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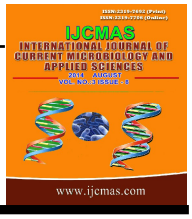

Original Research Article

Citric acid: A prospective permeabilizer for treatment of VISA infections

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A B S T R A C T

In the backdrop of managing MRSA infections, orthopaedic departments are facing a setback to curb the antibiotic menace, especially in ambulatory settings. Vancomycin is one such antibiotic which has been a cornerstone therapy for MRSA infections. But in the last decade Vancomycin Intermediate *Staphylococcus aureus* (VISA) are emerging around the globe and is necessitating the need to find alternative treatments. Effect of potentiating agents on antibiotics was studied by determination of MIC of citric acid by Agar dilution method. Comparative activities of different antibiotics with and without subinhibitory concentration of citric acid were recorded with different MRSA/VISA isolates. Citric acid was found to be an effective permeabilizer and potentiating agent for VISA/MRSA isolates. All the VISA isolates (n=9) were susceptible to citric acid except one. Of the 16 MRSA isolates tested for potentiating activity of citric acid, 14 isolates showed an increase in zone diameter size. Two isolates showed an exceptional resistance to citric acid. Citric acid offers a novel prophylactic approach for controlling MRSA and VISA infections of wounds from orthopaedic patients. The use of citric acid may prevent the development of infections that will minimize antibiotic use, prevent development of resistance as well as promote healing.

K e y w o r d s

Citric acid resistance, MRSA, Potentiating agent, MIC, VISA

Introduction

MRSA is a nuisance pathogen and it is estimated that 100 to 200 million Indians are carriers of MRSA. Almost 90% of the population is unaware of the epidemiological role MRSA is playing. One such aftermath is the emergence of many vancomycin resistance cases in the last decade. Vancomycin resistance in *S.aureus* is not just a medical complication but an unpredictable scenario which is coming forth with most of the pharmaceutical industries mere agencies of profit making rather than indulging in research and development programmes (Monnet and Sørensen, 2001).

The ambulatory setting of orthopaedic departments are becoming a major source of nosocomial infections (Hornberg *et al*, 2001). The deteriorating condition of the outpatient department is at the stake of medical staff including the authorities and doctors who are little concerned about the hygienic conditions prevailing in the

department. The result of which is the global widespread of MRSA with 40-70% of inappropriate antibiotic use, increase in mortality rate, especially in orthopaedic units (Drori-Zeides *et al*., 2001).

Development of multidrug resistant forms, indiscriminate use of antibiotics over the last 80 years and huge expenses involved to discover new antibiotics is haunting the medical scenario. In this context, to solve the dilemma wisely and to explore the present resources – augmentation of the activity of the already existing antibiotic by using potentiating agents is the most effective way out. Potentiating agents act as permeabilizers which are chemicals that increase the permeability of cell wall of bacteria to antibiotics (Rusell and Chopra, 1996).

The efficacy of citric acid have been reported and commended by many researchers **(**Nagoba et al., 1998;1999;2002; Sebastian *et al.,* 2009; Ayres *et al*., 1999). Citric acid has been found highly effective against almost all bacterial pathogens like *Pseudomonas aeruginosa*, *S.aureus*, *E.coli.* No work till date has been recorded to see the role of citric acid on VISA nuisance and hence the present work is a step in this direction to see a world of better tomorrow.

Materials and Methods

Antibiotic impregnated discs of vancomycin, teicoplanin, oxacillin and linezolid (HiMedia laboratories, Mumbai) were used in the study. The antibiotics were selected on the basis of preference by orthopaedic doctors. The potentiating agent used was citric acid (Loba Chemie, Mumbai).

Microorganisms and Media

The microorganisms employed were 23 MRSA isolates of clinical origin (pus), recovered from surgical site infections of orthopaedic patients from ambulatory settings of various orthopaedic departments. The wounds were in the dirty wound category and were highly profused with pus. *S.aureus* were screened on the basis of growth on Mannitol Salt agar, Baird Parker agar, Staphylococcus agar no. 110, Coagulase test and Staph Latex test kit. Biotyping of the isolates were done for biofilm formation, pigment production, DNase test, protease activity, lipase and lecithinase production. Also antibiotic susceptibility test was done for each *S. aureus* isolate by the Kirby Bauer disc diffusion method against vancomycin (30 mcg), tobramycin (10mcg), clindamycin (2mcg), teicoplanin (30mcg) and linezolid (30mcg). Methicillin resistance was evaluated with 4% NaCl in MHA and using Methicillin (5mcg) discs. Resistance was studied for vancomycin and methicillin by Etest method. Antimicrobial susceptibility testing was performed on Mueller Hinton Agar (MHA) plates by the Kirby Bauer disc diffusion method and according to Clinical and Laboratory Standards Institute (CLSI) guidelines (Bauer *et al*., 1966; CLSI, 2006). Finally 23 MRSA isolates were selected for study on the basis of overall performance. The isolates were maintained as per standard guidelines.

