



Zoonotic multidrug-resistant microorganisms among non-hospitalized horses from Germany

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ARTICLE INFO

Keywords:

Multidrug-resistant organism
Epidemiology
Horses
Staphylococcus
MRSA
LA-MRSA
ESBL producing bacteria
Enterobacterales
Enterobacteriaceae
Risk factor
Germany
CC398
One health

ABSTRACT

Colonization with multidrug-resistant organisms (MDROs) belonging to the genus *Staphylococcus* and the order *Enterobacterales* poses a particular threat to populations at risk. While previous studies focused on MDRO carriage among livestock or companion animals, respective epidemiological data on the general equine population are limited. Here, carriage of methicillin-resistant *Staphylococcus aureus* (MRSA) and extended spectrum beta-lactamase (ESBL) producing *Enterobacteriaceae* (ESBL-E) in non-hospitalized horses living on private farms in the rural area in Northwest Germany was assessed. Intranasal and perianal swab samples were cultured on solid chromogenic media directly and after enrichment in tryptic soy broth, respectively. *S. aureus* isolates were *spa*-typed, MRSA and ESBL-E were further classified by phenotypic and molecular methods. Additionally, a subgroup of the first 20 samples was used to isolate and characterize staphylococci other than *S. aureus*. Among 223 horses, fifteen (6.8%) carried *S. aureus*. Two isolates were identified as MRSA (0.9% of all horses, *mecA*-positive) and classified as *spa* types t011 and t6867, both known as members of the livestock-associated MRSA MLST clonal complex 398. Nine horses (4.0%) were colonized by ESBL-*Escherichia coli* positive for *bla*_{CTX-M} and/or *bla*_{TEM}. ESBL-E carriage was associated with prior antibiotic treatment (4/31 vs. 5/183; *p* = 0.0362) and veterinary examinations (4/31 vs. 5/183; *p* = 0.0362). In the subgroup, nine different staphylococcal species other than *S. aureus* were found. The high prevalence of ESBL-E *coli* in non-hospitalized horses underlines the necessity to raise awareness for strain dissemination across different hosts in order to do justice to the “One Health” concept.

1. Introduction

Horses may carry opportunistic pathogens including multidrug-resistant organisms (MDROs), which can also be transmitted between horses and other host species including humans [1–3]. Of high interest are methicillin-resistant staphylococci (MRS), in particular *Staphylococcus aureus* (MRSA) and species of the *Staphylococcus intermedius* group (SIG), or extended spectrum beta-lactamase (ESBL) producing *Enterobacteriaceae* (ESBL-E). Co-colonization of antibiotic-resistant with non-resistant bacteria may facilitate the exchange of resistance genes contributing to the global antibiotic resistance threat [4,5]. Particularly genes of the *mec*-family (*mecA-D*) in *Staphylococcus* and *Macrococcus* species and of the *bla*-family (*bla*_{SHV}, *bla*_{TEM}, *bla*_{CTX-M}, *bla*_{CMY-2}) in species of the order *Enterobacterales*, encoding ESBLs, are of major

interest [6–10].

In this study, we assessed the prevalence of MRSA, other MRS and ESBL-E among non-hospitalized horses in a rural area in Northwest Germany and characterized the respective isolates using phenotypic as well as molecular methods.

2. Materials and methods

2.1. Sample collection

Between May 2015 and March 2016, samples were collected from 23 farms of 223 non-hospitalized horses living on private farms in the rural area around the city of Münster, North Rhine-Westphalia, Germany. Of these, samples came from 38 animals living with ≤10

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horses, 57 with 11–30 horses, and 128 with ≥ 30 horses on the same farm. Swab samples (Transwab Amies MW 172P, Medical Wire & Equipment, Corsham Wiltshire, England) were collected from each animal's nasal vestibules (both sides with one swab) and perianal area. Samples were always taken by a trained veterinarian with full consent of horse owners and a questionnaire was filled out for every horse. Data obtained regarded sex, age, reason for veterinary examination where applicable, antibiotic treatment within the last six months, stay in a veterinary clinic, and contact with livestock. Samples were stored at room temperature for a maximum of two days before being further processed.

