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Subgrouping of ESBL-producing *Escherichia coli* from animal and human sources: An approach to quantify the distribution of ESBL types between different reservoirs

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ABSTRACT

Escherichia (E.) coli producing extended-spectrum beta-lactamases (ESBLs) are an increasing problem for public health. The success of ESBLs may be due to spread of ESBL-producing bacterial clones, transfer of ESBL gene-carrying plasmids or exchange of ESBL encoding genes on mobile elements. This makes it difficult to identify transmission routes and sources for ESBL-producing bacteria. The objectives of this study were to compare the distribution of genotypic and phenotypic properties of *E. coli* isolates from different animal and human sources collected in studies in the scope of the national research project RESET. ESBL-producing *E. coli* from two longitudinal and four cross-sectional studies in broiler, swine and cattle farms, a cross-sectional and a case–control study in humans and diagnostic isolates from humans and animals were used. In the RESET consortium, all laboratories followed harmonized methodologies for antimicrobial susceptibility testing, confirmation of the ESBL phenotype, specific PCR assays for the detection of bla_{TEM}, bla_{CTX}, and bla_{SHV} genes and sequence analysis of the complete ESBL gene as well as a multiplex PCR for the detection of the four major phylogenetic groups of *E. coli*. Most ESBL genes were found in both, human and non-human populations but quantitative differences for distinct ESBL-types were detectable. The enzymes CTX-M-1 (63.3% of all animal isolates, 29.3% of all human isolates), CTX-M-15 (17.7% vs. 48.0%) and CTX-M-14 (5.3% vs. 8.7%) were the most common ones. More than 70% of the animal isolates and more than 50% of the human isolates contained the broadly distributed ESBL genes $bla_{CTX\text{-}M\text{-}1}$, $bla_{CTX\text{-}M\text{-}15}$, or the combinations $bla_{SHV\text{-}12} + bla_{TEM}$ or $bla_{CTX\text{-}M\text{-}1} + bla_{TEM}$. While the majority of animal isolates carried $bla_{CTX\text{-}M\text{-}1}$ (37.5%) or the combination $bla_{CTX\text{-}M\text{-}1} + bla_{TEM}$ (25.8%), this was the case for only 16.7% and 12.6%, respectively, of the human isolates. In contrast, 28.2% of the human isolates carried $bla_{CTX\text{-}M\text{-}15}$ compared to 10.8% of the animal isolates.

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When grouping data by ESBL types and phylogroups $bla_{CTX\text{-}M\text{-}1}$ genes, mostly combined with phylogroup A or B1, were detected frequently in all settings. In contrast, $bla_{CTX\text{-}M\text{-}15}$ genes common in human and animal populations were mainly combined with phylogroup A, but not with the more virulent phylogroup B2 with the exception of companion animals, where a few isolates were detectable.
When *E. coli* subtype definition included ESBL types, phylogenetic grouping and antimicrobial susceptibility data, the proportion of isolates allocated to common clusters was markedly reduced. Nevertheless, relevant proportions of same subtypes were detected in isolates from the human and livestock and companion animal populations included in this study, suggesting exchange of bacteria or bacterial genes between these populations or a common reservoir. In addition, these results clearly showed that there is some similarity between ESBL genes, and bacterial properties in isolates from the different populations. Finally, our current approach provides good insight into common and population-specific clusters, which can be used as a basis for the selection of ESBL-producing isolates from interesting clusters for further detailed characterizations, e.g. by whole genome sequencing.

Introduction

Most bacterial pathogens associated with human enteric illness originate from animals and can be transmitted directly from animals to humans or indirectly, e.g. through food of animal origin, contaminated water or a common reservoir (Schlundt et al., 2004; Newell et al., 2010). This multitude of potential exposure routes and sources complicates epidemiological investigations. There is plenty of interaction between living beings, including humans, animals and – at the microbial level – their commensal bacteria as well as their facultative and obligate bacterial pathogens, sharing the same environment. Moreover, the horizontal gene transfer facilitates the spread of resistance genes in and between various bacterial species. To better understand these links and to identify options to reduce the spread of resistance and infections in humans, an integrative approach like 'One Health' is needed (Parmley et al., 2013; Calistri et al., 2013). It is assumed that by application of a global integrative concept, which utilizes a cross-sectoral data interpretation of zoonotic disease information, the prevention, prediction and control of zoonotic diseases can be improved (Wendt et al., 2014; Carmena and Cardona, 2014).

Third and fourth generation cephalosporins are critically important antimicrobials as defined by the World Health Organisation (www.who.int). In Enterobacteriaceae, resistance to these antimicrobial agents is caused by the production of extended-spectrum beta-lactamases (ESBLs). Especially, ESBL-producing *Escherichia* (*E.*) *coli* represent an increasing problem for public health (ECDC, 2011a,b). Among ESBLs, the CTX-M-type enzymes are most common and their number has increased rapidly during the last 10 years (Cantón et al., 2012; Carattoli, 2013). Apart from the occurrence in hospitalized patients, the spread of ESBL-producing *E. coli* within the human general population seems to be quite important (Eller et al., 2012; Leistner et al., 2013; Valenza et al., 2013; Rogers et al., 2011). Furthermore, travel to endemic countries with high prevalence rates for ESBL-producing Enterobacteriaceae (Peirano et al., 2011; Von Wintersdorff et al., 2014) and contact to pets are described as risk factors for colonization (Meyer et al., 2012), while another study identified human-to-human contact as a key factor in the dissemination of ESBL-producing bacteria among humans (Wu et al., 2013). Livestock and animal-derived foods are considered relevant sources for the colonization of humans with ESBL-*E. coli* (Sharp et al., 2014a; Hille et al., 2014). The same ESBL genes, ESBL gene carrying plasmids or even the same ESBL-producing *E. coli* isolates could be detected in animals and the farmers taking care for them (Dierikx et al., 2013a; Moodley and Guardabassi, 2009; Hammerum et al., 2014). Furthermore, a higher incidence of ESBL-producing *E. coli* was described for horse owners and persons having contact to horses or several pet species (Huijbers et al., 2013). In a case–control study, consumption of pork was shown as a risk factor for colonization with ESBL-producing *E. coli* (Leistner et al., 2013). Recent studies have shown frequent colonization of poultry (Kola et al., 2012; Leverstein-van Hall et al., 2011; Overdevest et al., 2011; Kluytmans et al., 2012), cattle (Schmid et al., 2013; Reist et al., 2013), pigs (Friese et al., 2013; Von Salviati et al., 2014) and other animal species, e.g. dogs, horses, rats, wild birds (EFSA, 2011; Ewers et al., 2012, Guenther et al., 2011) with ESBL-producing bacteria. Based on comparison of the ESBL genes found there, it is assumed that foodborne exposure has an impact on the probability of human colonization and/or infection caused by ESBL-producing bacteria (Leverstein-van Hall et al., 2011; Overdevest et al., 2011; Depoorter et al., 2012).

