Clonal spread of highly successful ST15-CTX-M-15 Klebsiella pneumoniae in companion animals and horses

Christa Ewers1*, Ivonne Stamm2, Yvonne Pfeifer3, Lothar H. Wieler4, Peter A. Kopp2, K. Schønning5,6, Ellen Prenger-Berninghoff1, Sandra Scheufen1, Inka Stolle1, Sebastian Günther4 and Astrid Bethe4

1Institute of Hygiene and Infectious Diseases of Animals, Justus-Liebig-Universität Giessen, Giessen, Germany; 2Vet Med Labor GmbH, Division of IDEXX Laboratories, Ludwigsburg, Germany; 3Robert Koch Institute, FG13 Nosocomial Pathogens and Antibiotic Resistance, Wernigerode, Germany; 4Institute of Microbiology and Epizootics, Centre for Infection Medicine, Freie Universität Berlin, Berlin, Germany; 5Department of Clinical Microbiology 445, Hvidovre Hospital, Hvidovre, Denmark; 6Department of Clinical Medicine, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark

*Corresponding author. Tel: +49-641-9938300; Fax: +49-641-9938309; E-mail: christa.ewers@vetmed.uni-giessen.de

Received 6 February 2014; returned 5 March 2014; revised 7 May 2014; accepted 21 May 2014

Objectives: To investigate the clinical relevance and molecular epidemiology of extended-spectrum β-lactamase (ESBL)-producing Klebsiella species in animals.

Methods: Antimicrobial susceptibilities and presence of ESBLs were examined among Klebsiella spp. (n = 1519) from clinical samples (>1200 senders from Germany and other European countries) mainly from companion animals and horses from October 2008 to March 2010. Multilocus sequence typing (MLST) and PFGE were performed including human isolates for comparative purposes.

Results: The overall ESBL rate was 8% for Klebsiella pneumoniae subsp. pneumoniae. Most K. pneumoniae subsp. pneumoniae ESBL producers were isolated from soft tissue infections (29.3%) and urinary tract infections (14.9%). The major ESBL type was CTX-M-15 (85.4%), located on different plasmid scaffolds (HI2, I1, FIA, FIB, FII, A/C, R and N). Other ESBL genes, such as blaCTX-M-1 (5.6%), blaCTX-M-3, blaCTX-M-9, blaSHV-2 and blaSHV-12 (1.1% each), were also detected. Additional resistances, e.g. to fluoroquinolones (89.9%), were frequently present. ST15-CTX-M-15, a clonal group that recently emerged in humans, accounted for 75.8% of the strains analysed by MLST and there was evidence for nosocomial events in five veterinary clinics. Human ST15-CTX-M-15 strains shared PFGE clusters with animal isolates, suggesting the dissemination of this clonal group between both populations.

Conclusions: Our data indicate a wide spread of ST15-CTX-M-15 K. pneumoniae subsp. pneumoniae, which should be considered as a zoonotic agent of high clinical relevance for humans and animals. Further research should be undertaken to unravel both microevolutionary and biological aspects probably contributing to this global success.

Keywords: ESBLs, antimicrobial resistance, wound infections, urinary tract infections

Introduction

Extended-spectrum β-lactamase (ESBL)-producing Escherichia coli and Klebsiella pneumoniae pose a serious threat to public health as they frequently cause severe infections, commonly leaving only limited therapeutic options.1 Also, in the veterinary field there is growing concern over the frequency of ESBL-producing bacteria, as these microorganisms pose a dual risk as infectious agents for animals and as putative zoonotic pathogens.2 So far, researchers preferentially put efforts into unravelling ESBLs produced by E. coli, whereas fewer data exist about the occurrence of multidrug-resistant Klebsiella spp., particularly in animals.3–6 ESBL-producing strains have been isolated from bovine mastitis and a variety of clinical conditions in horses, dogs, cats, swine and broilers.7–11 In contrast to the medical field, where epidemic clones such as K. pneumoniae ST15-CTX-M-15 and other frequently appearing phylogenetic lineages have been identified,1,12–14 the population biology of Klebsiella spp. from animal sources has been addressed only scarcely.9–11,15–17 Here, we report on the frequency, genotypic and phylogenetic characteristics of ESBL-producing K. pneumoniae from veterinary samples.

