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Faecal occurrence and emissions of livestock-associated methicillin-resistant *Staphylococcus aureus* (laMRSA) and ESBL/AmpC-producing *E. coli* from animal farms in Germany

**Summary**

The occurrence of laMRSA (livestock-associated methicillin-resistant *Staphylococcus aureus*) and extended-spectrum β-lactamase (ESBL) and/or plasmid-mediated AmpC β-lactamase-producing (AmpC) *Enterobacteriaceae* in healthy livestock herds is known for some time. The spread of these bacteria in the environment is discussed critically. The object of this study was to determine the presence of these microorganisms in faeces of livestock as well as the discussion about a potential faecal emission. Therefore, faeces samples from 37 different MRSA positive livestock holdings were tested for MRSA. Furthermore, faeces samples from 50 farms with an unknown status regarding ESBL/AmpC-producing *E. coli* were screened for those resistant bacteria. LaMRSA was detected in samples of turkey (2/7, 40%) and broiler fattening farms (1/4, 25%) as well as in pig farms with higher detection frequencies in fattening farms (11/17, 13.3%) than in breeding farms (4/12, 33.3%). ESBL/AmpC-producing *E. coli* was found in all investigated eight broiler farms (100%), in nine out of 16 (56.3%) breeding pig as well as in six out of 10 (60%) dairy cattle herds and in seven of 16 (43.8%) fattening pig holdings. This presents the first detection of ESBL/AmpC-producing *E. coli* originating from healthy pigs, turkeys and broilers in Germany. In addition, samples of fertilized field surfaces were studied exemplarily for the presence of MRSA (n = 4) as well as ESBL/AmpC-producing *E. coli* (n = 2). Furthermore, slurry samples from four broiler and five pig farms were analysed for the latter. Both MRSA and ESBL/AmpC-producing *E. coli* could be detected on the field surfaces, the last also in slurry samples. Faecal emissions from animal husbandry seem to be one possible route for the spread of these resistant microorganisms in the environment.

**Keywords:** resistant microorganisms, MRSA, ESBL, livestock, environmental health, slurry

Zusammenfassung

Introduction

Antibiotics are powerful therapeutic tools to treat bacterial diseases of humans and animals. However, constant application in human as well as in veterinary medicine accelerates the development of resistance in bacteria. Rising rates of resistant and multidrug-resistant microorganisms in animal holdings are a steadily upcoming problem (Coque et al., 2008). Consequently, problems can arise in the treatment of infectious diseases caused by these resistant pathogens. Especially laMRSA (livestock-associated methicillin-resistant Staphylococcus aureus) and extended-spectrum β-lactamase (ESBL) and/or AmpC β-lactamase-producing (AmpC) Enterobacteriaceae in food producing animals are in the focus of public attention and might be relevant for public health. In Germany, both laMRSA and ESBL/AmpC-producing Enterobacteriaceae occur in animal husbandry. LaMRSA could be found on German cattle farms with mastitis problems (Spohr et al., 2011), in healthy pigs (Meemken et al., 2008; Friese et al., 2012), turkey (Richter et al., 2012) and broilers (Friese et al., 2013). Also ESBL/AmpC-producing Enterobacteriaceae seem to be widespread among healthy livestock like pigs (Mesa et al., 2008), cattle (Liebana et al., 2006; Rodriguez et al., 2009) or poultry (Brinas et al., 2003; Mesa et al., 2006; Bortolaia et al., 2010; Overdevest et al., 2011) in Europe. Although published data for Germany are rare, ESBL/AmpC-producing E. coli could be detected in healthy cattle (Wieler et al., 2011a) and clinical samples of livestock (Ewers et al., 2012) as well as companion animals (Wieler et al., 2011b). The transfer of MRSA between livestock and humans has been reported earlier (Cuny et al., 2009; Graveland et al., 2011) and a transmission of ESBL-producing gram-negative bacteria between food-producing animals and humans via direct contact or meat is supposed (Smet et al., 2009). Another important topic is the spread of these pathogens into the environment, for example via the application of contaminated faeces on fields as fertilizer. According to the federal statistical office in Germany liquid manure was applied to 5.9 Mio ha agricultural land and solid manure to 2.3 Mio ha in 2010. Therefore, faeces of pigs, cattle and poultry are commonly used. This study presents data of two different, in parts ongoing, studies and discusses the capability of an emission of laMRSA and ESBL/AmpC-producing E. coli in the vicinity of animal farms in Germany.

