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AN INVITRO STUDY ON EFFICACY OF CHLOROQUINE AGAINST PSEUDOMONAS, AND STAPHYLOCOCCI.

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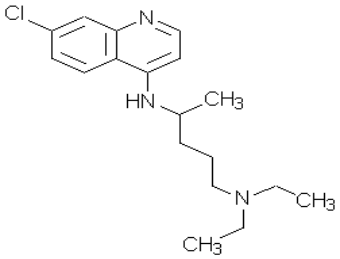
ABSTRACT

Chloroquine is a 4-aminoquinoline drug used in the treatment or prevention of malaria. Staphylococcus aureus is a Gram-positive coccal bacterium that is a member of the Firmicutes, and is frequently found in the human respiratory tract and on the skin. Pseudomonas aeruginosa is a common bacterium that can cause disease in animals, including humans. It is citrate, catalase and oxidase positive. It is found in soil, water, skin flora, and most man-made environments throughout the world. Antibiotic sensitivity is the susceptibility of bacteria to antibiotics. Antibiotic susceptibility testing (AST) is usually carried out to determine which antibiotic will be most successful in treating a bacterial infection in vivo. The objective of this study is to establish the efficacy of Chloroquine against common pathogenic bacteria such as pseudomonas and Staphylococci. Results: Chloroquine phosphate was found to show a broad range antibacterial activity. Staphylococcus aureus has almost same efficacy when compared to that of the standard amoxicillin-clavulanic acid combination. Pseudomonas is the most sensitive of all the organisms that were studied showing more susceptibility to Chloroquine than to the standard drug combination used here to compare.

Keywords: Chloroquine, Antimicrobial Susceptibility Test, Kirby-Bauer, Staphylococcus aureus, Pseudomonas.

INTRODUCTION

Chloroquine is 7-chloro-4-(4-diethylamino-1-methylbuylamino) quinoline. It has the following structural formula:[1]



Recent kinetic studies indicate that radio-labeled Chloroquine, quinidine, and mefloquine bind first to heme and then prevent further heme polymerization by incorporating as heme-quinoline complexes into growing heme polymer chains.[2,3] This unifying model also may apply to Amodiaquine, Quinacrine and Quinine but not to Primaquine.[4,5] Chloroquine is well absorbed from the gastrointestinal tract and rapidly from intramuscular and subcutaneous sites. The drug distributes relatively slowly into a very large apparent volume (over 100liters/kg).[6] The unique therapeutic value of Chloroquine for *extra intestinal amoebiasis* ,hepatic amoebiasis and also used in the suppression of Lepra Reaction.[3] In infectious mononucleosis it affords symptomatic relief. It is also used in the treatment of photogenic reactions. In Discoid Lupus Erythematosus where it is found to be very effective though less valuable in Systemic Lupus Erythematosus, and in Sjogren's syndrome. Side effects include gastrointestinal problems, stomach ache, itch, headache, hypotension, nightmares and blurred vison.[1,3]

Pseudomonas[8,9] are Gram-negative, aerobic (able to consume oxygen) rods. Most are flagellated so they can move around. Most produce a slime layer that cannot be phagocytosed, and which aids in the production of surface-colonising biofilms. Pseudomonas is able to grow in unexpected places. They have been found in areas where a lot of pharmaceuticals are prepared. Any carbon source, such as soap residue or cap liner adhesives is a suitable place for them to thrive. Other unlikely places where they have been found include antiseptics such as ammonium compounds and bottled mineral water. Most Pseudomonas spp. are naturally resistant to penicillin and related beta-lactam antibiotics, but will succumb to piperacillin, imipenem, tobramycin or ciprofloxacin. Their resistance to most antibiotics is attributed to their rapid efflux pumps which pump out the antibiotics before they are able to work.[10]

