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# Potential Use of Chlorpheniramine as a Curing Agent for Multi-Drug Resistant *Staphylococcus aureus*

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## ABSTRACT

The study was aimed to investigate the potential of chlorpheniramine as a curing agent in comparison to a standard curing agent, acridine orange, for multi-drug resistant *Staphylococcus aureus* (MDRSA). Urine samples from 50 patients were screened for *Staphyloccocus aureus.* Seventeen (17) different strains were isolated and labelled $S_1$-$S_{17}$. The susceptibility patterns of the 17 isolates were determined in order to further isolate MDRSA and as determine a baseline pattern. The intrinsic antibacterial activity of chlorpheniramine was also determined. The plasmid in the MDRSA were then removed or inhibited by curing with acridine orange or chlorpheniramine. The susceptibility pattern of the MDRSA isolates were then determined after curing. Ten (10) out the 17 *S. aureus* isolates were MDRSA. Chlorpheniramine showed no appreciable antistaphylococcal activity when used alone. Chlorpheniramine and acridine orange were able to inhibit the replication of resistant markers for gentamicin, cotrimoxazole and nitrofurantoin resistance, with reversal of resistance to gentamicin and cotrimoxazole and increased susceptibility to nitrofurantoin. In addition, chlopheniramine was able to reverse resistance in 3 of the 10 MDRSA strains. In each case, better results were obtained with chlorpheniramine than with acridine orange. In conclusion, chlorpheniramine showed promise as an alternative curing agent for MDRSA. Also, since it is an already approved drug it may be beneficial in combination therapy with an antibiotic that has resistance problems.

**KEYWORDS**: *Chlorpheniramine, antibiotics, MDRSA, Plasmid curing, Increased susceptibility*

## INTRODUCTION

*Staphylococcus aureus* is one of the medically and pharmaceutically important bacteria. Due to its membership of the normal body flora, it is major contaminant of pharmaceutical raw materials and drugs, and causes various opportunistic community and healthcare-associated infections [1]. It also causes food poisoning with resistant *S. aureus* being detected in locally produced drinks [2]. *S. aureus* is among the most prevalent causes of clinical infections globally and with much public interest due to increasing mortality associated with its multidrug resistance profile [3]. *S. aureus* has developed resistance to most classes of antibiotics [4].

When an organism acquires resistance to at least one agent in three or more antimicrobial categories that organism is said to be multi-drug resistant (MDR) [5,

6]. Methicillin-resistant *S. aureus* (MRSA) is the most common MDR organism causing healthcare-associated infections (HAIs). MRSA can cause a range of infections from mild infections like skin infections to severe infections of the blood, lungs and surgical sites [4, 5].

Drug resistance by microorganisms is a growing global health challenge. Even the newer and very potent antibiotics are not spared from the emergence of resistant strains of organisms [7]. Antibiotic resistance is mostly due to the transfer of resistant plasmids from one bacterium to the other either through transformation or conjugation. Majority of the clinically significant antibiotic resistance is generally mediated through conjugation [8]. Conjugation involves cell to cell contact, whence a sex pilli are coded for by the donor cell, which it uses to attach itself to the recipient cell. The sex pilli also function as tube through which the plasmids are passed on. Curing agents or treatment have the ability to remove the plasmid in a population of microorganism by inhibiting plasmid replication, directly or indirectly, without affecting cell reproduction, or selectively affecting the growth of the plasmid bearing segregants of the population, whilst allowing the plasmidless ones to become predominant. Some other mechanisms of curing have been suggested. Examples of curing agents include; mutagens, UV and ionizing radiation, thymine starvation, antibiotics, growth above optimal temperature. Acridine orange and sodium dodecyl sulphate are examples of mutagens that can be used. [9].

