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EFFLUX-PUMP EXPRESSION IN MULTIDRUG-RESISTANT BACTERIA: CASE IN A UNIVERSITY TEACHING HOSPITAL OF CAMEROON

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ABSTRACT

In line with the high multidrug-resistance rates observed in bacteria and the importance of resistance mechanisms in the management of infectious diseases, the present investigation was conducted to detect with an efflux pump inhibitor (reserpine) efflux pump-expressing bacterial isolates from clinical specimens at the Laboratory of Microbiology of the Université des Montagnes Teaching Hospital. Standard disk diffusion and minimal inhibitory concentration techniques were used as recommended by CASFM, 2016. Susceptibility tests were performed on a total of 51 multidrug-resistant isolates with and without reserpine at the sub-inhibitory concentration. Out of these, 30 eventually selected as potential efflux pump-positive isolates further underwent the micro-dilution at varying concentrations of antibiotic/reserpine combinations. The minimal inhibitory concentration test values recorded with antibiotic/reserpine combination revealed improved effectiveness for Erythromycin, Tetracycline, Gentamicin, Azithromycin, Ciprofloxacin and Ofloxacin. 1024 µg/mL for reserpine was sub-inhibitory, but when it was used at 32µg / mL with Ofloxacin the MIC value recorded dropped by about 1/8 compared to the value recorded when Ofloxacin was used alone on some isolates; and about 1/2 to 1/4 with other antibiotics. Overall, 47% of isolates were positive for efflux pump expression, overwhelmed by *Enterobacteriaceae* (62.7%) and *Micrococaceae* (17.6%) and, namely *Enterobacter* (78%), *Escherichia* (57%), *Staphylococcus* (56 %), and *Klebsiella* (25%). Since organisms from these genera are most frequent etiologies of IDs further work is necessary to detect efflux pump-positive isolates in the routine process.

KEYWORDS: Efflux pumps, multidrug-resistance bacteria, reserpine.

INTRODUCTION

Resistance to antimicrobial agents is a leading cause of concern that challenges all public health (PH) systems across the globe.[1–3] In bacteria, its impact on infectious disease (ID) prevention and control has overwhelmed that of professional pathogenic species previously regarded as the most serious threat to human, animal and plant health. In fact, resistant bacteria strains can be detected in both pathogen and common environmental hosts that may become potent etiologies of infectious diseases (IDs); matching thereby the current paradigm about resistance selection and dissemination of unusual phenotypes that pictures this phenomenon as related to a "tool set" from with bacteria can select the necessary genetic determinant likely to favor fitness at individual and population levels, regardless of the presence of virulence gene in the bacterial genome. Furthermore and in connection with the rampant poverty and connected human disasters like famine, wars, low hygiene, and flood in several parts of the world, and the global increased life expectances, IDs caused by multidrug-resistant non-pathogens have become a special focus with regards to the increased rates of opportunistic infections caused by endogenous bacteria in susceptible hosts. Bacterial resistance actually became a PH concern soon after the Second World War in the 1945s when the development of tolerance to Penicillin was observed in *Staphylococcus*. In the 1961s on *Staphylococcus pyogenes*, other events further shaded light on resistance that could develop naturally or be selected by antimicrobial agents through one or several unrelated resistance mechanisms and cause tolerance to

penicillin.[4] These bacterial behaviors were later found to be favored by the high flexibility of bacterial genome and the bacterial growth rate, paramount parameters to addressing the stochastic changing occurring to improve fitness in prokaryotic populations. In line with advances in both fundamental and applied sciences, common hosts of the environment and endogenous microflora were found either responsible for, or associated with arrays of life threatening human conditions. It is in fact from the 1980s that reports on the resistance in *Enterobacteriaceae* actually opened ways to intensive research initiatives through antibacterial resistance (ABR), with emphasis on extended spectrum beta-lactamases (ESBL) production. Such researches require human and financial resources that can hardly be afforded in many settings. In low-income communities, microbial infections are generally treated with available antimicrobial agents according to the probabilistic protocols in force in other settings (CAS-SFM, 2016), with no real relationship with the otherwise scarce holistic data.[5] This improper use of antimicrobials is recognized as a major engine for selection of resistance phenotypes in bacteria. In the 1960s, selection of resistance to one antibiotic by another one was largely known. If it is also largely recognized that five key resistance mechanism are used by bacteria to tolerate antibacterial agents activity and that they may act in combination, studies on the role of specific mechanism are not common.[6,7]

In the routine process at the Université des Montagnes Teachnig Hospital (West-Cameroon), close to 70% of bacteria isolates express resistance against more than 86% of antibiotics (unpublished data), but the mechanisms used are not yet fully addressed. Subsequent the previous work by Kamga *et al.*(2015)[7], the present study aims at detecting efflux pump-expressing strains in multidrug-resistant bacteria isolated in this healthcare facility. Clearly, reserpine will be used to detect efflux pump-positive strains in the multi-drug resistant (MDR) bacterial population isolated from clinical specimens. This detection will guide further initiatives focusing intermediate and long run antibiotic association in caretaking.

