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INHIBITION OF SWARMING BY UREA AND ITS DIAGNOSTIC IMPLICATIONS AMONG UROPATHOGENIC PROTEUS SPECIES FROM LAGOS, NIGERIA

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The anti-swarming property of urea and effects on antibiotic susceptibility among 52 uropathogenic *Proteus* strains from Lagos, Nigeria were investigated. Urea caused a reduction in swarming and number of swarmed cells at 0.5% (n = 42, DOCZ = 15.5mm), 0.75% (n= 24, DOCZ = 10.7mm), 1% (n = 17, DOCZ = 3.4mm) and 1.25% (n = 8, DOCZ = 1.7mm). Compared to DOCZ obtained at 0.5% urea, the further reduction in DOCZ at other urea concentrations was found to be significant ($p < 0.05$). Urea at less than 0.75% allowed identification of *E. coli, K. pneumoniae and S. saprophyticus* in mixed cultures containing *Proteus spp,* while colonies of *Pseudomonas aeruginosa* were distinctly identified at 1% urea with swarming restrained at 1.25% urea. At 1.25% urea, antibiotic susceptibility testing by agar diffusion method revealed significant increase and decrease in the number of *Proteus* strains that showed resistance to amoxicillin and nitrofurantoin. Compared with the control, significant increases in the MICs of gentamicin or nitrofurantoin and streptomycin were found at ≥ 0.5% and ≥ 0.75% urea respectively ($p<0.05$). The identification of extended spectrum beta lactamases (ESBL) producing strains were unaffected by urea. This study has demonstrated urea induced swarming inhibition of uropathogenic *Proteus in vitro.* However, results suggest the use of urea with great caution in diagnostic practices for optimal clinical and public health benefits in Nigeria.

Keywords: Antibiotic susceptibility, anti-swarming, urea, uropathogenic proteus, Nigeria, DOCZ (= Diameter of outermost colony zone mean value)

INTRODUCTION

Proteus spp are Gram-negative facultative anaerobic rods of tremendous clinical and public health importance particularly in developing countries (1). These bacteria are frequently implicated as aetiologic agents of urinary tract infections (UTI), which may lead to kidney damage and complicate pregnancies if untreated (2, 3). In Nigeria, several hospital and community based studies have put the isolation rates of *Proteus spp* at 3-8%, with *Proteus mirabilis* and *P. vulgaris* as predominant nosocomial pathogens among patients with indwelling catheters, benign prostatic hypertrophy, and vesicoureteral reflux (4, 5, 6). Of great concern is the mortality rate report of 17.1% in African neonates with UTIs that are untraceable by the radiologic examination of the urinary tract (7).

One of the unique characteristic features of *Proteus spp* on tolerable culture media is the ability to spread and form a thin film with distinct colonial zones on agar surfaces. This phenomenon called swarming, involves the differentiation of vegetative motile cells to hyperflagellated-elongated cells capable of coordinated and

concerted mass population migration (8). The susceptibility of catheterized patients to *Proteus* associated UTI has been demonstrated in vitro using Foley catherters (9). Swarming has also been shown to be one of the requirements for the colonization of urothelial cells *in vivo* and *in vitro* (10, 11). One of the drawbacks of *Proteus* swarming in the laboratory is the inherent difficulty in the detection of other pathogens in polymicrobial infection cases. Fons *et al* (12), reported the difficulty in distinguishing colonies of *Pseudomonas aeruginosa* among *P. mirabilis* swarm cells on agar plates.

In routine diagnostic laboratories, the use of nutrient and blood agar media for *Proteus* culture storage and antibiotic susceptibility testing, as practiced in developing countries, may compromise purity of stocks for genetic studies and many cases of polymicrobial infections involving *Proteus* may not be noticeable. This may hinder the use of drug combination to implement effective clinical cure, provide a reason for the incidence recurrent bacteruria in treated patients and promote antibiotic resistance of these 'silent' organisms. False antibiotic susceptibility outcome of pathogens *in vitro* also contributes to the spread of drug resistant strains in a community and promotes clinical failure.

Urea, P-nitrophenylglycerol (PNPG), and activated charcoal have experimentally been demonstrated to possess anti-swarming properties and recommended for routine laboratory usage (12,13,14). However the use of PNPG in culture media for antibiotic susceptibility testing has been queried (15). In Nigeria, urea is commonly used in culture media designed for the identification of pathogens of UTIs including *Proteus spp* (4, 5). However, reports have been silent on *Proteus* swarming prevention possibilities and consequences on antibiotic susceptibility outcome. This study investigated the clinical importance associated with the use of urea in *Proteus* identification media in terms of pattern of swarming inhibition, the effect on antibiotic susceptibility and extended spectrum beta lactamase (ESBL) classification.

