Indian J Med Res 147, January 2018, pp 97-101
DOI: 10.4103/ijmr.IJMR_1407_16

Quick Response Code:

Use of succinic & oxalic acid in reducing the dosage of colistin against New Delhi metallo-β-lactamase-1 bacteria

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Received August 23, 2016

***Background & objectives*: New Delhi metallo-β-lactamase 1 (NDM-1) cleaves the beta-lactam ring, and confers bacterial resistance against most of the beta-lactam antibiotics, except tigecycline and colistin. Among these two antibiotics, colistin is considered toxic, and therefore, its clinical use and dosage need cautious approach. In the present study, six organic acids were screened individually and in combination of two acids for their effectiveness against NDM-1 *Escherichia coli* and a combination of colistin and oxalic or succinic acid was tested to find out the potential of combination therapy for reducing the dose of toxic colistin.**

***Methods*: Antibacterial activity of the organic acid and their combinations was tested by disc diffusion method against NDM-1 *E. coli*, and minimum inhibitory concentration (MIC) was determined by broth dilution method. Synergistic effect between organic acids and colistin was tested by checkerboard method.**

***Results*: Oxalic acid showed the highest zone of inhibition (15±1 mm) followed by succinic acid, tartaric acid, fumaric acid, citric acid and malic acid. The combination of two acids did not increase the zone of inhibition significantly. MIC was found to be the lowest with oxalic acid and succinic acid (320 µg/ml). In the presence of 160 µg/ml oxalic acid or succinic acid, MIC of colistin was reduced from 8 to 4 µg/ml, indicating synergistic effect.**

***Interpretation & conclusions*: Our findings showed that combination therapy using colistin and oxalic acid or succinic acid might find safe clinical application of this antibiotic in controlling infections due to NDM-1 bacteria.**

Key words Antimicrobial activity - *Escherichia coli* - inhibition zone - minimum inhibitory concentration - New Delhi metallo-β-lactamase-1 - organic acid

Prevalence of multidrug-resistant bacteria limits the effectiveness of almost all the antibiotics and causes public health concern worldwide[1]. Even though pharmaceutical industries have developed several antibiotics in the past, bacterial resistance to these drugs is increasing. Multidrug-resistant bacteria have evolved due to the indiscriminate use of broad-spectrum antimicrobial drugs for the treatment of various infectious diseases[2]. Infections due to such multidrug-resistant bacteria are highly challenging to control using the existing antibiotics[3]. Bacteria not only acquire resistance to the drugs but also have the ability

to transmit the same to other bacteria[4]. The β-lactams antibiotics such as penicillins, cephalosporins, carbapenems and monobactams are an important class of antibiotics for treating the infections by multidrug-resistant bacteria[5]. Strains of *Escherichia coli* and *Klebsiella pneumonia*, which are highly resistant to all antibiotics, except tigecycline and colistin, were identified from hospital-derived infections[6]. These strains produced metallo-β-lactamase (MBL) enzyme but were negative for the previously known MBL genes. A new MBL enzyme encoded by bla_{NDM-1} gene, which shared very little identity with other MBLs, was found in these strains, and was named as New Delhi metallo-β-lactamase 1 (NDM-1)[7].

Several antimicrobial compounds have been tested against NDM-1 bacteria. Captopril with the 3-mercapto-2-methylpropanoyl fragment and a proline residue effectively inhibited NDM-1 with an IC_{50} value of 7.9 μM[8]. One of the captopril derivatives and its structural analogue were also found to inhibit NDM-1 with an IC_{50} value of 15 and 10 μM, respectively. The captopril structural analogue compound, being a clinically used antidote for metal poisoning, was proposed as a potential safe chemical to treat bacterial infections due to NDM-1 bacteria[9]. Aspergillomarasmine A, a fungal natural product, was found to inhibit the activity of NDM-1 and restore the activity of meropenem against *Enterobacteriaceae*, *Acinetobacter* sp. and *Pseudomonas* sp[10].