MATERIAL AND METHODS

General test scheme

Selection of potential efflux pump producers (EPP) was based on several considerations. The first was resistance to more than 65% of the antibiotics used. For this, susceptibility tests were conducted according to the standard protocol by CA-SFM, 2016 [5] for agar diffusion, on a total of 95 bacterial strains. In the second step, the 51 isolates that complied with this primary selection criterion underwent another agar diffusion test in association with reserpine. In this, 15 µL of a 1024 µg/mL reserpine solution was added to each antibiotic disk previously applied on the standard preparation in Petri dishes. Increased inhibition diameter with addition of reserpine was regarded as the likelihood of efflux pump expression. After this second selection, part of complying isolates further underwent micro-dilution tests for the minimal inhibitory concentration (MIC) with 14 antibiotics. The aim of the third test was to select from the set used, the antibiotics that could serve more effectively in the detection of potential efflux pump-expressing isolates. Finally, the EPP tests were performed by micro-dilution with selected antibiotics in association with reserpine.

Various tests in short

1. Susceptibility testing

The tests were carried out on 24 h bacterial culture. For this fresh bacterial population to be obtained, isolates were streaked on nutrient agar and incubated at 37°C overnight. From each resulting bacterial population, a suspensions equivalent to 0.5 McFarland was prepared and diluted to obtain the final opacity required for standard susceptibility tests by agar diffusion (Kirby Bauer) on Mueller Hinton agar as recommended by the "Comité de l'Antibiogramme de la Societé Française de Microbiologie (CA-SFM, Eucast, 2016)".[5] Eighteen antibacterial agents were selected for the procedure. They included representative of major families of antibiotics of interest in Cameroon. Namely, they were Penicillin (10 U), Oxacillin (1 µg), Amoxicilline (30 µg), Amoxicillin/Clavulanic acid (20/10 µg), Cephalotin (30 µg), Cefuroxime (30 µg), Ceftriaxone (30 µg), Nalidixic acid (30 µg), Ciprofloxacin (30 µg), Nitrofurantoin (300 µg), Trimethoprim/Sulfamethoxazole (Co-trimoxazole, 1.25/23.75 µg) and Gentamicin (5 µg). For identification and susceptibility tests, *E. coli:* ATCC 25922 and *S. aureus*: QC 1625 were the reference bacterial strains used for quality control.

2. Broth dilution testing

Both antibiotics and reserpine solutions were prepared to the concentrations that ranged from 1024 µg/mL through 16 µg/mL. As in the agar diffusion tests, the bacteria suspension were prepared at the equivalent opacity of 0.5 McFarland from fresh bacterial population. Into each well of the sterile 96-wells plate (in triplicate), 100 µL of sterile MH broth were dispensed first. Into the first wells of the upper line, 100 µL of the antibiotic (at 1024 µg/mL) was added, reducing thereby the concentration to 512 µg/mL in the first raw for each antibiotic. A serial dilution was thereafter conducted by transferring 100 µL of the conveniently mixed solution from one well into the next, right to the well number eleven. Next, 100 µL of a fresh bacterial suspension was added to each of the antibiotic concentration (from 512 through 16 µg/mL). The positive, negative and test controls consisted respectively of broth plus 100 µL of bacterial suspension, broth plus antibiotic at 1024 µg/mL and broth plus 100 µL sterile distilled water. All preparations were incubated overnight then revealed with addition 15 µL of 0.2% INT per well. Color changing to pink indicated positive tests, referring to bacterial growth (absence of inhibition).

The MIC was identified as the lowest of the series of the antibiotic dilutions that did not allow bacterial growth (that is, the concentration at which the pink color was not recorded upon addition of reserpine).

3. Reserpine testing

The MH broth and antibiotics were prepared as indicated in the above antibiotic test alone. In addition, 50 µL of reserpine at the sub-inhibitory concentration (128 µg/mL) were successively dispensed in all suspensions (final reserpine concentration = 32 µg/mL), except the negative and the test control wells. Incubated at 37°C overnight, above conditions were observed to detect growth or growth inhibition upon completion of incubation.

