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Effect of Dyes on Multi-drug Resistant *S. enterica* Harbouring Plasmids

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Authors' contributions

This work was carried out in collaboration between all authors. Authors ENO and OEA designed the study, performed the statistical analysis, wrote the protocol. Author ENO wrote the first draft of the manuscript and author COJ managed literature searches. Authors COJ, SDK and OEM managed the analyses of the study and literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aim: To determine and compare the antiseptic property of dyes such as acridine orange and ethidium bromide on Multi-drug resistant (MDR) *Salmonella enterica* in this part of Nigeria.
Study Design: The stool samples of patients with symptoms of enteric fever were isolated and investigated from four hospitals in the south-east region of Nigeria
Place and Duration of Study: The study was carried out in the Department of Microbiology and Biotechnology, Nigerian Institute for Medical Research, Lagos, between August and November, 2012.
Methodology: The 100 *S. enterica* were recovered using microbact® identification kit-12E. PCR analysis and chromogenic nitrocefin sticks were used to detect isolates with $Blactx_{-M}$ and β-lacatamase enzyme respectively, while alkaline lysis method was adopted for the plasmid extraction. Tube dilutions of the subinhibitory concentration of the dyes and the antibiotics Minimum

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Inhibitory Concentration [MIC] susceptibility were used to determine the antiseptic effects of the dyes on the isolates.

Results: The *S. enterica* recovered indicated direct dissemination of these organisms among hospitalized patients and transfers of R-plasmids. Low molecular weight plasmids recovered were 9(9%), 13(13%) of the MDR *S. enterica* harbored $Bla_{CTX\text{-}M}$ gene. Few strains of *S. enterica* had increased susceptibility to the MIC's of the antibiotics at 1.25 µg/ml and 0.625 µg/ml of the ethidium bromide dyes, while acridine dyes had reduced effects of the resistant plasmid at 2.50 µg/ml.

Conclusion: These dyes were confirmed to display an antiseptic activity on the resistant plasmid DNA of *S. enterica* with MDR. There were high significant differences at ($p<0.05$), in the performance of the selected antibiotics on the MDR *S. enterica*.

Keywords: Mutagen; multi-drug resistance; S. enterica; Plasmids; dye.

1. INTRODUCTION

Multidrug-resistant *Salmonella typhi* is emerging in developed countries like the United States and United Kingdom [1,2]. The bacterium can survive in contaminated water, but does not have any host other than man [3]. There are over 1,000 different strains of the bacterium of which only a few cause typhoid [4]. Salmonellae are Gram negative, motile aerobic rods that characteristically ferment glucose and manose but fail to ferment lactose or sucrose, they are pathogenic for humans or animals by the oral route [5, 3].

The genes for antibiotic resistance in *S. typhi* and *S. paratyphi* are acquired from *Escherichia coli* and other gram-negative bacteria via plasmids. The plasmids contain cassettes of resistance genes that are incorporated into a region of the Salmonella genome called an integron. Some plasmids carry multiple cassettes and immediately confer resistance to multiple classes of antibiotics [2]. This explains the sudden appearance of MDR strains of *S. typhi* and *S. paratyphi*, often without intermediate strains that have less-extensive resistance. [2]. *Salmonella* infections represent a major health problem worldwide, particularly in the developing countries where they are recognized as the most frequent cause of morbidity and mortality [6]. Recent data indicate that different resistance determinants can amass in linked clusters, such that antimicrobials of a different class or substances such as disinfectants or heavy metals may select for MDR in bacteria [7]. Resistance in particular MDR, appears to be most serious in certain serotypes, but this situation may be shifting.