Staphylococcus aureus (which is occasionally given the nickname golden staph.) is a bacterium, frequently living on the skin or in the nose of a healthy person, that can cause illnesses ranging from minor skin infections (such as pimples, boils, and cellulitis) and

abscesses, to life-threatening diseases such as pneumonia, meningitis, endocarditis and septicemia[7]. Each year millions of patients in hospitals around the world contact a staphylococcal infection. It is a spherical bacterium. Staphylococcus aureus[8,9] forms a fairly large yellow colony on rich medium. Staphylococci are facultative anaerobes that grow by aerobic respiration or by fermentation that yields principally lactic acid. The bacteria are catalase-positive and oxidase-negative. Nearly all strains of S. aureus produce the enzyme coagulase: Staphylococci are perfectly spherical cells about 1 micrometer in diameter. They grow in clusters because staphylococci divide in two planes. The configuration of the cocci helps to distinguish staphylococci from streptococci, which are slightly oblong cells that usually grow in chains (because they divide in one plane only). The catalase test is important in distinguishing streptococci (catalase-negative) from staphylococci, which are vigorous catalase-producers. The test is performed by adding 3% hydrogen peroxide to a colony on an agar plate or slant. Catalase-positive cultures produce O_2 and bubble at once.[10]

Bacteriological Sensitivity Testing,[11] is done by disk-agar diffusion method using standardized concentrations of antibiotics based on clinically attained plasma concentrations of these. As such, they serve only as guides and cannot be blindly extrapolated to the clinical situation in every patient and for every organism. Broth cultures with break point concentration (concentration that demarcates between sensitive and resistant bacteria) of antibiotics probably yield more reliable results. Break point concentrations are based on clinically attainable serum concentrations of the antibiotic. *Minimum Inhibitory Concentration* (MIC)[12] i.e. the lowest concentration of an antibiotic which prevents visible growth of a bacterium determined in microwell culture plates using serial dilutions of the antibiotic is more informative, but not estimated routinely. By definition, MIC is the lowest concentration that completely inhibits visible growth of the organism as detected by the unaided eye after an 18-24-hour incubation period. Although MIC is a useful predictor of the potency of the drug-microorganism interaction, it has both pharmacokinetic and pharmacodynamic disadvantages. From the pharmacokinetic point of view, it overlooks two important factors: tissue distribution and protein binding. From the pharmacodynamic point of view, the MIC approach does not provide information on the rate of bactericidal activity and whether increasing antimicrobial concentrations can enhance this rate. Time-kill curves are another method of assessment of the antimicrobial activity of agents. Time-kill curves can follow microbial killing and growth as a function of both time and antibiotic concentration.

This method has more meaningful information about the interaction between bacteria and antibiotics.

Minimum Bactericidal Concentration[13] (MBC) of the antibiotic is determined by sub-culturing from tubes with no visible growth. If the organism is killed no growth will occur, but if it was only inhibited in the parent culture – it will grow on sub-culturing in antibiotic free medium. MBC is the concentration of the antibiotic which kills 99.9% of the bacteria. A small difference between MIC and MBC indicates that antibiotic is primarily bactericidal, while a large difference indicates bacteriostatic action. MBC is not used to guide selection of antibiotics in clinical practice.

MATERIALS AND METHODS

The present study was conducted at the Department of Microbiology, JJM Medical College, Davangere. The drugs used in this study, Chloroquine and amoxicillin-clavulanic acid combination were procured from the local market (medical shop). Chloroquine was available in the form of a diphosphate salt as a base. The diphosphate is a water soluble, white crystalline, powder bitter in taste. The drugs were powered into dry powder and dissolved in distilled water. The dissolved drugs were then used immediately, they were not stored Antimicrobial susceptibility test of these isolates were performed by using Kirby-Bauer's method. A total of hundred bacterial isolates from clinical samples such as Escherichia coli and Proteus vulgaris were used in the study. These organisms were available in the Microbiology Department.

