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Ascorbic acid inhibitory activity on resistant strains of *Enterobacter* spp.: *in vitro* study

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ABSTRACT

Enterobacter species, members of Enterobacteriaceae family that belong to the ESKAPE group, were expressed as the principal rise of nosocomial resistant infections. This study was aimed to test the antibacterial activity of ascorbic acid on *Enterobacter* clinical strains. Four *Enterobacter* strains were collected and identified by both biochemical and MALDI-TOF methods. Antibiotic susceptibility testing of commercially available antibiotics was performed using disc diffusion method. Antibacterial activity of ascorbic acid was tested by using agar well diffusion and broth microdilution methods. The collected strains were identified as three strains *Enterobacter aerogenes* and one strain *Enterobacter cloacae*. High resistance rates toward both cefotaxime and nalidixic acid were obtained by disc diffusion method. By well diffusion, 100 mg/ml and 50 mg/ml concentrations of ascorbic acid were found to be effective. While by using microbroth dilution, concentrations of 1.5, 3.125 and 6.25 mg/ml of ascorbic acid were detected as minimum inhibitory concentrations. Our results demonstrated a good antibacterial activity of ascorbic acid on *Enterobacter* strains and further studies on ascorbic acid/antibiotics combinations are in need.

Keywords: *Enterobacter aerogenes*; *Enterobacter cloacae*; ascorbic acid; MALDI-TOF.

1. INTRODUCTION

Nowadays, antimicrobial resistance (AMR) is of great concern, as it is common in hospitals where acquired infections can be placed. ¹ The increased AMR which had seen in recent years caused by bacteria suggest that the choice of antibiotic should be guided by sensitivity assays, and as a result monitoring of antibiotic resistance pattern has to be a rational way to reduce the antibiotic treatment failure risk. ²⁻⁴ Multidrug-resistant Gram-negative bacteria [MDR-GN] involving MDR Enterobacteriaceae has taken place to be predominantly in charge of most drug-resistant contagions that arise in healthcare facilities. ⁵ *Enterobacter* species, members of Enterobacteriaceae family that belong to the ESKAPE group, were expressed as the principal rise of nosocomial resistant infections. ⁶

Enterobacter species (*E. aerogenes* and *E. cloacae*) present highly preserved regulation mechanisms acting to modulate porins expression integrated into the outer membrane. The pathogenic mechanisms and virulence factors contributing to the disease are not understood yet; this could be due to the scarcity and dispersion of information available. Its ability to form biofilms and to secrete various cytotoxins like: enterotoxins, hemolysins, and pore-forming toxins is important for its pathogenicity. ⁷ *Enterobacter* spp. outer membrane contains lipopolysaccharides from which Lipid-A, an endotoxin which is the major stimulus for the release of cytokines which are the mediators of systemic inflammation and its complications. ⁸

The ongoing pandemic spread of resistant bacteria illustrates that the problem can only be addressed through international cooperation. We may very well be forced with unimaginable setbacks medically, socially, and economically within just a few years. ¹ Treatment of infections caused by *Enterobacter* spp. is difficult and broad resistant to antibiotics is an increasing problem. As *Enterobacter* spp. are multi-drug resistant, a combination of antibiotics are prescribed simultaneously for serious infections. Despite its relevance to nosocomial infections, its pathogenic mechanisms and factors involved in causing disease are not understood. ⁹

Vitamins are compounds that are required in small amounts but that cannot be synthesized in quantities large enough to meet the normal needs.¹⁰ In order to find an alternative to antimicrobial usage or reduce antibiotics effective doses, new antimicrobial agents need to be tested. Vitamin C presents one such alternative, because it is cheap, easily available and has few or no adverse effects and also because it is frequently prescribed as a nutritional supplement as it has established antioxidant effects and has been used as an adjuvant in cancer chemotherapy.¹¹ Many other health benefits have been attributed to ascorbic acid (Vitamin C) such as an immuno-modulator, anti-atherogenic, prevents cold, and more other benefits.¹² Because of all that, the aim of this study was designed to test the activity of vitamin C as antibacterial agents against *Enterobacter* strains.