If resistant plasmids can be removed, there will be reversal of drug resistance or increased susceptibility of the multi-drug resistant organisms to the different antibiotics. Therefore, this study provides an opportunity to forestall the coming of the post-antibiotic era by investigating the ability of an already approved drug, chlorpheniramine, to reverse bacterial drug resistance. Chlorpheniramine, an antihistaminic agent [10], is not reported to be a classical antibiotic.

## MATERIALS AND METHODS

### *Staphylococcus aureus* isolates

The work was done according to Ethical Standards of the Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University-Awka, Nigeria. Urine samples were gotten from fifty (50) patients selected at random at the Bishop Shanahan hospital, Nsukka, Enugu State, Nigeria, from 2nd to 9th of May, 2014, with the assistance of the Medical Laboratory personnel of the Hospital after obtaining the consent of the Management and also the patients. Seventeen (17) *Staphylococcus aureus* isolates were obtained from the urine samples by culturing in mannitol salt agar plates and then subculturing the positive plates onto ordinary nutrient agar. The identities of the isolates were confirmed by Gram staining, Catalase and Coagulase tests [11, 12]. Each characterized isolate was transferred aseptically to nutrient agar slants. The agar slants were incubated at 37$^{o}$C for 24 hours after which they were stored in the refrigerator at a temperature of 2-8$^{o}$C. All characterized isolates were standardized to McFarland's 0.5 turbidity standard in Muller Hinton broth prior to carrying out any microbiological assay.

### Culture media

Culture media used in this study include; Nutrient agar, Mannitol salt agar, Mueller Hinton agar, Muller Hinton broth and Nutrient Broth (all from Oxoid, UK) and they were all prepared according to the manufacturer's instructions.

### Antibiotic discs and drug

The following antibiotic discs were used: chloramphenical (Clf, 30 µg), gentamycin (GN, 10 µg), nitrofurantoin (Nft, 200 µg), ceftriaxone (Ctx, 30 µg), co-trimoxazole (Cotr, 25 µg) and tetracycline (Tcn, 25 µg).The antibiotic discs were manufactured by Jireh Laboratories, Nigeria. Chlopheniramine sterile powder used in this study was a generous gift by Juhel Nigeria Limited, Anambra State, Nigeria, and it complied with BP standards.

Different concentrations of chlorpheniramine discs were prepared aseptically by cutting Whatman filter paper No. 1 into 7 mm discs. The discs were sterilized in a hot air oven (Genlab, UK) at 170° C for 1 hr and 100 µL of chlorpheniramine solution with the following concentrations: 3 mg/ml, 1.5 mg/ml, 0.75 mg/ml, 0.375 mg/ml, and 0.1875 mg/ml, were introduced into the discs to give total quantities of 300 µg, 150 µg, 75 µg, 37.5 µg, and 18.8 µg, respectively. The discs were then allowed to dry in Petri dishes that were later sealed with foil and stored at 5 ± 2° C until use.

### Reagents

Reagents used in this study are: Gentian violet, iodine, alcohol, safranin, oil-immersion, hydrogen peroxide ($H_2O_2$), acridine orange (Sigma-Aldrich, Germany).

### Antibiogram of the isolated organisms

This was done by the disk diffusion method [13]. The Petri dishes were divided into four sections, two of which were labelled according to the isolates (2 plates for each isolate). Aliquot of 15 ml of sterile Mueller-Hinton agar was poured into the plates and allowed to solidify. A sterile swab stick was used to streak the surface of the agar in the plates with the suspension of the isolates. The agar plates were allowed to stand for seven minutes ajar near the Bunsen flame and then a pair of sterile forceps was used to place the separated discs into each of the sections of the plates, one section for one antibiotic disc, and the middle. This process was repeated for all the isolates. All the plates were closed and allowed to pre-diffuse for about 15 minutes. Thereafter the plates were incubated at 37 $^{o}$C for 18 hours and the resultant inhibition zone diameters were measured and recorded.