MATERIALS AND METHODS

Bacterial strains

Fifty-two *Proteus spp* isolated from randomly selected 408 mid stream urine samples of in-patients and outpatients at different clinics and hospitals in Lagos, were used in this study. The isolates were identified on McConkey, Blood agar and composite media using criteria which included non-lactose fermentation, swarming ability,

urease and phenylalanine deaminase production (16). For mixed culture assay, pure strains of *P.aeruginosa, Klebsiella pneumoniae, Staphylococcus saprophyticus* and *E. coli* were obtained from the Microbiology Laboratory of the Nigerian Institute of Medical Research (NIMR), Lagos.

Swarming inhibition assay

Nutrient agar plates containing 5% sheep erythrocytes and supplemented with and without 0.5-1.25% urea (Sigma, USA) were used to grow the selected *Proteus* strains. A loopful of standardized inoculum (2×10^4 CFU/spot) of each strain was concentrically inoculated at the center of the agar and incubated at 37°C for 24 hours under aerobic conditions. Urea negative plates were used as controls. The swarming profile of *P. mirabilis* ATCC49565 was examined in parallel with those of the test organisms. The degree of swarming was measured as the diameter of the outermost colonial zone (DOCZ). DOCZs were interpreted as mean ± standard deviation, to allow statistical deductions using student's t- test and chi-square analysis. P value less than 0.05 was indicated as significant.

Mixed culture assay

Four swarmed isolates of the tested *Proteus spp* were rapidly selected and suspended in Mueller-Hinton broth containing at least any two of *P.aeruginosa, Klebsiella pneumoniae, Staphylococcus saprophyticus* and *E. coli.* The resulting mixed cultures were then used to inoculate Blood agar supplemented with urea (0.5-1.25%). Mixed culture plates containing a reference swarming strain of *P. mirabilis* ATCC49565 was also examined. Mixed culture plates without urea also served as control.

Antibiotic susceptibility testing

The response of untreated *Proteus* isolates and urea survivors to 13 antibiotics used in Nigeria was investigated by agar diffusion method according to Bauer *et al* (17). The antibiotics from Abtek Biological Limited, Liverpool, England mounted on inoculated plates were tetracycline 10 µg, ampicillin 25 µg, amoxicillin 25 µg, co-amoxyclav 30 µg, cotrimoxazole 25 µg, nitrofurantoin 200 µg, ceftazidime 30 µg, ceftriazone 30 µg, nalidixic acid 30 µg, streptomycin 25 µg, gentamicin 10 µg, ciprofloxacin 10 µg, and ofloxacin 5 µg. Diameters of zones of inhibition were measured to the nearest millimeters and values were interpreted as resistant and susceptible based on comparison with antibiotic susceptibility profile of *E. coli* ATCC 25922, a drug sensitive strain.

Determination of minimum inhibitory concentration (MIC) and selection of Extended Spectrum Beta Lactamase (ESBL) producing strains

The MICs of the selected antibiotics were determined by a microdilution method. *Proteus* strains from urea containing and deficient plates were first grown overnight on cysteine lactose electrolyte deficient (CLED) agar at 37°C under aerobic condition. Four distinct colonies of each strain were then subcultured in 4 ml of IsoSensitest broth (Oxoid, UK) and incubated at 37°C to attain a turbidity that was adjusted to 10^6 CFU/ml with the broth. Stock solutions of the antibiotics were prepared fresh at 128 mg/L for ampicillin, amoxycillin, co-amoxiclav, nitrofurantoin, streptomycin and tetracycline; 64 mg/L for cotrimoxazole; 32 mg/L for nalidixic, acid, gentamicin, ceftazidime and ceftriaxone; and 4 mg/L for ciprofloxacin and ofloxacin. In a 12 by 8 wells tray, 50 µL of antibiotic solution occupied the first two rows and subsequently double diluted to fill the remaining rows except the 12^{th} row, which served as a positive control. 50 µl of inoculum at 5 x 10^5 CFU/well were then added to the wells in increasing order of antibiotic concentrations. Antibiotic susceptibility of a control strain of *P. mirabilis* ATCC 49565 was examined in parallel with test organisms. All the plates were sealed and incubated at 37°C for 24 hours. Growth was assessed as turbidity observed on transillumination. The MIC of each antibiotic was defined as the lowest concentration that inhibits growth. Interpretation of MICs as resistant or susceptible was in line with NCCLS break points of the antibiotics tested (18). The significance of the mean MIC value differences between urea treated *Proteus* and untreated isolates was also evaluated statistically. Extended beta-lactamase producing strains were selected as those with ceftazidime: ceftazidime-clavulanate ratio greater than or equal to 16 according to Livermore and Yuan (19). Values of 8 were regarded as indeterminate.