2.2. Cultivation

Nasal swabs were streaked onto a selective chromogenic medium (chromID® *S. aureus*, bioMérieux, Marcy l'Étoile, France), then suspended in 5 ml of tryptic soy broth (TSB, Becton Dickinson, Franklin Lakes, NJ, USA) supplemented with 6.5% NaCl. Solid and liquid cultures were incubated at 37 °C for 24 h. From enriched liquid cultures, 10 μ l were inoculated on (i) chromID® *S. aureus* medium (bioMérieux), and (ii) chromogenic medium selective for MRSA (chromID® MRSA, bioMérieux). Another 1 ml was transferred into an MRSA enrichment broth, i.e. phenol red mannitol broth supplemented with ceftizoxim/aztreonam (9 ml, PHMB+C/AZ, Mediaproducs BV, Groningen, The Netherlands). Cultures were further incubated at 37 °C for 24 h (chromID® *S. aureus* and PHMB+C/AZ) or 48 h (chromID® MRSA), respectively. From the PHMB+C/AZ culture, 1 ml was given onto chromID® MRSA (bioMérieux) and further incubated at 37 °C for 48 h. Moreover, nasal swabs sampled from the first 20 horses were additionally streaked onto Columbia CAP selective agar (containing colistin and aztreonam) with 5% sheep blood (Oxoid, Wesel, Germany) and incubated at 37 °C for 24 h in order to isolate and characterize staphylococci other than *S. aureus*.

Perianal swab samples were suspended in TSB and incubated at 37 °C for 24 h. The enriched culture (10 μ l) was inoculated on a chromogenic selective medium (chromID® ESBL, bioMérieux) and further incubated at 37 °C for 24 h.

2.3. Identification of isolates

Colonies grown on solid media were tentatively isolated based on conventional phenotypic characteristics, such as colony morphology, pigmentation, Gram staining and production of clumping factor (Pastorex Staph Plus; bioMérieux). Further incubation of pure cultures was carried out on Columbia blood agar (Becton Dickinson) at 37 °C for 24 h.

Isolates were identified down to the species level applying matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry (MALDI-TOF MS, Bruker Daltonics, Billerica, MA) as described elsewhere [11]. Briefly, material from freshly grown single colonies was transferred onto a ground steel target plate (Bruker Daltonics) and covered with 1 μ l of alpha-cyano-4-hydroxycinnamic acid (HCCA) dissolved in 50.0% acetonitrile and 2.5% trifluoroacetic acid. Co-crystallized samples were analyzed in flexControl 3.3 (Bruker Daltonics). Evaluation via MALDI Biotyper® 4.0 (Bruker Daltonics) was set to an *m/z* range of 4000–10,000 Da and a score threshold ≥ 2.0 for unambiguous assignment on species level.

Bacterial isolates yielding scores ≤ 2.0 in MALDI-TOF MS analysis were further intended for biochemical identification in the VITEK® 2 automated system (bioMérieux) applying VITEK® 2 GP ID cards for nasal and VITEK® 2 GN ID cards (bioMérieux) for perianal samples, respectively.

For isolates with ambiguous identification results by both MALDI-TOF MS and VITEK® 2, 16S rRNA gene sequencing was performed. Extraction of total genomic DNA was performed with the QIAamp DNA MiniKit according to the manufacturer's instructions (Qiagen, Venlo,

Netherlands). V1-V4 hypervariable regions of the 16S rRNA gene were amplified using primers 27f and 907r(m) [12,13]. PCR products were purified with the MinElute Kit (Qiagen) following the manufacturer's instructions. The cycle sequencing technology on an ABI 3730XL sequencing machine was used (Eurofins Genomics, Ebersberg, Germany). Sequences obtained were compared against data from validly described type strains of the RDP-II database [14]. The threshold for assignment on species level was set to $\geq 98\%$ similarity.