### Test for Sensitivity of the isolated MDRSA to chlorpheniramine

The *S. aureus* isolates that were determined to be MDRSA were selected and used for this study. The susceptibility pattern of MDRSA to chlorpheniramine was determined by the Kirby Bauer disk diffusion method [13] Briefly, Petri dishes was divided into four sections. An aliquot of 15 ml of sterile Mueller-Hinton agar was poured into the plates and allowed to solidify. A sterile swab stick was used to streak the surface of the agar in the plates with the suspension of the isolates. The agar plates were allowed to stand for seven minutes ajar near the Bunsen flame and then a pair of sterile forceps was used to place the chlorpheniramine discs into each of the sections of the plates, one section for one disc, and then the middle. The experiment was done in duplicate. This whole process was repeated for all the MDRSA isolates. All the plates were allowed to pre-diffuse closed for about 15 minutes. Thereafter the plates were incubated at 37 °C for 18 h and the resultant inhibition zone diameters were measured and recorded.

### Plasmid Curing

This was done using acridine orange according to the protocol written by Esimone and Gugu, 2010 [14]. The MDRSA isolates were grown in Mueller Hinton broth and diluted in Double Strength Mueller Hinton broth. Twenty test tubes of diluted MDRSA isolate was made, two for each isolate for replications. A predetermined quantity of acridine orange solution in sterile distilled water was added to the test tube to give a final concentration of 0.25 mg/ml; and the Muller Hinton broth became single strength. The experiment was repeated using the same concentration of chlopheniramine. All the test tubes were incubated at 37 °C for 24 h. Following the incubation, the test tube containing the highest concentration of the curing agent and with visible growth of microorganism was plated on a nutrient agar plates. The plates were incubated for 24 h at 37$^{o}$C. The sensitivity of the recovered isolates in the plates were then determined using agar diffusion method.

## RESULTS AND DISCUSION

### Antibiogram of *S. aureus* isolates

Six different classes of antibiotics (chloramphenicol, β-lactam, fluoroquinolone, aminoglycoside, tetracycline and sulphonamides) were used to determine the susceptibility of *S. aureus* isolates as shown in Table 1 below. MDRSA exists when *S. aureus* develops resistance to at least one agent in three or more classes of antibiotics to which it was initially susceptible [5].

Results were interpreted using the CLSI break point IZD's [8]. For 30 µg ceftriaxone disc, IZD of 13 mm or less indicates resistance while 21 mm or more indicates susceptibility. For 30 µg tetracycline disc, IZD of 14 mm or less is resistance while 19 mm or more indicates susceptibility. For 30 µg chloramphenicol disc, 12 mm or less is resistance while 18 mm or more indicates susceptibility. For 300 µg nitrofurantoin disc, 14 mm or less is resistance while 17 mm or more indicates susceptibility. For 10 µg gentamycin disc, 12 mm or less is resistance while 15 mm or more indicates susceptibility. For 23.75 cotrimoxazole µg disc, 10 mm or less is resistance while 15 mm indicates susceptibility. Values in between the upper and lower limits are termed 'Intermediate'.

The isolated *S. aureus* strains were resistant to five of the six antibiotic classes except for two strains that

were resistant to four, with intermediate resistance to gentamicin and tetracycline. They were all sensitive to nitrofurantoin, an old drug, which in recent times is not a very popular antibiotic people come to ask for as "OTC's" in Nigeria and so is hardly misused. Furthermore, a study concluded that in the presence of therapeutic levels of nitrofurantoin, the growth of resistant mutants of *E. Coli* are so affected such that they are probably unable to become enriched and establish an infection [13]. This can be extrapolated to mutant *S. aureus*. Therefore based on their resistance profile, all the strains were classified as MDRSA.