Material and Methods

Animal farms

A total of 37 conventional farms (15 fattening pig, twelve breeding pig including weaner-to-grower-farms, five turkey, four broiler and one cattle farm) in Germany with a positive MRSA-status were examined for the presence of these bacteria in pooled faeces samples. The pig farms were distributed in the northern and eastern region of Germany, turkey farms in the southern and eastern part, the broiler farms were in the northwest as well as northeast and the cattle farm was in eastern Germany. A positive status of MRSA means that except the faecal sample at least one other sample of animals and/or environment inside the barn was tested positive for MRSA simultaneously. In addition to this, boot swabs taken from the surface of previously fertilized fields (within the last three months) around three selected pig farms and one turkey fattening farm were analysed. For pooled faeces samples around 250 g faeces from at least five different locations inside the barn were collected. For sampling the field ground surface a 50-metre distance was stepped with the boot swabs. These results are partly published in Friese et al. (2012) and Schulz et al. (2012). Furthermore, for the ESBL/AmpC study pooled faecal samples of 50 conventional farms with an unknown ESBL/AmpC status distributed regionally in the northern and eastern part of Germany (16 fattening pig, 16 breeding pig including weaner-to-grower-farms, eight broiler and ten dairy cattle farms), slurry samples of five selected pig fattening and four broiler farms, and boot swabs from surfaces of fertilized fields (within the last six weeks) around one pig and one broiler farm were investigated for ESBLs/AmpC-producing E. coli. The positive...
status of these two selected farms was known before sampling. Nine out of all investigated pig farms were tested for MRSA and ESBLs/AmpC-producing E. coli simultaneously.

### Analysis of MRSA

Boot swabs or 25 g faeces were inoculated in 225 ml Mueller Hinton broth (Oxoid Ltd., UK) with 6.5% NaCl (MHB+) and homogenized using a stomacher (230 rpm, 2 min). After 24 h of incubation at 37°C 2.5 ml of MHB+ was transferred into 22.5 ml tryptone soy broth (583439, Oxoid Ltd., UK) including 75 mg/l aztreonam and 3.5 mg/l cefoxitin (TSB+). This selective broth was incubated for 17 h at 37°C. A loop-full of TSB+ was streaked onto chromogenic MRSA screen agar (CHROMagarMRSA™, MAST Diagnostica GmbH, GER) and aerobically incubated at 37°C for 24 h. Characteristic colonies were transferred onto sheep blood agar (Oxoid, CM 0351, GER) and tested for coagulase reaction. Furthermore, positive results were confirmed by the detection of the nuc-gene specific for S. aureus and mecA-gene specific for the methicillin resistance (Pasanen et al., 2010). To emphasise the livestock association, exemplarily, eleven MRSA isolates originating from faeces of pigs, turkey and broilers were tested concerning their belonging to the clonal complex 398 as described before (Stegger et al., 2011).

### Analysis of ESBL/AmpC-producing E. coli

Boot swabs, 25 g faeces or 25 g slurry were inoculated in 225 ml Luria-Bertani(LB)-broth (Merck kGaA, GER) and aerobically incubated at 37°C for 24 h. A loop-full of LB-broth was streaked onto MacConkey agar plates (Oxoid, CM 0115, Wesel, GER) containing 1 mg/l cefotaxime and incubated equally. For each sample one characteristic E. coli colony was chosen randomly and the species was confirmed using MALDI TOF (Bruker Daltonik GmbH, Bremen). Subsequently the existence of the β-lactamase genes blaCTX-M, blaTEM, blaSHV and the plasmid-mediated AmpC β-lactamase gene blacMY2 was examined by PCR as described before (Guerra et al., 2001; Zhao et al., 2001; Weiß et al., 2004; Batchelor et al., 2005). For isolates in which either the blaTEM and/or the blaSHV gene was detected solely, a disk diffusion test of LB-broth was streaked onto MacConkey agar plates (Oxoid, CM 0115, Wesel, GER) containing 1 µg cefotaxime and cefotaxime 30 µg and cefotaxime 30 µg + clavulanic acid 10 µg (D62C, MAST Diagnostica GmbH, Reinfeld, GER) was applied. An increase of the inhibition zone about more than 5 mm in the presence of clavulanic acid was considered to be indicative for the presence of extended-spectrum β-lactamases.

### Results

LaMRSA as well as ESBL/AmpC-producing E. coli could regularly be found in pooled faeces samples of different farm animals. Moreover, a detection of ESBL/AmpC-producing E. coli was possible in samples of slurry and boot swabs of fertilized fields. MRSA was also found in boot swab samples from fertilized fields around three pig farms and one turkey farm.

#### Pooled faecal samples

Table 1 summarises the detection frequencies of laMRSA and ESBL/AmpC-producing E. coli in pooled faecal samples of different animal farms. LaMRSA could regularly be found (48.6%) in pooled faecal samples of different farms with a positive MRSA-status. Especially in fattening pig farms the detection of MRSA was very high (73.3%). In breeding pig farms the resistant pathogen was detected sporadically and significantly less than in fattening farms ($\chi^2$-test, $p = 0.038$). Also in samples of turkey and broiler farms the microorganisms could be found although the number of investigated farms is low. The faecal sample of the only sampled MRSA-positive cattle farm was negative. Ten out of eleven (90.9%) tested MRSA isolates originating from the pooled faeces samples belong to the clonal complex 398 and are thus livestock-associated (Stegger et al., 2011). One isolate of a broiler farm was negative for that.

