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Efflux Pump Mediated Multi Drug Resistant *Serratia marcescens* Isolated from Human Subjects and Food in Ogun State, Nigeria

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Abstract: This study evaluated the presence of efflux mediated resistance in *Serratia marcescens* using MIC technique in the presence of CCCP (carbonyl cyanide m-chlorophenyl hydrazine). The strains of *Serratia marcescens* showing resistance to ≥ 1 antibiotic were examined for their ability to transfer the resistance using conjugation method. Results obtained showed that the MIC values of the tested antibiotics were reduced in the presence of CCCP while some of the resistance patterns of the studied isolates were transferred genetically through conjugation to the transconjugants. It can therefore be inferred that the efflux mediated multi drug resistance observed in this study may be located on a conjugative plasmids.

Key words: MIC · CCCP · Efflux Pump · *Serratia marcescens*

INTRODUCTION

Serratia marcescens is a Gram-negative, rod-shaped motile bacterium belonging to the family Enterobacteriaceae that are commonly involved in nosocomial infections, particularly catheter-associated bacteremia, urinary tract and wound infections [1] and is responsible for 1.4% of bacteremia cases in the United States [2]. It is commonly found in the respiratory and urinary tracts of hospitalized adults and in the gastrointestinal system of children. It can grow in temperatures ranging from 5-40°C and in pH levels ranging from 5 to 9. This organism was described for the first time in 1819 and thought to be a non-pathogen until the latter half of the 20th century [3].

Nowadays, this microorganism is an accepted clinical pathogen, causing pneumonia, urinary tract infections, septicemia and meningitis, particularly in high risk settings [4]. Antimicrobial resistance represents a real challenge for hospital settings, considering that many microorganisms including S. marcescens carry both chromosomal and plasmid-mediated resistance determinants, such as the presence of efflux pump system. Resistance determinants can easily spread from a species to another, a further reason to implement infection control measures in hospital settings [5].

Multidrug efflux systems in bacteria are of particular concern for the treatment of patients with infectious diseases, since the substrates of many multidrug transporters include antimicrobials used for therapy [6,7]. Exposure to one substance that is a substrate of the efflux pump can favor its overexpression and the consequence may be cross-resistance to all other substrates which may include clinically relevant antimicrobials [8].

Although genes encoding efflux pumps may be present in plasmids, those found in chromosome are often related to the intrinsic resistance mechanisms and enable the bacteria to survive in hostile environments, as for instance in the presence of antimicrobials [8]. Multidrug transporters can therefore be associated to intrinsic and to acquired antimicrobial resistance [6, 8]. Acquired multidrug resistance can occur via three mechanisms via;

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mutation and amplification of genes encoding multidrug transporters which alter their level of expression [9] or their activity level [10], mutation in specific genes or in global regulatory genes, resulting in an increased expression of multidrug transporters, as for example the mutation in *mex R* of *P. aeruginosa* OCR1 [11] and intercellular transfer of resistance genes, such as *qacE*, by plasmids or transposons [12]. This study was aimed at determining the presence of efflux pump system in multi-drug resistant *Serratia marcescens* isolated from primary school pupils and grounded melon in Ijebu North Local government area of Ogun State, Nigeria

MATERIALS AND METHODS

Isolation and Identification of *Serratia marcescens:* One loopfull of suspension of grounded melon samples (83 samples) was plated on blood agar and MacConkey's agar and then incubated at 37°C for 24 h. 100 urine samples obtained from the primary school pupils in Ijebu North Local government areas were processed following standard procedures [13]. The identification was done using the API 20E kit.

Antimicrobial Susceptibility Testing: The antimicrobial susceptibility pattern of the isolates was determined using the Kirby-Bauer-National Committee for Clinical Laboratory Standard (NCCLS) modified disc diffusion technique [13]. All the strains were tested for their sensitivity to the following antibiotics: Penicillin (5 µg), Ampicillin (25 ug), Gentamycin (10 ug), Ofloxacin, nalidixic acid, ciprofloxacin (2 ug) and Cloxacillin(5 ug) (All from Abtek, U.K.). The zones of inhibition were recorded and classified as "resistant", intermediate" or "sensitive" based on the interpretative chart updated according to the current NCCLS Standards. *Escherichia coli* ATCC 25922 was used as control.