RESULTS

Survival and variations in the ability of urea at 0.5- 1.25% to refrain swarming of *Proteus* isolates were highlighted in Figure 1. All the *Proteus* strains studied survived urea at 0.5-1.25% concentrations. At 0.5% urea, 42 out of the 52 isolates swarmed and produced an average diameter of outermost colony zone (DOCZ) value of 15.5 mm. At 0.75% urea, 24 isolates swarmed producing mean DOCZ value of 10.7 mm. The number of swarmed *Proteus* further decreased

from 17 to 8 following 1-1.25% urea treatments. Mean DOCZ values of 3.4 mm and 1.7 mm were produced respectively. Urea at 0.5% was observed to allow distinct identification of *E. coli* and *K. pneumoniae* in mixed culture assay. *Staphylococcus saprophyticus* colonies were identified at 0.75% urea while at 0.75 and 1%, *Pseudomonas aeruginosa* was identified, with swarming inhibited (Table 1).

However, the number of cells that displayed resistance to amoxicillin, gentamicin, nitrofurantoin and ofloxacin by disk diffusion method at 1-1.25% urea differed (Table 2) and those of amoxicillin and nitrofurantoin were statistically significant ($p<0.05$). Table 3 summarized data for MICs of the 13 antibiotics tested. Significant increases in the MICs of nitrofurantoin or gentamicin, and streptomycin were obtained at ≥ 0.5 and ≥ 0.75% urea respectively ($p<0.05$). Furthermore, two *Proteus* strains were identified as extended beta lactamases producers in plates devoid of urea and those containing 0.5 - 1.25% urea. However, no isolate was identified as indeterminate for ESBL production among the strains cultured without urea, whereas, between one and two indeterminate identifications were recorded among the urea treated strains (Table 4).

Table 1: Isolates identification from mixed cultures containing swarmed *Proteus* strains.

Mixed culture assay	Isolate identification scheme
1.	*Proteus* strains
2.	*Proteus* strains, *E. coli, K. pneumoniae,*
3.	*Proteus* strains, *E. coli, K. pneumoniae, S. saprophyticus, P. aeruginosa*[a]
4.	*Proteus* strains, *E. coli, K. pneumoniae, S. saprophyticus, P. aeruginosa*[b]
5.	*Proteus* strains, *E. coli, K. pneumoniae, S. saprophyticus, P. aeruginosa*[c].

Keys: 1 = Assay without urea; 2 = Assay containing 0.5% urea; 3 = Assay containing 0.75% urea; 4 = Assay containing 1% urea; 5 = Assay containing 1.25% urea. *a, P. aeruginosa* not distinctly identified; *b, P. aeruginosa* swarmed cells identified distinctly; *c, P. aeruginosa identified without swarming* .

Table 2: Resistance to antibiotics in the presence and absence of urea by the 52 Proteus strains using disk diffusion method.

	Proteus spp.				
	E	a	b	c	d
Antibiotics	n (%)	n (%)	n (%)	n (%)	n (%)
Ampicillin	32 (61.5)	32 (61.5)	32 (61.5)	32 (61.5)	32 (61.5)
Amoxicillin	31 (59.6)	31 (59.6)	31 (59.6)	31 (59.6)	36 (69.2)*
Co-amoxiclav	29 (55.8)	29 (55.8)	28 (53.8)	28 (53.8)	28 (53.8)
Cotrimoxazole	48 (92.3)	48 (92.3)	48 (92.3)	48 (92.3)	46 (88.6)
Ceftazidime	3 (5.8)	3 (5.8)	3 (5.8)	3 (5.8)	3 (5.8)
Ceftrixone	3 (5.8)	3 (5.8)	3 (5.8)	3 (5.8)	3 (5.8)
Ciprofloxacin	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Gentamicin	2 (3.8)	2 (3.8)	5 (9.6)	5 (9.6)	7 (13.5)
Nalidixic acid	2 (3.8)	2 (3.8)	2 (3.8)	2 (3.8)	2 (3.8)
Nitrofurantoin	7 (13.5)	7 (13.5)	7 (13.5)	6 (11.5)	4 (7.7)*
Ofloxacin	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Streptomycin	34 (65.4)	34 (65.4)	34 (65.4)	32 (61.5)	32 (61.5)
Tetracycline	50 (96.2)	50 (96.2)	50 (96.2)	50 (96.2)	50 (96.2)

Keywords:Urea supplementation: (E, without urea; a, 0.5% urea; b, 0.75% urea; c, 1.0% urea; d, 1.25% urea), n (%), number and percentage of antibiotic resistant strains.
* = significant at 95% confidence limit by chi square analysis.