2.4. Characterization of isolates

Phenotypic antimicrobial susceptibility was tested for all isolates using the VITEK® 2 system (bioMérieux) according to the manufacturer's instructions with test cards AST-P632 for staphylococci and AST-N214 for *Enterobacteriaceae*. Evaluation of AST test results was carried out according to the clinical breakpoints for humans of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) [15].

In staphylococci, presence of the genes *mecA*, *mecB* and *mecC* conferring methicillin resistance was tested by PCR as introduced elsewhere [6,16]. Genotyping of *S. aureus* isolates was done by *spa* typing; MRSA were additionally classified by multilocus sequence typing (MLST) as previously described [17,18].

For *Enterobacteriaceae*, ESBL-production indicated by VITEK was confirmed using the MASTDISCS™ ID Extended-Spektrum- β -Laktamasen (ES β L)-Set (CPD10) D67C (MAST Diagnostica, Reinfeld, Germany). Genetic basis of antibiotic resistance was determined by PCR-based analysis of the genes *bla*_{SHV}, *bla*_{TEM}, *bla*_{CTX-M} and *bla*_{CMY-2} [19,20] and applying the eazyplex® SuperBug assay (AmplexDiagnostica GmbH, Gars am Inn, Germany) for detection of carbapenemase-producing *Enterobacteriaceae* (CRE).

Fisher's exact test (GraphPad Prism v.5.00, GraphPad Software, La Jolla, CA, USA) was carried out to analyze association of ESBL-E and MRSA carriage with potential risk factors. The probability value of *p* < 0.05 was considered significant in order to reject the null hypothesis.

3. Results

3.1. Sample group

Altogether, nasal and perianal samples from 223 horses originating from 23 different farms located in six different rural districts around Münster, North Rhine-Westphalia were included in this study. The group comprised 94 mares, 25 stallions and 104 geldings with a mean age of 9.2 years (range 0–31 years). The majority of horses was healthy (188/223; 84.3%) whereas 35/223 animals (15.7%) were sampled before undergoing veterinary examination. Moreover, 34/223 horses (15.2%) had received systemic antibiotics and 31/223 horses (13.9%) had been admitted stationary to a veterinary clinic within six months prior to sampling. Of all horses, 158 (70.9%) were held without close contact to livestock, i.e. no livestock was being kept on the farm with the horses. The remaining horses were held with pigs (32/223; 14.3%), cattle (8/223; 3.6%) and poultry (25/223; 11.2%) on the same farm. Further information is given in Table 1.

3.2. Colonization with ESBL-E

Colonization with ESBL-E was detected in the perianal samples from 9/223 horses (4.0%) living on 7/23 farms (30.4%). Four of these farms held 11–30 horses, 3 farms ≥ 30 horses. All isolates were found to belong to the species *Escherichia coli*. Overall, 3/94 mares (3.2%), 1/25 stallions (4.0%) and 5/104 geldings (4.8%) were colonized. Their mean age was 12.4 years (range 4–31 years) and 4/9 animals were introduced to a veterinarian, either for internal diseases (2/4), orthopedics (1/4) or surgical interventions (1/4). Moreover, 4/9 horses had received antibiotics within six months prior to sampling. One gelding was kept

Table 1
Metadata of horses sampled.

Factor	Mare	Stallion	Gelding	Total
Age (y)				
- Mean	9.8	6.9	9.1	9.2
- Range	0–31	0–18	3–30	0–31
Reason for veterinary examination				
- Complicated foaling	1	0	0	1
- Dental treatment	0	0	2	2
- Internal disease ^a	3	2	3	8
- Surgical intervention ^b	8	0	5	13
- Orthopedic disease ^c	4	2	5	11
Antibiotics ^d				
- Systemic	14	4	16	34
- No antibiotics	80	21	88	189
Stay in veterinary clinic ^d				
- Yes	15	4	12	31
- No	79	21	92	192
Contact with livestock				
- Pigs	11	6	15	32
- Cattle	3	2	3	8
- Poultry	14	1	10	25
Total	94	25	104	223

^a Internal disease; disease of digestive tract, urogenital tract, circulatory system, nervous system, skin, eyes, ears, or metabolism;

^b Surgical intervention; sterilization, wound management, orthopedic surgery, or tumor resection;

^c Orthopedic disease; diagnosis of lameness, radiography, or bandage management;

^d Within six months prior to sampling.

together with pigs, cattle and poultry, one mare together with poultry and another mare together with pigs.