Genetic Transfer: *Serratia marcescens* strains showing resistance to = 1 antibiotic were selected (donor cells) and examined for their ability to transfer the resistance. Conjugation was performed using the strain of *E.coli* J53 (as recipient cell) [14]. Aliquots of overnight cultures of donor and recipient organisms were mixed in a final volume of 4ml Luria-Bertoni medium. The mixture was incubated at 37°C for 2h. Ten-fold serial dilutions of conjugation mixture were made and 0.1ml of each dilution was spread on the agar surface of Mueller Hinton agar supplemented with ampicillin (100µg/ml and sodium azide 200µg/ml, for counting the total number of transconjugants.

Effect of Efflux Inhibitor on Minimum Inhibitory Concentration (MIC) Levels of Quinolones: To determine the extent of the efflux pump mediated resistance in *Serratia marcescens* isolates, MIC levels for the quinolones were determined using broth dilution method in the presence and in the absence of efflux pump inhibitor [CCCP (Sigma, USA)]. CCCP are proton motive force inhibitors. Stock solution of CCCP was prepared in DMSO to make a final concentration of (1 mg/l).

RESULTS

Table 1 depicts the distribution of *Serratia marcescens* isolated from primary school pupils in Ijebu North Local government area of Ogun State, Nigeria, as well those obtained from grounded melon. As shown in the table, fourteen (14) and four (4) multidrug resistant *Serratia marcescens* were isolated from primary school pupils and grounded melon respectively. All these isolates from grounded melon showed hundred percent resistance to the tested antibiotics while the isolates recovered from the primary school pupils displayed alarming rate of resistance to all the tested fluoroquinolones and Beta lactam antibiotics. The level of resistance observed against gentamicin was relatively very low when compared to the other tested antibiotics (table 2). The antibiotic resistance patterns of the studied isolates are recorded in table 3. Results obtained reveals

Table 1: Distribution of isolated *Serratia marcescens*

Sources	N	n	%
PSP	100	14	14
GM	83	4	4

PSP = Primary school pupils, GM = Grounded melon, N= Total number of isolates = number of resistant isolates

Table 2: Prevalence of Antibiotic resistant *Serratia marcescens*

Antibiotic	PSP (14)	GM (4)
Penicillin	7(50)	4(100)
Ampicillin	5(35.7)	4(100)
Gentamicin	4(28.6)	4(100)
Ofloxacin	10(71.4)	4(100)
Nalidixic acid	10(100)	4(100)
Ciprofloxacin	14(100)	4(100)
Cloxacillin	11(78.6)	4(100)

Table 3: Antimicrobial resistance patterns of *Serratia marcescens* isolates from clinical samples

Isolates designation	Antimicrobial Resistance Pattern: Original	Antimicrobial Resistance Pattern: Transferred
A21	P, AMP,GN,OFL,NAL,CIP	P, OFL, NAL, CIP
L6	P, GN,OFL, NAL,CIP	-
B13	P,AMP, CX,OFL,NAL,CIP	-
A23	P, AMP, CX,OFL, NAL,CIP	-
A24	P, CX, OFL,NAL,CIP	-
B12	P, CX,OFL,NAL,CIP	P, OFL,NAL,CIP
C16	P,OFL,CIP,NAL P,	OFL,CIP,NAL
L13	P,OFL, NAL, CIP	P,OFL, NAL, CIP
C18	P, NAL, CIP, OFL	-
D12	P, CIP, OFL, NAL	-
E6	P, CIP, NAL, OFL	-
R8	P, CIP, NAL, OFL	P, CIP, NAL, OFL
AA1	P, CIP, OFL, NAL	P, CIP, OFL, NAL
AA2	P, CIP, GN, NAL,OFL	P, CIP, GN, NAL,OFL
BC3	P, OFL, CX,NAL,OFL	OFL, NAL
AD9	P,CIP,CX,NAL,OFL	NAL,CIP,OFL
AD4	P, CIP, GN, NAL, OFL	-
L63	P,GN, CX, NAL,OFL, CIP	-

P = Penicillin, GN= Gentamicin, CX= Cloxacillin, NAL = Nalidixic acid, OFL = Ofloxacin, CIP = Ciprofloxacin, AMP = Ampicillin

