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

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ABSTRACT

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 $bla_{CTX\text{-}M\text{-}14}$-positive, cephalosporin-resistant *Enterobacteriaceae* human infections and compared resistance and pathogenicity markers with veterinary isolates.

Epidemiologically related human (n = 18) and veterinary (n = 4) $bla_{CTX\text{-}M\text{-}14}$-positive *E. coli* were fully characterised. All were typed by *Xba*I pulsed field gel electrophoresis and ST. Chromosomal/plasmidic locations of $bla_{CTX\text{-}M\text{-}14}$ were deduced by S1-nuclease digestion, and association with IS*Ecp1* was investigated by sequencing. Conjugation experiments assessed transmissibility of plasmids carrying $bla_{CTX\text{-}M\text{-}14}$. 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. $bla_{CTX\text{-}M\text{-}14}$ was found ubiquitously downstream of IS*Ecp1* upon conjugative plasmids (25–150 kb). $bla_{CTX\text{-}M\text{-}14}$ 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 $bla_{CTX\text{-}M\text{-}14}$ and virulence determinants was demonstrated.

There is evidence of clonal spread of $bla_{CTX\text{-}M\text{-}14}$-positive *E. coli* involving community patients and farm livestock. $bla_{CTX\text{-}M\text{-}14}$ 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.

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/). $bla_{CTX\text{-}M\text{-}14}$ is the precursor of the CTX-M Group 9 cluster, showing 100% homology to the chromosomal β-lactamase, KLUY-1, from *Kluyvera georgina* (Olson et al., 2005). CTX-M-14 is encoded by two distinct genetic sequences; $bla_{CTX\text{-}M\text{-}14a}$ (GenBank Accession No. AF252622) and $bla_{CTX\text{-}M\text{-}14b}$ (GenBank Accession No. D1359215) separated by 2 silent mutations (Navarro et al., 2007). On a global persp ective, $bla_{CTX\text{-}M\text{-}14}$ is the second most commonly reported CTX-M, after $bla_{CTX\text{-}M\text{-}15}$ (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 $bla_{CTX\text{-}M\text{-}14}$ 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 $bla_{CTX\text{-}M\text{-}14}$ linked with IS*CR1* elements (Bae et al., 2007),

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downstream association with IS*Ecp1* remains $bla_{CTX\text{-}M\text{-}14}$'s prevailing genetic context. Additionally, $bla_{CTX\text{-}M\text{-}14}$ is found upon a diverse range of conjugative plasmid types, including IncA/C, IncHI2, IncI, but favouring IncF and 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, $bla_{CTX\text{-}M\text{-}14}$ 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 $bla_{CTX\text{-}M\text{-}14}$-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 3rd generation cephalosporin (3GC) resistant *Enterobacterieaceae*.

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

2. Methods

2.1. Bacterial isolates

Cefpodoxime-resistant *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 $bla_{CTX\text{-}M}$ was carried out by multiplex PCR (Woodford et al., 2006) and subsequent sequence analysis.

$bla_{CTX\text{-}M\text{-}14}$ *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, marbofloxacin 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 $bla_{CTX\text{-}M\text{-}14}$

CTX-M Group 9-producing *E. coli* isolates identified were screened for the presence of $bla_{CTX\text{-}M\text{-}14}$ using the primers CTX-M-14-F (5′-ggt gac aaa gag agt gca acg-3′) and CTX-M-14-R (5′-ccg

Table 1
Characteristics relating to phenotype & epidemiology of antibiotic resistance of $bla_{CTX\text{-}M\text{-}14}$-positive *E. coli*.

