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Antimicrobial effect of copper on multidrug-resistant bacteria

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Summary

Copper has been used for centuries as a therapeutic agent in various cultures around the globe. With the emergence and spread of antibiotic resistance, the use of metallic copper alloys to control pathogenic microorganisms is attracting increasing attention. The antimicrobial effects of copper surfaces have been repeatedly demonstrated in both laboratory studies and clinical trials and copper surfaces have been attributed with great potential to limit the transmission and spread of pathogenic microbes. We investigated the antimicrobial effect of copper on three multidrug-resistant bacterial strains: methicillin-resistant *Staphylococcus aureus* sequence type 398, CTX-M-15 producing *Escherichia coli* and NDM-1 producing *Klebsiella pneumoniae*. Copper coupons were inoculated with bacterial cell suspensions and incubated at room temperature. At set time points, bacteria were resuspended and plated onto nutrient agar and colony-forming units were counted. Results show a more than fivefold log-reduction of viable bacteria for CTX-M-15 producing *E. coli* and NDM-1 producing *K. pneumoniae* after 60 min of incubation on metallic copper compared to stainless steel. The same reduction of viable

Zusammenfassung

Antimikrobielle Wirkung von Kupfer auf multiresistente Bakterien

Einleitung

Kupfer fand über Jahrhunderte hinweg medizinische Anwendung, doch mit Einführung der Antibiotikatherapie trat die Erforschung seiner antimikrobiellen Eigenschaften in den Hintergrund. Die in den letzten Jahren zunehmende Resistenzproblematik ließ alternative Methoden zur Infektionskontrolle wieder in das Augenmerk rücken. Dazu zählt auch der Einsatz von Kupferoberflächen in sensiblen Bereichen des Gesundheitssystems. Laborstudien sowie anwendungsorientierte klinische Arbeiten konnten eindrucksvoll die antimikrobielle Aktivität von Kupfer und Kupferlegierungen unter Beweis stellen.

Material und Methoden

Wir untersuchten die antimikrobielle Wirkung von Kupfer auf drei multiresistente Bakterienstämme: Methicillin-resistenter *Staphylococcus aureus* Sequenztyp 398, CTX-M-15 prduzierende *Escherichia coli* und NDM-1 produzierende *Klebsiella pneumoniae*. Kupferplättchen wurden mit den entsprechenden Zellsuspensionen inokuliert und bei Raumtemperatur inkubiert. Nach definierten Zeitpunkten erfolgten eine Resuspendierung der Keime und die Bestimmung der Keimzahl mittels Plattengussmethode.

Ergebnisse

Die Ergebnisse zeigen eine mehr als fünffache log-Reduktion für CTX-M-15 produzierende *E. coli* und NDM-1 produzierende *K. pneumoniae* nach 60 min Inkubation. Eine äquivalente Reduktion an lebensfähigen Keimen wurde für *S. aureus* Sequenztyp 398 nach 120 min beobachtet.

Schlussfolgerung

Unsere Daten ergänzen die bisherige Literatur in Bezug auf antimikrobielle Eigenschaften von Kupfer hinsichtlich multiresistenter Erreger, und lassen den Einsatz von Kupferoberflächen in ausgewählten Bereichen als sinnvoll erscheinen.

Abbreviations: CTX-M = cefotaxime-resistant - Munich; Cfu = Colony forming unit; ESBL = Extended spectrum beta-lactamase; MDRB = Multidrug-resistant bacteria; MRSA ST398 = Methicillin-resistant *Staphylococcus aureus* sequence type 398; NDM-1 = New Delhi metallo-beta-lactamase-1; PBS = Phosphate-buffered-saline; SHV = sulfhydryl variable; TEM = Temoneira; TSB = Tryptic Soy Broth; UTI = Urinary tract infection

bacteria could be demonstrated for methicillin-resistant *S. aureus* sequence type 398 after 120 min of incubation. Our data complement scientific evidence for copper´s antimicrobial properties on multidrug-resistant bacteria and suggest that the use of copper surfaces constitutes an approach to support the control of these organisms.

