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Occurrence and molecular composition of methicillin-resistant *Staphylococcus aureus* isolated from ocular surfaces of horses presented with ophthalmologic disease

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ABSTRACT

Severe infections due to methicillin-resistant *Staphylococcus aureus* (MRSA) have been increasingly recognized in virtually all fields of veterinary medicine. Our objective was to study the occurrence, phylogenetic relationships and antimicrobial resistance properties of MRSA isolated from ocular surfaces of horses prior to invasive procedures. Within a 49-week sampling period, ocular swabs obtained from 46 eyes of 44 horses, including eyes with clinical signs of conjunctivitis/blepharitis, keratitis or uveitis were screened for the presence of *S. aureus*. As a result, seven samples were positive for *S. aureus* (15.2%), with six of them being classified as MRSA (13%). In addition, all isolates were resistant or showed reduced susceptibility to tetracyclines, the aminoglycosides gentamicin and kanamycin, fluoroquinolones, and the combination sulfamethoxazole/trimethoprim. Since a very close relationship between the MRSA isolates was assumed after pulsed-field gel electrophoresis employing the restriction endonuclease ApaI, whole genome sequencing (WGS) was used to shed more light on the phylogenetic relationships and the molecular composition of all MRSA isolates. Analysis of WGS data revealed closely related MRSA belonging to sequence type 398, *spa* type t011 and *dru* type dt10q, harboring an SCC*mec* IV element and the *Staphylococcus aureus* pathogenicity island SaPIbov5. Moreover, all MRSA were positive for a beta-hemolysin converting phage carrying genes of the immune evasion cluster (IEC). Since cases of eye infections due to MRSA were often associated with fatal outcomes, more research is needed with respect to the origin of MRSA isolated from ocular surfaces to implement sufficient barrier and infection control measures.

1. Introduction

In veterinary medicine, methicillin-resistant *Staphylococcus aureus* (MRSA) were frequently associated with healthcare-associated infections (HAI) (Walther et al., 2017). Considering animal welfare and work place safety for veterinary personnel, sufficient control strategies to minimize the occurrence of HAI are needed (Walther et al., 2017). While severe eye infections associated with MRSA were regularly reported from human ophthalmology (Asbell et al., 2008; Chuang et al., 2012), such reports about ocular disease associated with MRSA in companion animals are scarce. However, since previous reports included MRSA-positive eye swabs (Cuny et al., 2010; LoPinto et al., 2015), MRSA may represent an important pathogen in ocular diseases as well. Descriptions of conjunctival and corneal microbial flora in healthy horses commonly showed a predominance of opportunistic and non-pathogenic Gram positive bacteria (Johns et al., 2011), which might comprise also resistant variants like MRSA. A study from Sicily already reported a detection rate of 8.7% MRSA for conjunctival swabs obtained from 46 healthy donkeys (Foti et al., 2013). This finding consequently raised the question whether prior presence of MRSA on equine ocular surfaces might be a reason for the development of severe infectious eye diseases after invasive ocular procedures. Many factors, such as season, geography, bedding, habitat and husbandry, were discussed in terms of their respective influence on the microbial burden in

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healthy as well as in diseased horse eyes (Andrew et al., 2003). However, once the outer eye barriers of protection were breached by opportunistic resident and/or transient microbes, a fulminant infection might occur (Andrew et al., 2003). In human medicine, *S. aureus* is an important cause of various purulent eye infections, and reported proportions of methicillin-resistant isolates range from 30.7% to 55% in different studies (Asbell et al., 2008; Chuang et al., 2012).

Although there have been numerous studies on MRSA in horses (Abdelbary et al., 2014; Anderson et al., 2009; Bergstrom et al., 2012; Cuny et al., 2006) in the recent past, little is known about the occurrence and genetic background of these antibiotic-resistant bacteria residing on ocular surfaces of horses. Thus, the present explorative study focuses on the occurrence, the phylogenetic relationships and molecular characteristics of MRSA isolated from equine ocular surfaces prior to invasive ophthalmological manipulation.

2. Material and methods

2.1. Equine patients and screening procedure

Horses with a medical history of acute or chronic ocular disorders, which presented between Oct 09, 2015 and Sep 01, 2016 at the Equine Clinic, Surgery and Radiology, Freie Universität Berlin, Germany, for invasive procedures, such as paracentesis, biopsy and surgery, were included in this prospective study.

Eyes of horses presented with clinical signs of either conjunctivitis/blepharitis, keratitis or uveitis were clinically inspected and routinely screened for the presence of pathogenic and opportunistic bacteria, including MRSA, by use of ocular swabs (Mast Diagnostica, Rheinfeld). Surrounding skin surfaces were cleaned with sterile cotton gauze slightly wetted with sterile saline (0.9% NaCl) solution before specimen collection. Sterile transport swabs (HEINZ HERENZ, Hamburg, Germany) were moistened by using sterile water (BRAUN, Melsungen, Germany). A single swab was taken by a skilled person (T.S.) from the eyelid margin and the lower conjunctival fornix immediately prior to an ophthalmological procedure.