All *E. coli* isolates were susceptible to ertapenem, imipenem, meropenem, tigecycline and piperacillin/tazobactam. Molecular tests confirmed presence of the genes *bla*_{TEM} and *bla*_{CTX-M} in 7/9 ESBL-E exhibiting either a single gene type (*bla*_{CTX-M}; n = 2) or a compound-gene type (*bla*_{TEM} and *bla*_{CTX-M}; n = 5) (Table 2). Neither *bla*_{TEM} nor *bla*_{CTX-M} were detected in the remaining two isolates phenotypically confirmed as ESBL-E.

3.3. Colonization with *S. aureus*

In the group of 223 horses, 15 horses (6.7%) living on 10/23 farms (43.5%) carried *S. aureus* in the nose. One of these farms held ≤10 horses, three held 11–30 horses, and six held ≥30 horses. One isolate was picked directly from SAID medium, fourteen were isolated after enrichment. Among the colonized horses, there were six mares (6/94; 6.4%), two stallions (2/25; 8.0%) and seven geldings (7/104; 6.7%).

Table 2
Resistance profiles of ESBL-*E. coli* found in 223 horses.

Animal host	Sampling site	Resistance genes	Phenotypic antimicrobial susceptibility test profile	
			ESBL ^a -screening	Other resistances ^b
Gelding	Perianal	<i>bla</i> _{CTX-M} , <i>bla</i> _{TEM}	POS	AMP, AMS, CPO, CTA, CUR, TRS
Gelding	Perianal	<i>bla</i> _{CTX-M}	POS	AMP, CTZ, CIP, CPO, CTA, CUR, GEN, MOX, TRS
Gelding	Perianal	<i>bla</i> _{CTX-M} , <i>bla</i> _{TEM}	POS	AMP, AMS, CPO, CTA, CUR, GEN, TRS
Stallion	Perianal	<i>bla</i> _{CTX-M} , <i>bla</i> _{TEM}	POS	AMP, AMS, CPO, CTA, CUR, GEN, TRS
Gelding	Perianal	<i>bla</i> _{CTX-M}	POS	AMP, CPO, CTA, CUR, GEN, TRS
Gelding	Perianal	<i>bla</i> _{CTX-M} , <i>bla</i> _{TEM}	POS	AMP, AMS, CPO, CTA, CUR, GEN, MOX, TRS
Mare	Perianal	n.a.	POS	AMP, CIP, CPO, CTA, CTZ, CUR, MOX, TRS
Mare	Perianal	n.a.	POS	AMP, CPO, CTZ, MOX, TRS
Mare	Perianal	<i>bla</i> _{CTX-M} , <i>bla</i> _{TEM}	POS	AMP, AMS, CPO, CTA, CUR, GEN, TRS

AMP, ampicillin; AMS, ampicillin-sulbactam; CIP, ciprofloxacin; CPO, cefpodoxime; CTA, cefotaxime; CTZ, ceftazidime; CUR, cefuroxime; GEN, gentamicin; MOX, moxifloxacin; TRS, trimethoprim-sulfamethoxazole; n.a., not assigned.

^a as determined by VITEK® 2 automated system (bioMérieux) and MASTDISC™ ID ESBL-Set (CPD10) D67C (MAST Diagnostica). POS = positive.

^b MICs were detected with VITEK® 2 (bioMérieux) and evaluated using breakpoints provided by EUCAST [15].