*Xba*I Type[e]	Isolate #	Source[a]	ST Type	$bla_{CTX\text{-}M\text{-}14}$ Variant	$bla_{CTX\text{-}M\text{-}14}$ Location[b]	Plasmid Inc Type	Conjugative?	IS*Ecp1*[c]	MIC (μg/ml)		
									CTX/Clav	CAZ/Clav	CIPRO
I	#5714	Bangor (C)	538	a	75 kb, Ch	F/FIB/I1	+	U2	32/0.125	2/0.5	0.023
	#5722	Bangor (C)	538	a	75 kb	F/FIB/I1	+	U2	32/0.25	8/4	0.32
II	#5715	Bangor (C)	718	a	100 kb & Ch	K/F	+	U2	>128/0.5	2/2	32
	#5740	Bangor (C)	718	a	100 kb	K/F	+	U1	64/0.125	4/0.5	32
III	#3336	Wrexham (C)	405	a	150 kb	F	+	U1	64/0.5	4/0.5	>128
	#3021	Newport (H)	405	a	75 kb	F	+	U2	32/0.25	2/0.25	0.5
	#6478	(V)	10	a	75 kb & Ch	F	+	U2	128/0.062	128/0.062	16
	#6479	(V)	10	a	25 kb & 75 kb	K & F	+	U2	32/0.062	64/0.062	8
IV	#3081	Newport (C)	38	b	Ch	–		U2	>128/0.125	16/1	128
	#3202	Cardiff (C)	38	a	Ch	–		U2	128/0.25	4/0.5	0.5
–	#6480	(V)	10	a	75 kb	F	+	U2	>128/0.25	>128/0.25	0.023
–	#3018	Newport (H)	38	a	75 kb	F	+	U2	64/0.5	2/0.5	0.5
–	#5729	Bangor (C)	410	a	75 kb	F/FIB/I1	+	U2	32/0.125	4/1	0.16
–	#2813	Aberystwyth (C)	1193	a	100 kb	I1	+	U2	128/0.125	32/4	32
–	#2864	Carmarthen (C)	73	a	100 kb	K	+	U2	>128/0.25	16/1	0.032
–	#3221	Cardiff (C)	641	a	100 kb	K/F	+	U2	32/0.5	4/4	128
–	#3253	Cardiff (C)	648	a	100 kb	K	+	U1	64/0.125	8/0.5	128
–	#5682	Bangor (H)	131	a	100 kb	K	+	U2	32/1	2/0.25	32
–	#6477	(V)	226	a	100 kb & Ch	K	+	U2	128/2	>128/2	32
–	#5737	Bangor (C)	131	a	150 kb	F/FIA/FIB	+	U2	4/0.008	0.25/0.5	0.008
–	#3341	Wrexham (C)	38	a	Ch	–		U2	>128/0.5	8/1	0.015
–	#5720	Bangor (C)	38	b	Ch	–	+	U2	32/0.062	4/0.125	0.12

[d] MICs of ciprofloxacin (CIPRO), cefotaxime (CTX) and ceftazidime (CAZ) with/without clavulanate (Clav).
[a] Indicates collection site and origin of specimen (C-community, H-hospital, V-veterinary).
[b] Plasmidic (kb) or chromosomal (Ch) location of $bla_{CTX\text{-}M\text{-}14}$.
[c] Presence of full (U2) or truncated (U1) copy of the IS*Ecp*1 insertion element upstream of $bla_{CTX\text{-}M\text{-}14}$.
[e] *Xba*I/PFGE types. *E. coli* shown to be clonal were grouped **I**,**II**, **III** and **IV**.

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- $bla_{CTX\text{-}M\text{-}14a}$: DQ304479, $bla_{CTX\text{-}M\text{-}14b}$: FJ668792). Bionumerics (Applied Maths, USA) and NCBI applications were used for sequence annotation.

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

$bla_{CTX\text{-}M\text{-}14}$-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 *Xba*I restriction enzyme. Digested fragments were separated by PFGE (9 °C, 6 V, 5 to 45s, for 22 h) and digest patterns visualised under UV. $bla_{CTX\text{-}M\text{-}14}$ copy number was assessed by hybridisation of the PFGE gel with a ^{32}P radio-labelled $bla_{CTX\text{-}M\text{-}14}$ 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 $bla_{CTX\text{-}M\text{-}14}$

Presence of IS*Ecp*1 upstream of $bla_{CTX\text{-}M\text{-}14}$ was confirmed using primers IS*Ecp*1-U1 (5′-aaa aat gat tga aag gtg gt-3′) (Leflon-Guibout et al., 2004) and ISEcp1-U2 (5′-gca ata ct acct 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 ^{32}P radio-labelled $bla_{CTX\text{-}M\text{-}14}$ DNA. Subsequent analysis allowed identification of chromosomal and/or plasmidic locations of the $bla_{CTX\text{-}M\text{-}14}$ genes. Replicon types of plasmids carrying $bla_{CTX\text{-}M\text{-}14}$ 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 $bla_{CTX\text{-}M\text{-}14}$-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 $bla_{CTX\text{-}M\text{-}14}$ 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- *sfaS*, *focG*, *fimH*, *papA*, *papG*I. Multiplex 2- *papC*, *papEF*, *bmaE*, *fyuA*, *papG*II & III. Multiplex 3- *kpsMT* K1, *iutA*, *nfaE*, *hlyA*. Multiplex 4- *kpsMT* K5, *kpsMT* II, *cnf1*, *cvaC*, PAI. Multiplex 5- *traT*, *kpsMT* III, *afa/draBC*, *gafD*.