## Sensitivity of MDRSA to Chlorpheniramine

Table 2 shows the activity of chlorpheniramine against MDRSA. At 18.8 µg there was no activity recorded in 9 out of the 10 isolates. Even at double this concentration the highest recorded IZD was 8 mm while there was 0 value for four of the isolates. The values recorded at 300 µg were not much different from the values recorded for 37.5 ug. Chlorpheniramine can thus be said to have a poor activity against MDRSA from 37.5 ug, with activity very slightly increasing as the doses doubled [8]

**Table 1: Antibiogram of the isolated *S. aureus* showing Inhibitory zone diameter (IZD) in mm**

| Isolate | IZD (mm)* for | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Clf | | GN | | Ntf | | Ctx | | Cotr | | Tcn | |
| $S_1$ | 0 | R | 13 | R | 24 | S | 0 | R | 0 | R | 0 | R |
| $S_2$ | 0 | R | 13 | R | 22 | S | 0 | R | 0 | R | 0 | R |
| $S_3$ | 0 | R | 11 | R | 24 | S | 0 | R | 0 | R | 0 | R |
| $S_5$ | 0 | R | 13 | R | 23 | S | 0 | R | 0 | R | 0 | R |
| $S_7$ | 0 | R | 12 | R | 22 | S | 0 | R | 0 | R | 0 | R |
| $S_9$ | 0 | R | 14 | I | 20 | S | 0 | R | 0 | R | 0 | R |
| $S_{11}$ | 0 | R | 12 | R | 23 | S | 0 | R | 0 | R | 0 | R |
| $S_{12}$ | 0 | R | 13 | R | 24 | S | 0 | R | 0 | R | 0 | R |
| $S_{16}$ | 0 | R | 13 | R | 22 | S | 0 | R | 0 | R | 16 | I |
| $S_{17}$ | 0 | R | 12 | R | 23 | S | 0 | R | 0 | R | 0 | R |

**Codes**: Resistant (R), sensitive (S), Intermediate susceptibility (I), chloramphenicol (Clf, 30 µg), gentamycin (GN, 10 µg), nitrofurantoin (Nft, 200 µg), ceftriaxone (Ctx, 30 µg), Co-trimoxazole (Cotr, 25 µg) and tetracycline (Tcn, 25 µg). *The IZD were interpreted using the Clinical Laboratory Standard Institute (CLSI) break point IZD [8].

**Table 2: Sensitivity of the isolated MDRSA to chlorpheniramine**

| Isolate | IZD (mm)* for | | | | |
|---|---|---|---|---|---|
| | 300 µg | 150 µg | 75 µg | 37.5 µg | 18.8 µg |
| $S_1$ | 9.5 | 8.5 | 8.0 | 8.0 | 0.0 |
| $S_2$ | 9.0 | 8.0 | 8.0 | 7.5 | 0.0 |
| $S_3$ | 8.0 | 8.0 | 7.5 | 0.0 | 0.0 |
| $S_5$ | 9.5 | 8.5 | 8.0 | 7.75 | 0.0 |
| $S_7$ | 8.25 | 7.75 | 0.0 | 0.0 | 0.0 |
| $S_9$ | 8.75 | 8.5 | 8.0 | 7.75 | 7.5 |
| $S_{11}$ | 8.0 | 7.0 | 0.0 | 0.0 | 0.0 |
| $S_{12}$ | 9.0 | 9.0 | 8.5 | 7.75 | 0.0 |
| $S_{16}$ | 8.75 | 7.5 | 0.0 | 0.0 | 0.0 |
| $S_{17}$ | 8.5 | 8.0 | 8.0 | 8.0 | 0.0 |

## Plasmid Curing and susceptibility studies after the curing

This suggest that the resistant gene that codes for resistance to gentamicin, cotrimoxazole and even nitrofurantoin, lies in the plasmid whose replication can be inhibited by acridine orange. It means that the resistance to these drugs is plasmid- mediated. Acridine orange removes plasmids by inhibiting the

replication of F plasmid responsible for the transfer of all other plasmid to other cells via conjugation. It can also act by selectively inhibiting the growth of plasmid carrying segragants of a population of bacteria. The efficiency of plasmid curing varies for the different agents and bacteria under study [9].