Concerning ESBL/AmpC the majority of pooled faecal samples (60%) was tested positive for ESBL/AmpC-producing E. coli (Tab. 1). In detail, all broiler farms were classified positive for this resistant pathogen. In many dairy cattle farms as well as in breeding pig farms ESBL/AmpC-producing E. coli could be found. In fattening pig farms the detection frequency was lowest (43.8%), however, there was no significant difference between breeding and fattening pig farms ($\chi^2$-test, $p = 0.48$). All of the four mentioned β-lactamase genes blaCTX-M, blatem, blashv or blacMY2 could be found in the E. coli isolates with different extent. Among isolates originating from fattening and breeding pig farms the CTX-M-type enzyme was dominant in 14 out of 16 positive isolates whereas five isolates produce both the CTX-M and TEM-type β-lactamases. The remaining two isolates from fattening pig farms carried the blatem gene. Interestingly, in one of these pig fattening farms also the first carbapenem resistant E. coli from livestock with the blavIM gene was found in parallel (Fischer et al., 2012). Concerning the eight broiler flocks five isolates had a plasmid-mediated AmpC β-lactamase, three of them in combination with a blatem gene. In two isolates only the TEM-type β-lactamase could be detected and in one only the SHV-type. Isolates from cattle farms showed the CTX-M-type of enzyme in two samples, the TEM-type in two, the AmpC-type in one sample and one isolate contained a combination of the three genes blacTX-M, blatem and blashv.

#### Slurry samples

In all slurry samples (100%) from four broiler farms and five pig fattening farms phenotypic suspected ESBL/AmpC-producing E. coli could be isolated on

### Table 1: Occurrence of MRSA and ESBL/AmpC-producing E. coli in pooled faeces samples from different animal farms in Germany

<table>
<thead>
<tr>
<th>Farm Type</th>
<th>MRSA in faeces (%)</th>
<th>ESBL/AmpC-producing E. coli in faeces (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>All farms</td>
<td>48.6 (18/37)</td>
<td>60 (30/50)</td>
</tr>
<tr>
<td>Fattening pig farms</td>
<td>73.3 (11/15)</td>
<td>43.8 (7/16)</td>
</tr>
<tr>
<td>Breeding pig farms</td>
<td>33.3 (4/12)</td>
<td>56.3 (6/11)</td>
</tr>
<tr>
<td>Turkey farms</td>
<td>40 (2/5)</td>
<td>-</td>
</tr>
<tr>
<td>Broiler farms</td>
<td>25 (1/4)</td>
<td>100 (8/8)</td>
</tr>
<tr>
<td>Dairy cattle farms</td>
<td>0 (0/1)</td>
<td>60 (6/10)</td>
</tr>
</tbody>
</table>

The number of positive samples (detection of nuc- and mecA-gene for MRSA, detection of blatem, blashv, and blacMY2 gene for ESBL/AmpC-producing E. coli) from the total number of samples is in parenthesis.

1. Partially published in Freise et al. (2012)
2. Farms with known positive MRSA status but with unknown ESBL/AmpC status
3. Including weaner-to-grower farms
MacConkey agar. In two samples originating from broiler farms and in two from pig farms the bla\textsubscript{TEM} gene was detected. In one broiler farm sample the bla\textsubscript{CTX-M} gene and in another the bla\textsubscript{CMY-2} gene was found. In the remaining slurry samples originating from three pig farms none of the four genes was detected by testing only this one randomly chosen \textit{E. coli} isolate.

**Boot swabs of fertilized fields**

Both MRSA and ESBL/AmpC-producing \textit{E. coli} could be detected on the surface of previously fertilized fields. MRSA was found around three pig farms. More details concerning these results were shown by Schulz et al. (2012). Phenotypic suspected ESBL/AmpC-producing \textit{E. coli} occurred on three different locations on a fertilized field around one pig farm. In these three samples the bla\textsubscript{TEM} and bla\textsubscript{SHV} genes were detected. The bla\textsubscript{TEM} gene was also found in the isolate of the associated slurry sample of this farm. The environment of the sampled broiler farm showed also positive results for ESBL/AmpC-producing \textit{E. coli} in the boot swab of the surface of a fertilized field. There the bla\textsubscript{SHV} gene could be detected.

**Disk Diffusion**

All isolates with an exclusive detection of bla\textsubscript{TEM} or bla\textsubscript{SHV} gene tested in disk diffusion test showed an increase of inhibition zone about more than 5 mm in the presence of clavulanic acid and could be therefore proved as ESBL-producing \textit{E. coli}.