Materials and methods

Bacterial strains and determination of phenotypic and genotypic resistance

From October 2008 to March 2010, 1519 Klebsiella spp. strains were isolated from clinical samples (Table 1) from veterinary clinics in Germany (n = 1211) and 15 other European countries (n = 308). Species identification
Klebsiella pneumoniae ST15-CTX-M-15 in animals

Table 1. Klebsiella species isolated in the current study and rate of ESBL-producing isolates according to animal host and clinical background

<table>
<thead>
<tr>
<th>Category [total] (n/% ESBL isolates)</th>
<th>K. pneumoniae subsp. pneumoniae</th>
<th>K. pneumoniae subsp. ozaenae</th>
<th>K. oxytoca</th>
<th>other Klebsiella species</th>
</tr>
</thead>
<tbody>
<tr>
<td>Host</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>dog [658] (50/7.6)</td>
<td>504 (46/9.1)</td>
<td>20 (1/5.0)</td>
<td>130 (2/1.5)</td>
<td>4 (0)</td>
</tr>
<tr>
<td>cat [168] (27/16.1)</td>
<td>117 (25/21.4)</td>
<td>2 (0)</td>
<td>48 (2/4.2)</td>
<td>1 (0)</td>
</tr>
<tr>
<td>horse [160] (5/3.1)</td>
<td>112 (5/4.5)</td>
<td>19 (0)</td>
<td>26 (0)</td>
<td>3 (0)</td>
</tr>
<tr>
<td>bird [189] (2/1.1)</td>
<td>117 (2/1.7)</td>
<td>15 (0)</td>
<td>52 (0)</td>
<td>5 (0)</td>
</tr>
<tr>
<td>ruminants [38] (0)</td>
<td>30 (0)</td>
<td>4 (0)</td>
<td>4 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>rodents/lagomorphs [101] (2/2.0)</td>
<td>62 (2/3.2)</td>
<td>5 (0)</td>
<td>31 (0)</td>
<td>3 (0)</td>
</tr>
<tr>
<td>reptiles/amphibians/fish [152] (1/0.7)</td>
<td>62 (1/1.6)</td>
<td>7 (0)</td>
<td>80 (0)</td>
<td>3 (0)</td>
</tr>
<tr>
<td>primates [10] (0)</td>
<td>5 (0)</td>
<td>1 (0)</td>
<td>3 (0)</td>
<td>1 (0)</td>
</tr>
<tr>
<td>pig [10] (0)</td>
<td>9 (0)</td>
<td>1 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>other hosts [33] (2/6.1)</td>
<td>18 (2/11.1)</td>
<td>2 (0)</td>
<td>12 (0)</td>
<td>1 (0)</td>
</tr>
<tr>
<td>Clinical presentation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>skin/soft tissue infection [139] (28/20.1)</td>
<td>92 (27/29.3)</td>
<td>5 (0)</td>
<td>40 (1/2.5)</td>
<td>2 (0)</td>
</tr>
<tr>
<td>urinary tract infection [146] (19/13.0)</td>
<td>114 (17/14.9)</td>
<td>4 (0)</td>
<td>28 (2/7.1)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>respiratory tract infection [358] (13/3.6)</td>
<td>237 (10/4.2)</td>
<td>22 (1/4.5)</td>
<td>95 (2/2.1)</td>
<td>4 (0)</td>
</tr>
<tr>
<td>enteritis [506] (12/2.4)</td>
<td>335 (12/3.6)</td>
<td>31 (0)</td>
<td>130 (0)</td>
<td>10 (0)</td>
</tr>
<tr>
<td>infection of eyes or ears [70] (7/10.0)</td>
<td>53 (7/13.2)</td>
<td>2 (0)</td>
<td>13 (0)</td>
<td>2 (0)</td>
</tr>
<tr>
<td>genital tract infection/mastitis [93] (0)</td>
<td>76 (0)</td>
<td>5 (0)</td>
<td>12 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>other diseases [207] (10/4.8)</td>
<td>129 (10/7.8)</td>
<td>7 (0)</td>
<td>68 (0)</td>
<td>3 (0)</td>
</tr>
<tr>
<td>Total: 1519 (89/5.9)</td>
<td>1036 (83/8.0)</td>
<td>76 (1/1.3)</td>
<td>386 (5/1.3)</td>
<td>21 (0)</td>
</tr>
</tbody>
</table>