The mean age of this group was 12.1 years (range 5–25 years) and 3/15 animals had been admitted to a veterinary clinic because of internal (1/3) or orthopedic (1/3) diseases or complicated foaling (1/3). Four of the *S. aureus*-carrying horses (4/15; 26.7%) had received antibiotics within a time period of six months before samples were taken. Moreover, 2/15 horses (13.3%) were kept in close contact to livestock: one mare was held together with pigs and one stallion was held together with pigs and cattle. Only one nasal *S. aureus* strain was detected per horse.

S. aureus strains (n = 15) were assigned to *spa* types t091 (n = 3), t127, t549, t1166, t3218 (each n = 2), t011, t233, t768 and t6867 (each n = 1). Methicillin resistance was found in 2/15 isolates (2/223 horses; 0.9%) and was always confirmed by the detection of the *mecA* gene. Isolates belonged to *spa* types t011 and t6867, known as part of the livestock-associated (LA) MRSA clonal complex (CC) 398. Both LA-MRSA isolates showed further resistance against gentamicin, tetracycline and trimethoprim-sulfamethoxazole when tested with the VITEK® 2 system (Table 3). MRSA t011 was additionally resistant against clindamycin and erythromycin. Both LA-MRSA-carrying animals were healthy mares not undergoing veterinary examination. They were five and ten years of age and had not received any antibiotics in the time before sampling. One of the mares, colonized with MRSA t011, was kept together with pigs.

3.4. Association of risk factors with ESBL-*E* and MRSA carriage

Regarding the equine colonization with ESBL-*E. coli*, antibiotic treatment (4/31 vs. 5/183; p = 0.0362) and veterinary examinations (4/31 vs. 5/183; p = 0.0362) were identified as risk factors. Coinciding numbers of animals treated with antibiotic substances and those undergoing veterinary examination were not based on identical composition of the groups. None of the other risk factors assessed was associated with ESBL-*E. coli* or MRSA carriage (p > 0.05 for all variables).

3.5. Colonization with other staphylococci

The subgroup of horses (n = 20) analyzed for staphylococcal species other than *S. aureus*, consisted of six mares, three stallions and eleven geldings. A total of nine different staphylococcal species other than *S. aureus* including one coagulase-positive and eight coagulase-negative members could be detected in this group (Fig. 1).

The most prevalent species was *Staphylococcus xylosum* (14/20 animals; 70%), followed by *Staphylococcus vitulinus* (10/20 animals; 50%), *Staphylococcus sciuri* (8/20 animals; 40%) and *Staphylococcus succinus* (6/20 animals; 30%). *Staphylococcus haemolyticus* and *Staphylococcus lentus* were only found in stallions (each in 1/3), whereas *Staphylococcus equorum* and the coagulase-positive species *Staphylococcus delphini* (member of the *S. intermedius* group) were only present in geldings

Table 3
Resistance profiles of MRSA found in 223 horses.

Animal host	Sampling site	spa type	Resistance genes	Phenotypic antimicrobial susceptibility test profile	
				CXI ^a -screening	Other resistances ^b
Mare	Nasal	t011	<i>mecA</i>	POS	BEN, CLI, ERY, GEN, OXA, TET, TRS
Mare	Nasal	t6867	<i>mecA</i>	POS	BEN, GEN, OXA, TET, TRS

BEN, benzylpenicillin; CLI, clindamycin; CXI, ceftioxin; ERY, erythromycin; GEN, gentamicin; OXA, oxacillin; TET, tetracycline; TRS, trimethoprim-sulfamethoxazole.

^a as determined by VITEK® 2 automated system (bioMérieux). POS = positive.

^b MICs were detected with VITEK® 2 (bioMérieux) and evaluated using breakpoints provided by EUCAST [15].

(each in 2/11; 18.2%). None of these staphylococcal isolates were methicillin-resistant.

