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The Role of Haemolysin Transport System in Antimicrobial Resistance of Haemolytic Strains of *Escherichia coli* and the Effect of Potential Efflux Inhibitors

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The study aimed to investigate the role of haemolysin transport system in resistance of haemolytic strains of *Escherichia coli* to antimicrobials and biocides and the effect of some potential efflux inhibitors on their susceptibility. Twenty clinical isolates of *E. coli* were tested for haemolysin production and susceptibility to different antimicrobial agents. High haemolysin activity was demonstrated in twelve isolates that correlated with multiple drug resistance. Leaky mutants comprising seven haemolytic and three non-haemolytic mutants were derived, by UV mutagenesis, from one isolate demonstrating concomitant high antimicrobial resistance and haemolytic activity. The leaky haemolytic mutants demonstrated increased susceptibility to antimicrobial agents and biocides, compared with the parent isolate. The non-haemolytic leaky mutants exhibited further increase in susceptibility compared with haemolytic mutants. The increase in susceptibility was high for ethidium bromide (EB), rhodamine123, azithromycin, crystal violet, ciprofloxacin, proflavine, tetraphenyl arsonium, busulphan, and erythromycin. Efflux pump inhibitors markedly increased the susceptibility of parent isolate and the haemolytic leaky mutant to antimicrobials, but had little effect on that of non-haemolytic mutant. Dinitrophenol (DNP) and N, N-dicyclohexylcarbodiimide were the most active in lowering MICs, followed by reserpine, verapamil and promethazine, while ascorbic acid, phenothiazine, retinol and selenium were the least effective. The efflux pump function was tested by EB accumulation test. The non-haemolytic mutant accumuled more EB compared with the haemolytic mutant and the parent isolate that accumulated EB only in presence of DNP. The present data reveals a positive role of haemolysin protein transporter in resistance to unrelated antimicrobial agents and the potentials of tested chemicals in inhibiting such effect.

Key words: Bacteria, *Escherichia coli*, Haemolysin, Efflux inhibitor, antimicrobial agent, biocide, resistance.

Resistance to antimicrobials is mediated by several mechanisms including: i) enzymatic inactivation, ii) reduction of uptake, iii) alteration of drug targets, and iv) efflux by multidrug transporters[1,2]. Multidrug efflux pumps or transporters are membrane proteins that recognize structurally unrelated toxic compounds and pump them out of cells, and hence they are responsible for multidrug resistance[3].

Haemolysin is among the virulence factors of uropathogenic *E. coli*[4]. Haemolysin secretion machinery consists of two inner membrane proteins, haemolysin B (HlyB) and haemolysin D (HlyD), a linker protein of the membrane fusion family (MFP), which is involved in release of haemolysin from the outer membrane and an outer membrane protein, TolC[5].

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The haemolysin B, which is responsible for extrusion of haemolysin protein, is a transport protein of the ATP-binding Cassette family (ABC) that is related to resistance to toxic compounds[6].

Inhibition of multidrug efflux pumps by multidrug efflux inhibitors can increase the intracellular accumulation of drugs or biocides inside resistant bacterial cells, and hence restore the activities of these compounds[7-10]. The present study aimed to investigate (i) any possible correlation between haemolysin protein transporter of uropathogenic *E. coli* and resistance to antimicrobial and cytotoxic agents, and (ii) the effect of some potential efflux inhibitors including some natural antioxidants (like ascorbic acid, retinol and selenium) on resistance of haemolysin-producing *E. coli* to these agents.

MATERIAL AND METHODS

Chemicals and culture media

Tetraphenyl arsonium, ascorbic acid, reserpine, chloropromazine, selenium dioxide, retinol acetate, rhodamine 123, busulfan, and isopropyl-β-D-thiogalacto- pyranoside (IPTG), ciprofloxacin, norfloxacin, cefotaxime, cefoperazone, polymyxin B, gentamicin, chloramphenicol, streptomycin, tetracycline, ofloxacin, azithromycin, erythromycin, 5-bromo-4-chloro-3-indolyl phosphate (X-P), ethidium bromide (EB), sodium N-lauryl sarcosinate and Coomassie Brilliant Blue (CBB) were obtained from Sigma, St. Louis, USA. Dinitrophenol (DNP), and N, N-dicyclohexylcarbodiimide (DCCD) were from Fluka Chemie GmbH, Sigma-Aldrich chemie GmbH, Steinheim, Germany.

Brain heart infusion agar (BHA) and broth (BHB) media, MacConkey agar, Müller-Hinton broth (MHB) and agar (MHA), and trypticase soya broth (TSB) were obtained form Oxoid (Basingstoke, UK). Luria-Bertani (LB) agar was the product of Difco, Detroit, MI, USA).

