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# Effectiveness of 5-Pyrrolidone-2-carboxylic Acid and Copper Sulfate Pentahydrate Association against Drug Resistant *Staphylococcus* Strains

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Bacterial resistance is an ongoing challenge for pharmacotherapy and pharmaceutical chemistry. *Staphylococcus aureus* is the bacterial species which makes it most difficult to treat skin and soft tissue infections and it is seen in thousands of hospitalization cases each year. Severe but often underrated infectious diseases, such as complicated nasal infections, are primarily caused by MRSA and *S. epidermidis* too. With the aim of studying new drugs with antimicrobial activity and effectiveness on drug resistant *Staphylococcus* strains, our attention in this study was drawn on the activity of a new association between two natural products: 5-pyrrolidone-2-carboxylic acid (PCA), naturally produced by certain *Lactobacillus* species, and copper sulfate pentahydrate (CS). The antimicrobial susceptibility test was conducted taking into account 12 different *Staphylococcus* strains, comprising 6 clinical isolates and 6 resistant strains. PCA 4%, w/w, and CS 0.002%, w/w, association in distilled water solution was found to have bactericidal activity against all tested strains. Antimicrobial kinetics highlighted that PCA 4%, w/w, and CS 0.002% association could reduce by 5 $\log_{10}$ viable bacterial counts of MRSA and oxacillin resistant *S. epidermidis* in less than 5 and 3 minutes respectively. Microscopic investigations suggest a cell wall targeting mechanism of action. Being very safe and highly tolerated, the natural product PCA and CS association proved to be a promising antimicrobial agent to treat *Staphylococcus* related infections.

**Keywords:** 5-Pyrrolidone-2-carboxylic acid, Copper sulfate pentahydrate, *Staphylococcus aureus*, MRSA, *Staphylococcus epidermidis*, Cell damage, Nasal infections, EN 12054.

Bacterial resistance to antimicrobial agents is constantly on the increase. It has been estimated that the annual economic cost of resistant infections exceeds $20 billion in the United States and 1.6€ billion in the European Union, causing more than 10 million additional hospital days [1].

*Staphylococcus aureus* (SA) is the bacterial species that causes most difficulties in treating infections, and it is the pathogen involved in 150,000 hospitalization cases per year in Europe [2]. This is because, due to abuse and misuse of currently available antibiotics over recent years, SA resistant strains emerged and, thus, SA has become the predominant pathogenic bacterial species in health care facilities [3]. Methicillin was used to treat resistant SA, but quickly methicillin resistant SA strains (MRSA) emerged and, currently, they are one of the hardest challenges among infectious diseases [4]. The SA ideal habitat is skin surface which means that hand by hand transmission is very common [5]. SA also easily colonizes hard to reach soft tissues such as the nose narix and, thus, SA and MRSA are the infectious agents that primarily cause severe, but often underrated infectious diseases, such as complicated nasal infections [6]. The nasal microbiome also comprises other species of *Staphylococcus*, particularly *S. epidermidis* and *Streptococcus* species [7]. Conventional treatment of nasal infections to date consists in spray or nasal drops containing antibiotics such as tobramycin [8], whereas amoxicillin is regularly administered orally, even if resistance to these classes of antibiotics is growing fast [9].

The research of new antimicrobial drugs for resistant bacterial strains is today pivotal, but it is worthy of note that large companies are not very interested in studying new products, since they would only be typically used for a few days and they should be cost effective for patients and, especially for national sanitary systems [10].

Bearing this in mind, our research group has been studying a new, innovative, natural, well tolerated and effective antimicrobial agent over the last years. In previous studies, 5-pyrrolidone-2-carboxylic acid (PCA) and copper sulfate (CS) association turned out to be an excellent candidate for this purpose, by proving very good antibiotic activity on many bacteria species, including different SA strains [11].

PCA is naturally present in many fruits and vegetables, in fermented soybean and in cereals [12]; PCA is also produced by several *Lactobacillus* species as an antimicrobial agent to eliminate other bacterial species and to guarantee their survival [13]. CS, on the other hand, is a well-known natural compound with antimicrobial and antifungal activity, used in agriculture and health care [14].

Our previous works revealed the antimicrobial inhibitory effectiveness of the association PCA + CS (40000 mg/L + 20 mg/L) against Gram-positive and Gram-negative species and yeasts, with the association being synergistic and thus more effective than PCA and CS alone [11].