In vivo infection models used larvae of the wax moth *Galleria mellonella* and were infected with bacterial loads of approximately 1.2×10^7, 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 $bla_{CTX\text{-}M\text{-}14}$-positive *E. coli*.

*Xba*I Type[d]	Isolate #	Source[a]	ST Type	Phylo Group[b]	Larvae Survival vs Infection Challenge			VFs[c]
					10^5cfu/larva	10^6cfu/larva	10^7cfu/larva	
I	#5714	Bangor (C)	538	B2	10%	0%	0%	*fimH*, *fyuA*, PAI, *cvaC*, *traT*, *kpsMT* II, *kpsMT* K1
	#5722	Bangor (C)	538	D	0%	0%	0%	*fimH*, *fyuA*, PAI, *cvaC*, *traT*, *kpsMT* II, *kpsMT* K1
II	#5715	Bangor (C)	718	D	100%	80%	20%	*fimH*, *iutA*, *cvaC*, *traT*
	#5740	Bangor (C)	718	A	90%	60%	10%	*fimH*, *iutA*, *cvaC*, *traT*
III	#3336	Wrexham (C)	405	D	100%	70%	30%	*fimH*, *fyuA*, *traT*
	#3021	Newport (H)	405	D	90%	70%	30%	*fimH*, *fyuA*, PAI, *traT*
	#6478	(V)	10	A	0%	0%	0%	*fimH*, *fyuA*, *iutA*, *traT*, *papA*, *papC*
	#6479	(V)	10	A	0%	0%	0%	*fimH*, *fyuA*, *iutA*, *traT*, *papA*, *papC*
IV	#3081	Newport (C)	38	D	20%	20%	0%	*fimH*, *fyuA*, *iutA*, PAI, *traT*, *kpsMT* II
	#3202	Cardiff (C)	38	D	70%	20%	0%	*fimH*, *fyuA*
–	#6480	(V)	10	B2	0%	0%	0%	*fimH*, *bmaE*, *iutA*, *cvaC*, *traT*, *papA*, *papC*
–	#3018	Newport (H)	38	D	100%	90%	90%	*fimH*, *fyuA*, *traT*
–	#5729	Bangor (C)	410	A	30%	0%	0%	*fimH*, *fyuA*, *iutA*, *cvaC*, *traT*
–	#2813	Aberystwyth (C)	1193	D	20%	0%	0%	*fimH*, *fyuA*, *iutA*, PAI, *kpsMT* II
–	#2864	Carmarthen (C)	73	D	100%	100%	20%	*fimH*, *fyuA*, *hlyA*, PAI, *cnf1*, *traT*, *kpsMT* II
–	#3221	Cardiff (C)	641	D	100%	30%	10%	*fimH*, *fyuA*, *traT*, *papC*
–	#3253	Cardiff (C)	648	D	20%	20%	0%	*fimH*, *fyuA*, PAI, *traT*
–	#5682	Bangor (H)	131	D	20%	0%	0%	*fimH*, *fyuA*, *iutA*, PAI, *afa/draBC*, *traT*
–	#6477	(V)	226	A	20%	0%	0%	*fimH*, *bmaE*, *iutA*, *traT*
–	#5737	Bangor (C)	131	D	0%	0%	0%	*fimH*, *fyuA*, *iutA*, PAI, *traT*
–	#3341	Wrexham (C)	38	D	0%	0%	10%	*fimH*, *fyuA*, *papC*
–	#5720	Bangor (C)	38	D	100%	100%	20%	*fimH*, *traT*

[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] *Xba*I/PFGE types. *E. coli* shown to be clonal were grouped **I**, **II**, **III** and **IV**.

with larvae being checked every 24 h 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 $bla_{\text{CTX-M-14}}$ 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 $bla_{\text{CTX-M-27}}$ (n = 1) and $bla_{\text{CTX-M-9}}$ (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 ceftazidime. 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 $bla_{\text{CTX-M-14}}$ 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.

