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Study of cross-resistance mediated by antibiotics, chlorhexidine and *Rhizoma coptidis* in *Staphylococcus aureus*

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ABSTRACT

This study aimed to determine whether selection by antibiotics, chlorhexidine (CHX) and *Rhizoma coptidis* extract (RCE) would lead to cross-resistance or decreased susceptibility in *Staphylococcus aureus*. The *S. aureus* reference strain ATCC 25923 as well as 14 clinical isolates were exposed to antibiotics, CHX and RCE at sublethal doses for up to 14 days. Changes in susceptibility were determined by analysis of minimum inhibitory concentrations (MICs). All isolates were cross-resistant to more than one other antibiotic following tetracycline exposure, and increased resistance (≥4-fold MIC increase) to RCE and CHX was observed in six and three isolates, respectively. Following selection by CHX, most of the treated strains showed no significant change in sensitivity to CHX. However, all strains developed cross-resistance to at least one antibiotic, and decreased susceptibility (≥4-fold MIC increase) to RCE appeared in seven strains. Following exposure to RCE, 11 isolates showed cross-resistance to at least one antibiotic. In addition, three RCE-exposed strains showed reduced susceptibility to CHX (4- or 8-fold MIC increase). The results obtained in this study imply that antibiotics, biocides and antimicrobial Chinese herbs might employ some of the same mechanisms of action against bacteria, triggering mutual cross-resistance to further foster the development of bacterial resistance.

1. Introduction

Antibacterial agents have been used by humans for decades. They are widely used to eliminate pathogens directly or to prevent their growth in order to protect public health. Nevertheless, bacteria have generated some mechanisms to avoid the danger of being killed [1–5]. Acquired bacterial resistance is defined as when bacteria become resistant to antibacterial agents to which they were originally sensitive. When bacteria acquire resistance to certain kinds of drugs, they may also develop new resistance to other drugs with similar mechanisms of action or similar structures [6,7]. Drug-resistant bacteria have seen widespread and swift growth, resulting in bacterial infectious diseases outbreaks in some areas. The rate of bacterial resistance amongst pathogens is on the rise globally, bringing about increased mortality, longer hospitalisation and huge economic losses [8,9]. Since the emergence of methicillin-resistant *Staphylococcus aureus* (MRSA), multidrug-resistant *Escherichia coli* in Europe and the NDM-1 superbacteria, the development of antibacterial resistance has entered a new stage, potentially accelerating us into the 'post-antibiotic era' [10].

Antibiotics and biocides, e.g. chlorhexidine diacetate (CHX), are vitally important antibacterial agents. Antibiotics are used widely as chemotherapeutic drugs to prevent and treat bacterial infections. Biocides are used as antiseptics, disinfectants or preservatives for eliminating microbes in media. It was pointed out that antibiotics and biocides might have some similarities in their mechanisms of action [11–13]. Cross-resistance between antibiotics and biocides has been reported in some laboratory studies. As is known to us, when bacteria become insensitive to certain kind of antibiotics, this may trigger cross-resistance to other kinds of antibiotics, or reduced susceptibility to biocides at low concentration. Similarly, the insensitivity of bacteria to biocides at low concentration may also lead to cross-resistance to antibiotics [14,15].

Antimicrobial Chinese herbs (ACHs) have been widely used in China and other Asian countries for treating infectious diseases for more than 4000 years [16]. Their chemical components are complicated, and even in the extracts there are many antibacterial components attacking different target sites on the bacterial cell [17–19]. Therefore, ACHs are usually considered unable to select for

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drug resistance when used for antibacterial action and thus were supposed as a new way to solve the problem of bacterial resistance [20,21]. *Rhizoma coptidis*, a typical ACH, has attracted great attention from academics because of its outstanding antibacterial activity [22,23]. It is very complicated to completely verify all antibacterial components in *Rhizoma coptidis*, although some have been separated and identified, such as berberine, coptisine and palmatine [24–26].

Bacterial resistance was considered as being driven by the inappropriate use of antibacterial agents for human and animal health as well as food production, especially the long-term abuse of antibiotics [11,27,28]. The relationship between biocide use and clinical antibiotic resistance has been revealed previously at the laboratory level [14,29,30]. The aim of this work was to examine whether concentrations of antibiotics, biocides and ACHs below the minimum inhibitory concentration (sub-MIC) could lead to mutual cross-resistance or decreased susceptibility in bacteria. In this study, 15 strains of *S. aureus*, including 14 clinical isolates (12 resistant and 2 sensitive isolates) and the reference strain *S. aureus* ATCC 25923, were cultured continuously in sub-MIC concentrations of ciprofloxacin (CIP), gentamicin (GEN), tetracycline (TET), CHX and *Rhizoma coptidis* extract (RCE) for 14 days. Potential cross-resistance was assayed by testing changes in susceptibility.