Microorganisms

Clinical isolates of *Escherichia coli*

Tewnty haemolytic clinical isolates of *Escherichia coli* recovered from urine specimens taken from patients in Zagazig University Hospitals with clinically diagnosed urinary tract infection were used in the study.

Determination of susceptibility to antimicrobial agents

Determination of minimum inhibitory concentration (MIC) by the agar dilution method

The agar dilution method was used according to the NCCLS recommendations[11]. Two-fold serial dilutions of the antimicrobial agent were prepared in MH agar medium. Standardised suspensions of the test organisms (equivalent to the 0.5 McFarland) were prepared from overnight cultures in MHB. Five-microlitre aliqots of the test organisms were spot inoculated onto the agar surface using micropipette (10^4 CFU/spot). The plates were incubated at 37°C for 18 h. The minimum inhibitory concentration was taken, as the lowest concentration of antimicrobial agents at which there was no visible growth of the organism. The results were recorded and interpreted according to NCCLS[11].

Determination of minimum inhibitory concentrations (MIC) by the broth dilution method MICs were determined for antimicrobials and efflux inhibitors by broth microdilution using twofold dilution in MHB medium according to the standard procedures[11].

Haemolysin assay

Quantitative estimation for haemolysin produced by E. coli isolates was performed for isolates according to Shibl and Gemmell[12]. Haemolysin production was followed over 24-hour period in 250 ml of BHI cultures in one-litre flask incubated in an orbital shaker, with shaking speed of 150 rpm. Flasks were inoculated with overnight cultures to give approximate densities of 10^7 CFU/ml. Samples taken at different time intervals (4, 8, 12, 16 and 24 hours) were assayed for haemolytic activity. Two-fold serial dilutions of the culture supernatants after centrifugation were made in a diluent of 3:1 mixture of saline and nutrient broth containing 0.001 % w/v phenylmercuric nitrate. One-half ml of 2% washed erythrocyte suspension in phosphate buffered saline, pH 7.3, containing 10 mM $CaCl_2$ was added and the tubes were incubated for 60 minutes in a shaking water bath at 37ºC. Six replicates were made for each dilution and haemolysis was inspected visually. The highest dilution that caused 50% haemolysis of the tubes

was considered to contain one haemolytic unit per ml (1 HU/ml).

Induction of leaky mutants

A heavy bacterial suspension in saline (approximately of 1.2×10^8 CFU/ml) was exposed to ultraviolet irradiation (254 nm) at a distance of 17 cm in the dark for a time that kills approximately 95% of the cells (from predetermined survivor's curve). Appropriate dilutions of irradiated bacterial suspensions were surface plated on LB agar plates that were incubated for 24-48 h at 37ºC. Plates with 30-100 colonies were served as master plates that were examined for the desired leaky mutants by replica plating (13), on blood agar, MacConkey agar, LB agar containing sub-MIC concentrations of selected antibiotics, or LB agar containing 200 µg/ ml X-P. Leaky mutants that fail to grow on antibiotic containing plates and produce blue colonies with blue halos on media containing X-P[14] were selected.

Effect of sub-inhibitory concentrations of multidrug efflux inhibitors on susceptibility of *Escherichia coli* to antimicrobial and cytotoxic agents

MICs of different antimicrobials and biocides were determined in presence of sub-MIC of each efflux inhibitor (one-quarter of MIC, as determined by broth microdilution method described in Section 2.3.2). Folds decreases in MIC were calculated by dividing the MICs of antimicrobial agents in absence of efflux inhibitor by that in its presence.

Ethidium bromide efflux test

Efflux pump function was tested by observing the accumulation of EB inside *E. coli* cells, in presence or absence of efflux inhibitor, using standard methods[9,15] after modifications. Ten ml of LB broth was inoculated with test organism (10^5 CFU/ml) and incubated for overnight at 37°C. The cells were harvested by centrifugation (8000 rpm for 1-2 minutes) and resuspended in 2ml of 100 mM phosphate buffer (50mM KH_2PO_4 and 50 mM K_2HPO_4). Twenty µl aliqots of EB (1 µg/ ml) were added to cell suspension (ml), either alone or in presence of sub-MIC of DNP. Controls of cell suspensions to which either 25mM (0.4%) glucose, or a drop of toluene was added were included. After 15 min, cells were collected by centrifugation, resuspended in 10 µl of potassium phosphate buffer and 5µl aliquots of each suspension were spotted on the surface of agarose gel and examined over ultraviolet trans-illuminator. Bright fluorescence indicates intracellular accumulation of ethidium bromide.