Source attribution methods allocate cases of foodborne disease to the vehicle food or other sources responsible for illness or colonization. Identifying and quantifying the contribution of the different sources to zoonotic human infection or colonization is an important first step in efforts to reduce the exposure of the consumer to zoonotic pathogens. The principle of the microbial subtyping approach is to compare the subtypes of isolates from different animal and food sources with the subtypes of bacteria isolated from humans (Hald et al., 2007; Sharp et al., 2014b). Recently, a first approach was described to quantify the proportion of human isolates which match isolates found in animal populations (Valentin et al., 2013). More traditional, epidemiological and statistical methods have been used to compare different data sets and to estimate the proportion of similarity between the different data sets. The detection of bacteria with the same properties may provide hints towards a possible transmission between different populations or production stages (e.g. along the food processing chain) or a common exposure source. Recently, a proportional similarity index was calculated when comparing typing patterns from methicillin-resistant *Staphylococcus aureus* (MRSA) isolates from two different populations (Vossenkuhl et al., 2014). In another approach, a statistical method was described to investigate linkages in phenotypic resistance profiles in a population sample of *Salmonella* Typhimurium isolates from sporadic salmonellosis cases which can be used to identify linked resistance in isolates from different populations (Ruddat et al., 2012).

The objectives of the present study were to analyze the genotypic and phenotypic properties of ESBL-producing *E. coli* isolates from different animal and human sources and to identify common and distinct isolate subgroups based on typing results. Different questions were addressed: (i) which subgroups may be identified; (ii) which subgroups are common to humans and to specific animal populations and how are these subgroups distributed among the species and (iii) are there subgroups of ESBL-producing *E. coli* isolates which are unique for a population? The outcome of this study may support the decisions which isolates should be selected for an in-depth characterization, e.g. by whole genome sequencing, since it may show the relationship of common subtypes and identify the properties of unique subtypes in a strain collection.

Material and methods

Origin of the isolates

The national interdisciplinary research project RESET (www.reset-verbund.de) is a consortium of ten partners from veterinary medicine, human medicine and public health institutes. In the scope of the RESET project, ESBL-producing *E. coli* from humans and animals were investigated in several studies since 2011. In livestock populations, two longitudinal and four cross-sectional studies were performed to collect potential ESBL-producing *E. coli* using MacConkey agar containing 1 mg/L cefotaxime as selective medium. In the longitudinal studies, faecal and environmental samples from seven broiler and seven swine farms were taken at the beginning, in the middle and at the end of their fattening period (Laube et al., 2013; Von Salviati et al., 2014; Friese et al., 2013). In one cross-sectional study, faecal samples, boot swabs, and dust samples from 30 mixed dairy and beef cattle farms (3 age groups) and 15 beef cattle farms (2 age groups) were sampled in the southern part of Bavaria, Germany (Schmid et al., 2013). In other cross-sectional studies following the same protocol, 34 broiler, 48 pig and 42 cattle farms (2 age groups if available) representing different regions of Germany were investigated (Hering et al., 2014a,b,c). In each of the studies, different samples were tested, which made several isolates available for further typing. For each farm included in the studies duplicates showing the same pattern were identified. Only one isolate of each pattern per farm was used for this analysis.

To include also clinical isolates in the analysis, *E. coli* isolates from routine monitoring programs on antimicrobial resistance in animal pathogens (GE*RM*-Vet, Bft-GermVet), but also from diagnostic submissions were used. These isolates covered livestock populations (poultry, swine, cattle) and companion animals (dogs and horses) from all regions of Germany. The isolates were already available in strain collections of the Federal Office of Consumer Protection and Food Safety, Berlin, the Institute of Farm Animal Genetics, Friedrich-Loeffler-Institut, Neustadt-Mariensee, and the Institute of Hygiene and Infectious Diseases of Animals, Justus Liebig University Gießen (GERMAP, 2008; Schink et al., 2011, 2013; Schmiedel et al., 2014).

Within further studies ESBL-producing *E. coli* isolates were collected from healthy humans, outpatients and hospitalized patients. The presence of ESBL-producing *E. coli* was determined among 3344 study participants from the German community (Valenza et al., 2014). Moreover, isolates from hospitalized patients were collected within a matched case–control study at the Charité University Hospital Berlin. Cases were defined as patients colonized with community-acquired ESBL-producing *E. coli* identified <72 h after hospital admission (Leistner et al., 2013). Furthermore, ESBL-producing *E. coli* isolates from ambulant and nosocomial infections were collected by several laboratories and were submitted to the Robert Koch Institute and the Institute for Medical Microbiology of the Justus Liebig University Gießen (Pfeifer and Eller, 2012; Pfeifer et al., 2013; Schmiedel et al., 2014).

Overall, a rather comprehensive set of isolates was available from several epidemiological as well as molecular-epidemiological studies, which represents the most common ESBL-producing *E. coli* strains in each of the animal populations as well as the human population investigated.

Antimicrobial susceptibility testing and molecular typing

In the consortium RESET, all different laboratories followed a harmonized methodology. The potentially ESBL-producing *E. coli* isolates were tested for their antimicrobial susceptibility to cephalosporins (CLSI, 2012, EUCAST (www.eucast.org)) and all isolates showing resistance to 3rd generation cephalosporins (cefotaxime and/or ceftazidime) were subjected to further characterization (Guerra et al., 2012; Pfeifer et al., 2013). First, the ESBL phenotype of the potentially ESBL-producing *E. coli* isolates was confirmed by antimicrobial susceptibility testing (CLSI, 2012; EUCAST; Michael et al., 2014). Then, the ESBL-producing isolates were characterized by different pheno- and genotypic methods, including (i) antimicrobial susceptibility testing by the CLSI broth microdilution method (CLSI, 2012), the CLSI disc diffusion method (CLSI, 2012) or VITEK®2 compact system with AST N117 cards (bioMérieux) and Etest® stripes (Liofilchem®) containing ertapenem, cefepime, chloramphenicol and nalidixic acid, (ii) specific PCR assays for the detection of ESBL bla_{TEM}, bla_{CTX}, and bla_{SHV} genes and sequence analysis of the complete ESBL gene (Rodríguez et al., 2009), and (iii) a multiplex PCR for the detection of the four major phylogenetic groups of *E. coli* (Clermont et al., 2000). Sequence analysis of bla_{TEM} genes focused on those isolates, in which no ESBL variants of bla_{CTX-M} or bla_{SHV} genes were present.

For the purpose of this study, results of the antimicrobial susceptibility testing of gentamicin, chloramphenicol and sulfamethoxazole/trimethoprim were interpreted using common breakpoints, derived from EUCAST clinical breakpoints (www.eucast.org). For the analysis of antimicrobial resistance pattern, isolates were classified as susceptible (below or equal to breakpoint) or non-susceptible (above breakpoint for intermediate or resistant). For the latter, the term 'resistant' is used through this document. The results obtained with these typing methods were used for the categorization of the isolates, which is explained in the following sub-section.