**Table 3: The antibiogram of the isolated MDRSA after curing the plasmid with acridine orange (0.25 mg/ml)**

| Isolate | IZD (mm)* for | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Clf | | GN | | Ntf | | Ctx | | Cotr | | Tcn | |
| $S_1$ | 0 | R | 20 | S | 29 | S | 0 | R | 22 | S | 0 | R |
| $S_2$ | 0 | R | 17 | S | 27 | S | 0 | R | 19 | S | 17 | R |
| $S_3$ | 0 | R | 19 | S | 24 | S | 0 | R | 22 | S | 0 | R |
| $S_5$ | 0 | R | 17 | S | 24 | S | 0 | R | 20 | S | 0 | R |
| $S_7$ | 0 | R | 17 | S | 27 | S | 0 | R | 19 | S | 15 | R |
| $S_9$ | 0 | R | 19 | S | 25 | S | 0 | R | 23 | S | 13 | R |
| $S_{11}$ | 0 | R | 20 | S | 27 | S | 0 | R | 20 | S | 0 | R |
| $S_{12}$ | 0 | R | 18 | S | 24 | S | 0 | R | 22 | S | 0 | R |
| $S_{16}$ | 0 | R | 17 | S | 27 | S | 0 | R | 19 | S | 0 | R |
| $S_{17}$ | 0 | R | 18 | S | 27 | S | 0 | R | 22 | S | 18 | R |

**Codes**: Resistant (R), sensitive (S), Intermediate susceptibility (I), chloramphenicol (Clf, 30 µg), gentamycin (GN, 10 µg), nitrofurantoin (Nft, 200 µg), ceftriaxone (Ctx, 30 µg), co-trimoxazole (Cotr, 25 µg) and Tetracycline (Tcn, 25 µg). *The IZD were interpreted using CLSI break point IZD [8].

**Table 4: The antibiogram of the isolated MDRSA after curing the plasmid with chlorpheniramine (0.25 mg/ml)**

| Isolate | IZD (mm)* for | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Clf | | GN | | Ntf | | Ctx | | Cotr | | Tcn | |
| $S_1$ | 0 | R | 19 | S | 30 | S | 15 | R | 25 | S | 0 | R |
| $S_2$ | 0 | R | 21 | S | 29 | S | 13 | R | 23 | S | 19 | S |
| $S_3$ | 0 | R | 19 | S | 31 | S | 14 | R | 20 | S | 0 | R |
| $S_5$ | 0 | R | 20 | S | 28 | S | 13 | R | 24 | S | 0 | R |
| $S_7$ | 0 | R | 19 | S | 30 | S | 15 | R | 22 | S | 18 | R |
| $S_9$ | 0 | R | 19 | S | 29 | S | 13 | R | 25 | S | 20 | S |
| $S_{11}$ | 0 | R | 21 | S | 31 | S | 12 | R | 25 | S | 0 | R |
| $S_{12}$ | 0 | R | 18 | S | 28 | S | 15 | R | 23 | S | 0 | R |
| $S_{16}$ | 0 | R | 21 | S | 30 | S | 14 | R | 22 | S | 19 | S |
| $S_{17}$ | 0 | R | 18 | S | 30 | S | 15 | R | 23 | S | 0 | R |

**Codes**: Resistant (R), sensitive (S), Intermediate susceptibility (I), Chloramphenical (Clf, 30 µg), Gentamycin (GN, 10 µg), Nitrofurantoin (Nft, 200 µg), Ceftriazone (Ctx, 30 µg), Co-trimoxazole (Cotr, 25 µg) and Tetracycline (Tcn, 25 µg). *The IZD were interpreted using CLSI break point IZD [8].