2. Materials and methods

2.1. Chemicals and strains

Amongst the six tested antibiotics, amikacin (AMK), cefepime (FEP) and meropenem (MEM) were purchased from Shanghai Aladdin Biochemical Polytron Technologies Inc. (Shanghai, China) and TET, CIP and GEN were commercially provided by Tianjin Silan Technology Co., Ltd. (Tianjin, China). CHX was commercially provided by China Pharmaceutical Biological Products Analysis Institute (Beijing, China). *Rhizoma coptidis* was purchased from the First Affiliated Hospital of Guangxi University of Chinese Medicine (Nanning, Guangxi, China) and was identified according to standard Chinese herbal identification procedures [31].

S. aureus ATCC 25923 was obtained from the National Institutes for Food and Drug Control (Beijing, China). Fourteen clinical isolates of *S. aureus* were kindly supplied and identified by the First Affiliated Hospital of Guangxi University of Chinese Medicine.

2.2. Preparation of Rhizoma coptidis extract (RCE)

RCE was prepared with a standard procedure by aqueous extraction [32,33] and was concentrated to 1.0 g/mL, which corresponds to a dose of 1.0 g crude herb per millilitre.

2.3. Minimum inhibitory concentration (MIC) assays

The MICs of the 15 strains to various drugs were determined by geometric microdilution in Mueller–Hinton broth according to Clinical and Laboratory Standards Institute (CLSI) recommendations [34]. Susceptibility categorisation was assessed according to current susceptibility and resistance breakpoints of the European Committee on Antibiotic Susceptibility Testing (EUCAST) [35].

2.4. Exposure of strains to drugs at sub-MICs

This experiment was performed by a previously reported method with slight modifications [13]. Bacteria were grown in the continuous presence of drug concentrations corresponding to 0.5× MIC. The change in MIC of the bacteria to drugs was examined daily, followed by a corresponding increase in drug concentration for up to 14 days. Samples were withdrawn directly and were stored in 20% glycerol at −80 °C for further analysis [16].

3. Results and discussion

3.1. Tetracycline (TET) selection

In this experiment, strains exposed to sub-MICs of TET showed enhanced resistance to TET, with a ≥4-fold MIC increase, except for one strain (SA czx) that showed decreased susceptibility with only a 2-fold MIC increase (Table 1). In addition, *S. aureus* ATCC 25923 showed cross-resistance to CIP and FEP. Correspondingly, 11 TET-exposed isolates became cross-resistant or showed further reduced susceptibility to another 5 antibiotics, and the remaining 3 isolates (SA czx, SA lqq and SA zxr) turned out to be cross-resistant to another two or three antibiotics, respectively. These results related to cross-resistance to antibiotics were similar to previous studies [36,37].

Noteworthy, decreased susceptibility to CHX and RCE at a low level was also observed in some of the strains. Three isolates (SA cp, SA hzj and SA xzl) exhibited less sensitivity to CHX (4-fold MIC increase) and six strains (ATCC 25923, SA czx, SA hzj, SA lqq, SA xzl and SA dgx) showed decreased susceptibility to RCE (4- to 8-fold MIC increase). The results were also similar to those for CIP and GEN exposure (data not shown), and they have not been reported previously.

Although the experiments were performed at the laboratory level and they are hard to repeated in parallel in the natural environment, the obtained results implied that abuse of antibiotics, causing sublethal concentrations for bacteria in the micro-environment, could not only result in decreased susceptibility or cross-resistance to antibiotics [11,38], but also in lower sensitivity to biocides and ACHs.

3.2. Chlorhexidine (CHX) selection

Following culture in CHX at sub-MIC doses for 14 days continuously, most of the strains showed no obvious change in susceptibility to CHX (<4-fold MIC increase) except for six isolates (SA hzj, SA zxr, SA mch, SA zsl, SA lzy and SA xzl) that increased their MICs by 4 or 8 times (Table 2).

However, cross-resistance at least one antibiotic was observed in all strains following CHX exposure. *S. aureus* ATCC 25923 exhibited cross-resistance to TET and FEP. Correspondingly, all isolates became cross-resistant or showed increased resistance to TET. Remarkably, a >512-fold increase in MIC to AMK, TET and GEN was found in one isolate (SA ljb). No obvious change in susceptibility to CIP, GEN, AMK, FEP and MEM was observed only in four, five, two, three and five isolates, respectively (Table 2). In addition, seven strains (ATCC 25923, SA zdw, SA cp, SA czx, SA hzj, SA lqq and SA zsl) became less susceptible to RCE (≥4-fold MIC increase).

It is possible that biocides used widely for household work are ultimately discharged to the living surroundings creating a suitable diluted concentration for bacteria, resulting in cross-resistance to antibiotics and decreased susceptibility to ACHs. Based on the presented data, it is rationally presumed that biocides are not only factors causing antibiotic resistance, but are also responsible for decreased susceptibility to ACHs.

3.3. Rhizoma coptidis extract (RCE) selection

The active components of *Rhizoma coptidis* are quite complicated [25]. Alkaloids as an important group of active components have been determined partially, including berberine etc. [24]. Berberine exhibited outstanding antibacterial activity with an MIC