Introduction

Early records on the medical use of copper date back to the 2nd millennium B.C., when its use to disinfect wounds and drinking water was described in an ancient Egyptian medical text, the so-called Smith Papyrus (BREASTED, 1930). Greeks, Romans, Aztecs and numerous other peoples have empirically used copper or copper compounds for the treatment of ailments such as headaches, burns and skin infections, as well as for general hygiene (DOLLWET and SORENSON, 1985). Later, copper's medical potency was attributed to its antimicrobial properties and its therapeutic use became widespread in the 19th and early 20th century, until the advent of commercially available antimicrobials in the 1930s opened new doors for the treatment of infectious diseases. With the increasing use of antibiotics in both human medicine and agriculture, the spread of antibiotic-resistant bacteria in health care settings, food processing plants and livestock farming became a challenge for public health. The search for alternative approaches to impede the propagation of pathogenic microorganisms was initiated and copper once again received attention as an antimicrobial substance for contact surfaces with the aim of reducing their microbial colonization.

Copper is an essential trace element that is vital to all organisms and crucial for the proper functioning of organs and metabolic processes. Many enzymes depend on copper as a cofactor, with the element serving as an electron donor/acceptor by alternating between the redox states Cu(I) and Cu(II). On the other hand, the redox properties of copper can also cause cellular damage, with the formation of reactive hydroxyl radicals possibly being a pivotal initial step (GRASS et al., 2011).

Regarding the antimicrobial effect of copper surfaces, contact-mediated killing on copper and copper alloy surfaces has repeatedly been demonstrated (ESPIRITO SANTO et al., 2008). The mechanisms underlying the effect are not completely understood; the generation of reactive oxygen species, followed by the oxidation of cellular proteins and lipids, are possible key reactions (GRASS et al., 2011).

The human pathogens most widely tested against copper surfaces include methicillin-resistant *Staphylococcus aureus* (MRSA), *Clostridium difficile*, *Escherichia coli* and *Pseudomonas aeruginosa* (GRASS et al., 2011). These studies have revealed a remarkable reduction of viable bacteria after incubation on metallic copper, suggesting the use of copper surfaces for control of these bacteria in health care environments.

In the last decades, multidrug resistance has emerged in many frequently encountered pathogenic bacteria, sometimes culminating in resistance to any licensed antibacterial agent (TADESSE et al., 2012). Since its first appearance in 1960, MRSA has become widespread in hospitals and intensive care units and, mainly responsible for hospital-acquired infections. However, the epidemiologic situation changed fundamentally with the advent and spread of community-acquired MRSA, appearing in humans with no health-care-associated risk factors and in animals (SPRINGER et al., 2009).

The scenario of the emergence and spread of community-acquired MRSA has recently been paralleled by the emergence of community-onset infections caused by gram-negative bacteria that produce extended-spectrum beta-lactamases (ESBLs). From the late 1990s, multidrug-resistant Enterobacteriaceae, predominantly ESBL-producing *E. coli*, have emerged within the community setting as an important cause of infections of the urinary tract (UTI) and the bloodstream (LAUPLAND et al., 2008).

ESBLs are able to hydrolyze nearly all types of penicillin and cephalosporin and empirical treatment of infections caused by EBSL-producing organisms was shown to be associated with higher morbidity and mortality (TUMBARELLO et al., 2007; ROTTIER et al., 2012). Most ESBLs can be assigned to one of three groups: TEM, SHV and CTX-M. Organisms producing CTX-M enzymes have been increasingly reported as spreading in European countries, often leading to difficulties in antibiotic treatment, as there is frequent co-resistance to co-trimoxazole, tetracycline, gentamicin and ciprofloxacin (LAUPLAND et al., 2008). CTX-M-15-harboring *E. coli* are receiving growing attention as a cause of community-onset UTIs and bloodstream infections (NICOLAS-CHANOINE et al., 2008; PITOUT et al., 2009).

ESBLs have been repeatedly described in bacterial populations circulating in animals (CARATTOLI, 2008; SMET et al., 2009; EWERS et al., 2011). Resistant bacteria of animal origin may therefore be transferred to humans through close contact or by consumption of animal meat.