4. Discussion

In recent decades, veterinary healthcare settings have been described as places of origin for outbreaks caused by a variety of multi-drug-resistant microorganisms (MDROs) [21–24]. Both MRSA and ESBL-E are notorious resistance phenotypes in equine hospitals [21,24–26]. Overall, 2.7–9.3% of horses carry MRSA upon admission to veterinary hospitals [27,28]; 2.6% and 10.7% tested positive for nasal and fecal colonization with ESBL-E, respectively [25]. Regarding MRS other than *S. aureus*, a study that included 42 healthy horses from four different farms in Poland found that 28.6% were colonized in the nasal cavities [29]. However, less data is available on the distribution of these MDROs in the general equine population in Germany. Of note, the region in which the sampling was performed has a particularly high density of animal production. As both MRSA and ESBL-E are widespread among regional livestock, these farms may represent potential sources for MDRO spread in non-hospitalized horses [30–32].

In the study group of 223 horses, we found nine horses colonized with ESBL-E (4.0%), all identified as *E. coli*. This prevalence resembles data from another study carried out in the UK, where 6.3% of 650 fecal samples from non-hospitalized horses were positive for ESBL-E *coli* [33]. Studies on equine patients in veterinary clinical settings report on even higher rates of 10.7% and 34.2% of ESBL-E carrying animals, respectively [22,25] indicating the high potential of strain

dissemination and horizontal gene transfer among ESBL-encoding genes under opportune environmental conditions. In the study group, ESBL-*E. coli* carriage was shown to be significantly associated with antibiotic treatment. This finding is in line with data obtained from the human population where antibiotic therapy has been identified as a risk factor for the colonization and infection with ESBL-E in numerous studies [34–36]. Even though strains recovered in this study were only present as commensals in the respective horses, under favorable conditions, an infection of the host or other animals and humans in contact, is a likely scenario. The high potential of strain dissemination in veterinary clinics was recently reported by Walther and colleagues who investigated the suspected spread of an ESBL-*E. coli* clone across three equine patients [24]. Furthermore, in an earlier study, the conjugal horizontal transfer of *bla*_{CTX-M-1} genes between *E. coli* and *Salmonella* strains was shown in vitro [51]. Accordingly, the horse stable environment needs to be more acknowledged as a complex biocenosis consisting of a plethora of microorganisms (i.e. the microbiota) including pathogenic species and their resistome, which comprise not only those of equine sources, but also those from humans, which are in contact with the horses. This is of particular importance in veterinary facilities where antibiotic treatment generates selection pressure favoring the generation and distribution of (multi-)resistant organisms, which may easily cross host-species barriers.

Altogether, the prevalence of *S. aureus*-positive animals (6.7%) in the group of 223 horses is comparable to data from Canada (7.9%) and remains below the most recent data from healthy horses in Denmark (13.5%) [37,38]. Of interest, half of the *spa* types identified in our study