Determination of Minimum Inhibitory Concentration (MIC) for citric acid by agar dilution method

Sterilized gradient plates of citric acid (range 0.1- 2 gm %) in MHA were prepared. A citric acid-free control plate was also included as control. Gradient plates containing different concentration of citric acid and the control plate without citric acid were spot inoculated with 10 µl suspensions with a micropipette and thereafter incubated at 35˚C for 24hrs. MIC representing the

lowest concentration of antimicrobial at which there was complete inhibition of growth was recorded. In reading the end points, a barely visible haze of growth or a single colony was disregarded.

Determination of comparative activities of different antibiotics with and without subinhibitory concentration of citric acid

Sterilized MHA plates containing subinhibitory concentration of citric acid for each isolates were prepared. A control plate without any citric acid was also included. Agar surface of the plates were swabbed with the inoculum. Antibiotic discs of vancomycin, teicoplanin, linezolid and methicillin were placed equidistance on all the plates and incubated at 35˚C for 24hrs. Zone diameters were measured with the help of zone scale at 24 and 48 hrs following CLSI criteria [11]. The isolates were considered resistant as according to the reference chart provided by the manufacturer. Zone diameters were measured at 24 hrs in transmitted light for linezolid.

Determination of Minimum Inhibitory Concentration for citric acid by disc diffusion method

Two isolates which did not record potentiating effect with citric acid for any antibiotic and one isolate which did not record any activity with methicillin were selected for determination of MIC for citric acid by disc diffusion method. Concentrations of citric acid were selected (range 0.025- 1.6gm %) on the basis of their MIC values against the particular organism obtained by agar dilution method. Sterilized MHA plates were prepared and inoculum (0.5 McFarland standard) was spread on their respective plate. Sterile paper discs were placed equidistance on the plate. Ten µl of different concentrations of citric acid was added onto each paper disc. The plates were allowed to set at room temperature and kept in the incubator at 37˚C for 24 hrs for incubation. Inhibitory zones were recorded and results interpreted.

Results and Discussion

The *S.aureus* isolates basically used in this study were all MRSA, for which the MIC of citric acid was determined. The MICs at which 50 and 90% of isolates were inhibited by citric acid were 0.2 gm%. Citric acid inhibited all isolates in the range of 0.05-0.2 gm% (Table 1). Comparative activities of different antibiotics with and without subinhibitory concentration of citric acid against 16 MRSA isolates were studied. Fourteen (87.5%) MRSA isolates demonstrated potentiation with citric acid and two (12.5%) MRSA didn't show any potentiation (Table 2). Determination of MIC for citric acid by disc diffusion method for the two citric acid resistant isolates displayed no inhibitory zones around the discs. All the VISA isolates (n=9) were susceptible to citric acid except one.

MRSA are vulnerable worldwide threat paralyzing especially the orthopaedic departments, where long term antibiotic treatment is a curse. Vancomycin is one such preferred drug of choice against MRSA infections which reduces the hospitalization of a patient by a week. Well known as a pet antibiotic among orthopaedic doctors vancomycin is being used extensively without any limitation. Hence surfacing of VISA/hVISA infections is representing the beginning of vancomycin resistance era and highlights the consequence of treatment failures of 'superbugs'.

In 1996 the first case of hVISA was reported in Japan and till now many cases have been reported worldwide. VISA accounts for distorted cellular physiology such as

reduced peptidoglycan cross-linking, cell-wall turnover and autolysis causing the thickening of cell wall due to mutations in regulatory systems that control cell wall homeostasis (VraSR, GraSR, and WalKR) (Hiramatsu *et al*., 1997; McAleese *et al*., 2006). Citric acid treatment of chronic infected wounds, projects as an exceptional permeabilizer that enhance intracellular accumulation and have a valuable role to play against multidrug resistant bacteria in the clinical settings (Nagoba et al., 1998;1999;2002; Ormerod *et al*., 2011). The activity of citric acid against pathogens is attributed to reduction in pH of microbial cell by ionization of undissociated acid molecules and altering the cell membrane permeability or reduction of proton motive force (Beuchat, 1998). Citric acid is also documented to alter the size and shape of *S.aureus* on exposure to inhibitory concentrations Raju *et al*., 2007)