Approach for the categorization of isolates and analysis

The basis of these comparisons is a series of standardization and harmonization within the RESET consortium, as well in laboratory techniques as in the documentation and statistical analyses of the data generated. Based on this, all data were stored in a common database and used for this analysis.

For the analysis of the data, the sources were categorized in different ways. Distinction was made (i) between samples collected within epidemiological studies (screening) or diagnostic investigations (diagnostic) and (ii) in some approaches between livestock and companion animals or the individual animal species (livestock: chicken, cattle, pigs; companion animals: dogs, horses). Human ESBL-producing *E. coli* isolates were collected from the general population, outpatients (ambulant isolates) and hospitalized patients (nosocomial isolates). An overview is given in Table 1.

The following genotypic and phenotypic properties of the isolates from all studies were used for the analyses: (1) presence of ESBL genes of the CTX-M- and/or SHV-families and/or TEM-family irrespective if this was specific for ESBL-production, (2) *E. coli* phylogenetic group and (3) additional resistance to gentamicin (R > 2 mg/l), chloramphenicol (R > 8 mg/l) and sulfamethoxazole/trimethoprim (R > 4 mg/l). The ESBL genes give insight into the genetic background for the phenotypically observed production of ESBLs and the genes are transferable between plasmids and bacterial strains. The phylogenetic grouping was used to obtain some preliminary information on the genetic background of these bacteria and the pathogenicity of the *E. coli* strains. Additional information on resistance to gentamicin, chloramphenicol and sulfamethoxazole/trimethoprim was used, as the related resistance genes are usually located within multi-drug resistance regions on conjugative plasmids, which might be transferred together with the ESBL genes between bacterial isolates.

Preliminary analysis of resistance data had shown that each of these antimicrobial agents split isolates into a susceptible and resistant class of sufficient size (data not shown). The variable

Table 1
Overview of the isolates (n = 1329) used for pattern analysis and categorization in a study subgrouping ESBL-producing *E. coli* from animal and human sources in Germany.

Group	Study type	Number of isolates per study type	Source	Number of isolates per source
Livestock	Screening	297	Chicken	38
			Cattle	120
			Pigs	139
	Diagnostic	287	Chicken	11
			Cattle	203
			Pigs	73
Companion animals	Diagnostic	110	Dogs	29
			Horses	81
Human	Screening, diagnostic	635	General population	213
			Ambulant cases	145
			Nosocomial cases	277

'resistance pattern' used in the analysis was defined by combining the results for the two substances and the combination of substances tested (gentamicin, chloramphenicol, sulfamethoxazole/trimethoprim) to one term, e.g. RRS for an isolate with values above the breakpoint for gentamicin and chloramphenicol, but below the breakpoint for sulfamethoxazole/trimethoprim. Similarly, the ESBL genes ($bla_{CTX\text{-}M}$; bla_{SHV}) and presence of any bla_{TEM} gene were combined to one variable 'genes', e.g. '1.12.+' for an isolate producing the ESBL-specific enzymes CTX-M-1 and SHV-12 and a not further characterized TEM.

Four different approaches to categorize the isolates were applied:

(A) Presence of different ESBL types (CTX-M; SHV) and any TEM type
(B) Combination of the presence of different ESBL types, any TEM type and the phylogenetic group
(C) Combination of the presence of different ESBL types, any TEM type and the resistance pattern to the aforementioned two antimicrobials and the combination of substances
(D) Combination of the presence of different ESBL types, any TEM type, the phylogenetic group and the resistance pattern.

Besides considering each animal population separately, different animal groups were defined for the analysis. Priority was given to the reason for sampling, i.e. only diagnostic isolates or screening isolates were grouped together, and a separation between livestock animals and companion animals resulting in three groups, namely livestock screening, livestock diagnostic and companion animals diagnostic (Table 1). All human isolates, irrespective of the type of study, were grouped together reflecting the focus of this analysis.

To compare strain patterns, different combinations of the animal groups were tested for their similarity with human isolates. Details on the combinations compared are given in Table 2.

Results

Availability of strains by source and typing data

A total of 1329 *E. coli* isolates showed the presence of at least one ESBL gene and were included in the analysis. They were originating from screening livestock populations (297 isolates), diagnostic samples of livestock (287 isolates) or companion animals (110 isolates). Furthermore, 635 isolates from humans originating from different regions of Germany were used (Table 1). In total, the data set comprised 15 different variants of the ESBL $bla_{CTX\text{-}M}$ gene (CTX-M-1, -15, -14, -2, -3, -27, -55, -9, -24, -32, -79, -97, -8, -104, -117 in order of frequency), three variants of the ESBL bla_{SHV} gene (SHV-12, -2a, -2), and at least four variants of the bla_{TEM} gene (TEM-52, -1, -12, -20 in order of frequency). Most common were the enzymes CTX-M-1 (n = 625, 47.0%), CTX-M-15 (n = 428, 32.2%) and CTX-M-14 (n = 92, 6.9%). Phylogenetic grouping revealed the presence of *E. coli* belonging to the groups A, B1, B2 and D in different quantities for every study population. Overall *E. coli* of phylogenetic groups A (n = 534; 40.2%), B1 (n = 301; 22.6%), B2 (n = 218; 16.4%) and D (n = 276; 20.8%) were detected.

In the analyses of the resistance data, eight resistance patterns were observed. Resistance to sulfamethoxazole/trimethoprim was most frequent (1019 isolates, 76.7%), followed by resistance to chloramphenicol (612 isolates, 46.0%) and gentamicin (453 isolates, 34.1%). Among the animal isolates, the proportion of resistant isolates was highest among companion animals for all three antimicrobials, followed by diagnostic isolates from livestock. There was no common tendency when all human and animal isolates were compared (resistance to sulfamethoxazole/trimethoprim 85.7% for human isolates vs. 68.4% for animal isolates; chloramphenicol 40.0% vs. 51.6%; gentamicin 32.3% vs. 35.7%), but resistance rates for each antimicrobial in screening isolates from livestock were below those from human isolates. Among livestock, isolates from cattle tended to have the highest resistance rates.