$bla_{\text{CTX-M-14}}$-positive *E. coli* were variably assigned to phylogenetic groups A, D and B2 (Tables 1 and 2). Sixteen different *Xba*I clonal types of *E. coli* were identified, amongst 12 different STs (Table 2, Supplementary Fig. 1). Here, $bla_{\text{CTX-M-14}}$ 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 $bla_{CTX\text{-}M\text{-}14}$

75 kb IncK and 100 kb IncF plasmids were the most common host constructs of $bla_{\text{CTX-M-14}}$ (Fig. 1, Table 1), both found in 36.4% (n = 8) of isolates. In addition, a 150 kb $bla_{\text{CTX-M-14}}$-positive plasmid was found in isolates #5737 and #3336, and a 25 kb $bla_{\text{CTX-M-14}}$-positive plasmid in isolate #6479. S1-digests identified the location of $bla_{\text{CTX-M-14}}$ 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 $bla_{\text{CTX-M-14}}$-positive plasmids were conjugative in nature and transferred $bla_{\text{CTX-M-14}}$ successfully to the GFP-*E. coli* HB101 recipient. In three transconjugants, $bla_{\text{CTX-M-14}}$ was located on a plasmid construct different in size to that of its parent. *E. coli* #3021 carries $bla_{\text{CTX-M-14}}$ 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 $bla_{\text{CTX-M-14}}$ gene or re-structuring of plasmids. $bla_{\text{CTX-M-14}}$ was conjugatively transferred to the GFP-recipient from #5720, despite only identification of a chromosomal copy of the gene within the donor strain. $bla_{\text{CTX-M-14}}$ was found ubiquitously downstream of IS*Ecp*1 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 fimbriae 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 *focG*, *sfaS*, *rfc*, *nfaE*, *gafD*, *papEF*, *papG* I, II & III, *kpsMT* III & K5 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.

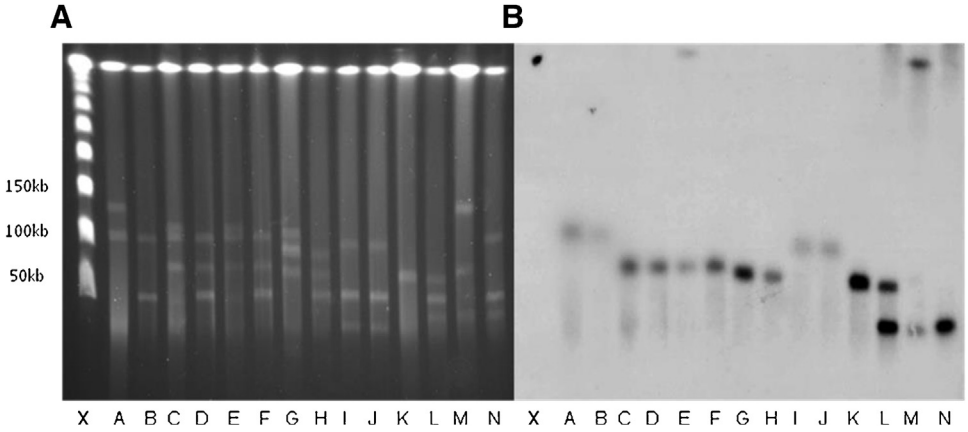

Fig. 1. A) PFGE of S1-nuclease Digestion of CTX-M-14-producing *Enterobacteriaceae* and corresponding transconjugants B) Autoradiograph of PFGE Gel after hybridisation with ^{32}P- radiolabelled $bla_{\text{CTX-M-14}}$ DNA.
X: Molecular Weight Lambda Marker. L1: #5682-Wild Type. L2: #5682-Transconjugant. L3: #5714-WT. L4: #5714-T. L5:#5722-WT. L6:5722-T. L7: #5729-WT. L8: #5729-L. L9: #5740-WT. L10: #5740-T. L11: #3021-WT. L12: #3021-T. L13: #2867-WT. L14: #2867-T.