The present work showcases sufficient evidence to base state-of-the-art inferences about the merits of citric acid on MRSA and VISA treatment with 87.5% of MRSA isolates showing an increase in diameter of inhibitory zone. All the VISA isolates (as determined by Etest method earlier) except one were potentiated by citric acid indicating that antibiotic alone is insufficient to deal in contemporary wound dressings and that supplementary aids promoting wound healing are required. Pus samples were collected from wounds which were dirty and persistent from more than three months. Sakoulas also documented that high bacterial load and low concentration of vancomycin in serum resulted in persistent infection (Sakoulas *et al*., 2006) Thickening of the cell wall of VISA strains traps the vancomycin m-olecules and prevents them to reach their target site (Hiramatsu *et al*., 1997). This might be the reason for long vancomycin treatment i.e. above three months. hVISA strains were recovered from three patients. Also it was documented by workers that vancomycin treatment gave poor response when infection was caused by hVISA strains (Moore, 2003). Potentiating effect of citric acid with vancomycin has been shown by Ayres and coworkers against multidrug resistant *P. aeruginosa (*Ayres *et al.*,1999*).* Cell wall thickening is one of the attributes of VISA isolates and susceptibility of VISA isolates to citric acid in our work proves that citric acid overcomes the cell wall barrier to produce its effect on the pathogen. Only one VISA isolate (SA26) was resistant to citric acid, which is an exceptional behavior. This isolate was resistant to all the antibiotics studied and was a strong biofilm former. The physiological attributes of biofilm organisms bestow an inherent resistance to antimicrobial agents and makes it less susceptible to antibiotics and antiseptics which might be the reason of the exceptional behavior (Costerton *et al*., 1999; Vuong *et al*., 2004). On the contrary another citric acid resistant isolate (SA06) was simply MRSA and as to which factor was responsible for its resistance to citric acid could not be articulated.

Citric acid inhibited all isolates in the range of 0.05-0.2 gm% with the MIC_{50} andMIC_{90} value of 0.2 gm%. The MIC of the *S.aureus* isolated from non healing ulcer was 900 µg /ml by Nagoba and coworkers (Nagoba *et al*., 2008; Vukušić *et al*., 2011). The MIC_{90} value in our work was much higher representing that high concentration of citric acid was effective in the case of MRSA/VISA isolates due to cell wall thickening.

Table.1 Minimum Inhibitory Concentration of citric acid against *S.aureus* isolates (n=23)

% of isolates susceptible at MIC (gm %)												Concentration (gm %)		
0.01	0.03	0.05	0.07	0.10	0.12	0.14	0.16	0.18	0.20	0.22	0.24	Range	MIC_{50}	MIC_{90}
0	0	4.34	13.04	0	0	0	0	1.73	60.86	0	0	0.05-0.20	0.20	0.20

Table.2 Comparative activities of different antibiotics with and without subinhibitory concentration of citric acid against MRSA (Inhibition zone in mm)

Isolate no.	Submic value of citric acid (gm %)	Methicillin (5mcg)		Vancomycin (30mcg)		Linezolid (30mcg)		Teicoplanin (30mcg)	
		Without citric acid	With citric acid	Without citric acid	With citric acid	Without citric acid	With citric acid	Without citric acid	With citric acid
SA03	0.09	09	16	15	17	29	34	14	17
SA04	0.10	10	15	15	16	29	32	14	16
SA05	0.10	07	12	14	15	28	30	14	15
SA06	0.05	08	08	18	18	35	35	15	15
SA13	0.09	07	09	13	13	26	31	13	13
SA20	0.035	07	08	12	14	24	26	12	13
SA21	0.10	09	09	13	18	26	33	13	18
SA22	0.10	07	09	13	14	25	28	12	13
SA24	0.09	NZ	12	12	17	25	29	13	17
SA26	0.05	NZ	NZ	13	13	28	26	12	12
SA37	0.10	NZ	08	13	14	28	30	13	14
SA39	0.035	09	13	15	17	32	34	15	16
SA48	0.09	07	10	12	16	24	27	12	14
SA49	0.035	07	09	14	16	30	34	12	14
SA57	0.10	08	11	14	17	29	34	13	20
SA59	0.10	NZ	11	15	17	29	31	15	16
SA61	0.06	08	09	13	14	29	30	13	13
SA63	0.10	09	14	13	14	26	31	14	14
SA65	0.10	NZ	10	15	17	28	31	14	16

To talk about implementation of actual concentration of citric acid applied for wound healing- Nagoba *et al* effectively cured the ulcer using cotton pads soaked with 3% citric acid and AD Ormerod *et al* demonstrated eradication of MRSA carriage from wounds using a topical formulation of 4.5% of citric acid and 3% sodium nitrite creams co-applied for 5 days and cleared of 60% of wounds

(Nagoba *et al*., 2008; Ormerod *et al*., 2011)**.**

The wide range of study on citric acid suggests that it would be a valuable option in the therapy of MRSA/VISA infections and in topical application in wound care. No reports of bacteria resistant to citric acid have been reported so far. Also it has long being used as a food preservative and S B Vukusic gave findings that cotton fabric treated with citric acid showed significant antibacterial activity against MRSA and *and hence can* be successively used for the making of disposable materials used by clinicians (Vukušić *et al*., 2011). Nevertheless it can be explored in all possible ways to curb Hospital associated MRSA infections in the long run.

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