Outer membrane proteins analysis

The outer membrane proteins were extracted according to Kunin *et al*[16]. Cells grown overnight in 10 ml of TSB with vigorous shaking at 37°C were collected by centrifugation and washed, twice with sterile distilled water, and twice with sterile 0.15 M phosphate buffered saline (pH 7.2) and centrifuged at 3,000 rpm for 15 minutes. Cells were resuspended in 2% sodium N-lauryl sarcosinate, placed in ice bath and sonicated three times each for one minute at 80 Watts with 30 seconds apart. Crude sonicates were centrifuged for 20 minutes at 8,000 rpm and supernatant was analysed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using 12% SDS-polyacrylamide gel (17). Protein aliquots (50 ìl) were diluted 1:1 (v/v) with 2X SDS sample loading buffer and kept for 5-10 minutes in a boiling water bath. Aliquots containing approximately 50-100 µg protein were loaded into the gel. The gel was first run at 10 mA/cm in the stacking gel, until the bromophenol blue tracking dye entered the separating gel, and then the current was raised to 15 mA/cm. The current was shut off when the bromophenol blue tracking reached the bottom of separating gel. The gel was stained by Coomassie Brilliant Blue. A pre-stained multicomponent wide range reference protein marker (Bio-rad laboratories, Hercules, California, USA) with known M.W. was loaded and run parallel with the samples.

RESULTS

Among 20 haemolysin producing *E. coli* isolates, 12 produced high titres (table 1). MICs (µg/ml) of 12 selected antimicrobial agents were determined for the isolates by agar dilution method (Section 2.3.1). The results (table 1) show that all tested isolates were resistant to erythromycin, and chloramphenicol. Most of the isolates showed multiple resistance to tetracycline, cephalosporins, and fluoroquinolones, while two isolates showed complete resistance to all tested antimicrobials except gentamicin.

A highly resistant isolate demonstrating the highest haemolytic activity (isolate No. 14) was used to derive leaky mutants by UV mutagenesis. Leaky mutants demonstrating blue halos around the blue colonies (Fig. 1) on agar medium containing X-P substrate were obtained at frequency of approximately 1×10^{-6}. Ten leaky mutants, representing seven haemolytic and three non-haemolytic were selected for further investigations.

The MICs of the antimicrobial and cytotoxic agents against parent E. coli isolate and its haemolytic and non-haemolytic leaky derivatives, as determined by the agar dilution method (table 2), show that leaky mutants were significantly ($P<0.05$) more susceptible to quinolones, macrolides, crystal violet, proflavine, busulphan, tetraphenyl arsounium, rhodamine 123 and ethidium bromide compared with the parent isolate. Folds decrease in MICs for individual mutants, compared with the parent isolate were calculated by dividing the MIC for the parent by that of mutant (Table 3). The haemolytic leaky mutants demonstrated a decrease in MICs of antimicrobial agents by up to 32 folds, but much higher decrease (up to 256 folds) was obtained in non-haemolytic leaky mutants. On the other hand, the sensitivity of these mutants to hydrophilic agents (e.g., streptomycin) did not show significant decrease in MICs for both parent isolate and the leaky mutants ($P> 0.05$). The effect of leakiness alone or combined leakiness and haemolysin production were determined by dividing the MIC of the parent isolate by that of haemolytic leaky mutant and non haemolytic leaky mutant, respectively (Table 4). The effect of haemolysin production on susceptibility was calculated by dividing the folds decrease in MIC for non haemolytic leaky mutants by that of haemolytic leaky mutants (Table 4).

To test the effect of some potential efflux inhibitors on haemolysin production-linked resistance, the MICs of individual antimicrobial

Table 1. Haemolytic activity and susceptibility of *Escherichia coli* isolates to antimicrobial agents by agar dilution method.

Isolate's No.	Haemolytic activity[a] after		MIC of antimicrobial agents[b] (μg/ml)											
	4h	12 h	TE	CTX	CFP	CIP	NFX	OFX	SM	PX	GM	AZM	ERY	C
1	8	32	32	64	64	0.5	1	1	2	2	1	4	64	128
2	8	32	32	64	64	0.5	1	1	2	2	1	4	64	128
3	32	128	64	128	64	0.5	1	1	2	2	1	4	64	128
4	32	128	64	128	64	4	16	8	2	2	1	4	32	128
5	8	32	64	128	64	8	16	16	2	8	1	8	64	128
6	16	128	64	64	128	4	32	16	2	2	1	8	64	128
7	16	128	32	64	128	8	32	8	64	2	64	8	32	128
8	2	16	1	2	4	0.5	2	0.5	64	2	64	8	32	128
9	16	128	128	128	128	8	32	16	2	2	1	4	64	128
10	32	128	128	64	128	4	32	8	1	8	1	4	64	128
11	4	32	64	64	128	4	16	8	1	4	1	4	64	128
12	8	32	64	64	64	4	16	8	1	64	1	4	64	128
13	32	128	32	64	64	0.5	0.5	0.5	2	2	2	4	32	128
14 (P)	64	256	128	64	64	16	16	8	32	64	2	32	64	128
15	8	32	2	2	2	0.25	0.5	0.5	16	2	1	8	32	128
16	16	128	128	128	64	8	32	16	16	2	2	8	64	128
17	32	128	64	128	128	8	32	16	64	64	2	32	64	128
18	2	16	1	2	2	0.25	2	1	64	64	1	32	64	128
19	16	128	2	0.5	2	0.25	2	1	32	2	1	4	32	128
20	32	128	64	64	64	8	32	16	32	2	2	4	32	128

a; Haemolytic Units per ml. b; CIP, ciprofloxacin; AZM, azithromycin; C, chloramphenicol; SM, streptomycin; CFP, cefoperazone; TE, tetracycline; NFX, norfloxacin; OFX, ofloxacin; CTX, cefotaxime; ERY, erythromycin; PX, polymyxin-B.