All the strains became sensitive to gentamicin and cotrimoxazole after curing with chlorpheniramine, and there was increased susceptibility to nitrofurantoin (Table 4), as seen with acridine orange. Three strains of MDRSA became sensitive to tetracycline, suggesting that a higher dose of chlorpheniramine could have potentially removed all the resistance plasmids in all the strains as sub-inhibitory concentration is used for plasmid curing. The results suggest that the resistance gene for gentamicin, cotrimoxazole, nitofurantoin and even tetracyclin lies in the plasmid whose replication can be inhibited by

chlorpheniramine. It thus means that the resistance to these drugs is plasmid-mediated. Several studies have reported that resistance is plasmid-borne if the bacterium under study becomes sensitive to an antibiotic after curing including a study reported that the resistance of Gram negative bacteria isolated from infected wounds at the University of Benin Teaching Hospital was plasmid mediated since the organism became sensitive after curing [15]. Resistance to those antibiotics with little or no activity after curing is indicative of chromosomally-borne resistance factors.

Better antibiotic activity was obtained with chlorpheniramine than with acridine orange (Table 5). This strongly suggests better or at least equivalent plasmid curing ability of chlorpheniramine as compared to acridine orange. A study by Olalubi et al, 2013, provided evidence that chlorpheniramine, verapamil or promethazine is able to reverse resistance in *Plasmodium falciparium* when it is combined with amodiaquine [16]. A similar mechanism could be at play here.

No work has before now been reported on chlorpheniramine as a curing agent in multidrug resistant bacteria. In this study, we investigated if chlorpheniramine can increase susceptibility in MDRSA by inhibiting the replication of their plasmid that may harbour the resistant genes. Chlorpheniramine showed slightly improved curing than the more common acridine orange, suggesting that it could be a better alternative to acridine orange laboratory operations involving plasmid curing.

We recommend that more studies be done on the MIC of chlorpheniramine to determine the best concentration that can be used for curing. The mechanism of the plasmid curing activity of chlorpheniramine can be determined. Investigations on the potential of chlorpheniramine to cure other bacteria can be carried out. Further investigations on the potential of chlorpheniramine to cure other bacteria can also be carried out.

**Table 5: Comparison of effects of acridine orange and chlorpheniramine**

| Isolate | IZD (mm)* for | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | GN | | | Ntf | | | Cotr | | |
| | Initial | AO | Chlf | Initial | AO | Chlf | Initial | AO | Chlf |
| $S_1$ | 13 | 20 | 19 | 24 | 29 | 30 | 0 | 22 | 25 |
| $S_2$ | 13 | 17 | 21 | 22 | 27 | 29 | 0 | 19 | 23 |
| $S_3$ | 11 | 19 | 19 | 24 | 24 | 31 | 0 | 22 | 20 |
| $S_5$ | 13 | 17 | 20 | 23 | 24 | 28 | 0 | 20 | 24 |
| $S_7$ | 12 | 17 | 19 | 22 | 27 | 30 | 0 | 19 | 22 |
| $S_9$ | 14 | 19 | 19 | 20 | 25 | 29 | 0 | 23 | 25 |
| $S_{11}$ | 12 | 20 | 21 | 23 | 27 | 31 | 0 | 20 | 25 |
| $S_{12}$ | 13 | 18 | 18 | 24 | 24 | 28 | 0 | 22 | 23 |
| $S_{16}$ | 13 | 17 | 21 | 22 | 27 | 30 | 0 | 19 | 22 |
| $S_{17}$ | 12 | 18 | 18 | 23 | 27 | 30 | 0 | 22 | 23 |

**Codes**: Gentamycin (GN, 10 µg), nitrofurantoin (Nft, 200 µg), co-trimoxazole (Cotr, 25 µg), acridine orange (AO), chlorpheniramine (Chlf).

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## AUTHORS' CONTRIBUTIONS

The authors declare no conflict of interest. Dr Uduma Osonwa designed and supervised the work. He also organised the final draft. Anagu wrote the first draft of

the manuscript and participated in the interpretation of results. Gugu and Okoye carried out the microbial isolation, characterisation and analyses. All the authors read and approved the final draft of the manuscript

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