Among the 109 antibacterial drugs that were approved from 1981 to 2010, 69 per cent were derived from natural products[11]. Organic acids have played a significant role in food preservation by their ability to control microbial growth[12]. These acids may act by acidification of the cytoplasm or accumulation of the dissociated acid anions to a toxic level[13]. Antimicrobial effect of the organic acids is mainly associated with the ratio of undissociated forms and reduction in *p*H[14]. Microbial toxicity of organic acids is based on the export of protons and depletion of the energy of microbial cells. Organic acids may damage outer or cytoplasmic membrane, which inhibits the macromolecular synthesis or denature the nucleic acids and proteins[15]. Less direct antibacterial activity of organic acids includes interference with nutrient transport, cytoplasmic membrane damage resulting in leakage, disruption of the outer membrane and influencing macromolecular synthesis[16,17]. Benzoic acid and ascorbic acid have been reported as active inhibitors of bacterial growth[18]. Acetic acid, fumaric acid, propionic acid and lactic acid have been shown to significantly delay the microbial growth[19,20]. Organic acids such as acetic acid, citric acid and lactic acid were found to inhibit *Shigella* species of bacteria effectively[21]. *Salmonella* species in meat and poultry products, which are responsible for salmonellosis, could be controlled using acetic acid, citric acid, lactic acid, propionic acid, succinic acid, tartaric acid, and malic acid[22]. In this study, antimicrobial property of citric acid, fumaric acid, malic acid, oxalic acid, succinic acid and tartaric acid was tested against NDM-1 *E. coli*. Subsequently, combination of colistin and oxalic acid or succinic acid was studied to explore the potential of combination therapy for reducing the dosage of colistin for its safe clinical use.

Material & Methods

The study was conducted in the Genomics laboratory of SRM University, Chennai, India, during January to July 2016.

NDM-1-positive *E. coli* strain (NDM-1 *E. coli*) was obtained from Dr David Livermore, UK (through Dr Karthikeyan Kumarasamy, Department of Microbiology, Institute of Basic Medical Sciences, Taramani, Chennai). DNA sequencing of 16S rDNA and NDM-1 gene was done to verify the strain. Nutrient broth and Mueller-Hinton agar and MacConkey agar were purchased from HiMedia Laboratories Pvt. Ltd., Mumbai, India. The strain was sub-cultured on MacConkey agar plates and maintained at 4°C. Citric acid was purchased from Sigma, USA; L-malic acid, fumaric acid, succinic acid, oxalic acid, L-tartaric acid and dimethyl sulphoxide were purchased from HiMedia.

Determination of antimicrobial activity: Antibacterial activity of the organic acid and their combinations was tested using the disc diffusion method[23]. Organic acids (1.0 mg/disc) were applied on 6 mm sterile discs and placed on the agar plates, which were inoculated with NDM-1 *E. coli*. The plates were incubated at 37°C for overnight (16 h); the zone of inhibition was measured in millimetres to determine the antimicrobial activity. Colistin (Sigma-Aldrich, USA) was used as positive control at a concentration 10 μg/disc.

Determination of minimum inhibitory concentration (MIC): Minimum inhibitory concentration of the organic acids alone and in combination with colistin against NDM-1 *E. coli* was determined by broth dilution method[24]. Briefly, organic acids (1.0 mg/ml)

were serially diluted with the nutrient broth and inoculated with 1.0×10^8 colony-forming units (cfu)/ml of NDM-1 *E. coli*. The plates were incubated at 37°C for 24 h. MIC was calculated as the lowest acid concentration at which no growth of NDM-1 *E. coli* was observed.

Synergistic effect of organic acids with antibiotic: The bacterial cultures were grown to OD 0.5 in nutrient broth at 37°C, and used for determining the synergistic effect between organic acids and colistin. The least MIC value of organic acids (*i.e.* 160 µg/ml) was considered and tested in combination with colistin, which was serially diluted from its MIC (8 µg/ml).

Checkerboard method: Standard forms of succinic acid and oxalic acid were freshly prepared. The stock solutions and serial two-fold dilutions of each drug to at least double the MIC were prepared according to the recommendations of Clinical and Laboratory Standards Institute (CLSI) immediately before testing[25]. A total of 50 µl of Mueller-Hinton broth was distributed into each well of the microdilution plates. The first combination of organic acid was serially diluted along the ordinate, while the antibiotics were diluted along the abscissa. An inoculum of 0.5 McFarland turbidity was prepared from NDM-1 *E. coli* isolate in Mueller-Hinton broth. Each microtitre well was inoculated with 3 µl of a bacterial inoculum of 5×10^5 cfu/ml, and the plates were incubated at 37°C for 48 h under aerobic conditions. The resulting checkerboard contains each combination of organic acids and antibiotics, with tubes that contain the highest concentration of each antibiotic at opposite corners.