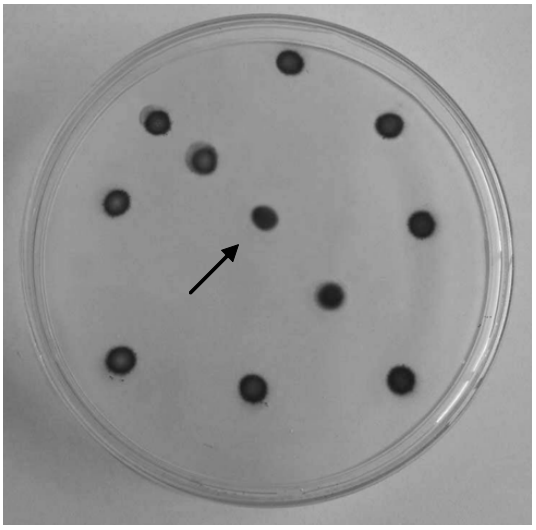

Fig. 1. Demonstration of the appearance of leaky mutants on LB agar plate containing X-P substrate. Heavy cell suspensions were applied onto LB agar plate containing 200 µg/ml X-P. After 18-hr incubation at 37°C, the colonies of leaky mutants appear blue with blue halos. The parent isolate (P 14), indicated by arrow grew as blue colony without halo.

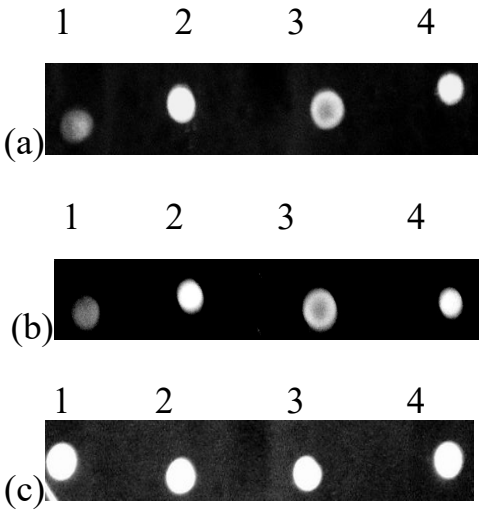

Fig. 2. Effect of dinitrophenol on accumulation of ethidium bromide in the cells of parent isolate (a), haemolytic leaky mutant (b), and non haemolytic leaky mutant (c). Cells treated with EB; (1) in presence of glucose, (2) in the presence of glucose and DNP, (3) in absence of both, and (4) in the presence of toluene.

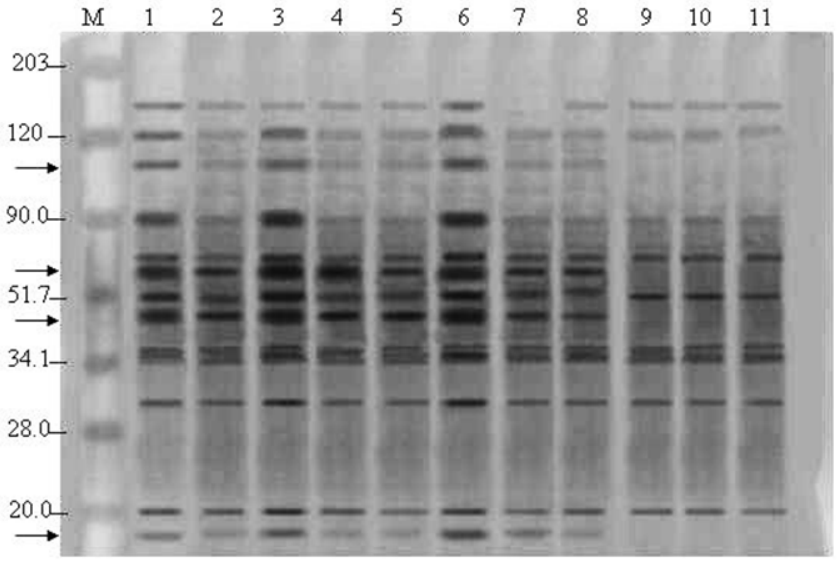

Fig. 3. Gel electrogram of SDS-polyacrylamide denatured gel electrophoresis of OMPs of parent *E. coli i*solate and its haemolytic and non haemolytic leaky mutants. Lane M, prestained molecular weight marker; lane 1, parent isolate (P 14). Lanes 2-8, haemolytic leaky mutants, H1-H7, respectively. Lanes 9-11, non haemolytic leaky mutants N8-N10, respectively.