In this study we have been focusing our attention on anti-*Staphylococcus* activity of the association PCA + CS. We tested PCA + CS activity (40000 mg/L + 20 mg/L) against six *Staphylococcus* strains of the Goteborg University bacterial collection (CCUG): one SA, two MRSA strains, two *S. epidermidis* (SE) strains and one oxacillin resistant SE strain (ORSE) and 6 *Staphylococcus* clinical isolates: two SA isolated, one SE, two

MRSA and one ORSE. MIC and MBC according to EUCAST recommendations were evaluated [15]. The PCA 40000 mg/L + CS 20 mg/L association was found to be inhibitory and bactericidal against all tested strains. This study confirmed that the association of PCA + CS is equally effective on SA, SE, MRSA and ORSE, CCUG and clinical isolates, overcoming the pivotal issue of microbial resistance (see table1).

**Table 1:** PCA + CS bactericidal effectiveness at 40000 mg/L + 20 mg/L for different strains of *Staphylococcus* spp.

| Strain | bactericidal activity |
|---|---|
| **SA** | + |
| **SE** | + |
| **SE** | + |
| **MRSA** | + |
| **MRSA** | + |
| **ORSE** | + |
| **SA*** | + |
| **SA*** | + |
| **SE*** | + |
| **MRSA*** | + |
| **MRSA*** | + |
| **ORSE*** | + |

*Clinical isolates.

The killing time kinetics were then determined for IC MRSA and IC ORSE, as they were the most interesting strains among the *Staphylococcus* tested strains: MRSA colonies were reduced by $3\log_{10}$ after 3 minutes, and by $5\log_{10}$ after 5 minutes; ORSE colonies were reduced by $3\log_{10}$ after 2 minutes and by $5\log_{10}$ after 3 minutes.

The PCA + CS association exhibited a very fast killing action against *Staphylococcus* strains and results highlighted that the association is suitable to reduce MRSA and ORSE viable counts in practical conditions.

Thanks to the interesting results obtained, the antimicrobial mechanism of action was finally evaluated by observing under digital microscope MRSA and ORSE treated with PCA + CS for 1 and 5 minutes. Experiments were repeated using sterile distilled water as a negative control. Figure 1 shows the differences between control and treated colonies of MRSA and ORSE: after 1 minute morphological changes in microbial colonies are evident, in particular for MRSA, suggesting that cell walls and membranes suffered from structural alterations. The observation was repeated 5 minutes after exposure to the treatment (see Figure 2): colony damage is evident and the MRSA colony organization was completely disassembled.

The PCA + CS association proved to be a strong antimicrobial agent, particularly against MRSA and ORSE, and we suggest a cell wall targeting mechanism of action. Thus, PCA + CS association could be considered for clinical application.

One can see that other than its antimicrobial effectiveness, the PCA + CS association shows interesting additional characteristics that make this combination of active ingredients suitable to treat soft tissue infections caused by resistant *Staphylococcus* strains, particularly nose infections. In fact PCA + CS is water soluble, colorless and odorless, very safe and with a moisturizing effect [16]. Moreover, the PCA + CS association was found to have a peculiar persistent antimicrobial effect [17]. Due to these positive features, the PCA + CS association could conveniently be used for spray and nasal drop formulation. Finally, an observational clinical investigation was recently conducted on fifty adult patients affected by cold dry air rhinitis [18]. Treatment with two nasal drops per

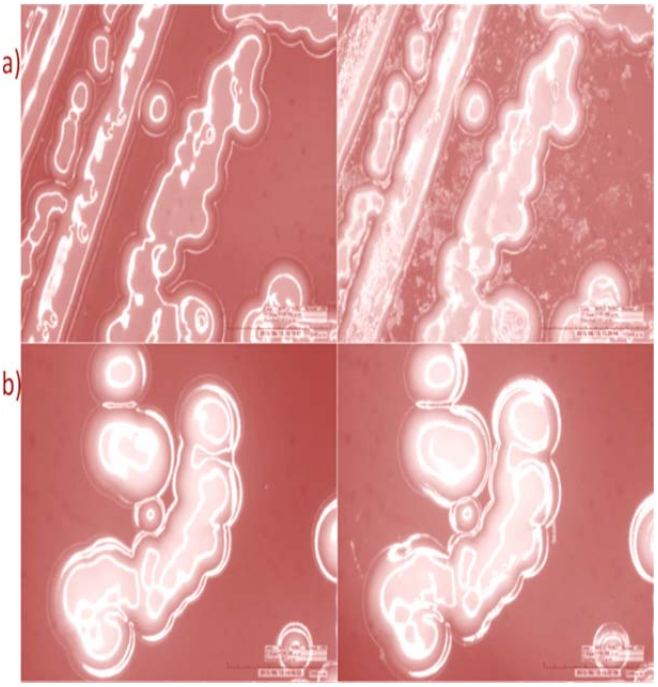


**Figure 1:** Microscope photos of MRSA (a), ORSE (b) colonies before (left) and after (right) 1 minute of PCA + CS exposure.