2. MATERIALS AND METHODS

2.1 Bacterial isolates

Four different bacterial strains of *Enterobacter spp.* were kindly collected from Kasr El-Ainy hospital, Egypt from October 2015 to December 2015. Isolates were defined as *Enterobacter spp.* according to routine biochemical testing.¹³ After confirmation of isolates, they were collected on glycerol broth and stored at -80 freezer until been used.

2.2 Bacterial identification by MALDI-TOF

Direct transfer-formic acid method was used,¹⁴ bacteria were applied as a thin film onto a 384-spot polished steel target plate and allowed to air dry. To the bacterial spot 1 µl of 70% formic acid was added and allowed to air dry. Then, 1 µl of MALDI matrix (α -cyano-4-hydroxycinnamic acid saturated solution (HCCA) in 2.5% trifluoroacetic acid and 50% acetonitrile) was applied to each bacterial colony and allowed to dry. Analysis was operated in the positive linear mode (mass over charge ratio (m/z) ranging from 2,000 to 20,000) under control of FlexControl software. Spectra were compared to fingerprint database by using the Bruker Biotyper 3.1 software and library of 5,623 entries.

2.3 Antibiotic sensitivity test

Antimicrobial susceptibility of the *Enterobacter* isolates was determined by the disk diffusion as recommended by CLSI.¹⁵ The commercial antibiotics tested were imipenem (10 µg), meropenem (10 µg), cefotaxime (30 µg), cefepime (30 µg), ceftazidime (30 µg), gentamicin (10 µg), amikacin (30 µg), tobramycin (10 µg), nalidixic acid (30 µg), ciprofloxacin (5 µg), levofloxacin (5 µg) and amoxicillin/clavulanic (30 µg). Results were interpreted according to CLSI criteria.

2.4 Cluster analysis

Based on the results of antibiotic sensitivity of different *Enterobacter* strains Hierarchical cluster analysis was carried out using SPSS software (SPSS Inc. v. 12). Antibiotic results for each were coded as '1; Resistant' and Non-Resistant coded as '0'. The analysis was presented graphically to find the strains that are most similar according to antibiotic profile, clustering of the samples was performed based on average linkage, and the branch length represents the distance between the strains.¹⁶

2.5 Antibacterial activity of ascorbic acid and sodium ascorbate

Different concentrations of both ascorbic acid and sodium ascorbate were prepared according to recommendations.

2.5.1 Well diffusion method

The method was performed according to that previously reported.¹⁷ A sterilized Muller Hinton Agar was poured into sterile plates, after solidification, 100 µl of fresh culture of *Enterobacter* strains (0.5 MacFarland) were swabbed on the respective plates. Standard wells were made on the agar plate. 100 µl of ascorbic acid and sodium ascorbate at different concentrations of 100 mg/ml, 50 mg/ml, 25 mg/ml and 12.5 mg/ml were added into the wells and incubated for 24 h at 37 °C. Then the diameters of the zones of inhibition were measured.

2.5.2 Minimum Inhibitory Concentration (MIC)

The effect of ascorbic acid was studied using the method reported with modifications.¹⁸ Briefly, 100 µl (0.5 MacFarland bacterial cultures) was dispersed into each well of microplate, and then was mixed with 100 µl twofold dilutions of treatment concentrations for 24 h at 37 °C. Final concentrations were 0.1875–100 mg/ml for both ascorbic acid and sodium ascorbate. Well containing 100 µl of fresh broth with 100 µl of bacterial culture was used as a positive control, whereas negative control was broth only. The lowest concentration of the tested antimicrobial agents that inhibited visible growth is considered MIC.

2.5.3 Minimum Bactericidal Concentration (MBC)

MBC was evaluated by plating 5 µl from each well that showed no growth on nutrient agar medium after incubation at 37 °C for 24 h, at which the lowest concentration that cause no growth on agar is considered MBC.¹⁹

3 RESULTS AND DISCUSSION

High resistance to several antibiotics became a common phenomenon among gram-negative bacteria specially those of the Enterobacteriaceae family. ESKAPE are pathogens that responsible for many nosocomial infections and are capable of "escaping" the biocidal effect of antimicrobial agents.²⁰

Therefore, updating knowledge about resistance rates of those infectious agents has become an important role to achieve success with antibiotics treatment.