**Discussion**

The increasing presence of multiresistant bacteria is a problem in veterinary as well as in human medicine. Especially the occurrence in healthy farm animals is discussed to be a possible source for transmission of laMRSA and ESBL/AmpC-producing \textit{Enterobacteriaceae} to humans or the environment. This study presents the first results of ongoing research on the spread of multiresistant organisms in the environment of animal farms via the faecal route. To the best of our knowledge this is the first detection of ESBL/AmpC-producing \textit{Enterobacteriaceae} in faecal samples of healthy pigs, turkeys and broilers, slurry and the environment of farms in Germany. Our results show a regular detection of MRSA and ESBL/AmpC-producing \textit{E. coli} in faeces and exemplarily in slurry as well as on fertilized field surfaces in the vicinity of farms. There were a few isolates suspected to produce ESBL/AmpC in which merely the bla\textsubscript{TEM} gene or bla\textsubscript{SHV} could be detected by PCR. This is no evidence for the presence of extended-spectrum \textbeta-lactamases. To underline the results of these isolates disk diffusion tests were applied.

The detection of ESBL/AmpC-producing \textit{E. coli} in pooled faeces samples originating from various animal farms is quite high (43.8% to 100% for the different farm animals) and indicates the importance of the faecal spread of these multiresistant pathogens. Similar results were found by Mesa et al. (2006). This group investigated ten broiler and ten pig farms in Spain by testing pooled faecal samples and found the resistant bacteria also in every broiler and in eight pig farms. Furthermore, five examined broiler farms were positive for ESBL-producing \textit{E. coli} in Italy (Bortolaia et al., 2010) as well as in Belgium (Smet et al., 2008). However, many other studies in Europe had different study designs focused on either the prevalence of ESBL-producing \textit{E. coli} in animal samples originating from one individual farm or different slaughterhouses (Costa et al., 2009; Dieriks et al., 2010; Goncalves et al., 2010). Since our study examined many different farms by investigating one pooled sample the data are hardly comparable. Concerning MRSA, there exist only a few other studies about the detection especially in faeces. Interestingly, samples from fattening farms had a significantly higher MRSA detection rate than those from breeding farms. This phenomenon can also be observed in other sample matrices (Friese et al., 2012) and maybe correlates with a higher animal exchange and the mixing of pigs from different suppliers in fattening farms (Broens et al., 2011). Pletinckx et al. (2011) isolated MRSA in cloacal swabs of broilers from three different farms, Anukool et al. (2011) in a rectal swab of one pig and Szabo et al. (2011) in rectal swabs of experimentally infected swine. Although no manure samples were analysed, the occurrence of viable laMRSA in it is very likely.

However, a few slurry samples were studied exemplarily for the presence of ESBL/AmpC-producing \textit{E. coli} and lead to positive results. In addition to this, a detection of these microorganisms as well as MRSA was possible in the direct environment of some selected farms on fertilized fields. A French study investigated cattle farms and their vicinity and led to similar results (Hartmann et al., 2012). They found CTX-M-producing \textit{E. coli} in faecal samples as well as in soil of the environment of the barns. Cobbold et al. (2006) detected multiresistant \textit{Salmonella} in faecal and environmental samples on dairy farms, too. This indicates a potential emission of these multiresistant bacteria in the vicinity of different animal farms. Manure has been applied on the fields which were tested for ESBL/AmpC-producing \textit{E. coli} in this study within six weeks before our measurements. Regarding the fields around the three pig barns investigated for MRSA fertilization within the last three months before our measurements was performed. However, at the time of sampling no residue of manure was visible. In the study of Hartmann et al. (2012) there has not been any application of manure since one year. That all means that MRSA as well as \textit{E. coli} originating from livestock as a potential carrier of genes for ESBL/AmpC seem to survive in the environment for longer times. Until now there is no specific knowledge about the tenacity of MRSA or \textit{E. coli} on different grounds and under different environmental conditions. Factors like temperature, rain, ultraviolet radiation, structure of the soil, concentration of heavy metals or the presence of other microorganisms could influence the viability of these pathogens (Chapple et al., 1992; Wang et al., 2007). Apart from the contamination of the surfaces via faeces other routes are conceivable. An emission via the airborne way is possible and has already been described for laMRSA (Friese et al., 2013; Schulz et al., 2012). They detected laMRSA in exhaust air of pig barns. Furthermore, rodents or other wild animals as carriers of laMRSA or ESBL/AmpC-producing \textit{E. coli} could spread these microorganisms (van de Giessen et al., 2009; Guenster et al., 2011). However, a risk for farms, animals or humans living in the vicinity can currently not be estimated.
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