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Increase of zinc resistance in German human derived livestock-associated MRSA between 2000 and 2014

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ABSTRACT

Problem addressed: Livestock-associated methicillin-resistant *Staphylococcus aureus* (LA-MRSA), particularly of the clonal complex (CC) 398, emerged as zoonotic pathogens predominantly among humans with direct or indirect livestock contact, but also in healthcare settings. The factors contributing to the success of LA-MRSA are only poorly understood.

Objective: During the past years, the use of heavy metal compounds as feed-supplements was found to influence the co-selection of LA-MRSA in pig herds. This study aimed to determine the prevalence of zinc resistance among MRSA CC398 isolated from patients of a German university hospital located in a pig farming-dense area.

Methods and approach: In comparison to concurrent healthcare-associated MRSA (HA-MRSA), LA-MRSA CC398 comprising isolates from their first appearance in 2000 to recent isolates from 2014 were included.

Results: Among MRSA CC398, the overall resistance rate towards zinc chloride was 57% compared to only 3% among concurrently isolated HA-MRSA. Zinc resistance correlated with the presence of the *czrC* gene in 100% of the MRSA CC398 and in 67% of the HA-MRSA.

Conclusions: The zinc resistance rate in MRSA CC398 significantly increased from 2009 to 2014 with a maximum in 2014. Alarmingly, zinc resistance has become a frequent phenotype of human LA-MRSA in Germany potentially facilitating co-selection of antibiotic resistance genes.

1. Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) emerged in livestock production during the past decade (Cuny et al., 2010). In Europe, most livestock-associated (LA-) MRSA belong to the clonal complex CC398, but other clonal complexes have also been described (Köck et al., 2010). Multiple epidemiological investigations described that LA-MRSA break the species barrier and are transmitted from livestock to humans (Wulf et al., 2008; Köck et al., 2012, 2013). Thus, exposure to livestock is a major risk factor for LA-MRSA colonization among humans, but a spread of MRSA CC398 independently from direct livestock contact has also been described (Köck et al., 2009; Deiters et al., 2015). Consequently, LA-MRSA are introduced in the human healthcare system and the general population especially in regions with a high density of livestock production (van Loo et al., 2007; van Alen et al., 2017; Becker et al., 2017a). Furthermore, a considerable proportion of MRSA CC398 colonization and infections were described in humans without livestock contact (Lekkerkerk et al., 2012; Wulf et al., 2012; Deiters et al., 2015). This raises concern as MRSA CC398 are found to cause a range of human infections that is as broad as the many infection types typically associated with *S. aureus* (Becker et al., 2017b). They include *e.g.* bacteremia, skin and soft tissue infections, pneumonia, osteomyelitis, endocarditis and blood-stream related infections (Wulf et al., 2012; Köck et al., 2013). Since LA-MRSA CC398 constitutes a hazard for humans, investigations of factors promoting the success of LA-MRSA are of major importance (Becker et al., 2017b).

Heavy metal compounds, such as zinc oxide or copper sulphate, are used as feed supplements in livestock as an alternative for in-feed antibiotics for growth promotion and prevention of gastro-intestinal disorders (Monteiro et al., 2010; Adamse et al., 2011). The large-scale application of these heavy metal compounds is often discussed as a risk factor involved in the selection of MRSA (Cavaco et al., 2010; Slifierz et al., 2014, 2015). In 2010, Aarestrup et al. (2010) found that most MRSA CC398 isolates recovered from pig farms in Denmark had reduced susceptibilities to zinc chloride with minimum inhibitory concentration (MIC) values > 2 mM. Zinc resistance was also found to be highly prevalent among MRSA CC398 isolates from animals in Denmark and Germany due to the acquisition of the zinc and cadmium resistance

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gene *czrC* (Cavaco et al., 2010; Argudín et al., 2016). The *czrC* gene has been found within the staphylococcal cassette chromosome *mec* (SCC*mec*) type V (5C2&5), which is often found among MRSA CC398 isolated from livestock (Li et al., 2011). SCC*mec* types IV, V variants and VIII may also carry *czrC* (Zhang et al., 2009; Li et al., 2011; Argudín et al., 2016). Thus, the use of zinc oxide in livestock is considered as a risk factor for the co-selection of methicillin-resistant *S. aureus* in animal husbandry (Cavaco et al., 2011; Slifierz et al., 2015).

The purpose of this retrospective single-center study at a German university hospital, which is located in a region where MRSA CC398 is endemic, was to investigate the zinc susceptibility in MRSA CC398 isolates recovered from human specimens. Compared to concurrent MRSA belonging to "classical" healthcare-associated (HA-) MRSA clonal lineages, CC398 isolates continuously collected since their first appearance were analyzed over a 15-year period (2000–2014).

2. Materials and methods

2.1. Strain collection

At the University Hospital Münster, a routine, universal nasopharyngeal MRSA admission screening was established in 2006. For identification of isolates, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS, Microflex-LT system, MALDI-Biotyper 3.0, Bruker Daltonics, Germany) was applied as previously described (Idelevich et al., 2014; Kaspar et al., 2016). Phenotypic and genotypic characterization of methicillin resistance was assessed by using the VITEK-2 automated system (bioMérieux Nürtingen, Deutschland) applying the antimicrobial susceptibility test card AST-P632 and via *S. aureus*-specific PCR targeting the *mecA* and *mecC* genes as described before (Becker et al., 2006; Kriegeskorte et al., 2012) and additionally by applying the GenoType MRSA assay (Hain-Lifescience, Germany). In general, every first MRSA isolate of each patient was genotyped by *S. aureus* protein A (*spa*) sequence-based typing (Harmsen et al., 2003; Becker et al., 2006). This was implemented in 2006, but performed retrospectively for all isolates collected between 2000 and 2006. All typing data are stored in a central database. The "Based Upon Repeat Pattern" (BURP) was used to cluster MRSA isolates into *spa*-clonal complexes (Mellmann et al., 2008) and *spa*-CCs were used as indicators for MRSA CC398 and HA-MRSA clonal lineages. Data about the prevalence of MRSA CC398 at the study hospital and its emergence between 2000 and 2014 have been published elsewhere (van Alen et al., 2017).