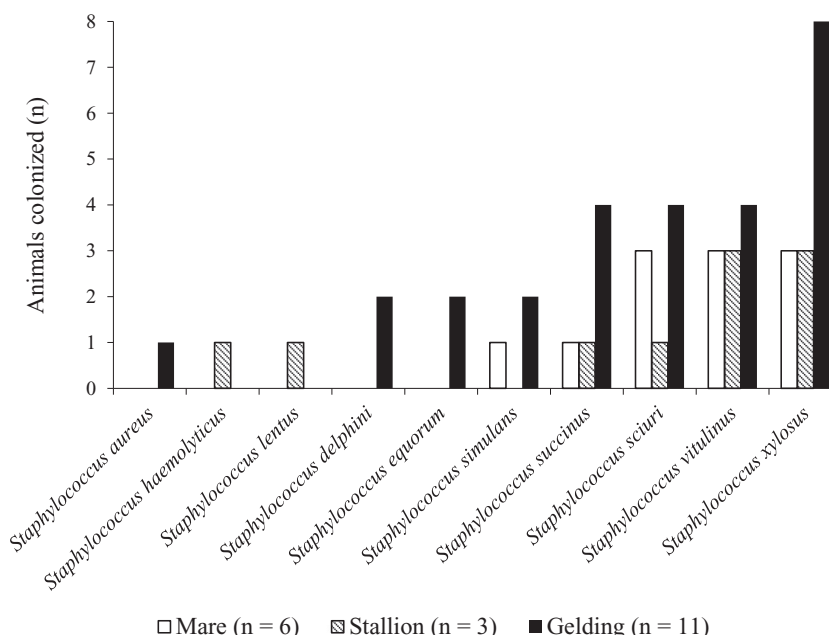


Fig. 1. Absolute numbers of mares, stallions and geldings of a subgroup (n = 20) analyzed for colonization with any staphylococcal species.

were also present in the Danish equine population [38]. This pattern is also reflected in the colonization by methicillin-resistant strains. Even though the number of MRSA-positive horses documented here (0.9%) rather resembles numbers from studies investigating healthy horses in other European countries (0.53–1.7%) than data obtained in Denmark (4.2%), the major fraction of MRSA detected in Denmark was found to belong to the typically livestock-associated clonal complex (CC) 398 [33,38–40]. This was also true for the two isolates (t011 and t6867, both ST398) detected in our study. In 2017, the Danish population accounted for about 5.7 million people and over 31 million pigs were bred and slaughtered [41,42]. In 2016, about 88% of the Danish pig farms tested positive for MRSA CC398 [43]. Accordingly, high proportions of CC398 can be found in MRSA isolated from human infections in Denmark. In 2014, 16% of MRSA-related bloodstream infections and 21% of soft-tissue infections were caused by MRSA CC398 [44]. Correspondingly, in the rural regions around Münster, in 2018, 4210 farms with over four million pigs were registered [45] and at the local university hospital, a relatively steady proportion of about 30% of MRSA detected at hospital admission of the patients belonged to CC398 between 2010 and 2014. Of these, between 4% and 11% were obtained from specimen associated with infections [46]. Moreover, in a recent study on MDRO prevalence in companion animals also living in districts around Münster, but without obvious contact to livestock, all MRSA detected were also assigned to livestock-related clonal lineages [47].

Regarding staphylococcal species other than *S. aureus*, we found nine different species in the subgroup of 20 horses. All of them were methicillin-susceptible, which is not surprising because mainly healthy horses outside of healthcare facilities were enrolled. Among these, the coagulase-positive species and SIG member *Staphylococcus delphini* was detected in one horse. Resistant strains of this species have been described in equine samples before [48]. Apart from *S. aureus*, other staphylococci are often neglected when screenings for methicillin-resistant strains are performed. This can be problematic, as, for example, carriage of MRS in horses has been shown to occur at a high frequency and strains are also capable of causing disease and transferring methicillin resistance genes towards more pathogenic species, such as *S. aureus* [33,49,50]. Accordingly, even though in this study group no MRS besides MRSA was found, particular attention needs to be paid to these strains in future analysis.

5. Conclusion

In this study, a considerable number of multiresistant pathogenic species, in particular ESBL-*E. coli*, was detected in a group of non-hospitalized, mainly healthy horses living in rural districts surrounded by livestock production farms. Although only infrequently found, the exclusive presence of MRSA isolates belonging to the livestock-associated CC398 lineage underlines the impact of livestock farming on the geographic distribution of epidemic strains. Transmission of MDROs to humans which are in close contact to MDRO-colonized horses and their environment may be an underestimated risk. A consequent realization of the “One Health” concept is crucial for the containment of antibiotic resistances.

Funding

This work was supported by the German Federal Ministry for Education and Research (BMBF), within the research consortia MedVet-Staph [grant number 01KI1301A to KB and RK], RESET [grant number 01KI1013C to UR], and #1Health-PREVENT [grant number 01KI1727A to KB and RK] as part of the Research Network on Zoonotic Infectious Diseases.

Conflict of interest

None.

Acknowledgements

We thank Melanie Bach, Damayanti Kaiser and Martina Schulte for excellent technical assistance.

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