4. Minimal bactericidal concentration

To address this parameter, sub-cultures from the wells in which no growth was reported were performed on Mueller Hinton agar. For this, 5 µL of the conveniently mixed well's content was streaked on MH agar in Petri dish and allowed to incubate aerobically for 24 hours at 37°C. Absence of significant growth (< 3 colonies) indicated bactericidal potential of the antibiotic/reserpine combination. Efflux pump expression was then characterized by decrease in the antibiotic MIC. Otherwise, a strain was regarded as EP-positive when the MIC value in the test with reserpine was significantly lower than the one recorded when the antibiotic was used alone.

RESULTS

From the total of 95 multidrug-resistant isolates, 51 expressed resistance to at least 65% of antibiotics and were further submitted to the antibiotic/reserpine (A/R) test. They included *Staphylococcus* (9), *Enterococcus* (05), *Bacillus* (03) *Pseudomonas* (02) and *Enterobacteriaceae* (32). After addition of reserpine to the standard disks, significant increased diameters was recorded with Gentamicin (70 % of the isolates), Erythromycin (67 %) Co-trimoxazole and Penicillin (60 % each), Ciprofloxacine and Rifampicin (57 % each), and Tetracyclin (53 %). Table I provides detailed information about the 30 isolates for which increased inhibition diameters were recorded with the antibiotic/reserpine association. Out of these, *Enterobactereaceae* and *Staphylococcus* represented 70% and 20% of the total.

Table I: Rates of isolates in which increased inhibitory diameter was recorded after addition of reserpine to standard antibiotic disks

Bacteria	N	Antibiotic Resistance rates (%)																	
		PG	AX	AMP	AMC	CX	IPM	CXM	CRO	CAZ	TE	CN	E	CIP	OFX	VA	RP	SXT	F
Staphylococcus	**6**	67	33	67	50	50	17	67	17	50	50	67	67	83	50	50	17	67	17
Enterobacteria	**21**	62	52	33	52	29	43	24	43	33	48	71	62	48	43	43	62	57	33
Bacillus	**1**	0	0	0	0	0	0	0	0	0	100	100	100	0	100	0	100	100	0
Pseudomonas	**2**	50	50	50	50	100	100	100	50	0	100	50	100	100	0	100	100	50	50
Total	**30**	**60**	**47**	**40**	**50**	**37**	**40**	**37**	**37**	**33**	**53**	**70**	**67**	**57**	**43**	**47**	**57**	**60**	**30**

PG: Penicillin (10 U); AX: Amoxicillin (30µg) ; AMP; Ampicillin 2 µg; AMC: Amoxicillin/ Clavulanic Acid (20/10 µg); CX: Cloxacillin (1µg); IPM : Imipenem (10µg); CXM : Cefuroxime (30µg); CRO: Ceftriaxone (30µg); CAZ: Ceftazidime (30µg); TE: Tetracyclin (30µg); CN: Gentamicine (10µg); E: Erythromycin (15µg); CIP: Ciprofloxacin (30µg); OFX: Ofloxacin (5 µg); VA: Vancomycin (30µg); RP:Rifampicin (5µg) ; SXT : Co-trimoxazole (1.25/23.75); F: Nitrofurantoin (5 µg); N: frequency

For the choice of antibacterial agents to be used on these major groups, micro-dilution test results for MIC values were recorded and displayed as presented in table II. In this test reserpine was used at the sub-inhibitory concentration.

Table II: Micro-dilution tests on selected isolates

Drug Family	drug	Bacteria			
		***Klebsiella* spp**	***Escherichia adecarboxylate***	***Staphylococcus* spp**	***Enterobacter aerogenes***
Beta-lactams	Penicillin G	-	-	-	-
	Amoxicillin	-	-	-	-
	Ampicillin	-	-	-	-
	Cefuroxim	-	-	-	-
	Cefixim	-	-	-	-
	Ceftriaxon	-	-	-	-
Aminosides	Gentamicin	64	128	8	8
Cyclins	Tetracyclin	128	128	128	128
Macrolides	Erythromycin	-	128	256	256
	Azythromycin	64	32	8	16
Quinolones	Ciprofloxacin	32	16	32	32
	Ofloxacin	64	32	128	16
Phenicol	Chloramphenicol	-	-	-	-
Nitro-imidazol	Metronidazol	-	-	-	-
FPI	Reserpine	-	-	-	-

EPI: Eflux pomp inhibitor; -: no inhibition observed

Overall five antibiotics revealed higher inhibitory potential on all the tested isolates. These included Gentamicin, Tetracyclin, Azythromycin, Ciprfloxacin and Ofloxacin. Good potential was also recorded for Erythromycin on three out of the four isolates under study. This activity was improved with Gentamicin, Azythromycin, Ciprofloxacin and Ofloxacin.