A collection of 123 MRSA CC398 clinical isolates obtained from patients at the University Hospital of Münster was analyzed regarding its heavy metal susceptibility. For the years 2000–2004, all MRSA CC398 isolates available (n = 23) were tested, whereas in the later years (since 2005) the first ten MRSA CC398 isolates of each year were systematically analyzed. In parallel to the MRSA CC398 isolates included in the study, the first ten HA-MRSA isolates of each year were chosen from 2005 to 2014. In the early study period (2000–2004), the respective number of HA-MRSA isolates as it was found for MRSA CC398 was included in the study (n = 23; first four to five isolates per year). Only one isolate per patient was included in the study. All MRSA isolates were cultivated on Columbia blood agar (Becton Dickinson, Franklin Lakes, USA) supplemented with 5% sheep blood (Oxoid, Wessel, Germany).

2.2. Susceptibility testing

Determination of heavy metal MICs was performed by broth microdilution method using Mueller-Hinton broth with a pH adjusted to 5.5 as described elsewhere (Aarestrup and Hasman, 2004). Zinc chloride (Sigma Aldrich, Mw: 136.3 g/mol) was applied in concentrations ranging from 0.25 mM to 64 mM in twofold dilutions. *S. aureus* ATCC 29213 was used as an internal control for testing procedures (MIC: 1 mM or 2 mM). Results were read after 24 h incubation at 37 °C without shaking. Staphylococci were classified as susceptible or resistant to zinc chloride according to the tentative threshold values described in literature with MIC values > 2 mM being resistant (Aarestrup and Hasman, 2004).

2.3. DNA isolation and PCR

Genomic DNA was extracted using the QIAamp DNA Mini Kit according to manufactures instructions (Qiagen, Hilden, Germany). For lysis of *S. aureus* cells, lysostaphin [20 µg/ml] (Wakchemie, Steinbach, Germany) was applied for 30 min at 37 °C. Detection of the *czrC* gene was performed by PCR using the following protocol: 5 min at 95 °C, then 35 cycles of 0.5 min at 95 °C, 0.5 min at 63 °C and 2.5 min at 72 °C following a final extension at 72 °C for 7 min. Oligonucleotides for the detection of *czrC* (*czrC*_f: 5′-AGGGTTAGTGAATACGGTTG-3′; *czrC*_r: 5′-CTCTGTCCTGGCATTATCT-3′) were designed on the basis of the genome sequence of *S. aureus* S0385 (EMBL AM990992). SCC*mec* typing was performed using a multiplex PCR according to Kondo et al. (2007).

2.4. Statistical analysis

Two-tailed Fisher's Exact test was used to compare zinc chloride resistance among MRSA CC398 and HA-MRSA over the years. *P*-values < 0.05 were considered significant. Linear regression was performed to analyze the resistance behavior of MRSA CC398 over the time. GraphPad Prism (version 5, GraphPad Software Inc.) was used for statistical analysis.

3. Results

3.1. Spa typing

Based on BURP clustering, nine CC398 associated and 14 HA-MRSA associated *spa* types were included in the study (Table 1). Most MRSA CC398 isolates belonged to the *spa* type t011 (n = 74), followed by t034 (n = 38), t108 (n = 4), t2576 (n = 2), t1793, t3423, t4208, t4652 and t571 (each n = 1). The majority of HA-MRSA included were characterized as *spa* types t032 (n = 63), t003 (t = 46), t022, t045 (each n = 2), t035, t1499, t1770, t294, t3247, t481, t557, t628, t6428 and t756 (each n = 1).

3.2. Zinc resistance

According to Aarestrup and Hasman (2004), staphylococci with MIC values higher than 2 mM were considered as resistant. Overall, in this study, the MIC values of zinc chloride for MRSA CC398 ranged from 1 mM to 4 mM whereas MIC values for HA-MRSA ranged between 0.25 mM and 4 mM. A decreased susceptibility towards zinc chloride with MIC values of > 2 mM was shown in 57% (70/123) of MRSA CC398 and 2% (3/123) of HA-MRSA isolates (Table 1). In the group of MRSA CC398, more than two thirds of the isolates of *spa* type t011 (72%, 53/74), but only one third (34%, 13/38) of *spa* type t034 were resistant. MRSA CC398 isolates of the *spa* types t1793, t3423 and t4652 (each n = 1) and one out of two isolates classified as *spa* type t2576 exhibited a zinc chloride-resistant phenotype. None of the isolates belonging to *spa* type t108 showed MIC values higher than 2 mM (n = 4). All HA-MRSA with decreased zinc chloride susceptibility (2%, 3/123) belonged to *spa* type t003. More than half of the HA-MRSA had MIC values of 1 mM (55%, 68/123).

The presence of the *czrC* gene correlated with the results of the MIC determination since all phenotypic resistant MRSA CC398 were found to carry it (Table 1). In total, 82% (101/123) of the MRSA CC398 harbored the *czrC* gene. Stratified according to the MIC values, 100% of MRSA CC398 with MIC values of 4 mM (70/123) as well as 25% of