blaCTX-M-14 by PCR (18 E. coli, see Table 1, and one Klebsiella pneumoniae, isolate #2867, absent from Table 1). Remaining CTX-M Group 9 members were found to carry blaCTX-M-27 (n = 1) and blaCTX-M-9 (n = 6; all collected from the same hospital).

3.2. Description of CTX-M-14-producers

Subsequent analysis was performed on CTX-M-14-producing E. coli from both nosocomial and veterinary sources (Tables 1 and 2). All CTX-M-14-producers were resistant to both cefotaxime and cefazidime. All expressed confirmed ESBL-phenotypes by synergy testing against potassium clavulanate (CLAV). Co-resistance to ciprofloxacin, and gentamicin was common in CTX-M-14-producers with rates of 52.2% and 43.5%, respectively. No resistance to nitrofurantoin or mecillinam was found (data not shown). Sources of nosocomial E. coli were geographically diverse across 6 different hospital sites (Table 1), and carriage rates of blaCTX-M-14 in cephalosporin-resistant populations increasing in correlation with the proximity to the Liebana et al. (2006) index study farm. 84% of human E. coli were isolated from community-sourced patients. Only three were classified as hospital-acquired infections.

blaCTX-M-14-positive E. coli were variably assigned to phylogenetic groups A, D and B2 (Tables 1 and 2). Sixteen different XbaI clonal types of E. coli were identified, amongst 12 different STs (Table 2, Supplementary Fig. 1). Here, blaCTX-M-14 was most commonly carried by E. coli of ST38. Clone I E. coli, (ST538, isolates #5714 and #5722), were both collected at Bangor hospital, as were Clone II E. coli (ST718, isolates #5715 & #5740) (Tables 1 and 2). Clone III E. coli (ST10, isolates #3336, #3021, #6478 & #6479) were found across various collection sites; the index North Wales dairy farm (Liebana et al., 2006; location withheld) Wrexham, Newport (separated by a distance of approximately 126 miles), respectively. Clone IV, E. coli (ST38, isolates #3081 and #3202), also originate from two locations, Newport and Cardiff (separated by a distance of 15 miles), respectively.

3.3. Genetic context of blaCTX-M-14

75 kb IncA and 100 kb IncF plasmids were the most common host constructs of blaCTX-M-14 (Fig. 1, Table 1), both found in 36.4% (n = 8) of isolates. In addition, a 150 kb blaCTX-M-14-positive plasmid was found in isolates #5737 and #3336, and a 25 kb blaCTX-M-14-positive plasmid in isolate #6479. S1-digests identified the location of blaCTX-M-14 upon the chromosome of many E. coli, either in isolation (isolates #3341, #5720, #3081, #3202) or in addition to a plasmidic copy of the gene (isolates #5714, #5715, #6477, #6478) (Fig. 1).

All blaCTX-M-14-positive plasmids were conjugative in nature and transferred blaCTX-M-14 successfully to the GFP-E. coli HB101 recipient. In three transconjugants, blaCTX-M-14 was located on a plasmid construct different in size to that of its parent. E. coli #3021 carries blaCTX-M-14 upon a 75 kb plasmid, and on an additional 25 kb plasmid in its corresponding transconjugant (Fig. 1). Similar phenomenon were seen for isolates #2813 and #3336, suggesting recombination events resulting in relocation of the blaCTX-M-14 gene or re-structuring of plasmids. blaCTX-M-14 was conjugatively transferred to the GFP-recipient from #5720, despite only identification of a chromosomal copy of the gene within the donor strain. blaCTX-M-14 was found ubiquitously downstream of IScep1 upon all constructs in our test sample, both in complete (n = 20) and truncated forms, as with E. coli #5740, #3336 and #3253.

3.4. Pathogenicity of CTX-M-14-producing E. coli

Distribution of virulence factors in our test sample can be seen in Table 2. The type 1 fimbiae gene fimH was the most commonly found virulence marker, present in all E. coli. The traT gene, involved in both serum resistance and plasmid mobilisation, was found in all but three E. coli (isolates #3202, #3341 and #2813). The genes focC, sfA, rfc, nfaE, gafD, papEF, papG1, II & III, kpsMT III & KS were not found in any isolates. All other virulence markers were found at varying rates throughout the sample, as outlined (see Table 2). The average virulence score of veterinary E. coli was 5.8, compared to 4.4 for E. coli originating from hospitalised patients. No significant correlation between virulence score and phylogenetic group was seen.
traT was conjugatively transferred to transconjugants in 14/19 of cases, transconjugants #5740-T, #3081-T, #2864-T, #3221-T and #3253-T the exceptions. Other genes conjugatively transferred included fyuA (yersiniabactin) from isolates #6479 and #5720, the P-fimbriae genes papA and papC (#6479), intimA (aerobactin) and cvcC (colicin V) from #5715, and finally pai (undefined pathogenicity island marker) and kpsMT II (group II LPS capsule marker) from isolate #5722.

Kaplan-Meier survival analysis showed veterinary E. coli to be significantly more virulent than nosocomial E. coli isolates when used as an infective challenge in the C. mellonella infection model (p < 0.05) (Fig. 2). 100% of veterinary E. coli reached LD90 rates at 10^5, 10^6 and 10^7 cfu/larva. Corresponding rates of nosocomial E. coli achieving LD90 were 22.2%, (4/22), 38.9% (7/18) and 66.7% (12/18), respectively. No correlation between virulence potential of the E. coli, and their relative phylogenetic group classification, was seen (p > 0.1). Additionally, E. coli of the highest pathogenic potential included ST538, ST10 and ST131. Interestingly, E. coli ST131 did not carry significantly higher pathogenic potential, or record higher VF scores, than many of our STs described.