Used in combination with reserpine, the tests with the five antibiotics that showed inhibitory effect within the concentrations range on 21 potential EPP isolates yielded data that were summarized as presented in table III.

Tableau III: Antibiotic/reserpine association on selected isolates

Genus	N	Azythromycin			Gentamicin			Tetracyclin			Ciprofloxacin			Ofloxacin		
		Inc	Red	Unc	Inc	Red	Unc	Inc	Red	Unc	Inc	Red	Unc	Inc	Red	Unc
Staphylococcus	6	2	1	3	1	3	2	4	1	1	1	2	3	2	3	1
Enterobacter	7	3	1	3	1	2	4	1	2	4	1	4	2	1	3	3
Escherichia	4	0	1	3	2	1	1	0	2	2	1	2	1	0	3	1
Klebsiella	4	2	1	1	3	1	0	2	1	1	3	0	1	2	1	1
Total	21	7	4	10	7	7	7	7	6	8	6	8	7	5	10	6

N: Frequency; MIC: Minimal inhibitory Concentration; Inc: increased; Red: Reduced; Unc: Unchanged

The EPP phenotype was most frequently recorded on isolates that belonged to the genus *Staphylococcus* with the highest rate observed with fluoroquinolones (Ciprofloxacin and Ofloxacin). This picture was similar with other bacterial types. More detail in bacteria susceptibility revealed no significant changes from one bacterial group to the other, while Gentamicin and Tetracyclin were more effective on *Staphylococcus* and *Escherichia*, respectively. Improved effectiveness of Ciprofloxacin and Ofloxacin was also frequently reported amongst members of the *Entrerobacteriaceae* family namely *Enterobacter* and *Escherichia*.

Ofloxacin was also the most effective antibiotic in the presence of reserpine. In fact, close to 56% of *Staphylococcus* and 54% of members of the *Enterobacteriaceae* family expressed EP against at least one antibiotic. Further and in decreasing order, these isolates belonged to the genera *Enterobacter*, *Escherichia* and *Klebsiella* with 78%, 57% and 25% of the strains, respectively.

DISCUSSION

The MDR rate was very high in the study bacterial population. Though yet to be clearly addressed, this tendency could be substantiated with activities in connection with human medicine, but better in animal husbandry and crop production; with regards to increased use of antimicrobial agents these recent years. In fact, the paradigm of “gene set” with advents of stochastic lateral transfer of genomic traits generated by the selection

pressure imposed in these domains can reliably be used as clue.

Primarily recognized as potentiated by misuse of drugs in human, resistance selection and spread evolves as a natural phenomenon that aims at improving fitness. Their amplification by human activities can undermine therapeutic efforts in clinical settings and in communities. Resistance in bacteria was first observed in the 1945s while its selection and dissemination were substantiated in the 1957- 1961s; when investigation results revealed that selected strains were extremely versatile and tended to lose their resistance when they underwent multiple sub-cultures in the absence of antimicrobials.[4]

In the present investigation, higher resistance rates were recorded with antibiotics that act and affect the bacterial cell wall. These findings could, accordingly, be expected because these drugs are also most available and affordable. However, these conclusions should not rule out advents of co-resistance that is known to emerge through mobilization of genetic elements like plasmids, transposons and integrons amongst both related and genetically distinct bacterial populations when other unrelated selective agents are used.[4,8,9] The indiscriminate use of Celbenin in the 1961s was shown to select resistance strains of *Staphylococcus pyogene* to Penicillin.[10] In the present study, no activity was obtained with Chloramphenicol, Metronidazole and β-lactams (Penicillin, Amoxicillin, Ampicillin, Cefuroxime, Cefixime and Ceftriaxone) when they were used at the highest values of the dilutions (256 mg/mL). Most frequent isolates belonged four major genera: *Staphylococcus, Klebsiella, Escherichia* and *Enterobacter*. These are also the most common etiologies of bacterial infections.[11,12] Their predominance is partly associated with the non stringent (less resource demanding) characteristics that favor their dissemination. It is also known that most lateral gene transfer is favored by bacterial density amongst pathogens and non-pathogens.[9] Addition of reserpine on standard antibiotic disks appeared to be not effective as EPP selection criteria. In fact, some strains for which increased diameter were observed yielded contradictory results in micro-dilution assay (the MIC rather increased). This may be explained by the fact that the agar diffusion of reserpine might not be effective. Actually, the role of reserpine rather appeared heterogeneous because, it did not improve inhibition in 36%, and seemed to inhibit the action of the antibiotic used in combination in 31% of the tested strains. Real inhibition was, however, recorded in 47% of cases.