Grouping data by ESBL types and bla_{TEM} gene (approach A)

In the dataset, the enzymes CTX-M-1 (63.3% of all animal isolates, 29.3% of all human isolates), CTX-M-15 (17.7% vs. 48.0%) and CTX-M-14 (5.3% vs. 8.7%) were the most common. Classification of the isolates by the presence of the ESBL gene types ($bla_{CTX\text{-}M\text{-}type}$, $bla_{SHV\text{-}type}$) and the bla_{TEM} genes revealed 35 different patterns (including combinations of the genes). More than 70% of the animal isolates and more than 50% of the human isolates contained the broadly distributed ESBL genes $bla_{CTX\text{-}M\text{-}1}$, $bla_{CTX\text{-}M\text{-}15}$, or the combinations $bla_{SHV\text{-}12} + bla_{TEM}$ or $bla_{CTX\text{-}M\text{-}1} + bla_{TEM}$ (categories 1a–c, Tables 2 and 3). The distribution of these four types between animal and human isolates was quite different. While the majority of animal isolates carried $bla_{CTX\text{-}M\text{-}1}$ (37.5%) or the combination $bla_{CTX\text{-}M\text{-}1} + bla_{TEM}$ (25.8%), this was the case for only 16.7% and 12.6%, respectively, of the human isolates. In contrast, 28.2% of the human isolates carried $bla_{CTX\text{-}M\text{-}15}$ compared to 10.8% of the animal isolates. Furthermore, in the category 1c, which required the presence of the genes only in the three screened livestock populations, some isolates from all origins belonged to the ESBL type $bla_{SHV\text{-}12} + bla_{TEM}$ (1.0% animal isolates vs. 0.6% human isolates).

Subtypes with limited distribution among the animal populations (categories 1d–h, Table 2), but present in the human isolates could also be identified, but each of the subtypes comprised only a few isolates (Table 3). Similarly, unique patterns found in specific animal populations and in the human population (categories 2a–h, Table 2) were detected among isolates from pigs (screening isolates; $bla_{CTX\text{-}M\text{-}24}$; $bla_{CTX\text{-}M\text{-}24} + bla_{TEM}$; $bla_{CTX\text{-}M\text{-}9}$), cattle (diagnostic isolates; $bla_{CTX\text{-}M\text{-}3} + bla_{TEM}$) and horses (diagnostic isolates; $bla_{CTX\text{-}M\text{-}97} + bla_{TEM}$). Only three animal isolates (one isolate from

Table 2
Definition of the categories and subgroups of animal populations for comparison purposes in a study subgrouping ESBL-producing *E. coli* from animal and human sources in Germany.

Category *	Screening samples			Diagnostic samples			Diagnostic samples		Screening or diagnostic samples
	Chicken	Cattle	Pig	Chicken	Cattle	Pig	Dog	Horse	Humans
1 a)	+	+	+	+	+	+	+	+	+
b)	+	+	+	+a	+a	+a	+b	+b	+
c)	+	+	+						+
d)	+	+	+	≠	≠	≠	≠	≠	+
e)	+	+	+	+			≠	≠	+
f)	+			≠a	≠a	≠a	≠b	≠b	+
g)	≠a	≠a	≠a	+b	+b	+b	≠c	≠c	+
h)	≠a	≠a	≠a	≠b	≠b	≠b	+c	+c	+
i)	≠a	≠a	≠a	≠b	≠b	≠b	≠c	≠c	+
j)	+a	+a	+a	+b	+b	+b	+c	+c	≠
2 a)	≠	+	≠	≠	≠	≠	≠	≠	+
b)	≠	≠	+	≠	≠	≠	≠	≠	+
c)	+	≠	≠	≠	≠	≠	≠	≠	+
d)	≠	≠	≠	≠	≠	≠	≠	+	+
e)	≠	≠	≠	≠	≠	≠	+	≠	+
f)	≠	≠	≠	≠	+	≠	≠	≠	+
g)	≠	≠	≠	≠	≠	+	≠	≠	+
h)	≠	≠	≠	+	≠	≠	≠	≠	+
3 a)	≠	+	≠	≠	≠	≠	≠	≠	≠
b)	≠	≠	+	≠	≠	≠	≠	≠	≠
c)	+	≠	≠	≠	≠	≠	≠	≠	≠
d)	≠	≠	≠	≠	≠	≠	≠	+	≠
e)	≠	≠	≠	≠	≠	≠	+	≠	≠
f)	≠	≠	≠	≠	+	≠	≠	≠	≠
g)	≠	≠	≠	≠	≠	+	≠	≠	≠
h)	≠	≠	≠	+	≠	≠	≠	≠	≠
4	≠	≠	≠	≠	≠	≠	≠	≠	+

[a] For the analysis, the three livestock populations (cattle, pigs, broiler) sampled by screening methods or within diagnostic investigations as well as the companion animals (dogs, horses) were either considered separately or as a group. This is reflected by either giving each species a separate sign (marked by + if considered, marked by ≠ if excluded; empty if not considered) or marking several species additionally with the same letter if considered as a group. For further explanation of the different categories, please refer to the text. Results are given referring to these different categories (combinations of animal species and humans) to assist understanding.

a pig; two isolates from chickens) showed unique patterns which were not present in any of the other populations (including human population) (categories 3a-3h, Table 2). In total, 25 isolates reflecting four subtypes belonged to patterns which were present in the three animal groups, but not in human isolates (category 1j, Table 2). Thirteen different subtypes, reflecting 48 human isolates, could not be identified in any of the animal isolates investigated (category 4, Table 2).

Grouping data by ESBL types, bla$_{TEM}$ gene and phylogenetic groups (approach B)

Overall *E. coli* phylogenic groups A (51.7% of all animal isolates, 27.6% of all human isolates), B1 (28.8% and 15.9%, respectively), B2 (2.7% and 31.3%, respectively) and D (16.7% and 25.2%, respectively) were detected. Using the combination of ESBL types, any bla$_{TEM}$ gene and phylogenetic groups, 89 different patterns were observed.

Table 3
Results of comparison of *E. coli* isolates on the basis of subgrouping approach A (ESBL subtypes) in a study subgrouping ESBL-producing *E. coli* from animal and human sources in Germany.