and testing for presumptive ESBL production was performed using Vitek® 2 (bioMérieux, Germany; AST-GN38 and AST-N062). ESBL production was verified by a double-disc confirmation test and the isolates were further screened for AmpC production by agar disc diffusion testing with cefoxitin discs and by PCR to detect acquired AmpC genes.18,19 ESBL producers were screened for blaTEM-type, blaSHV-type, blaCTX-M-type and blaOXA-type genes and for non-β-lactam resistance and integrase genes by PCR and sequencing.20 In case no common ESBL gene was identified, strains were tested for the blaTEM, blaSHV and blaOXA gene families (Table S1, available as Supplementary data at JAC Online). Sequences were analysed using Lasergene 6 (DNAStar, Germany) and Ridom SeqSphere 0.9.19 (http://www.pasteur.fr/mlst). Thirteen human ESBL-producing K. pneumoniae from Germany and Denmark were included for comparative purposes.3,13

Conjugation experiments

Transfer of β-lactam resistance was achieved by the filter mating method using recipient E. coli K12-J53 (AziR). Plasmid replicons were determined with a PCR-based typing kit (DIATEVA, Italy).

PFGE

Macrorestriction analysis was performed as described previously.21 Profiles were compared using BioNumerics (Version 6.6, Applied Maths, Belgium) and cluster analysis of Dice similarity indices based on UPGMA.

Multilocus sequence typing (MLST)

MLST was performed according to a published protocol.22 Allele sequences (LGC Genomics, Berlin, Germany) were analysed with Ridom SeqSphere in line with a database available online (www.pasteur.fr/mlst). eBURSTv2 analysis (http://eburst.mlst.net/9.asp) was performed to identify clonal complexes (CCs), defined as groups of two or more independent isolates sharing identical alleles at six or more loci.

Results

Klebsiella species distribution and ESBL rate according to host and clinical history

Of 1519 strains, 68.2% were K. pneumoniae subsp. pneumoniae, 25.4% Klebsiella ozaenae, 5.0% K. pneumoniae subsp. ozaenae and 1.4% other or undefined Klebsiella (Raoultella) species. Eighty-nine (5.9%) strains, predominantly K. pneumoniae subsp. pneumoniae, were confirmed as ESBL producers (Table 1). They were from seven European countries, including Germany (n = 72; 30 clinics in 27 towns), Italy (n = 10), France, Luxembourg (n = 2 each), Spain, Denmark and The Netherlands (n = 1 each) and were recovered from dogs (n = 50), cats (n = 27), horses (n = 5) and other animals (n = 7). ESBL producers were most frequent among skin/soft tissue infections (20.1%), followed by infections of the bladder/kidneys (13%) and eyes/ears (10%).

Genotypic and phenotypic resistance

ESBL-producing Klebsiella spp. predominantly harboured blaCTX-M-15 (85.4%) and less frequently blaCTX-M-1 (5.6%), blaCTX-M-3, blaCTX-M-9, blaSHV-2 and blaSHV-12 (1.1% each) (Figure S1, available as Supplementary data at JAC Online). Neither phenotypic cefoxitin resistance nor acquired AmpC
genes were detected. Overall, 76 strains possessed a blaSHV-type gene, mainly bla\textit{SHV-28} (88.2%); 57.3% strains carried bla\textit{TEM-1} and one isolate harboured bla\textit{TEM-82} in addition to the SHV-28 and CTX-M-15 genes. Other genes, conferring resistance to tetracycline [tet(A/B/C) (47.2%\%/11.2%\%/1.1%)], sulphonamides [sul1/2/3 (22.5%\%/75.3%\%/1.1%)], streptomycin [strA/B (93.3%\%/89.9%)], aadA1-like (59.6%)] and quinolones [aac(6\prime)-Ib-cr (92.1%), qoxA/B (87.5%\%/92.0%), qnrB2 (2.3%), qnrA1, qnrB1 and qnrB6 (1.2% each)] were detected as well. While \textit{intI2} was present in one \textit{K. oxytoca}, the class 1 integrate gene \textit{intI1} was highly prevalent (87.6%) and overall nine different integron gene cassettes were determined (Table S2, available as Supplementary data at \textit{JAC} Online). Self-transferability of a single ESBL plasmid (verified by plasmid preparation) was confirmed for 20 isolates of different sequence types (STs) and ESBL types. The plasmid replicon types were HI2, A/C, R, I1, N, FII, FIA and FIB (Table S3, available as Supplementary data at \textit{JAC} Online). Resistance towards fluoroquinolones (89.9%), tetracycline (43.8%), gentamicin (88.8%), tobramycin (86.5%) and trimethoprim/sulfamethoxazole (93.3%) was commonly observed (Table S4, available as Supplementary data at \textit{JAC} Online).