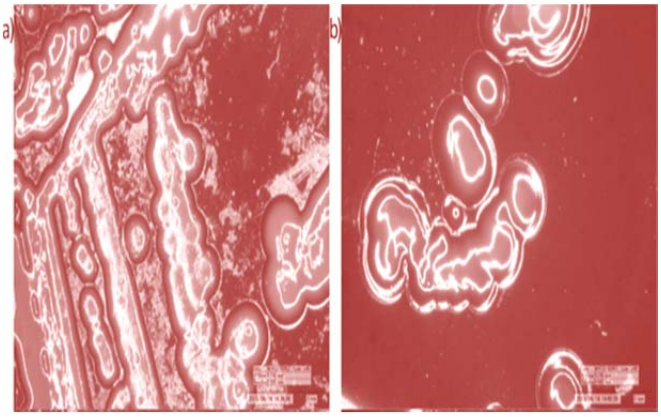


**Figure 2:** Microscope photos of MRSA (a), ORSE (b) colonies after 5 minutes of PCA + CS exposure.

nostril, three times a day for 7 days, showed that the PCA + CS association again at 40000 mg/L + 20 mg/L also demonstrated an effective virucidal activity against influenza and parainfluenza viruses and other common nasal pathogens, being at the same time a safe treatment for cold dry air rhinitis. The latter aspect is a key factor that highlights this product also as a treatment for common rhinitis considering that virus infections cause over 80% of overall rhinitis [19].

## Experimental

***Materials:*** PCA was kindly provided by Laboratori Baldacci, Pisa, Italy; CS was purchased from Carlo Erba, Milano, Italy. SA (CCUG 19207), MRSA (CCUG 41586 and CCUG 41879), SE (CCUG 39508 and CCUG 57787), ORSE (CCUG 35257) were furnished by the University of Goteborg (CCUG). Clinical isolates were obtained from Siena Hospital "Campostaggia" and collected and tested for drug resistance by Dr. Gianna De Fina and by Prof. Natale Figura, University of Siena. Brain Heart Infusion Medium (BHI), agar plates, 5% horse blood agar plates, multiwell plates and technical

material for microbiology were purchased from Biomerieux, Florence, Italy. Polysorbate Tween 20, soy lecithin, histidine and cysteine were purchased from Sigma-Aldrich, Milan, Italy. Sterile distilled water was used. Microscopy was performed using a digital 3D Hirox KH 7700 instrument.

***Methods:*** The antimicrobial susceptibility test was conducted using the suspension method on multiwell plates [20]. A water solution of PCA and CS (400000 mg/L + 200 mg/L) was freshly prepared and diluted tenfold in BHI broth (100 µL final volume in the wells). Five µL of different bacterial suspension containing 5 x $10^6$ CFU ca. were added to each well. All experiments were performed in triplicate. Plates were incubated at 37°C. After 24 h of incubation, plates were inspected for bacterial growth and, according to EUCAST recommendations [20], inhibitory activity of studied samples was stated when no bacterial growth was observed in the wells, compared with positive controls. Five µL from each well was transferred onto 5% horse blood agar plates, which were then incubated at 37°C for 24 h. Plates were then inspected for bacterial growth and bactericidal activity of PCA + CS association was recorded when subculture on agar yielded no colony development [20]. Killing study was conducted according to the method reported on prEN 12054 [21]. A bacterial suspension (1 x $10^7$ CFU/mL ca) was added to the sample solution in BHI and at 0 time and after 30 sec, 1, 2, 3, 5, 10, 30 and 60 min of contact times at room temperature, aliquots of the mixture were neutralized using a solution of Polysorbate Tween 20 (3%, w/w), soy lecithin (3%, w/w), histidine (0.1%, w/w) and cysteine (0.1%, w/w) and transferred to agar plates diluted 1, 10, 100 and 1000 fold in BHI. After 48 h of incubation at 37°C, surviving colonies were counted. The reduction of bacteria was calculated as the difference in viable counts before and after the application time and 3 and 5 $\log_{10}$ reduction time was calculated. Microscopy investigations were conducted directly on 5% horse blood agar plates where fresh bacterial strains were cultured. Ten µL of PCA + CS association (40000 mg/L + 20 mg/L) was poured on plates and colonies were observed for 10 min.

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