Acridine is also used to describe compounds containing the $C_{13}N$ tricycle. It is a raw material used for the production of dyes and some valuable drugs. Many acridines, such as proflavine, also have antiseptic properties [1, 8]. The use of acridine dyes such as acridine orange for curing and recognizing resistant plasmid in resistant *S. aureus* and other bacterial had been reported [1]. Apart from acridine, other agents used for curing are ethidium bromide and mepacrine [1]. The molar mass of ethidium bromide is 394.294 g/mol and the appearance is Purple-red solid [9]. Ethidium bromide is an intercalating agent commonly used as a fluorescent tag (stain) in molecular laboratories for techniques such as agarose gel electrophoresis. Ethidium bromide may be a mutagen, carcinogen or teratogen, although this depends on the organism and the conditions [10]. Reports have shown that the resistance of gastro enteric *Salmonella* strains to these antimicrobial agents is in large part, due to the production of extended-spectrum β-lactamases (ESBLs) encoded on plasmids, as well as on the chromosome, as reported [11,5,11,12]. The levels and degree of resistance vary globally and are influenced by antimicrobial use practices in humans, animals and geographical variations in the epidemiology of *Salmonella* infections [13]. Thus, there is a continuing need for increased surveillance of antimicrobial- resistant phenotypes in *Salmonella* isolates of animal and human origin on a global basis. Based on these reports, this research was carried out in other to investigate the activities of dyes such as acridine orange and ethidium bromide on Multi Drug Resistance *S. enterica* serovar. Typhi in the south east Nigeria.

2. MATERIALS AND METHODS

2.1 Study Design and Place of Study

The isolates are from the routine Section of the Medical Microbiology Laboratory in four hospitals from Southeast part of Nigeria, between June

and October, 2011. Twenty-five *S. enterica* isolates from stool of patients suspected to have *Salmonella* infection were collected from each of the four hospitals (one teaching hospital and three Federal Medical Centres).

2.2 Test Organism and Antimicrobial Sensitivity Testing

A total of 100 isolates from stool samples were randomly collected from patients diagnosed with enteric fever, using sterile plastic bottles from several units and wards in four hospitals. The hundred *Salmonella enterica* serovars were screened and confirmed (each from unrelated patients from the four hospitals) by standard biochemical characterization, using microbact® identification kit-12E (oxoid-England). The antibiogram of the isolates were analysed using selected antibiotics such as levofloxacin, ciprofloxacin, amoxicillin/clavulanic acid, cefotaxime and ceftriaxone (oxid, England) [14].

2.3 Beta-lactamase Production Test using Nitrocefin Sticks

The nitrocefin™ sticks (oxoid, India) is a chromogenic cephalosporin β-lacatamase indicator in a container [15]. The Procedure was as described [5]. A positive reaction was shown by the development of a pink/red color. No color change was observed with organisms that do not produce beta-lactamase. The unused nitrocefine stick was used as control. Then double disk synergy test was used to detect the presence of extended spectrum b-lactamase (ESBL) as described [6].

2.4 Plasmid DNA Isolation

The Alkaline lysis method was adopted for the extraction. A 200ul of buffer 1A (400 Mm Tris, 20 mM Na EDTA, acetic acid to pH 8.0) was added to the cell pellets and vortexed. Then 400 ul of the lysing solution (4% SDS (Sodium Dodocyl Sulphate) was added and the tubes were inverted 20 times at room temperature and 300ul ice cold buffer2B (3M Na Acetate, Acetic acid to pH 5.5) was added. The mixture was vortexed and kept on ice for 30 minutes. Then centrifuged at 3,000xg for 15 mins., after which 700 µl of chloroform was added to the supernatant and vortexed. Centrifuged again at 3,000xg for 10 mins. and transferred to 500 ul of the aqueous layer into a new tube. Then 1 ml of absolute ethanol was added and kept on ice for 1 hr. After Centrifuging at 3,000xg for 30 mins., the pellets were washed with 70% ethanol, decanted and dried. 50 ul of buffer 3C (10 Mm Tris, 2 Mm Na2 EDTA, acetic acid to pH 8.0.) was added and then kept on ice before electrophoresis.