2.2. MRSA screening and antimicrobial susceptibility testing (AST)

All samples were cultured on Columbia agar with 5% sheep blood (bioMérieux, Germany) and chromID® MRSA (bioMérieux, Germany). All swabs were transferred to enrichment broth for staphylococci (Hanselman et al., 2008), incubated overnight at 37 °C and subsequently cultivated on chromID® MRSA agar for a second time. A semi-quantitative assessment of staphylococcal growth was performed with the following categories: enrichment (growth after broth enrichment only), occasional (up to 5 colony forming units (cfu) grown per agar plate, + (up to 30 cfu/plate), + + (up to 100 cfu/plate) and + + + (> 100 cfu/plate) as described before (Jores et al., 2004). Identification of *S. aureus* was carried out for suspicious colonies using MALDI-TOF MS (Bruker, Germany) analyses. Antimicrobial susceptibility testing was performed using the VITEK®2 system (bioMérieux, Germany). For classification of the isolates as susceptible, intermediate or resistant, clinical breakpoints as laid down in the documents VET01-S (3rd edition) and M100-S28 of the Clinical and Laboratory Standards Institute (CLSI, 2015, 2018) were applied.

2.3. Molecular analysis and whole genome sequencing of equine MRSA

Molecular analysis of MRSA included pulsed-field gel electrophoresis (PFGE) performed as previously described employing the restriction endonuclease ApaI (Walther et al., 2009). The PFGE-based dendrogram was created using Bionumerics 7.6 (AppliedMaths, Belgium) with Dice coefficient and the unweighted-pair group method using arithmetic averages (UPGMA) with 1.3% band tolerance and 0.5% optimization. Since a very close relationship was observed after initial PFGE analysis, all MRSA isolates were subjected to whole-genome sequencing (WGS) using Illumina MiSeq 300 bp paired-end sequencing with an obtained coverage > 90 × . After quality control using the NGS tool kit13 (70% of bases with a phred score > 20), high-quality filtered reads were used for *de novo* assembly into contiguous sequences (contigs) and subsequently into scaffolds using SPAdes v3.11. All draft genomes were annotated using Prokka (Seemann, 2014). Two further MRSA-ST398 genomes, IMT33368 (PUTY00000000) and IMT37082 (PUXL00000000) representing two distinct molecular compositions frequently isolated from horse patients during routine hygiene screenings in the horse clinic in 2014–2015 (unpublished data) were included together with the livestock-associated MRSA-ST398 genome CP020019 (reference pig) as an outgroup (Makarova et al., 2017).

The determination of the maximum common genome (MCG) alignment was done by identifying those genes present in all nine genomes (von Mentzer et al., 2014). We therefore clustered the coding sequences based on the parameters sequence similarity (min. 70%) and coverage (min. 90%) and defined those 2404 genes that were present in each genome while fulfilling the threshold parameters of the MCG.

In a next step the allelic variants of these genes were extracted from all genomes by a blast-based approach, aligned individually for each gene and then concatenated which resulted in an alignment of 2.616 Mbp for these 9 strains. This alignment was used to generate a phylogenetic tree with RaxML version 8.1.14 with a General Time Reversible model and gamma correction for among site rate variation (Stamatakis, 2014).

WGS data were used for genotypic characterization including the determination of the sequence type (ST) (MLSTFinder (Larsen et al., 2012)), transferable resistance genes (ResFinder 2.1 (Zankari et al., 2012), threshold: 95% ID, 80% minimum length) and virulence factors (Joensen et al., 2014). *Spa*- and *dru* types of the isolates were deduced from the whole genome sequences.

2.4. Database accession numbers

This Whole Genome Shotgun project has been deposited at DDBJ/ENA/GenBank under the accession QITK00000000 (IMT38358), QITL00000000 (IMT38422), QITM00000000 (IMT38431), QITN00000000 (IMT38587), QITO00000000 (IMT38594), and QITP00000000 (IMT38653).

2.5. Statistical analysis

Data were stored in MS Excel. Statistical analyses were carried out using IBM SPSS (SPSS version 24). The association between categorical variables was investigated by Chi-Square test. Fisher's exact test was used if the number of cells with expected counts < 5 exceeded 25%. P-values below 0.05 were considered to be statistically significant.

3. Results

3.1. Detection of MRSA in equine ocular samples

Between Oct 09, 2015 and Sep 01, 2016, ocular swabs obtained from 46 eyes of 44 horses, including eyes with clinical signs of conjunctivitis/blepharitis (n = 8), keratitis (n = 9) or uveitis (n = 29), were screened for the presence of *S. aureus* (Supplemental Table 1). As a result, seven samples were positive for *S. aureus* (15.2%), with six of them being classified as MRSA (13%). Considering the ophthalmological disorders investigated here, MRSA-positive swabs were obtained from eyes showing clinical signs of keratitis (4/9; 44%) and from cases of uveitis (2/29; 6.9%), while MRSA-positive samples were absent in horses presented with signs of conjunctivitis/blepharitis. The maximum of MRSA-positive swabs per individual horse was one, even among horses with diseases involving both eyes (Supplemental Table 1).

Fisher's exact test revealed that the horse group investigated here