A few colonies of the organism to be tested were picked up with a wire loop from the original culture plate and introduced into a test tube containing 4 ml of tryptose phosphate.[14] These tubes were then incubated for four hours to produce a bacterial suspension of moderate cloudiness. The suspension was then diluted, with distilled water to a density visually equivalent to that of a standard prepared by adding 0.5ml of 1% Barium Chloride. Petri dishes containing Mueller-Hinton agar were used for anti-microbial sensitivity tests. The plates were dried for about thirty minutes before inoculation. The bacterial broth suspension was streaked evenly in three planes onto the surface of the medium with a cotton swab. Surplus suspension was removed from the swab by rotating it against the side of the tube before the plates were seeded. A stock solution containing 64mg Chloroquine phosphate mL was prepared with distilled water, serial dilution was prepared to obtain 5mL solution, each having different concentration of the drug. Whatman No.1 filter paper was used to prepare

discs of 6mm diameter. The discs were then sterilized and each dilution of the drug was added on to the disc at the volume of 10µlL per disc, using a micro pipette. Final concentration of Chloroquine phosphate per disc was 64, 53, 42, and 30 µg. Discs will be dried and stored at 4ºC. Discs of Amoxicillin/clavulanic acid combination containing 30µg discs were used as control. After the inoculums were dried, the discs were placed on the agar with flamed forceps and gently pressed down to ensure contact. Plates were then incubated immediately. After over night incubation, the zone diameters were measured on the undersurface with the help of a ruler. The complete inhibition of growth as determined by the naked eye was taken as the end point[15]. The zone diameter were recorded and interpreted accordingly.

RESULTS

Table 3: Pseudomonas

	Zone of inhibition		Difference between groups			
	Range	**Means ± SD**	**53 µg**	**42 µg**	**30 µg**	**A/C 30 µg**
64 µg	27 – 29	28.0 ± 0.6	P<0.01	P < 0.01	P < 0.01	P < 0.01
53 µg	26 – 28	27.0 ± 0.6	-	P < 0.01	P <0.01	P < 0.01
42 µg	24 – 26	25.0 ± 0.6	-	-	P < 0.01	P < 0.01
30 µg	17 –19	18.0 ± 0.7	-	-	-	P < 0.01
A/C 30 µg	7 – 9	8.0 ± 0.5	-	-	-	-

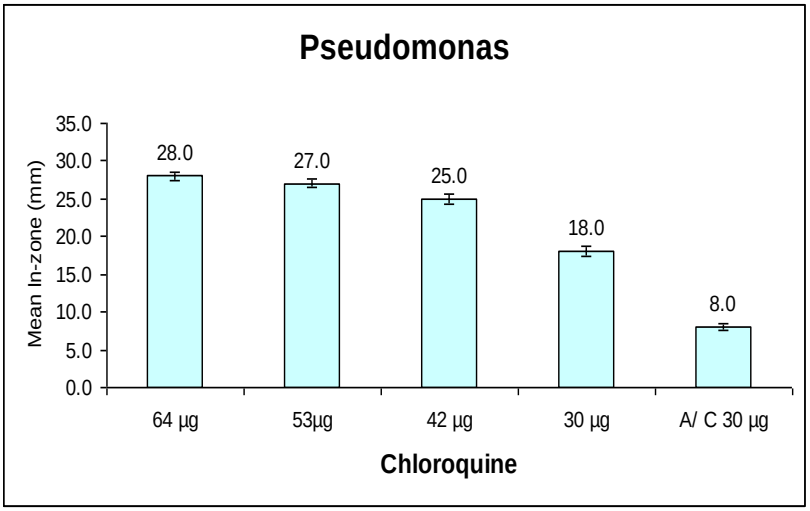
ANOVA, F = 4545.7 ve = 0.38

LSD: 0.48 ($p<0.05$)

0.58 ($p<0.01$)

In in-vitro tests of Chloroquine with Pseudomonas: The patterns of inhibition zone are observed at different doses of Chloroquine and the control drug combination of amoxicillin-clavulanic acid, and inter group comparisons are done. The range for zone of inhibition is around 27-29 mm in diameter for Chloroquine with strength of 64µg $mL^{-1,}$ with a mean of about 28mm. With strength of 53µg $mL^{-1,}$ the zone of inhibition is between 26-28 mm diameters, and with 42µg mL^{-1} and 30 µg mL^{-1} of Chloroquine the zone of inhibition is 24-26 mm and 17–19 mm respectively. There is an increase of inhibition zone along with the increase of strength of Chloroquine. The control drugs show an inhibition zone of about 7-9 mm diameter, less than that of Chloroquine. There is a significant difference between the Chloroquine and the control drug (strength of 30µg $mL^{-1)}$. When Chloroquine strength of 64µg mL^{-1} was compared with that of 53µg mL^{-1} and then with 42µg mL^{-1} and subsequently with 30µg mL^{-1} of Chloroquine a probability value of less than .01 was seen, indicating that

the results may be significant. The results of other strengths of Chloroquine were also compared with each other, and there was a definitive significance seen.