Category	Number of animal isolates out of 694 isolates matching the patterns (proportion of all isolates)	Number of human isolates out of 635 isolates matching the patterns (proportion of all isolates)	Number of subtypes (ESBL subtypes detected[a])
Broadly distributed subtypes in animal and human isolates (available in all animal populations considered and in the human population)			
1a) in each of the 8 animal populations	514 (74.1%) 179 (25.8%) 260 (37.5%) 75 (10.8%)	365 (57.5%) 80 (12.6%) 106 (16.7%) 179 (28.2%)	3 (CTX-M-1, TEM; CTX-M-1; CTX-M-15)
1b) in each of the 3 animal populations screened, in the diagnostic livestock sample and in the diagnostic companion animal sample	514 (74.1%) 179 (25.8%) 260 (37.5%) 75 (10.8%)	365 (57.5%) 80 (12.6%) 106 (16.7%) 179 (28.2%)	3 (CTX-M-1, TEM; CTX-M-1; CTX-M-15)
1c) in each of the 3 livestock populations screened (other animal populations are not considered)	521 (75.1%) 179 (25.8%) 260 (37.5%) 75 (10.8%) 7 (1.0%)	369 (58.1%) 80 (12.6%) 106 (16.7%) 179 (28.2%) 4 (0.6%)	4 (CTX-M-1, TEM; CTX-M-1; CTX-M-15; SHV-12, TEM)
Subtypes with limited distribution in animal and human isolates (available in only few animal populations considered and in the human population)			
1d) in each livestock population screened but not in diagnostic isolates (n = 381 isolates)	0	0	0
1e) in each livestock population screened and in diagnostic livestock isolates but not diagnostic isolates from companion animals	0	0	0
1f) only in any of the livestock populations screened, but not in any of the diagnostic isolates	6 (0.9%) 4 (0.6%) 1 (0.1%) 1 (0.1%)	5 (0.8%) 1 (0.2%) 1 (0.2%) 3 (0.5%)	3 (CTX-M-24, TEM; CTX-M-24; CTX-M-9)
1g) only in diagnostic isolates from livestock but not in livestock isolates from screening or from companion animals	1 (0.1%)	7 (1.1%)	1 (CTX-M-3, TEM)
1h) only in diagnostic isolates from companion animals, but not in any of the other animal populations	3 (0.4%)	1 (0.2%)	1 (CTX-M-97, TEM)

[a] TEM – as not all bla_{TEM} genes were sequenced, details for the TEM-type (even if available) are not given.

In total, 55 patterns were observed in animal populations and 69 patterns in human populations.

In line with the more diverse patterns observed, the number of isolates belonging to common patterns was lower (Fig. 1). More than 40% of the animal isolates and more than 20% of the human isolates contained broadly distributed subtypes (categories 1b-1c, Tables 2 and 4), namely $bla_{CTX\text{-}M\text{-}1}$ in combination with one of the phylogenetic groups or $bla_{CTX\text{-}M\text{-}15}$ in combination with phylogroup A (Table 4). The combinations of $bla_{CTX\text{-}M\text{-}1}$ and phylogroups A or B1 were more frequently present in animal isolates compared to human isolates (Table 4).

Subtypes with limited distribution among the animal populations, but present in human isolates (categories 1d-1h) could also be identified, but the proportion of all animal isolates was below 2% (Table 4). Ten isolates from diagnostic samples from companion animals belonged to subgroups which were not present in any livestock populations, but represented 12.6% of all human isolates (category 1h, Table 4). Among these findings were $bla_{CTX\text{-}M\text{-}15}$ in combination with phylogroup B2.

Unique patterns between one animal population and the human population (categories 2a-2h, Table 2) were detected in isolates from cattle, chicken, horses and dogs but not in isolates from pigs. Sixteen unique patterns, which were not present in any of the other defined populations, were seen among 24 animal isolates (categories 3a-3h, Table 2). Twenty patterns (44 isolates) were present in the three animal groups, but not in the human isolates (category 1j, Table 2). Finally, 34 different patterns, reflecting 118 human isolates (18.6%), could not be identified in any of the animal isolates investigated (category 4, Table 2).

Grouping data by ESBL types, bla_{TEM} gene and resistance patterns (approach C)

A total of 121 different combinations of ESBL genes, any bla_{TEM} gene and resistance profiles (combination of the three antimicrobials used for classification) were present in the data set. Only one pattern ($bla_{CTX\text{-}M\text{-}15}$ + RRR) could be identified, which was common in each of the eight animal populations (4.2% of all animal isolates) and in the human population (5.2% of all human isolates) (category 1a, Table 2, Fig. 2). In total 34.7% of the isolates from the three screening livestock populations and 20.6% of the human isolates belonged to the same subgroups (category 1c, Table 2, Fig. 2). Most frequently, isolates with the patterns $bla_{CTX\text{-}M\text{-}1}$ + SSS (susceptible to all three tested antimicrobials; 91 animal isolates, 13.1%; 20 human isolates, 3.1%) and $bla_{CTX\text{-}M\text{-}1}$ + SSR (resistance to sulfamethoxazole/trimethoprim; 67 animal isolates, 9.7%; 51 human isolates, 8.0%) were detected. There was one type ($bla_{SHV\text{-}12}$ + bla_{TEM} + SRR) unique for the screened livestock populations and humans (category 1d) and two patterns [$bla_{CTX\text{-}M\text{-}1}$ + bla_{TEM} + SSR, 50 (7.2%) animal isolates, 25 (3.9%)

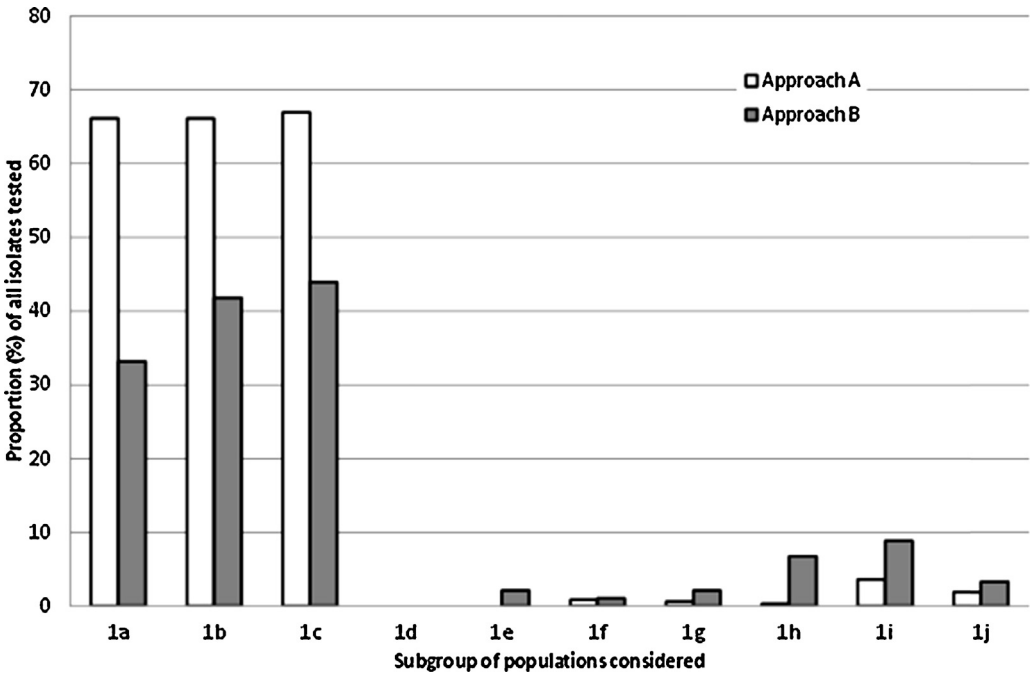

Fig. 1. Proportion of all isolates belonging to a subgroup on the basis of the subgrouping approaches. White bars show the approach A (ESBL-type only); gray bars show the approach B (ESBL-type and phylogenetic group) in a study subgrouping ESBL-producing *E. coli* from animal and human sources in Germany. The designations 1a-1j represent the subcategories in Table 2.

human isolates; $bla_{\text{CTX-M-1}}$ + SSS, 91 (13.1%) animal isolates, 20 (3.1%) human isolates] common in livestock and human isolates (category 1e).