\section*{Clonal analysis and phylogenetic relatedness of ESBL-Klebsiella}
Animal and human strains grouped into seven PFGE clusters (A–G) and 19 singletons (Figure S1). The largest cluster G contains 43 CTX-M-15-SHV-28 \textit{K. pneumoniae} exclusively from animal sources from 15 German clinics. In four of these clinics, the clone has been isolated from 3–10 different animals at different sampling times (Figure S1). Within cluster C (n = 29), strains from animals show high similarity to human strains.

We found nine STs among 62 \textit{K. pneumoniae}, with ST15 being the predominant one (85.5%). All strains typed within the major clusters C and G belonged to ST15. Further STs were ST70 (n = 2), ST101, ST188 and five novel STs (ST988–ST992) (n = 1 each). To put our MLST data into a global context, an in silico data-set was established that contained 2331 \textit{K. pneumoniae} from human and animal sources (Figure 1) and included 940 STs separated into 30 CCs and 471 singletons. We further defined clonal groups (CGs) of CC37, as this CC tended to merge, constituting a group of unrelated STs rather than one diversified from a single common ancestor. More than 90% of ST15 strains expressed

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{eBurst analysis of allelic profiles of 2331 \textit{K. pneumoniae} strains (phenotypic ESBL, 28%; carbapenemase production, 11%; and unknown resistance phenotype, 61%) from the MLST database (www.pasteur.fr/mlst) (n = 1580; isolates from animal (98), human (1427), environmental (30) and unknown (25) sources), previously published but not listed in the MLST database (n = 682; isolates from animals (17) and humans (665)) (see Supplementary data, references for Figure 1) and finally from this study (isolates from animal (62) and human (7) sources). STs including a minimum of 20 isolates and/or identified in this study are indicated by numbers; those identified in the present study are additionally labelled by arrows. The names of the CCs are based on the ST assigned as the founder genotype. The relative size of the circles indicates the prevalence of STs and lines between STs connect single locus variants (SLVs). According to Breurec et al., the straggly CC in the centre of the picture (predicted founder ST37, CC37) was further separated into CGs that were determined as subsets of this complex and include: (i) one central genotype; (ii) its SLVs; and (iii) its double locus variants (DLVs).
\end{figure}

Ewers et al.
CTX-M-15, which is currently the most common ESBL type in human *Klebsiella* spp. as well (>70% of human ESBL strains of the *in silico* dataset), abundantly occurring in ST15 strains (Figure S2, available as Supplementary data at JAC Online).

**Discussion**

This study shows that ESBL-producing *K. pneumoniae* are far from being a rare finding in veterinary clinical samples. Within the given study period, third-generation cephalosporin-resistant (3GCR) *K. pneumoniae* accounted for 12% of invasive isolates from humans in European countries. Supposed that almost two-thirds of 3GCR *K. pneumoniae* isolates represent ESBL producers, as recently stated by the European Centre for Disease Prevention and Control (ECDC), these 12% only marginally exceed the overall percentage of 8% of ESBL producers identified among our animal strains. The primary sites of isolation almost resembled the situation in the medical field, where high numbers of ESBL-positive *Klebsiella* spp. are likewise isolated from urine and wounds.