Table 2. Susceptibility of parent *E. coli* isolate and its haemolytic and non haemolytic leaky mutants to antimicrobial and cytotoxic agents

E. coli strains	Haemolysin production	MIC for antimicrobial agents[b] (µg/ml)																
		EB	AZM	CV	RDH	CIP	PF	BS	ERY	TPA	NFX	OFX	PX	CTX	CFP	TE	C	SM
P 14	+	256	32	512	512	16	512	512	64	256	16	8	64	64	64	128	128	32
H1	+	8	2	64	32	2	32	32	4	16	2	2	8	16	16	32	64	16
H2	+	32	8	128	128	4	128	128	16	64	4	2	16	32	32	64	64	32
H3	+	8	4	64	32	2	32	32	8	32	2	1	16	16	16	32	64	16
H4	+	8	2	32	32	1	32	32	4	32	2	2	8	16	16	32	32	16
H5	+	16	4	64	64	2	64	64	8	32	2	1	8	16	32	32	64	32
H6	+	8	2	32	32	1	32	64	8	32	2	1	8	8	16	32	64	32
H7	+	8	2	32	32	1	32	32	4	16	2	2	8	16	16	32	64	16
N8	-	2	0.5	8	8	0.25	8	8	1	4	0.5	0.25	4	4	4	16	16	16
N9	-	2	0.5	8	8	0.25	8	8	1	4	0.5	0.25	4	4	4	16	16	16
N10	-	1	0.25	4	4	0.25	8	8	1	4	0.25	0.25	4	4	4	16	16	16

a; P 14, parent isolate; H, haemolytic leaky mutant; N , non haemolytic leaky mutant b; CIP, ciprofloxacin; AZM, azithromycin; C, chloramphenicol; SM, streptomycin; CFP, cefoperazone; TE, tetracycline; CV, crystal violet; PF, proflavine; TPA, tetraphenyl arsonium; BS, busulphan; EB, ethidium bromide; RDH, rhodamine 123; NFX, norfloxacin; OFX, ofloxacin; CTX, cefotaxime; ERY, erythromycin; PX, polymyxin-B.

Table 3. Folds decrease in MICs of antimicrobial and cytotoxic agents for leaky mutants compared with parent *E. coli* isolate

E. coli strains	Folds decrease in MIC for antimicrobialsb with reference to parent (MIC of parent / MIC of mutant)																
	EB	AZM	CV	RDH	CIP	PF	BS	ERY	TPA	NFX	OFX	PX	CTX	CFP	TE	C	SM
P 14	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
H1	32	16	8	16	8	16	16	16	16	8	4	8	4	4	4	2	2
H2	8	4	4	4	4	4	4	4	4	4	4	4	2	2	2	2	1
H3	32	8	8	16	8	16	16	8	8	8	8	4	4	4	4	2	2
H4	32	16	16	16	16	16	16	16	8	8	4	8	4	4	4	4	2
H5	16	8	8	8	8	8	8	8	8	8	8	8	4	2	4	2	1
H6	32	16	16	16	16	16	8	8	8	8	8	8	8	4	4	2	1
H7	32	16	16	16	16	16	16	16	16	8	4	8	4	4	4	2	2
N8	128	64	64	64	64	64	64	64	64	32	32	16	16	16	8	8	2
N9	128	64	64	64	64	64	64	64	64	32	32	16	16	16	8	8	2
N10	256	128	128	64	64	64	64	64	64	32	32	16	16	16	8	8	2

a; P 14, parent isolate; H, haemolytic leaky mutant; N , non haemolytic leaky mutant b; CIP, ciprofloxacin; AZM, azithromycin; C, chloramphenicol; SM, streptomycin; CFP, cefoperazone; TE, tetracycline; CV, crystal violet; PF, proflavine; TPA, tetraphenyl arsonium; BS, busulphan; EB, ethidium bromide; RDH, rhodamine123; NFX, norfloxacin; OFX, ofloxacin; CTX, cefotaxime; ERY, erythromycin; PX, polymyxin-B.