Subtypes with limited distribution among one of the animal subgroups, but present in the human isolates (categories 1f-1h, Table 2) represented 19 distinct patterns (129 isolates) with different combinations of resistance.

Unique patterns (n = 15) comprising 74 isolates (21 from animals and 53 from humans) between one animal population and the human population were detected in isolates from pigs and cattle, but not in chicken (categories 2a-2h, Table 2). The patterns included 7 isolates, unique for horses and humans, with $bla_{\text{CTX-M-2}}$ + bla_{TEM} + SRR (category 2d, Table 2) but no isolates, unique for dogs and humans (category 2e, Table 2).

Thirty-four animal isolates (4.9%) showed unique patterns (n = 20), which were not present in any of the other populations (categories 3a-3h, Table 2). Twenty-eight patterns comprising 79 isolates (12.4%) were only present in livestock (screening, diagnostic) and companion animals, but not in humans (category 1j, Table 2). Finally, 44 different patterns among 80 human isolates (12.6%) could not be identified in any of the animal isolates investigated (category 4, Table 2). All details, together with the information on the phylogenetic groups are given in Supplementary Table 1.

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijmm.2014.07.015.

Grouping data by ESBL types, any bla_{TEM} gene, phylogenetic groups and resistance patterns (approach D)

Altogether in the data set, 254 different combinations of ESBL types, any bla_{TEM} gene, phylogenetic groups and resistance patterns, were present. The detailed results are shown in Supplementary Table 1. Overall, the number of unique types increased and the proportion of isolates with common characteristics decreased in several populations (Fig. 2). In total, 28.8% of the animal isolates and 13.2% of the human isolates belonged to one of the common subgroups present in the three screening livestock and the human populations (category 1c, Table 2). In this category, eight different patterns could be identified, containing $bla_{\text{CTX-M-1}}$ in combination with phylogroup A, B1 or D and the resistance patterns SSR or SSS, or $bla_{\text{CTX-M-15}}$ in combination with phylogroup A and the resistance pattern RRR. None of these types was unique for the screened livestock population and humans (category 1d).

In total, 91 animal isolates (13.1%) showed unique patterns (overall 61 patterns) which were not present in any of the other populations defined (categories 3a-3h, Table 2). Furthermore, 159 animal isolates (22.9%) exhibited 79 patterns, which were present in each of the three animal groups, but not in human isolates (category 1j, Table 2). Finally, 265 (41.7%) human isolates, reflecting 99 different patterns, could not be identified in any of the animal isolates investigated (category 4, Table 2).

Discussion

In the course of the 'One Health' approach, several studies are ongoing to show the potential of collaborative work from human and veterinary medicine. This study shows an example in this context from antimicrobial resistance research, namely on ESBL-producing *E. coli* in Germany. For this, recent data was generated with harmonized laboratory techniques from several populations of humans and animals to analyze ESBL-producing *E. coli* from different populations for similar and different properties. This approach may be recognized as a basic data description for a future study of transmission of resistance between populations and therefore was considered as a basis for a 'One Health' discussion in this context.

Overall, typing data of 1329 isolates from different animal and human sources were used to identify common and distinct patterns among the ESBL-producing *E. coli* isolates. All isolates included in the study were processed and documented following standardized

Table 4
Results of comparison of *E. coli* isolates on the basis of subgrouping approach B (ESBL subtypes and phylogenetic groups) in a study subgrouping ESBL-producing *E. coli* from animal and human sources in Germany.

Category	Number of animal isolates out of 694 isolates matching the patterns (proportion of all isolates)	Number of human isolates out of 635 isolates matching the patterns (proportion of all isolates)	Number of subtypes (ESBL subtypes and phylogroups detected[a])
Broadly distributed subtypes in animal and human isolates (available in all animal populations considered and in the human population)			
1a) in each of the 8 animal populations	304 (43.8%) 117 (16.9%) 94 (13.5%) 38 (5.5%) 55 (7.9%)	137 (21.6%) 37 (5.8%) 30 (4.7%) 22 (3.5%) 48 (7.6%)	4 (CTX-M-1, A; CTX-M-1, B1; CTX-M-1, D; CTX-M-15, A)
1b) in each of the 3 animal populations screened, in the diagnostic livestock sample and in the diagnostic companion animal sample	396 (57.1%) 117 (16.9%) 94 (13.5%) 38 (5.5%) 55 (7.9%) 92 (13.3%)	160 (25.2%) 23 (3.6%) 37 (5.8%) 30 (4.7%) 22 (3.5%) 48 (7.6%)	5 (CTX-M-1, A; CTX-M-1, B1; CTX-M-1, D; CTX-M-15, A CTX-M-1, TEM, A)
1c) in each of the 3 livestock populations screened (other animal populations are not considered)	407 (58.6%) 117 (16.9%) 94 (13.5%) 38 (5.5%) 55 (7.9%) 92 (13.3%) 11 (1.6%)	177 (27.9%) 23 (3.6%) 37 (5.8%) 30 (4.7%) 17 (2.7%) 22 (3.5%) 48 (7.6%)	6 (CTX-M-1, A; CTX-M-1, B1; CTX-M-1, D; CTX-M-15, A CTX-M-1, TEM, A; CTX-M-1, B2)
Subtypes with limited distribution in animal and human isolates (available in only few animal populations considered and in the human population)			
1d) in each livestock population screened but not in diagnostic isolates	0	0	0
1e) in each livestock population screened and in diagnostic livestock isolates but not diagnostic isolates from companion animals	11 (1.6%)	17 (2.7%)	1 (CTX-M-1, B2)
1f) only in any of the livestock populations screened, but not in any of the diagnostic isolates	6 (0.9%) 2 (0.3%) 3 (0.4%) 1 (0.1%)	8 (1.3%) 2 (0.3%) 2 (0.3%) 4 (0.6%)	3 (CTX-M-14, B1; SHV-12, TEM, D; TEM-52, D)
1g) only in diagnostic isolates from livestock but not in livestock isolates from screening or from companion animals	3 (0.4%) 1 (0.1%) 1 (0.1%) 1 (0.1%)	25 (3.9%) 1 (0.2%) 18 (2.8%) 6 (0.9%)	3 (CTX-M-14, TEM, B1; CTX-M-14, D; CTX-M-3, TEM, D)
1h) only in diagnostic isolates from companion animals, but not in any of the other animal populations	10 (1.4%) 1 (0.1%) 5 (0.7%) 1 (0.1%) 2 (0.3%) 1 (0.1%)	80 (12.6%) 76 (12.0%) 1 (0.2%) 1 (0.2%) 1 (0.2%) 1 (0.2%)	5 (CTX-M-15, B2; CTX-M-2, TEM, A; CTX-M-2, TEM, D; CTX-M-2, B1; CTX-M-97, TEM, A)

[a] TEM – as not all bla_{TEM} genes were sequenced, details for the TEM-type (even if available) are not given.

protocols, which distinguish these data from classical strain collections or even registers, among which the laboratory sampling procedures varied substantially and the documentation standards were narrow. To our knowledge, this is the first national strain and data collection of that magnitude handled in this way. Recently, 629 isolates from humans, animals and animal food products in Germany, The Netherlands and the UK were compared using a microarray approach (Wu et al., 2013). To our best knowledge, similar approaches, in which phenotypic and genotypic typing data from such a broad strain collection have been compared, have not yet been described.