The above graph shows the diameter of inhibition on the y-axis, and the strength on the x-axis. In the bar graph there is a small vertical line indicating the standard deviation (SD) the last bar in the graph on the right is that of the control drug combination amoxicillin-clavulanic acid. The graph shows that the mean inhibition zone by the test drug, Chloroquine increases with increasing strength to that of the control drug. The zone of inhibition for the test drug is greater than that of the test drug in case of pseudomonas.

Table 4: Staphylococcus aureus

	Zone of inhibition		Difference between groups			
	Range	**Means ± SD**	**53 µg**	**42 µg**	**30 µg**	**A/C 30 µg**
64 µg	22 – 24	23.0 ± 0.6	P<0.01	P < 0.01	P < 0.01	P < 0.01
53 µg	19 – 21	20.0 ± 0.6	-	NS	P <0.01	P < 0.01
42 µg	19 – 21	20.0 ± 0.6	-	-	P < 0.01	P < 0.01
30 µg	17 –19	18.0 ± 0.5	-	-	-	P < 0.01
A/C 30 µg	19 – 21	20.0 ± 0.4	-	-	-	-

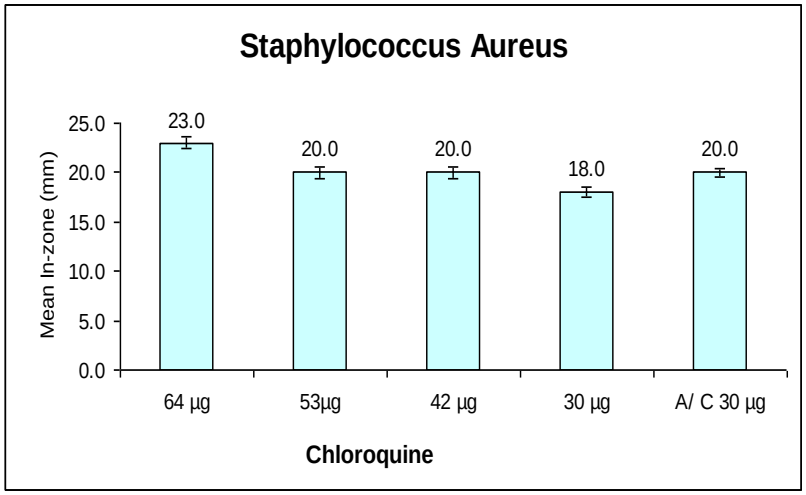
ANOVA, F = 282.4 p<0.001 ve = 0.28

LSD: 0.41 p<0.05

0.50 p<0.1

In in-vitro tests of Chloroquine with Staphylococcus aureus: The patterns of inhibition zone are observed at different doses of Chloroquine and the control drug combination of amoxicillin-clavulanic acid, and inter group comparisons are done. The range for zone of inhibition is around 22-24 mm in diameter for Chloroquine with strength of 64µg $mL^{-1,}$ with

a mean of about 23mm. With strength of 53µg $mL^{-1,}$ the zone of inhibition is between 19-21 mm diameters, and with 42µg mL^{-1} and 30 µg mL^{-1} of Chloroquine the zone of inhibition is 19-21 mm and 17-19 mm respectively. There is a slight increase of inhibition zone along with the increase of strength of Chloroquine. The control drugs show an inhibition zone of about 19-21 mm diameter. When Chloroquine strength of 64µg mL^{-1} was compared with that of 53µg mL^{-1} and then with 42µg mL^{-1} and subsequently with 30µg mL^{-1} of Chloroquine a probability value of less than .01 was seen, indicating that the results may be significant. The comparison between 53µg mL^{-1} and 42µg mL^{-1} of Chloroquine strength has shown no significance. Comparison between strengths of 53µg mL^{-1} and 42µg mL^{-1} with the control drug combination shows no significance in the probability. There is a slight difference in the zone of inhibition between the Chloroquine and the control drug (strength of 30µg $mL^{-1)}$.