4. Discussion

We aimed to characterise carriage of blaCTX-M-14 within cephalosporin resistant Enterobacteriaceae infections in Wales, in context to strains and findings of Teale et al. (2005) and Liebana et al. (2006). We describe transmissible blaCTX-M-14 genes within the microbial populations of both human and cattle specimens, seemingly via a combination of horizontal gene transfer and clonal dissemination.

Table 1 shows 16 varying XbaI types across 12 E. coli STs with 4 multi-isolate clones identified, as highlighted. This clonal diversity highlights the importance of sampling multiple and varied geographical locations in such work. Such clonal variation also reflects previous findings (Valverde et al., 2009; Pitout et al., 2005a). Our findings highlight the possible role of clonal propagation in disseminating resistance among patients of a single nosocomial environment (as is the case with Clones I & II), and within community/environmental settings as with isolates #3081 and #3021 (Clone IV. ST38) found at Newport and Cardiff, respectively. 84% of the blaCTX-M-14-positive E. coli were isolated from community-acquired infections, further alluding to this route of spread, reflecting global epidemiology of CTX-M-positive infections (Pitout et al., 2005a,b). It should be noted, however, that patients with community-acquired infections may have had significant contact with healthcare establishments. E. coli Clone III (ST10) was found in patients across two hospitals and also of veterinary origin. Evidence of zoonotic transfer of CTX-M-positive clones is notable but limited (Liebana et al., 2012), and in this case the nature and direction of these possible disseminations is unknown. To our knowledge this is the first report of bla CTX-M-14 with the strain backgrounds of E. coli ST538, ST718 and ST73.
bla\textsubscript{CTX-M-14} was ubiquitously downstream of an IS\textsubscript{Ecp1} element. In 3 \textit{E. coli}, \textit{bla\textsubscript{CTX-M-14}} was found downstream of a truncated copy of the element IS\textsubscript{Ecp1}, in accordance with previous findings by Eckert \textit{et al.} (2005). \textit{bla\textsubscript{CTX-M-14}} was primarily located upon 100 kb IncF plasmids, reflecting the plasmid characteristics of the index isolate (Liebana \textit{et al.}, 2006; Cottrell \textit{et al.}, 2011). 75 kb IncF plasmids were also described in both clinical and veterinary isolates. Of note is the discovery of these \textit{bla\textsubscript{CTX-M-14}} genes upon the chromosome, either in isolation or in addition to a plasmidic copy. Such findings are sparsely reported (Kim \textit{et al.} 2011) with CTX-M genes rarely chromosomal outside of \textit{Proteus} spp. (Song \textit{et al.}, 2011). Given rates of recombination and transposition associated with resistance elements and the bacterial genome as a whole, it should be of no surprise that such events may occur. Hybridisation of XbaI digests with radio-labelled \textit{bla\textsubscript{CTX-M-14}} DNA confirmed multiple gene copies within the \textit{E. coli} genome (Data not shown). This highlights the horizontal gene transfer of \textit{bla\textsubscript{CTX-M-14}} between multiple genetic constructs and locations in the dissemination of the gene.

Veterinary \textit{E. coli} carry a more significant pathogenic potential than their clinical counterparts in a \textit{C. mellonella} infection model (see Fig 1 and Table 2). Veterinary \textit{E. coli} also carry a higher mean VF score than that of clinical \textit{E. coli}. Does this represent an evolutionary trade-off between virulent and resistant phenotypes to successfully navigate their diverse niches? In nosocomial settings, where antibiotic selective pressure would be much greater, multi-resistance as opposed to a wholly pathogenic approach is of a greater survival advantage. The transfer of virulence markers in addition to the \textit{bla\textsubscript{CTX-M-14}} gene adds further significance to the study of genetic dissemination of such resistance determinants. The convergence of both pathogenic and resistance evolutionary strategies in this way may act to further promote the persistence and dissemination of multi-resistant organisms in hospitals and the community.

4.1. Concluding remarks

This study describes a \textit{bla\textsubscript{CTX-M-14}}-positive \textit{E. coli} population across microbial populations of both humans and livestock. Evidence has been presented for roles of horizontal gene transfer, via conjugative plasmids, and clonal expansion, in this dissemination. Growing evidence suggests the potential of livestock to act as both reservoirs and endpoints of antibiotic-resistant organisms, acting as vectors to facilitate this spread. However, conclusions regarding reservoirs of community-acquired and zoonotic infections are still contentious, and cannot be defined here. Use of antibiotics in livestock may be an important factor promoting emergence and spread of multidrug resistant infections, the risk factors evaluated extensively (Snow \textit{et al.}, 2012). Given our relatively intimate relationship with livestock and our environment, exchange between microbial populations and route to human infection cannot and should not be dismissed. Moreover, given the higher pathogenicity potential with veterinary \textit{E. coli} isolates alongside a multidrug resistant phenotype, surveillance is pertinent to understand and prevent further dissemination of multi-drug resistant organisms through livestock and human populations.

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Transparency agreement

None to declare.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vetmic.2016.02.007.

References