With regards to the fact that strains of interest were the ones on which either the inhibition diameters increased or reduced MIC was obtained upon addition of reserpine, the diversity of strains on which improved antibacterial activity was recorded with addition of reserpine and different levels of activity for several antibiotics on the same strain might be consistent with the ubiquitous and flexible nature of efflux pumps.[13] Otherwise, reserpine would act differently from one efflux pump protein to the other, regardless of substrate specificity.[14–17] Accordingly, findings from the present investigation are consistent with those reported by previous authors on *E. coli* and *Klebseilla* with fluoroquinolones and Gentamicin which were potent in detecting EPP strains.[18,19] Similar findings were reported with Gentamicin on *E. coli*.[19] In similar investigations, De Marrco *et al.* 2007[20] and Costa *et al.* (2016)[21] observed that resistance phenotypes in *Staphylococcus* were commonly associated with efflux pumps expression. These findings agree with developments recognizing resistance as a multi-factorial process in which different effectors actually operate at different amplitudes.

Because they may vary in their structure and activity, EP proteins behavior may be affected by some fluoroquinolones and not by the others.[14,22] In this regards and in connection with the findings from the current survey, reserpine likely adhere differently to targets that are otherwise diverse in their configuration, either by inhibiting the energy source, competiting with antibiotics for its extrusion, or reducing the antibiotics affinity for the EP targets.[22]

The reduced efficacy (higher MIC values) obtained with bacteria from the *Enterobacteriaceae* family like *Klebsiella* would reflect the presence of resistance mechanisms other than EP.[14] In Tehran, Pakzad *et al.*,(2013)[23] reported 77 % EPP in *Klebseilla* isolates which expressed resistance to Ciprofloxacine, Tetracyclin, Ceftazidim and Gentamicin.[23] Though yet to be fully understood, the putative role of complex mobile genetic elements could be anticipated in most resistance phenotypes selection and spread.

In this regards, Nikaido and Takatsuka (2009)[24] reported the presence of the *AcrAB-TolC* complex as responsible for EP expression in *E. coli.* It is also alleged that this *Acr AB-TolC* complex would be associated with extrusion of large numbers of drugs from the bacteria cytoplasm and periplasm. Other related genes include the *AcrD* which is thought to extrude Gentamicin from *E. coli* cell.[19,25] Likewise, *Staphylococcus* resistance to fluoroquinolones is likely mediated by the *NorA, NorB, NorC, MepA* and *MdeA* genes, consistent with the polymorphism of transporters as stated earlier, and with advents of cross- and co-selection favored by complex mobile genetic elements like plasmids and transposons as components of the "tool set".[21] The role of a particular determinant is difficult to address accurately, but might be unnecessary or, of little importance in instances of resource limitation. Rather, phenotypic characterization of resistance expressed by bacteria would appear paramount in such contexts to guide drug association in the course of an infection. It is clear that EP is polymorphic and ubiquitous in bacterial population. Like other resistance mechanisms, their selection is most

likely exacerbated by avoidable conducive factors including those cited earlier in the present discussion. It is unclear whether or not the high resistance to beta-lactams that couples with absence of inhibition upon addition of reserpine in the present work implies that resistance to beta-lactams does not involve EP. In line with above development on the polymorphic feature of the proteins involved in EP and the large varieties of documented EP potential inhibitors, study on beta-lactam resistance with several other EP inhibitors is necessary and will be major focus in future research projects based or related to investigations through the mechanisms used by bacteria to tolerate the presence of antibiotics.

CONCLUSION

The present investigation through resistance revealed that 47% of multidrug-resistant bacteria isolates under study expressed the efflux pump phenotype. This phenotype was also frequently detected in *Enterobacter* (78%), *Escherichia* (57%), *Staphylococcus* (56%), and *Klebsiella* (25%). The rates of detection varied with the antibiotic used, but Ofloxacin, Ciprofloxacin and Gentamicin were more effective for all the strains submitted to the study.

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