The above graph shows the diameter of inhibition on the y-axis, and the strength on the x-axis. In the bar graph there is a small vertical line indicating the standard deviation (SD) the last bar in the graph is that of the control drug combination amoxicillin-clavulanic acid. The graph shows that the mean inhibition zone by the test drug, Chloroquine is almost similar in diameter to that of the control drug, with marginal differences. The zone of inhibition increases with the increase in the strength of Chloroquine.

DISCUSSSION

Chloroquine has almost same efficacy as that of the standard amoxicillin-clavulanic acid combination, with which the comparisons have been done. At lower strength of Chloroquine (of 30µg mL^{-1}) the zone of inhibition is around 17 to 19 mm, which less than a similar

strength of the standard drug combination (zone of inhibition 19 – 21 mm) As the strength is decrease, the zone of inhibition also decreases but marginally. With strength of 53μg mL^{-1}, the zone of inhibition is between 19-21 mm diameters, and with 42μg mL^{-1} and 30 μg mL^{-1} of Chloroquine the zone of inhibition is 19-21 mm and 17-19 mm respectively. There is no really difference with the intermediate strengths being compared with that of amoxicillin-clavulanic acid combination. At the higher strength of 64μg mL^{-1} the zone of inhibition is around 22 to 24 mm. Considering the strength of Chloroquine has doubled the zone of inhibition is far lower.

The opportunistic pathogen, gram negative rod *pseudomonas* shows an interesting result, when compared with the control drug combination. : The patterns of inhibition zone were observed at different doses of Chloroquine and the control drug combination of amoxicillin-clavulanic acid. The range for zone of inhibition is around 27-29 mm in diameter for Chloroquine with strength of 64μg $mL^{-1.}$ With strength of 53μg $mL^{-1,}$ the zone of inhibition is between 26-28 mm diameters, and with 42μg mL^{1} and 30 μg mL^{-1} of Chloroquine the zone of inhibition is 24-26 mm and 17–19 mm respectively. There is an increase of inhibition zone along with the increase of strength of Chloroquine. The control drugs show an inhibition zone of about 7-9 mm diameter, less than that of Chloroquine.

We have seen that in many of these bacteria show susceptibility to Chloroquine, although less than the standard drug combination. We must realize that the patient is not necessarily a person who can absorb all costs. When the cost factor is looked at, it can be clearly seen that there is a cost benefit ratio here which is beneficial to the patient. The average company manufactured Chloroquine is far more affordable than the standard drug combination that we see being prescribed in clinical practice. Perhaps one day it may be available in the market for the benefit of the patient. But for this to be realized, further study needs to be taken.

Chloroquine at high concentrations inhibits protein, RNA and DNA synthesis; this has been reported in most literature. Therefore we could postulate that the mechanism of action could be in line with these pathways to cause the antibacterial activity. It could be said that further tests can be done on animal models to study the efficacy of Chloroquine *in-vivo*.

CONCLUSION

Chloroquine, a 4-aminoquinoline derivative is frequently used as an anti-malarial compound it has also been used in the treatment of *Acanthamoeba, Clonorchis sinesis*, tenia, fungal,

bacterial infection and rheumatoid arthritis. *It has also been used as an immunomodulator. On* assessment of the antibacterial activity of Chloroquine on certain pathogenic bacteria by using disc diffusion technique Chloroquine phosphate was found to show a broad range antibacterial activity. *Staphylococcus aureus* has almost same efficacy when compared to that of the standard amoxicillin-clavulanic acid combination. *Pseudomonas* is the most sensitive of all the organisms that were studied showing more susceptibility to Chloroquine than to the standard drug combination used here to compare.

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