Another resistance mechanism of increasing clinical relevance is the production of carbapenemases in gram negative bacteria. Many different Enterobacteriaceae have been identified among carbapenem-resistant bacteria, with *Klebsiella pneumoniae* and *E. coli* beeing the most prevalent (CORNAGLIA et al., 2011). Metallo-beta-lactamases show potent carbapenemase activity as well as resistance to clinical beta-lactamase inhibitors, thus conferring resistance to almost all beta-lactam antibiotics. Due to its genetics, the recently described New Delhi metallo-beta-lactamase (NDM-1) is more likely to spread between

various bacterial species than other carbapenem-resistance genes (YONG et al., 2009), and indeed dissemination has occurred, with human infections in countries throughout the globe (ROLAIN et al., 2010). NDM-1 is often associated with further resistance genes, sometimes leaving colistin or tigecycline as the final treatment options (NORDMANN et al., 2011).

To date, only few studies have investigated the antimicrobial effects of copper surfaces on multidrug-resistant bacteria (MDRB).

Material and Methods

Bacterial strains, growth conditions and alloys

The strains used in this work are a methicillin-resistant *S. aureus* sequence type 398, co-resistant to tetracycline (SPRINGER et al., 2009), a *bla*$_{CTX-M-15}$ positive *E. coli*, co-resistant to fluoroquinolones and co-trimoxazole (personal strain collection), and a *bla*$_{NDM-1}$ positive *K. pneumoniae*, showing resistance to all major classes of antibiotics except for tigecycline and colistin (YONG et al., 2009). Bacteria were grown on blood agar and incubated overnight at 37 °C. To prepare the stock solution, overnight cultures were concentrated in a Soybean-Casein Digest (TSB) broth (Oxoid Ltd., Basingstoke, England) comprising 10% glycerol. The bacterial density of the stock solution resulted in approx. $3.4x10^9$ colony forming units (cfu) after plating onto TSB agar. Until use, stock solutions were stored at -20°C. Controls of each stock solution were conducted immediately after thawing to determine cfu counts. Alloy CuZn-21Si3P (CuTouch, Diehl Metall Stiftung & Co. KG, Röthenbach, Germany) was chosen as a representative copper alloy. It contains 76% copper. All CuZn21Si3P samples used for testing had a surface finish made by blasting with glass pellets (by Wilhelm May GmbH, Velbert, Germany). Stainless steel 1.4301 (S30400) was used as reference material. Surface finishing of the stainless steel samples was done by brushing. Prior to testing, all metal coupons were cleaned and sterilized to standardize surface properties. Cleaning was performed manually. Coupons were washed with a detergent containing anionic tensides (Pril® Original, Henkel GmbH, Germany) and rinsed with water. Subsequent sterilization was performed by dry heat autoclaving at 122 °C for 20 min at 31.6 psi pressure. Coupons were stored in sterile bags until use.

Assay to determine survival on metal surface

For determination of bacterial survival, 100 µl of cell suspension from the stock solution were applied to the different test coupons made of copper alloy or stainless steel. In order to ensure equal distribution of the liquid, cell suspensions were plated evenly on the coupons using sterile bacterial spatulas. Coupons were