In our study, the four bacterial isolates were identified as *Enterobacter spp.* using biochemical tests, and confirmed by MALDI-TOF as three strains of *Enterobacter aerogenes* and one strain *Enterobacter cloacae*, however different prevalence have been reported in other studies.^{2,8} By using antibiotic sensitivity testing results interpretation (Table 1), high resistance rates were observed to cefotaxime (100%), nalidixic acid (100%) followed by cefepime (75%) and ceftazidime (75%). To compare the sensitivity and resistance of different strains toward antibiotics Hierarchical cluster analysis was performed

(fig. 1). *Ea1* and *Ea3* were found to be more sensitive toward the tested antibiotics than *Ea2* and *Ec1*.

Different reports from other countries were also founded. Among 61 *Enterobacter spp.*, the antibiotic susceptibility results revealed that all isolates were resistance to amikacin, and then mostly resistance to cefotaxime and gentamicin with 90.2% and 75.4%, respectively.²¹ High Susceptibility of *Enterobacter* species was reported for amikacin (91.22%), norfloxacin (92.98%) and imipenem (98%).² Over a period of six years (2010-2015), 939 *Enterobacter* species were isolated by Maraki *et al.*, reported that 83.3% sensitivity of these strains to trimethoprim/sulfamethoxazole.²²

Table 1: Resistance pattern of different commercial antibiotics among *Enterobacter* strains

Antibiotics			<i>Enterobacter aerogenes Ea1</i>	<i>Enterobacter aerogenes Ea2</i>	<i>Enterobacter aerogenes Ea3</i>	<i>Enterobacter cloacae Ec1</i>
Antibiotic groups	Carbapenems	MEM	S	R	S	I
		IPM	S	R	S	I
	Cephamycins	FOX	S	R	R	R
	Aminoglycosides	AK	S	R	S	R
		CN	S	R	S	R
		TOB	S	R	S	R
	Cephalosporins	CTX	R	R	R	R
		FEP	S	R	R	R
	Fluoroquinolones	NA	R	R	R	R
		CIP	I	R	I	R
		LVX	I	R	S	R
	Penicillins/β-lactamase inhibitors	AMC	S	R	S	S

IPM, imipenem (10µg); MEM, meropenem (10µg); CTX, cefotaxime (30µg); FEP, cefepime (30µg); FOX, ceftazidime (30µg); CN, gentamicin (10µg); AK, amikacin (30µg); TOB, tobramycin (10µg); NA, nalidixic acid (30µg); CIP, ciprofloxacin (5µg); LEV, levofloxacin (5µg); AMC, amoxicillin/clavulanic (30µg); S, susceptible; I, intermediate; R, resistant.

In 2009, Amin *et al.*, reported that among *Enterobacter* strains high sensitivity rates were reported to amikacin (94.4%).²³ By studying the susceptibilities to eight antimicrobial agents against ESKAPE pathogens, its observed that *Enterobacter spp.* from intra-abdominal

infections had the susceptibility to amikacin exceeded 95 %, while for isolates from urinary tract infections amikacin, imipenem and levofloxacin were the most active agents tested, with rates of susceptibility of 90.9, 87.0 and 81.8 %, respectively.²⁴

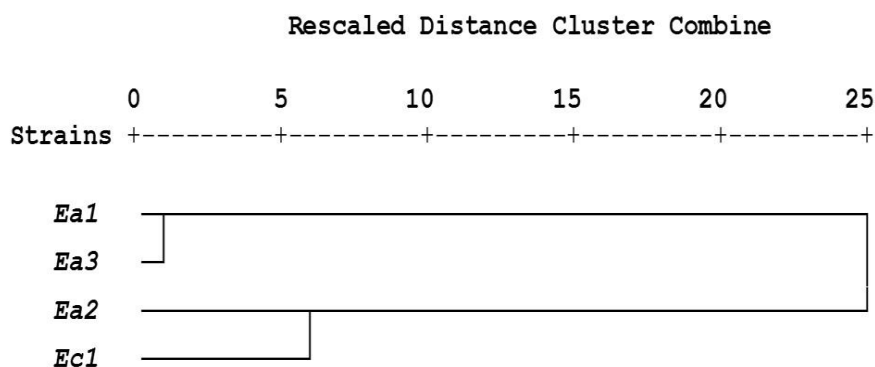


Fig.1: Hierarchical cluster analysis of different strains of *Enterobacter spp*

Different antibiotic resistance rates were often reported in different countries. In middle-income countries, which are rapidly converging to consumption levels found in high-income countries, poor sanitation, a continued high background burden of bacterial infections and the increasing use of antibiotics have facilitated the rapid growth and spread of AMR.²⁵ In addition to that the antibiotic exposure may provide the selection pressure for the creation of resistant mutants, the movement of people, lack of access to clean water, poor hygiene, vaccine availability, and healthcare facilities promote their broader dissemination.²⁶