Table 3: Minimum inhibitory concentrations of antibiotic resistant Proteus strains by microbroth dilution method.

	Proteus spp. MIC (mg/L)				
	E	a	b	c	d
Antibiotics	n ( )	n ( )	n ( )	n ( )	n ( )
Ampicillin	32 (75+33.1)	32 (74+33.9)	32 (76+32.3)	32 (76+32.3)	32 (77+34.3)
Amoxicillin	33 (81+33.5)	33 (81+33.5)	33 (82+32.4)	33 (83+34.3)	33 (80+31.5)
Co-amoxiclav	29 (77.2+33.7)	29 (77.2+33.7)	29 (79.4+34.9)	31(79.5+35.9)	31 (80.5+31.9)
Cotrimoxazole	48 (82.7+33.6)	48 (83.3+32.8)	48 (84+34.1)	48 (84.7+33.3)	48 (84+34.1)
Ceftazidime	3 (18.7+12.2)	3 (21.3+9.2)	3 (18.7+12.2)	3 (21.3+9.2)	3 (26.7+9.2)
Ceftrixone	3 (21.3+9.2)	3 (26.7+9.2)	3 (26.7+9.2)	3 (21.3+9.2)	3 (21.3+9.2)
Ciprofloxacin	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Gentamicin	2 (8.2+1.8)	2 (12.0+5.7)*	5 (20.8+10.7)*	5 (22.4+8.8)*	7 (21.7+10.0)*
Nalidixic acid	2 (12.0+5.7)	2 (12.0+5.7)	2 (16.0+0.0)	2 (12.0+5.7)	2 (12.0+5.7)
Nitrofurantoin	7 (59.4+34.2)	7 (73.1+40.1)*	7 (82.3+31.2)*	7 (73.1+40.1)*	7 (86.9+40.1)*
Ofloxacin	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Streptomycin	35 (85.9+33.6)	35 (85.9+33.6)	35 (90.5+33.3)*	35 (91.4+32.1)*	35 (92.3+33.6)*
Tetracycline	50 (90.9+33.8)	50 (90.9+33.8)	50 (92.2+34.0)	50 (92.2+34.0)	50 (92.2+34.0)

Keywords:Urea supplementation: (E, without urea; a, 0.5% urea; b, 0.75% urea; c, 1.0% urea; d, 1.25% urea), n ( ), number of antibiotic resistant strains, numbers in parentheses are mean ± SD of MIC values, * = significant at 95% confidence limit by Student's t-test.

Table 4: **Identification of the extended beta lactamases producing strains among the *Proteus spp.***

	Proteus spp.				
	E	a	b	c	d
ESBLs n (%)	2 (3.8)	2 (3.8)	2 (3.8)	2 (3.8)	2 (3.8)
Inderminate	0 (0)	1 (1.9)	1 (1.9)	1 (1.9)	2 (3.8)

Keywords: Urea supplementation: (E, without urea; a, 0.5% urea; b, 0.75% urea; c, 1.0% urea; d, 1.25% urea), n (%), number and percentage of ESBL producing strains or inderterminate result.