Table 4. Increase in susceptibility to antimicrobial agents due to leakiness and/ or lack of haemolysin

Chemical agent	Folds decrease in MIC) due to leakiness[a] (A	Folds decreasein MIC due to leakiness and haemolysin[b] loss (B)	Effect due tohaemolysin excretion[c] (B/A)
Ethidium bromide	8-32	128-256	4-32
Rhodamine 123	4-16	64-128	4-32
Azithromycin	4-16	64-128	4-32
Crystal violet	4-16	64-128	4-32
Ciprofloxacin	4-16	64	4-16
Proflavine	4-16	64	4-16
Tetraphenyl arsonium	4-16	64	4-16
Busulphan	4-16	64	4-16
Erythromycin	4-16	64	4-16
Cefoperazone	2-4	16	4-8
Norfloxacin	4-8	32-64	4-8
Ofloxacin	4-8	32	4-8
Cefotaxime	2-8	16	2-8
Chloramphenicol	2-4	8	2-4
Tetracycline	2-4	8	2-4
Polymyxin B	4-8	16	2-4
Streptomycin	1-2	2	1-2

a; MIC of parent isolate/ MIC of haemolytic leaky mutant.
b; MIC of parent isolate/MIC of non haemolytic leaky mutant.
C; B/A, low limit = the lowest "B" value/ the highest "A" value, the high limit = the highest "B" value/ the lowest "A" value.

agents and biocides for the parent multi-resistant, haemolytic isolate (P14), a most resistant haemolytic leaky mutant (H2), and the most sensitive non haemolytic leaky mutant (N10) in presence and absence of sub-inhibitory concentrations (1/4 MIC) of efflux inhibitors were determined by broth microdilution method (Section 2.3.2). Folds decrease in MICs in presence of inhibitors were calculated for the parent isolate (P14), the non haemolytic leaky mutant (N10) and the haemolytic leaky mutants (H2) by dividing the MIC in absence of inhibitor by that in its presence for individual antimicrobial agents (Table 5). The effects of individual inhibitors on haemolysin export associated resistance was determined by dividing folds decrease in MIC for haemolytic mutant by that of non haemolytic mutant for each antimicrobial agent (Table 6).

The function of efflux pump for the parent isolate (P14), the resistant haemolytic mutant (H2) and the sensitive non haemolytic mutant (N 10) was tested by EB efflux test (Section 2.7). The leaky non-haemolytic (sensitive) mutant accumulated EB even in the absence of DNP, whereas the parent isolate and the haemolytic leaky mutant (resistant) accumulated EB in presence of DNP (Fig. 2).

The outer membrane proteins analysis for the parent isolate (P14) and its haemolytic (H2-H7) and non-haemolytic (N8-N10) leaky mutants (Fig. 3) reveals the absence of some protein bands of approximately 18, 46, 62 and 110 KDa from the non haemolytic mutants.

Table 5. Effect of efflux inhibitors on MIC of biocides against parent *E. coli* isolate and its leaky mutants.

Efflux inhibitor	Strain	Folds decrease in MIC of											
		EB	AZM	CV	RDH	CIP	PF	BS	TPA	CFP	TE	C	SM
Dinitrophenol	P14	32	64	32	16	32	16	16	32	8	2	4	2
	H2	128	64	64	32	128	64	32	64	16	2	4	2
	N10	4	2	2	2	4	2	2	4	2	2	2	2
DCCD	P14	32	32	16	16	32	16	8	32	4	2	4	2
	H2	128	128	32	16	64	32	32	64	8	2	4	2
	N10	4	2	2	2	2	1	2	4	2	1	2	2
Promethazine	P14	32	32	8	16	16	8	8	16	4	2	4	1
	H2	64	64	32	16	32	16	16	32	8	2	4	2
	N10	4	2	2	2	2	2	2	2	2	2	2	1
Reserpine	P14	32	16	8	8	16	16	16	16	4	1	4	2
	H2	64	32	32	16	32	32	32	32	8	2	4	2
	N10	2	2	2	2	2	2	2	2	2	2	2	2
Verapamil	P14	32	16	16	8	8	8	8	16	4	2	4	1
	H2	64	16	32	16	16	16	8	32	8	2	4	2
	N10	2	2	2	2	2	1	1	2	1	1	2	2
Ascorbic acid	P14	16	16	8	8	8	8	8	16	4	1	2	2
	H2	32	16	16	16	16	16	8	32	4	2	4	2
	N10	2	1	2	1	2	2	1	2	1	2	2	1
Phenothiazine	P14	8	16	16	8	8	8	8	8	4	2	2	1
	H2	32	16	16	8	8	16	16	16	4	2	4	2
	N10	2	1	2	1	1	2	2	2	1	2	2	1
Retinol	P14	8	8	8	8	8	8	8	8	4	1	2	1
	H2	32	8	16	8	8	16	8	16	4	2	2	1
	N10	2	1	1	1	2	2	1	1	2	1	1	1
Selenium dioxide	P14	8	8	8	8	8	8	8	8	4	2	2	1
	H2	16	8	16	8	8	16	8	8	4	2	2	2
	N10	1	1	1	1	1	2	2	1	2	1	1	1

P14, parent isolate; H2, haemolytic leaky mutant No. 2 ; N10, non haemolytic leaky mutant No.10 ; DCCD, N,N-dicyclohexylcarbodiimide; EB, ethidium bromide; AZM, azithromycin; CV, crystal violet; RDH, rhodamine123; CIP, ciprofloxacin; PF, proflavine; BS, busulphan; TPA, tetraphenyl arsonium; CFP, cefoperazone; TE, tetracycline; C, chloramphenicol; SM, streptomycin.