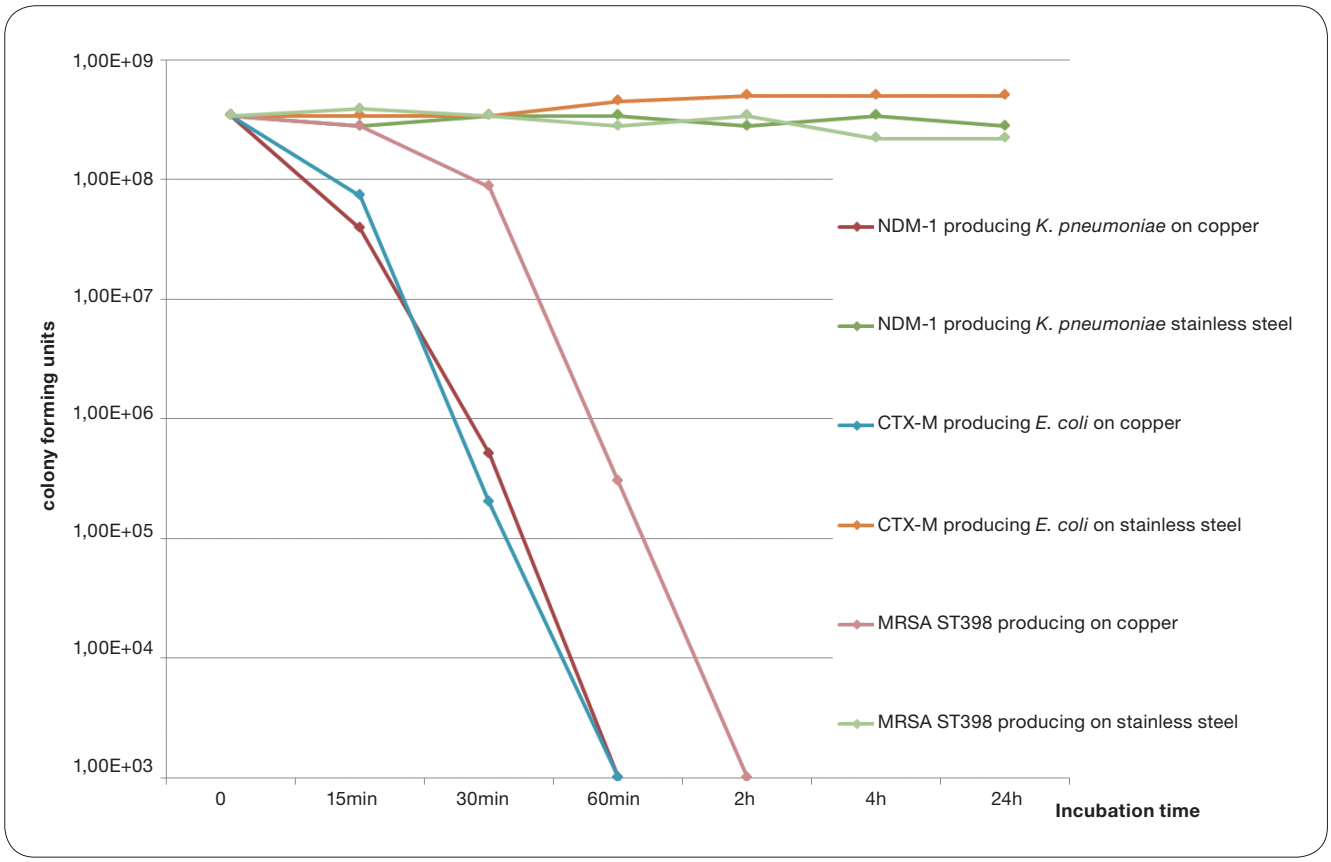

Fig. 1: Survival of different multidrug-resistant bacteria on copper surfaces and stainless steel

incubated in petri dishes at 22 °C. Incubation times were set at 15, 30, 60, 120 and 240 min and 24 h. Coupons were subsequently removed and placed in 100 ml phosphate-buffered saline (PBS) with approx. 40 glass beads (VWR International, Pennsylvania, USA). Upon shaking vigorously on a horizontal shaker (Model KS 15 B control, Edmund Bühler GmbH, Hechingen, Germany) for 5 min, samples were serially diluted in PBS to 10^{-6} and 1 ml was plated onto Soybean-Casein Digest agar (Oxoid Ltd., Basingstoke, England) by the pour plate method. Cfu were counted using a darkfield colony counting chamber (Model Buffalo 15, American Optical Company, New York, USA). Experiments were repeated in duplicate. Control coupons for copper alloy and stainless steel were removed immediately after inoculation at time zero to determine the initial number of bacteria. The lower detection limit was 10^3 cfu.