The level of homogeneity and heterogeneity was related to the number of typing results used for classification. With all four approaches, used for the classification of the isolates, common subgroups of isolates could be identified between animal and human populations. This points towards common resistance genes and/or plasmids in several or even all populations, which might be explained by an exchange between them or common exposure sources.

In addition, unique subtypes could be determined. For example, some ESBL genes, partly in combination with bla_{TEM} genes were present only in one population. If additional information (phylogenetic group, resistance pattern) was included, the number of distinct types increased. This reflects the huge diversity of combinations of the information considered, and may be interpreted as a signal for horizontal spread of genes or plasmids. It may be in part related to the still limited number of isolates per population covered in the study.

Dominating ESBL genes

In this study, more than 70% of the animal isolates and more than 50% of the human isolates contained broadly distributed ESBL genes. This is in accordance with previous reports in which $bla_{CTX\text{-}M\text{-}1}$, $bla_{CTX\text{-}M\text{-}15}$ and $bla_{SHV\text{-}12}$ were most frequently detected. Wu and coworkers reported that $bla_{CTX\text{-}M\text{-}group\text{-}1}$ genes were widespread in all host species and in the three participating countries. Genes of the $bla_{CTX\text{-}M\text{-}group\text{-}1}$ predominated among the

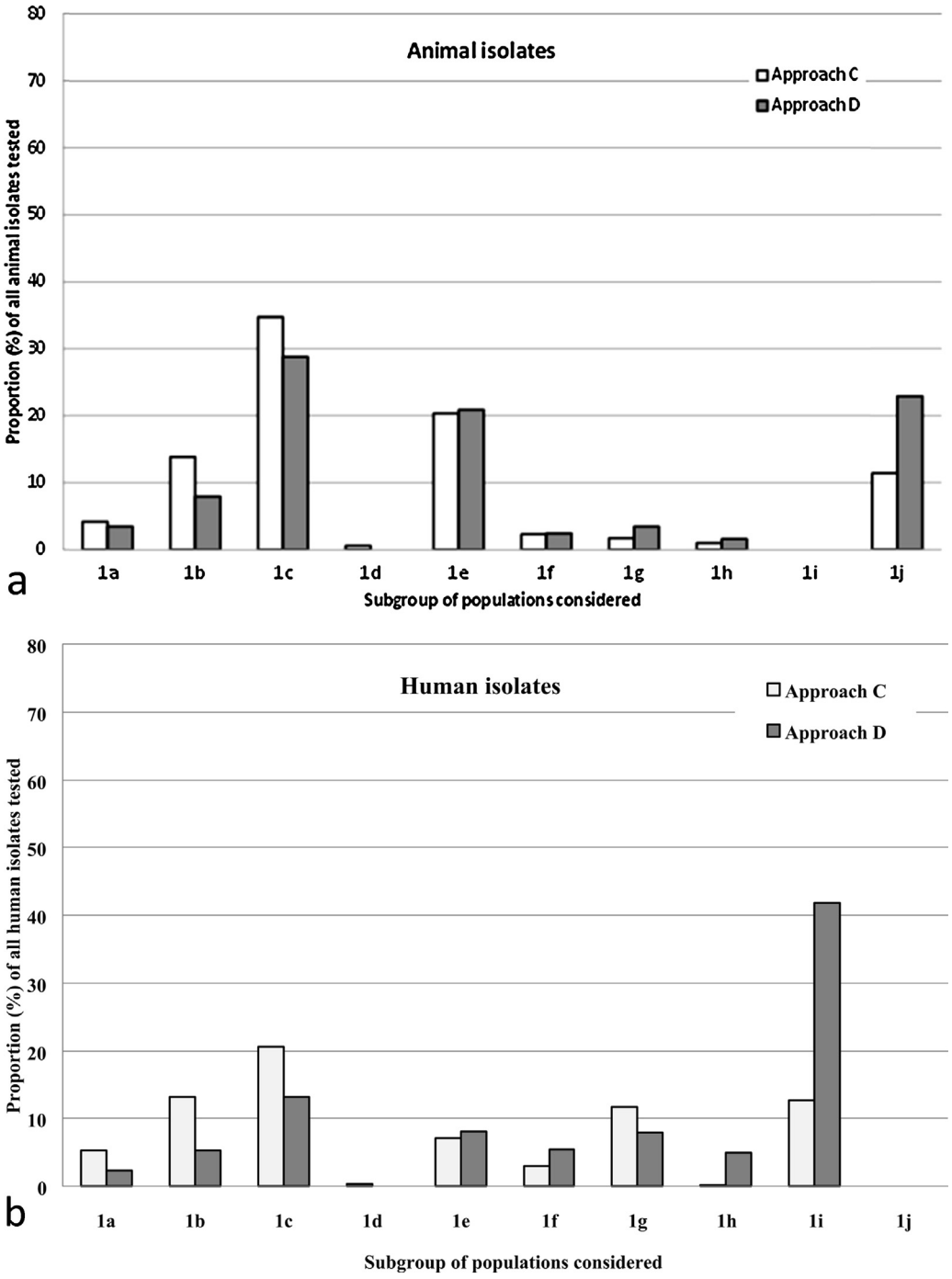

Fig. 2. Proportion of animal (a) or human (b) isolates belonging to a subgroup on the basis of the subgrouping approaches in a study subgrouping ESBL-producing *E. coli* from animal and human sources in Germany. The white bars show the approach C (ESBL-type and antimicrobial resistance) and the gray bars show the approach D (ESBL-type, phylogenetic group and antimicrobial resistance). The designations 1a-1j represent the subcategories in Table 2.