Table 6. Effect of efflux inhibitors on haemolysin export system mediated resistance[a] to antimicrobial agents

Efflux inhibitor	Folds decrease in MIC for haemolytic mutants/Folds decrease in MIC for non haemolytic mutants										
	AZM	EB	CIP	CV	PF	TPA	BS	RDH	CFP	C	SM	TE
DNP	32	32	32	32	32	16	16	16	8	2	1	1
DCCD	64	32	32	16	32	16	16	8	4	2	2	1
Promethazine	32	16	16	16	8	16	8	8	4	2	1	2
Phenothiazine	16	16	8	8	8	8	8	8	4	2	1	2
Reserpine	16	32	16	16	16	16	16	8	4	2	1	1
Verapamil	8	32	8	16	16	16	8	8	8	2	2	1
Ascorbic acid	16	16	8	8	8	16	8	16	4	2	1	2
Retinol	8	16	4	16	8	16	8	8	2	2	2	1
Selenium dioxide	8	16	8	16	8	8	4	8	2	2	2	2

a; expressed as folds decrease in MIC for haemolytic mutant/folds decrease in MIC for non haemolytic mutant,
DNP, Dinitrophenol ; DCCD, N,N-dicyclohexylcabrodiimide; EB, ethidium bromide; AZM, azithromycin; CV, crystal violet; RDH, rhodamine123; CIP, ciprofloxacin; PF, proflavine; BS, busulphan; TPA, tetraphenyl arsonium; CFP, cefoperazone; TE, tetracycline; C, chloramphenicol; SM, streptomycin.

DISCUSSION

The haemolysin A of E. coli is known to be excreted by an ABC export system. To investigate any possible role of haemolysin transport system in resistance to antimicrobial agents, twenty haemolytic clinical isolates of E. coli recovered from patients with urinary tract infections were used. The predominance of multiple drug resistance to unrelated antimicrobial agents in most of the isolates would propose the existence of efflux mechanism[18-20].

A positive correlation could be found between haemolytic activity and antimicrobial resistance, thus among 4 isolates (No. 8, 15, 18 and 19) sensitive to tetracycline, cephalosporins and fluoroquinolones tested, all but one (No. 19) were low haemolysin-producer. Also, among the 12 isolates resistant to the above drugs, only 3 (No. 5, 11 and 12) had low haemolytic activity. Furthermore, the most haemolytic isolate (No. 14) was highly resistant to all tested antimicrobials except gentamicin, and three isolates with maximum haemolysin production were also highly resistant. On the other hand, however, two highly haemolysin producing isolates (No. 3 and 13) were sensitive to fluoroquinolones.

All leaky mutants derived from the most resistant isolate No. 14, demonstrated higher susceptibility to the tested antimcirobials compared with the parent isolate**.** The increase in susceptibility of haemolytic mutants, expressed as folds decrease in MIC compared to parent isolate, varied for different agents, being maximum for EB (8-32 folds), then rhodamine 123, azithromycin, crystal violet, ciprofloxacin, proflavine, tetraphenyl arsonium, busulphan and erythromycin (4-16 folds each), but was low for cefoperazone, chloramphenicol and tetracycline (2-4 folds each) with little or no effect was obtained with streptomycin (1-2 folds). This increased sensitivity of leaky mutants to hydrophobic agents could be attributed to increased penetration due to absence of or defect in the outer membrane[21, 22].

The non haemolytic mutants expressed much higher sensitivity compared to the haemolytic ones, confirming a role of haemolysin export system in resistance to such agents. The calculated increase in susceptibility that could

be attributed to lack of haemolysin production was maximum for EB, azithromycin, crystal violet and rhodamine (4-32 folds each) followed by ciprofloxacin, tetraphenyl arsonium, erythromycin, proflavine and busulphan (4-16 folds each), and was intermediate for norfloxacin, ofloxacin and cefoperazone (4-8 folds each) and cefotaxime (2-8 folds). On the other hand, minimum or no of increased susceptibility were obtained with chloramphenicol, tetracycline and polymyxin-B (2-4 folds each), streptomycin and EDTA (1-2 folds each).

Further support to the presumed role of haemolysin in resistance to antimicrobials was inferred from the increased susceptibility of a resistant haemolytic isolate and a haemolytic leaky derivative of it compared with a non-haemolytic mutant to these agents in presence of efflux inhibitors.