Results

The antimicrobial effect of copper on the MRSA ST398 isolate reveals a more than fivefold log reduction within 120 min of incubation at room temperature (Fig.1). After 30 min of incubation, approx. $9x10^7$ cfu could be recovered and approx. $3x10^5$ cfu after 60 min. Copper's effect on CTX-M-15 producing *E. coli* was stronger, showing a more than fivefold log reduction within 60 min of incubation. After 30 min of incubation, bacterial killing on copper coupons resulted in a threefold log reduction of viable CTX-M-15 producing *E. coli*. Similarly, our tests with NDM-1 producing *K. pneumoniae* showed a more than fivefold log reduction after 60 min and a threefold log reduction after 30 min of incubation (Fig.1). No reduction in numbers of viable bacteria could be seen on stainless steel for any of the three strains. Subsequent incubation on copper (120 min, 240 min, and 24 h) resulted in cfu counts lower than 10^3.

Discussion

Infections due to MRSA can pose a serious challenge to antibiotic treatment, especially in strains carrying further resistance traits. In the last years, a new MRSA lineage has attracted increasing attention: MRSA sequence type (ST) 398 was shown to colonize farm animals with high prevalence and it was claimed that animals harboring this strain represent a potential source for the transmission of MRSA to humans (SPRINGER et al., 2009). Pig farmers and veterinary personnel were found to be at increased risk of MRSA ST398 colonization, with a prevalence of 8% nasal carriage, notably higher than the prevalence in the general population (WITTE et al., 2007; SPRINGER et al., 2009). The use of copper and copper alloy surfaces in livestock farming to control MDRB has not yet been considered, nor has the antimicrobial activity of copper against MRSA ST398 been tested. Our findings reveal a fivefold log reduction within 120 min of incubation at room temperature. NOYCE et al. investigated copper´s antimicrobial effect on different strains of MRSA and reported complete killing of a 10^7 cfu inoculum of MRSA (NTCT 10442) on copper within 45 min of incubation at 22 °C (NOYCE et al., 2006). However, metallic copper coupons were composed of 100% copper (C19700) versus 76% copper-containing metal used in the present study, which may have contributed to the different killing kinetics.

Copper's toxicity to ESBL-producing organisms has not yet been studied. Our experiments on the antimicrobial effect of copper surfaces on CTX-M-15 producing *E. coli* show a more than fivefold log reduction within 60 min of incubation at room temperature. In contrast, wild-type strain W3110 exposed to a 99% copper-containing alloy (C11000) was shown to be killed after only 1 min at 23 °C (ESPIRITO SANTO et al., 2008). The notable difference in the killing kinetics is likely to be due to the different copper contents of the alloys used, as well as to diverging assay protocols. In ESPIRITO SANTO's work, cells were first applied to a sterile cotton swab and subsequently spread across the metal coupons, instead of being applied in liquid solution.

We provide the first evidence on the effect of copper coupons on NDM-1 producing *K. pneumoniae*, revealing a more than fivefold log reduction after 60 min of incubation at room temperature (Fig.1). We presume that antibacterial copper surfaces in healthcare facilities can complement standard hygiene precautions and constitute an approach to support the control of healthcare-associated infections. MEHTAR et al. (2008) also reported complete killing of a 10^7 cfu inoculum of *K. pneumoniae* on 99.9% copper-containing metal coupons within 60 min of incubation at room temperature. Once again, the higher copper content of the test alloys used may have accounted for the faster killing. Future studies are needed to define the correlation between copper content and bacterial killing more precisely and to identify the best alloys for use in clinical settings. Regarding the hospital environment, doorknobs and other surfaces in close contact with patients are important sources of nosocomial infections because of their frequent contamination by opportunistic pathogens (WENGER et al., 1997). Stainless steel is the metal predominantly used in the healthcare environment. However, pathogens remain viable on stainless steel surfaces (KUSUMANINGRUM et al., 2002), questioning the use of this metal from a hygienic point of view. In addition to standard hygiene procedures, copper as a self-sanitizing material on surfaces could possibly help to decrease the spread of MDRB in both hospital and veterinary settings. Recent hospital trials have shown that copper has the potential to reduce the microbial contamination of surfaces (CASEY et al., 2010; MIKOLAY et al., 2010; SCHMIDT et al., 2012).

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