$bla_{CTX\text{-}M}$ genes and were detected in 66% of the isolates investigated (Wu et al., 2013). In the Netherlands, among 314 randomly selected and sequenced clinical *Enterobacteriaceae* of human origin, $bla_{CTX\text{-}M\text{-}15}$ was most prevalent (39%), followed by $bla_{CTX\text{-}M\text{-}1}$ (15%), $bla_{CTX\text{-}M\text{-}14}$ (5%), $bla_{SHV\text{-}12}$ (8%) and $bla_{TEM\text{-}52}$ (4%) (Voets et al., 2012). The $bla_{CTX\text{-}M\text{-}1}$ was the dominating ESBL gene detected in German pig and cattle farms (Guerra et al., 2012). In contrast to previous findings, the presence of $bla_{CTX\text{-}M\text{-}1}$ in human populations increased to 22% of the isolates typed (Leistner et al., 2014). Similarly, in livestock animals, an increase of the prevalence of ESBL-producing *E. coli* and *E. coli* carrying $bla_{CTX\text{-}M\text{-}1}$ genes was observed during the last years (Agersø et al., 2012; Dierikx et al., 2010). These common findings and trends point towards exposure pathways linking animal populations and humans, which should be investigated further in more detail. In a recent study, it was shown that $bla_{CTX\text{-}M\text{-}15}$-producing isolates from non-human sources are still rarely found in Germany (Fischer et al., 2014). The pandemic occurrence of $bla_{CTX\text{-}M\text{-}15}$-producing isolates in humans, observed in several studies, was explained by the dissemination with the O25:H4-ST131 *E. coli* clone (Rogers et al., 2011; Lau et al., 2008; Livermore and Hawkey, 2005; Hawkey and Jones, 2009). As a possible explanation for the wide dissemination of the ST131 clone, it

is assumed that this may be related to an enhanced fitness for colonization and transmission (Johnson et al., 2012; Vimont et al., 2012). In this study, there was no clear difference in the matching probability between animal isolates, collected in specific studies (screening healthy populations using selective media) as well as those collected from diagnostic samples on one hand and the human isolates on the other hand. Unique types were identified for each of the animal populations as well as for the human population, but unique types covered only a limited number of isolates (2.6% of the animal isolates, 7.3% of the human isolates). Thus, currently no major subtype can be identified which is specific for any of the animal populations.

Phylogenetic groups

Out of the four major *E. coli* phylogenetic groups (A, B1, B2, D), the phylogenetic groups A and B1 dominated in isolates of animal origin (46.9% of animal isolates, 16.5% of human isolates) whereas in human isolates, phylogenetic groups B2 and D were detected more frequently. Previous studies showed that most *E. coli* strains responsible for urinary tract infections and other extraintestinal infections in humans belong to group B2 or, to a lesser extent, to group D and that strains of phylogenetic group B2 and D often carry virulence determinants that are lacking in group A and B1 strains (Goullet and Picard, 1986; Johnson and Stell, 2000; Picard et al., 1999).

Nevertheless, isolates of the same phylogenetic group and common ESBL genes were found in the different populations. Within this study, more than 40% of the animal isolates and more than 20% of the human isolates contained broadly distributed ESBL genes, namely $bla_{\text{CTX-M-1}}$ in combination with any of the four phylogenetic groups or $bla_{\text{CTX-M-15}}$ in combination with phylogenetic group A. Interestingly, ten isolates from diagnostic samples of companion animals belonged to subgroups which were not present in any livestock populations, but represented 12.1% of all human isolates. This may reflect some intensive interaction between humans and companion animals or a common reservoir for both populations which needs further investigations.

When phylogenetic groups were considered for grouping, the proportion of unique subtypes increased from 2.6% to 5.7% for animal isolates and from 7.3% to 16.8% for human isolates, indicating different parent strains carrying ESBL genes in humans and animals. This might hint towards horizontal transfer of plasmids between these groups. Several previous studies comparing isolates from different origins reported similar results (Leverstein-van Hall et al., 2011; Overdevest et al., 2011).

Antimicrobial resistance patterns

Although only three antimicrobial agents were included for the subtyping approach involving antimicrobial resistance patterns, there were several different combinations of resistance patterns. Most of the isolates showed resistance to one or several of these antimicrobial agents and common patterns between isolates of the different populations could be observed. This may be interpreted as some hint towards an exchange of plasmids which carried several resistance genes. The proportion of isolates in common clusters was markedly reduced. Interestingly, 13.1% of the animal isolates and 3.1% of the human isolates belonged to the subtype $bla_{\text{CTX-M-1}}$ with resistance pattern SSS (fully susceptible). Quite worrying, the pattern $bla_{\text{CTX-M-15}}$ RRR was present in all populations, which points towards the widespread presence of a multiresistant subtype of *E. coli*, carrying an ESBL gene originally more commonly found in humans. The resistance pattern RRR was linked with phylogroup B2 in human isolates, and with phylogroup D in isolates from humans, companion animals and one isolate from cattle. This may reflect a direct or indirect transfer between humans and animals or the exchange of strains or plasmids while sharing the same environment. Several studies had shown that persons having direct contact to animals carry similar strains or strains with similar plasmids (Dierikx et al., 2013a; Moodley and Guardabassi, 2009; Huijbers et al., 2013; Meyer et al., 2012). The proportion of unique subtypes increased in this approach to 4.9% for animal isolates and 12.6% for human isolates when using several typing results for classification. Thus, the results show that the information on antimicrobial resistance patterns gives useful additional information to identify common and unique strains.

Conclusions

The study showed the benefit from following an integrated approach for analyzing isolates representing different populations for their similarity and possible relevance for exposure of other populations. A detailed understanding of these interactions is a prerequisite for the identification and implementation of appropriate control measures. One outcome from this research is the agreement on and application of common methods, applied on actively collected isolates with detailed knowledge and storage of data in one dataset.

Following different grouping approaches, a relevant proportion of indistinguishable subtypes was detected in the human and animal populations, reflecting that some interaction or exposure cannot be ruled out and need further investigation. For example, more than 70% of the animal isolates and more than 50% of the human isolates contained the broadly distributed ESBL genes $bla_{\text{CTX-M-1}}$, $bla_{\text{CTX-M-15}}$, or the combinations $bla_{\text{SHV-12}} + bla_{\text{TEM}}$ or $bla_{\text{CTX-M-1}} + bla_{\text{TEM}}$. Additionally, these results clearly showed that there are some similarities between genes and bacterial properties in isolates from the different populations. The ESBL gene $bla_{\text{CTX-M-1}}$ in combination with one of the phylogenetic groups, $bla_{\text{CTX-M-15}}$ in combination with phylogroup A or $bla_{\text{CTX-M-15}}$ in combination with resistance to gentamicin, chloramphenicol and sulfamethoxazole/trimethoprim were quite common among populations. Our current approach provides interesting insight into common clusters and specific clusters in ESBL-producing *E. coli* from distinct sources. These results will be used for selection of isolates with similar properties from different settings for further molecular characterization to confirm or exclude a transmission of resistant strains or resistance gene carrying plasmids between different reservoirs.

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