Some known efflux inhibitors like DNP, DCCD, and other chemical agents reported to affect efflux pumps like promethazine, reserpine, verapamil, ascorbic acid, phenothiazine, retinol, and selenium dioxide were tried. All tested agents induced increased susceptibility of the parent isolate and the tested mutants to antimicrobial agents by varying degrees.

The effect of potential efflux inhibitors on haemolysin associated resistance was inferred from comparing folds decrease in MICs for leaky haemolytic mutant with that of the non haemolytic mutant in presence of inhibitors. The increased susceptibility to antimicrobial agents in presence of efflux inhibitors although varied with individual agents yet maintained a more or less comparable profile. Some agents were highly affected like EB, azithromycin, tetraphenyl arsonium, crystal violet, proflavine, ciprofloxacin, and busulphan. Some others were fairly affected like, rhodamine 123 and cefoperazone. On the other hand, some other agents were little or not affected like chloramphenicol, tetracycline and streptomycin.

It can be noticed that most antimicrobial agents that were highly or moderately affected by efflux pumps inhibitors are hydrophobic (like EB, tetraphenyl arsonium, rhodamine 123, proflavine, crystal violet, ciprofloxacin, azithromycin and cefoperazone). Some of these compounds are cationic lipophiles (like EB, tetraphenyl arsonium, rhodamine 123, proflavine, and crystal violet) or zwitterionic lipophile (e.g. ciprofloxacin). On the contrary, other agents showing little or not affected are hydrophilic, like tetracycline and streptomycin.

The effect of efflux pump inhibitors on antimicrobial agents varied in potency, thus, DNP and DCCD were the most active efflux inhibitors. Following DNP and DCCD in activity were reserpine, verapamil and promethazine, while ascorbic acid, phenothiazine, retinol and selenium were the least active efflux inhibitors. DNP and DCCD had more or less similar profiles (differed only with crystal violet, rhodamine 123 and azithromycin). Promethazine and phenothiazine also showed more or less similar profiles, but with lesser effect for phenothiazine with some agents (e.g., ciprofloxacin, azithromycin, crystal violet and tetraphenyl arsonium). Both compounds demonstrated somehow similarity in effect with DNP and DCCD. Ascorbic acid demonstrated somehow similar profile to that of phenothiazine, but with higher effect on tetraphenyl arsonium and rhodamine 123. Except for little differences, reserpine demonstrated more or less similar profile to that of DNP, but with lesser effects on some agents (azithromycin, crystal violet, rhodamine 123, proflavine and ciprofloxacin). Likewise, verapamil demonstrated slightly different profile from that of reserpine.
Both selenium and retinol showed more or less similar profiles that were somehow different from that of the previous agents.

In accordance with the present data, it was found that phenothiazine did not potentiate the antimicrobial effect of tetracycline in haemolysin and non-haemolysin producing *E. coli*[23]. In the same line, sub-MICs of promethazine and verapamil were found to increase the sensitivity of tumour cells and haemolysin producing plasmid containing *E. coli* cells[6].

On the other hand, and in agreement with the current results, it was reported that two inhibitors of mammalian P-glycoprotein, reserpine and verapamil completely reversed the multidrug resistance (MDR) and markedly increased the sensitivity of *Bacillus subtilis* to rhodamine, EB, tetraphenyl phosphonium and acetyl trimethyl ammonium bromide[24].

It was also found that sub-MICs of reserpine decreased MICs of norfloxacin, EB and tetraphenyl phosphonium against *Staphylococcus aureus*[9]. Also, it was found that ascorbic acid had marked effect in aborting P-glycoprotein (P-gp) mediated drug resistance in *E. coli* leaky mutant harboring mdr1 gene, and that retinol and selenium produced similar but lesser effects[25]. Reserpine was also shown to dramatically decrease the MICs of unrelated compounds including EB, tetraphenyl phosphonium, rhodamine, benzalkonium chloride, erythromycin and ciprofloxacin by up to 8-fold in *E. coli* expressing P-gp [26].

Furher supporting evidence for the role of haemolysin in resistance was obtained by the DNP-induced accumulation of EB in haemolysin producing parent isolate.

In the present study, the SDS-PAGE of outer membrane proteins (OMPs) showed different major outer membrane proteins (OMPs) ranging from 18 to 162 KDa. Some outer membrane proteins were not present in non-haemolytic mutant derivatives. Four outer membrane proteins bands with M.W. of approximately 18, 46, 62 and 110 KDa were not detected in non-haemolytic leaky mutants. These could be related to the haemolysin transport proteins.

In conclusion, the present study provides several lines of evidence on the role of haemolysin exporting system in efflux to antimicrobial agents and biocides and resistance to such agents. Moreover, several multidrug efflux inhibitors were found to increase the susceptibility to such agents by inhibition of the haemolysin transport system. This conclusion may be support the idea of combining drugs with potenial efflux pump inhibitors with antimicrobial and anticancer agents to combat resistance.

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