Statistical analysis: All the experiments were performed in triplicates. Statistical analysis was done by one-way ANOVA, and $P<0.05$ was considered as significant.

Results

Antimicrobial activity of citric acid, fumaric acid, malic acid, oxalic acid, succinic acid and tartaric acid was tested individually and in a combination of two acids against NDM-1 *E. coli* using colistin as positive control. All the acids showed a clear zone of inhibition, and three acids showed a zone of inhibition larger than that observed with colistin. For quantitative assessment, the experiment was done in triplicates, and the zone of inhibition was analyzed. Oxalic acid, succinic acid and tartaric acid at a concentration of 1.0 mg/disc showed a significantly ($P<0.05$) larger zone of inhibition when compared to colistin at a concentration of 10 µg/disc. Effect of the combination of two organic acids was studied by combining 0.5 mg/disc of each organic acid to achieve a total organic acid concentration of 1.0 mg/disc. The zone of inhibition for the combination of two organic acids was found to be not significantly different when compared with either of the individual organic acids present in the combination. This indicated the absence of additive, synergistic or antagonistic effect between the acids that were tested in combinations.

Table. Checkerboard assay for succinic and oxalic acid (10-320 µg/ml) and colistin (0.5-8 µg/ml) at different minimum inhibitory concentrations

Organic acids (succcinic and oxalic acid) (µg/ml)	0.5	1	2	4	8
320					
160					
80					
40					
20					
10					

Colistin (µg/ml)

Shaded boxes denote combinations that showed growth inhibition of NDM-1 *E. coli*

MIC for the organic acids against NDM-1 *E. coli* ranged between 320 and 640 µg/ml. Oxalic acid and succinic acid showed the lowest MIC of 320 µg/ml. These two acids were individually combined with colistin to understand the effect of the combinations. Half the MIC of oxalic acid or succinic acid (160 µg/ml) was combined with MIC of colistin (8 µg/ml), and MIC for the combinations was determined by broth dilution method. In colistin plus oxalic acid as well as colistin plus succinic acid combinations, the MIC of colistin was reduced from 8 to 4 µg/ml, which indicated the synergistic effect between the acids and colistin against NDM-1 *E. coli*. Synergy between the organic acids and colistin was also evaluated using checkerboard assay. Complete growth inhibition of NDM-1 *E. coli* was observed in 0.5 and 2.0 µg/ml colistin when it was combined with 320 and 80 µg/ml organic acids, respectively (Table).

Discussion

NDM-1 bacteria are resistant to most of the current generation of antibiotics, except colistin and

tigecycline. Use of colistin was abandoned in the 1970s due to nephrotoxicity but it re-emerged as life-saving antibiotic to combat multidrug-resistant NDM-1 bacteria[26,27]. However, its clinical application requires extreme caution and careful consideration of safety and efficacy[28]. Therefore, there is an urgent need to identify new and safe antimicrobial compounds, which can control the growth of NDM-1 bacteria. Organic acids are known for their antimicrobial activity and therefore, are regularly used in food preservation[15,29]. Synergistic effect of antibiotics has been shown to reduce the usage and toxicity of antibiotics. In this study, the dosage of colistin was drastically reduced from 8 to 0.5 µg/ml by its synergistic action with organic acids, which in turn could decrease the toxicity of colistin during treatment. Further delivery modes and evaluation of dosage are the future prospects for safer treatments. Organic acids have been reported to control the growth of several bacterial species with varying levels of efficiency[30]. Since organic acids are naturally present in the fruits and vegetables, these are suitable for human use without any concern about toxicity.

In conclusion, the present study demonstrated that organic acids, which naturally prevent bacterial growth, could be a source to reduce the antibiotic usage. A possibility of combination therapy using colistin and oxalic or succinic acid is indicated by the synergistic effect between the antibiotics and organic acids against NDM-1 bacteria, which may help in the control of misuse of antibiotics in the future.

Acknowledgment

Authors thank the SRM University, Chennai, for providing the well-equipped laboratory facility.

***Financial support & sponsorship*:** This study was funded and supported by the Indian Council of Medical Research (ICMR), New Delhi, India (AMR/22/2011-ECD-I).

Conflicts of Interest: None.

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