CTX-M-15, the dominating β-lactamase type in the present study, is also considered the most common ESBL in *K. pneumoniae* from patients worldwide, indicating its global distribution across species. This supports previous findings about the generally higher abundance of CTX-M-15 in companion animal isolates, whereas CTX-M-1 is more commonly reported for livestock. Although CTX-M-15 was the predominant ESBL type in our isolates, they carried *bla*	extsubscript{CTX-M-15} on different plasmids, suggesting frequent horizontal transfer. Among the replicon types identified were IncFII, IncFIA and IncFIB, all of which are considered main vehicles of *bla*	extsubscript{CTX-M-15} dissemination in humans. Recently, *bla*	extsubscript{CTX-M-15} was found to be located on a novel plasmid scaffold widely distributed in *K. pneumoniae* from animals. The authors suggested that companion animals may be reservoirs for CTX-M-15-producing *K. pneumoniae* evolving separately from the human source of CTX-M-15 producers.

We found strong indications for nosocomial events in 5 of 45 veterinary clinics. This is in line with recent findings of clinically associated infections with OXA-48-ST15 *K. pneumoniae* in dogs in Germany and the spread of CTX-M-15-ST274 *K. pneumoniae* in a veterinary clinic in France. Another study reported about recurrent clinically acquired urinary tract infections in pets with ST15-CTX-M-15 *K. pneumoniae*, mirroring the situation observed for nosocomial infections in human hospitals. However, the predominance of one CG, namely CG15 (85.5%), among our ESBL producers may be not simply attributable to nosocomial dissemination, since 28 clinics were represented by one ST15-CTX-M-15 strain only. One case of pneumonia in a dog in Italy due to CTX-M-15-ST15 *K. pneumoniae* further hints to the emergence of this clone in other countries.

Diancourt *et al*. identified 40 STs among 67 predominantly human *K. pneumoniae*, hinting towards a high genetic diversity of this species. Weak clonality was also observed among 3GCR *K. pneumoniae* from major health institutions in Africa and Vietnam. However, CG15 (17%) was among the two major CGs identified, circulating in almost all participating centres but predominantly expressing CTX-M-1. Nevertheless, the spread of ESBL-producing *K. pneumoniae* is basically multiclonal, as >130 STs have been published as ESBL producers so far. Generally, CG15, CG23 and CG258 appear predominant among the entire *K. pneumoniae* population (Figure 1). CG15 is a well-known ‘ESBL clonal group’, whereas CG258 is particularly linked with the global dissemination of KPC-2 producers while ESBLs, including CTX-M-15, have been reported as well. In CG23, ESBL producers have been described only sporadically. Our data support the observation of the worldwide expansion of CG15 strains as we identified them among both epidemiologically related and unrelated animals. Less frequently observed STs among our isolates, e.g. ST101-CTX-M-15 and ST70-CTX-M-15 *K. pneumoniae*, were previously recovered from human patients as well and might also be of zoonotic relevance. Very recently, ST101-CTX-M-15 strains have also been reported as a major type among clinical cases in dogs and cats in Italy.

In conclusion, the frequent occurrence and nosocomial dissemination of multidrug-resistant CTX-M-15-producing *K. pneumoniae* determines animals as a relevant source of such strains. Whether they form a uniform pool with human isolates or represent a separate group of strains undergoing a parallel evolution remains unknown. Although there is no evidence for a direct transfer of multidrug-resistant *K. pneumoniae* between animals and humans, further studies should unravel relevant transmission paths of these life-threatening bacteria across both populations.

**Acknowledgements**

We thank the platform Genotyping of Pathogens and Public Health (Institute Pasteur, Paris, France) for coding MLST alleles and profiles and making them available at www.pasteur.fr/mlst. Control strains for virulence gene typing were kindly provided by Sylvain Brisse, Institute Pasteur, Paris. We would also like to express our thanks to Ines Diehl, Ursula Leidner, Christine Günther and Sybille Müller-Bertling for technical assistance.

**Funding**

This work was supported by a grant from the German Research Foundation to C. E. and S. G. (grants Ev 116/2-1 and GU 1283/3-1).

**Supplementary data**

Tables S1 to S4 and Figures S1 and S2 are available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/).

**References**