Table 4: Efflux positive *Serratia marcescens*

	MIC (mg/L) with/without CCCP					
	CIP		OFL		NAL	
Lab code	WITH	WITHOUT	WITH	WITHOUT	WITH	WITHOUT
A21	32	0.5	32	0.25	64	1
L6	64	1	32	1	64	0.25
B13	64	1	64	0.25	128	2
A23	16	0.06	32	2	128	1
A24	32	0.03	32	0.125	128	1
B12	32	0.25	32	0.03	128	1
C16	32	0.016	64	0.5	64	1
L13	32	0.016	64	0.5	64	1
C18	32	0.008	64	0.25	64	1
D12	32	0.008	16	0.5	64	0.5
E6	32	0.008	16	0.03	64	0.5
R8	8	0.06	64	0.016	64	1
AA1	16	0.06	64	0.008	128	1
AA2	16	0.03	32	0.06	128	4
BC3	32	1	32	0.03	64	0.25
AD9	32	1	32	0.03	64	0.25
AD4	16	0.008	32	0.016	64	1
L63	16	0.06	32	0.016	64	1

that some of the resistance patterns could be transferred genetically through conjugation. On the determination of the efflux positive organisms, the minimum inhibitory concentrations of the most resisted antibiotics (fluoroquinolones) against the *Serratia marcescens* isolates were determined both in the presence and in the absence of carbonyl cyanide m-chloro phenyl hydrazone (CCCP) and it was found that the MIC values were decreased in the presence of CCCP (Table 4).

DISCUSSION

The detection of *Serratia marcescens* in both primary school pupils and grounded melon circulating in the same local government area may be very informative and could mean that the consumption of improperly cooked melon is the major source of this organism in the studied area. This is because majority of the studied population are of low economic status and when questioned through a structured questionnaire admitted that they consumed soup containing melon at least once per week. The presence of this organisms has been linked to several episodes of bacteremia traced to infusion pumps [14-19]. In addition to bacteremia, *S. marcescens* can cause a wide spectrum of infectious diseases, including urinary, respiratory and biliary tract infections, wound infections, intravenous catheter-related infections, septic arthritis, osteomyelitis, infective endocarditis and peritonitis [20, 21]. The susceptibility rate of *S. marcescens* isolates to fluoroquinolone in this study was higher than expected. For instance, the isolates from urine showed 100 and 71.4% resistance to nalidixic acid and ofloxacin respectively while 100% resistance was observed to ciprofloxacin. This observation suggests a progression in the resistant rate of ciprofloxacin since the reporting of the Taiwan Surveillance of Antimicrobial Resistance study in 2000 [22, 23]. Sheng *et al*. [24] reported that the susceptibility rate of *S. marcescens* to ciprofloxacin decreased from 100% in 1985-1986 to 80% in 1996-1997. The continuous increase in fluoroquinolone resistance among clinically important Gram-negative bacilli poses a serious problem because of the widespread use of fluoroquinolones to treat both community-acquired and nosocomial infection. The resistance observed against the beta lactam antibiotics in this study may be suggesting that these isolates were producing ESBLs. The most common enzymes associated with resistance to third-generation cephalosporins in certain Gram-negative bacilli, including *S. marcescens,* are chromosomally encoded, inducible AmpC beta-lactamases. However, ESBLs were increasingly found among clinical *S. marcescens* isolates in the past decade. CTX-M-3, TEM-47 and SHV-5 were discovered in 19% of 347 *S. marcescens* isolates in Poland from 1996 to 2000 [25]. In Taiwan, Wu *et al.* [26] reported that 21 (62%) of 34 *S. marcescens* isolates non-susceptible to cefotaxime exhibited an ESBL-resistant phenotype and all possessed CTX-M-3, suggestive of the wide spread of such a beta-lactamase, at least among *S. marcescens*. The presence of ESBL will further limit the choice of appropriate antimicrobial therapy for cefotaxime-resistant *S. marcescens* bacteremia.

The transfer of antibiotic resistance pattern to the transconjugants is an indication that the isolates may be harbouring conjugative plasmid. Resistance to high levels of antibiotics has been ascribed in most instances to the presence of plasmids [27-30]. The fact that the MICs value of the fluoroquilononones were reduced in the presence of carbonyl cyanide m-chloro phenyl hydrazone disclosed that the level of resistance engineered to the antibiotics was caused by the presence of efflux pump system in the studied isolates. Exposure to one substance that is a substrate of the efflux pump can favor its overexpression and the consequence may be cross-resistance to all other substrates which may include clinically relevant antimicrobials [8]. In conclusion, the results of this study have shown that the major mechanism of fluoroquilononones resistance was due to the presence of efflux pump in the studied isolates.

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