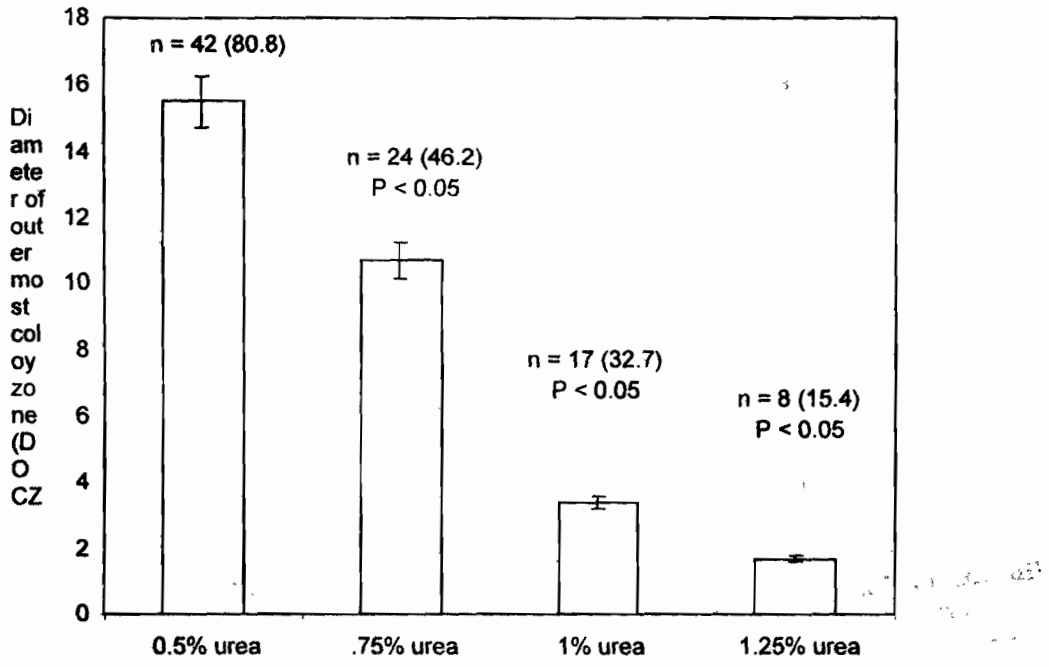

Figure 1: Effect of urea on swarming among *Proteus* isolates from Lagos, Nigeria.
Bars represent mean DOCZ valuesand projections on bars indicate deviations from mean.
n = number of swarmed *Proteus* strains, figures in parentheses indicate percentages.

DISCUSSION

Urea is primarily used in selective and composite media to identify urease-producing microorganisms (20). In recent times, the possibilities of exploiting the anti-swarming property of urea to aid isolation and identification of single colonies on solid media are being tested (12). In the present study, we found urea useful in refraining swarming of *Proteus spp* causing urinary tract infections in Lagos. *In vitro,* urea was also observed to allow isolation and identification of *K. pneumoniae, Pseudomonas aeruginosa; S. saprophyticus* and *E. coli. Pseudomonas aeruginosa* identified at 1% urea is higher than the 0.5% urea reported by Fons *et al* (12). The disparity in urea concentration could be attributed to strain variation and difference in swarming ability. Antigenic differences and varying capsular polysaccharide composition have been found among swarming *Proteus spp* (21). All the *Proteus* isolates examined survived urea at 1.25% and this suggests that urea is not inhibitory to cell growth at this concentration. This may not be unexpected since many media formulations for identification and speciation of bacteria contain up to 2% urea (22).

In this study, we found that the responses of our isolates to amoxicillin, nitrofurantoin, gentamicin and streptomycin were compromised following exposure to urea. This became more evident when the minimum inhibitory concentrations of these antibiotics were determined. In a study conducted by Ward *et al* (15), P-nitrophenylglycerol was found to increase the MICs of aminoglycosides; gentamicin and tobramycin and decrease the MICs of ticarcillin, ciprofloxacin and colistin against *Pseudomonas aeruginosa.* In Nigeria, several prospective studies have condemned the use of streptomycin and amoxicillin for empirical treatment of bacterial infections (23, 24). Recently, high amoxicillin resistant *Helicobacter pylori* strains were found in the biopsy samples of patients with gastritis and peptic ulcer in Western Nigeria (25). Although MICs of gentamicin and nitrofurantoin among the urea treated and untreated *Proteus* strains were above their respective break points (17), it is very important to adopt a cautionary use of urea when investigating susceptibility of *Proteus* to these antibiotics. The prevalence of bacterial pathogens that show resistance to these antibiotics in human infections is generally low in Nigeria (23). Therefore, discrepancies associated with the assessment of pathogens to these antibiotics may jeopardize control

measures and heighten the risk of multidr g resistant infections. The identification of pathogens as ESBL further provides an insight into their mechanisms of resistance to beta-lactam drugs. ESBL pathogens have been experimentally demonstrated to express stably de-repressed, constitutive chromosomal class 1β-lactamases, which hydrolyze most β-lactam antibiotics except carbapenems (26). This study has demonstrated the inability of urea at ≤ 1.25% to cause no discrepancies in ESBL classification and thus provides an additional credit to its diagnostic usefulness in clinical medicine and public health.

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