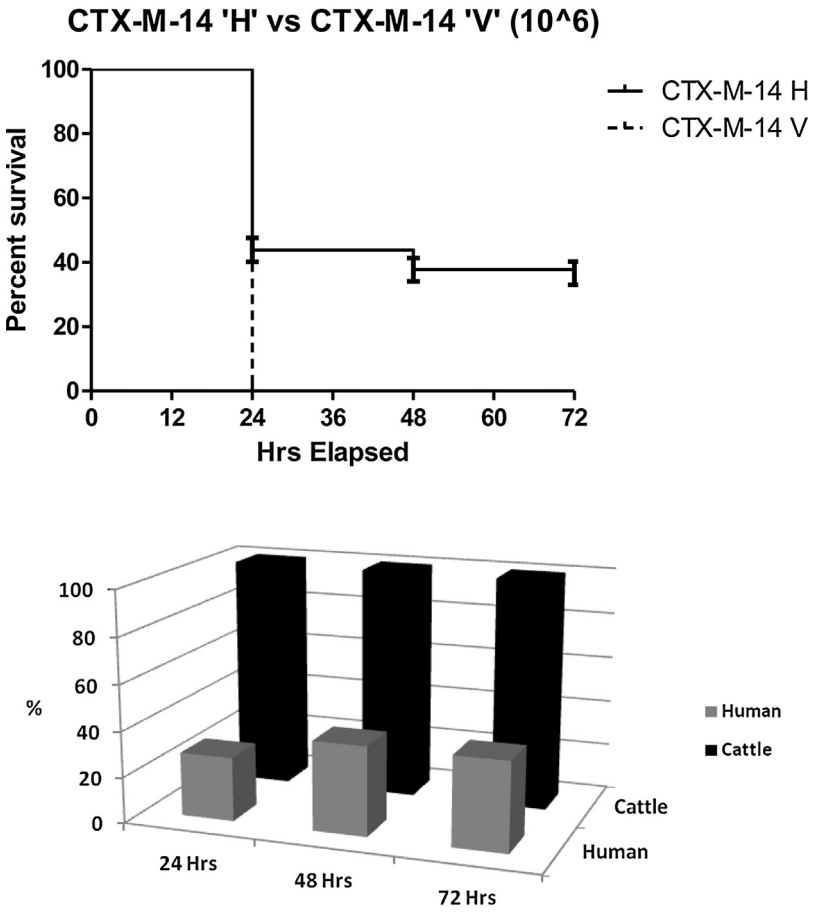

Fig. 2. A) Kaplan-Meier Survival Analysis of $bla_{CTX\text{-}M\text{-}14}$–carrying Human & Cattle *E. coli* at 10^6cfu/larva. B) Percent of $bla_{CTX\text{-}M\text{-}14}$-carrying Human & Cattle *E. coli* Reaching LD90 at 10^6cfu/larva. A) $bla_{CTX\text{-}M\text{-}14}$ Human Specimens: n = 18. $bla_{CTX\text{-}M\text{-}14}$ Cattle Specimens: n = 4. Log-rank (Mantel-Cox) test P value- 0.0001. Significant Difference?- Yes. B) $bla_{CTX\text{-}M\text{-}14}$ Human Specimens: n = 18. $bla_{CTX\text{-}M\text{-}14}$ Cattle Specimens: n = 4. For 24, 48 & 72Hrs respectively: $bla_{CTX\text{-}M\text{-}14}$ Human- 27.8%, 38.9% 38.9%. $bla_{CTX\text{-}M\text{-}14}$ Cattle- 100%, 100%, 100%.

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), *iutA* (aerobactin) and *cvaC* (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 *G. mellonella* infection model ($p \leq 0.05$) (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 $bla_{CTX\text{-}M\text{-}14}$ 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 $bla_{CTX\text{-}M\text{-}14}$ 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 *Xba*I 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 $bla_{CTX\text{-}M\text{-}14}$-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$_{CTX-M-14}$ was ubiquitously downstream of an IS*Ecp*1 element. In 3 *E. coli*, *bla*$_{CTX-M-14}$ was found downstream of a truncated copy of the element IS*Ecp*1, in accordance with previous findings by Eckert et al. (2005). *bla*$_{CTX-M-14}$ was primarily located upon 100 kb IncK plasmids, reflecting the plasmid characteristics of the index isolate (Liebana et al., 2006; Cottell et al., 2011). 75 kb IncF plasmids were also described in both clinical and veterinary isolates. Of note is the discovery of these *bla*$_{CTX-M-14}$ genes upon the chromosome, either in isolation or in addition to a plasmidic copy. Such findings are sparsely reported (Kim et al., 2011) with CTX-M genes rarely chromosomal outside of *Proteus* spp. (Song 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 *Xba*I digests with radio-labelled *bla*$_{CTX-M-14}$ DNA confirmed multiple gene copies within the *E. coli* genome (Data not shown). This highlights the horizontal gene transfer of *bla*$_{CTX-M-14}$ between multiple genetic constructs and locations in the dissemination of the gene.

Veterinary *E. coli* carry a more significant pathogenic potential than their clinical counterparts in a *G. mellonella* infection model (see Fig. 1 and Table 2). Veterinary *E. coli* also carry a higher mean VF score than that of clinical *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 *bla*$_{CTX-M-14}$ 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 *bla*$_{CTX-M-14}$-positive *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 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 *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.

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