Besides its role involving in different fields,²⁷⁻²⁹ Vitamin C has a well-known role as an antioxidant agent. It serves as a cofactor in several important enzyme reactions, such as those involved in cholesterol, amino acids, and certain peptide hormones synthesis.³⁰

In our study, different concentrations (100, 50, 25 and 12.5 mg/ml) were used, minimum inhibitory concentration (MIC) of ascorbic acid was detected for all strains (Table 2), while for sodium ascorbate the MIC was detected for only *Enterobacter aerogenes* Ea1. Minimum bactericidal activity of ascorbic acid was observed for both *Enterobacter aerogenes* Ea2 and *Enterobacter aerogenes* Ea3. Several studies have shown that vitamin C, in the form of ascorbic acid exerts significant antibacterial effects on pathogenic organisms alone or in combination with known antibiotics. Ascorbic Acid was reported to inhibit the growth of *P. mirabilis* planktonic

cells.³¹ It did not show any synergistic action in combination with ciprofloxacin when tested on uropathogenic *E. coli*. However, when tested alone, it significantly inhibited the growth of *E. coli*.³² Cursino *et al.*, indicated that multi-resistant *P. aeruginosa* was affected by the combination of Ascorbic acid and antibiotics except the antagonism observed only for Chloramphenicol.¹⁸

The antibacterial activity of ascorbic acid is not well understood, however Pandit *et al.*, propose that the inhibitory effect of vitamin C on biofilm formation proceeds by inhibition of quorum sensing and other stationary phase regulatory mechanisms underpinning biofilm development, which specifically leads to inhibition of polysaccharide biosynthesis.³³ Also, sensitivity of ciprofloxacin against *E. coli* has enhanced in the presence of ascorbic acid which observed by Srividya *et al.*, suggested to be due to its pro-oxidant activity or by altering the membrane permeability.³⁴ While by studying *Mycobacterium smegmatis*, Syal *et al.*, suggest that vitamin C has the potential to be developed as the inhibitor of (p)ppGpp synthesis which considered the master regulator of stress response and is responsible for bacterial survival under stress.³⁵ On the other hand, about the toxicity level, it was suggested that the high doses of vitamin C can be toxic, although excess ascorbate is normally excreted harmlessly in the urine, the excess formation of oxalate can accumulate in various organs in patients such as kidney transplanted patients and patients undergoing dialysis.³⁶

Table 2: Antibacterial assays using different methods against *Enterobacter* strains

Bacterial strains	Methods of antibacterial assay											
	Well diffusion (cm)								Microbroth dilution (mg/ml)			
	AA (mg/ml) ***				SA (mg/ml) ****				MIC*		MBC**	
	100	50	25	12.5	100	50	25	12.5	AA	SA	AA	SA
<i>Enterobacter aerogenes</i> Ea1	1	1	-	-	-	-	-	-	1.5	12.5	-	-
<i>Enterobacter aerogenes</i> Ea2	1.2	1	-	-	-	-	-	-	6.25	> 100	100	-
<i>Enterobacter cloacae</i> Ec1	1.3	1.2	1	1	-	-	-	-	3.125	> 100	-	-
<i>Enterobacter aerogenes</i> Ea3	1.2	1	0.8	0.6	-	-	-	-	6.25	> 100	6.25	-

*Minimum inhibitory concentration, ** Minimum bactericidal concentration, *** Ascorbic acid,**** Sodium ascorbate.

4. CONCLUSION

Our study suggested the strong antibacterial activity of the used concentration of ascorbic acid against *Enterobacter* strains. However further studies on their mechanism of action, toxicity and antibiotic combination are still in need.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this paper.

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