2.5 PCR Amplification and Agarose Gel Electrophoresis

The purified DNA template in Polymerase Chain Reaction (PCR) eppendorf tubes (2.0 µl DNA) was mixed with the Master mix ready to load (HOT FIREPol ® DNA polymerase with 2.5 mM Mgcl2, Solis Biodyne). The mixture of primers, DNA and the Master Mix was vortexed, and then centrifuged before introducing it into the PCR machine (Eppendorf-Germany). After amplification, the PCR products were then taken for electrophoresis (CBS.Scientific Company Inc.) on the agarose gel at 80-100volts and finally viewed on the UV light for visible amplified image of the genes. The Polymerase Chain Reaction (PCR) were performed under the following conditions with the master mix ready to load. The thermo cycling condition used for ESBL $Bla_{\text{ctx-M}}$ were 30 – 35 cycles of 95°C for 30secs, 72°C for 1min.,66.2 for1 min., 72°C for 1 min, 95°C for 30 sec.,(PCR timing 1.38- 2.58 hrs). The $Bla_{\text{ctx-M}}$ [accession number: 90303X1185C03] universal primer used were; $Bla_{\text{ctx-M}}$ - F(5'ATG TGC AGY ACC AGT AAR GTK ATG GC-3'), R(5'- TGG GTR AAR TAR GTS ACC AGA AYC AGC GG-3') where R in the sequence is purine,Y is pyrimidine, and S is G or C (this is to design to occupy for the ambiguity of sequence variation among the CTX-M types) [16].

2.6 Investigating the Activities of the Dyes on Isolates Carrying R-plasmids

An overnight culture of 0.5 ml mac Farland standard dilution of each resistant strain of *S. typhi* was obtained in 10 ml Mueller Hinton broth (5 test tubes per strain) containing 5, 2.5, 1.25, 0. 625, 0.3125 ug/ml of the dyes (acridine orange and ethidium bromide) respectively [5]. The mixture was incubated overnight at 37°C for 24 h. After incubation, each dye-exposed culture were placed on nutrient agar medium and incubated similarly. Colonies were randomly selected from each of the five plates per strain, for sensitivity testing after growing them overnight in 5 ml Muller Hilton broth nutrient broth (oxoid, India) and diluted to 10^{-2} in sterile distilled water. After shaking, 0.1 ml of the diluted (10^{-2}) was seeded into a molten Mueller Hinton agar (10 ml), swirled to mix. Then poured on a

sterile agar plate and allowed to solidify. The wells were then filled each with referenced MIC of each test antibiotics against each strains of the *Salmonella enterica* [1], for cefotaxim, ceftriazone, levofloxacin, amoxil-clavulanic acid, and ciprofloxacin. When necessary, the test concentrations of the antibiotics were increased up to x10 or more. After a pre-incubation diffusion period of 2 hr., plates were incubated at 37°C for 24 h and zones examined. Absence of growth was indicative of plasmids-mediated resistance while growth was indicative of chromosomal mediated. The plasmid DNA was re-extracted to check if the resistant plasmid were still intact.

3. RESULTS

The result of the investigation reported from a total of 100 isolates of *Salmonella enterica* serovar.*Typhi* recovered from clinical stool samples were as shown below.

4. DISCUSSION

The percentage prevalence rate of *S. enterica* recovered from various units in the hospitals from the different regions investigated was as shown [Table 1]. The wide-scale use of antimicrobial drugs has been implicated in microbial drug resistance, which is an adaptive response in which microorganisms are able to tolerate an amount of drug [5]. From this study, an epidemiological survey of multi-drug- resistant *S. enterica* serovars recovered from various institutions were multi–drug resistant(MDR) as they were resistant at varying degree to Chloramphenicol, Amoxicillin, and (Co-trimoxazole) trimethoprim-sulphamethoxazole [Fig.1]. However, this is not in line as reported [4] that these drugs are first-line antibiotics and effective alternative drugs of choice for the treatment of typhoid fever.

In order to confirm further that multidrug resistant *Salmonella typhi* is becoming a problem in a developing country like Nigeria, some of the isolates of *S. enterica* were further screened and found to be resistant to some members of the β-lactam antibiotic drugs such as the cephalosporins and also the floroquinolone, as this reports were in line with the works of [2]. A high number of this *S. enterica* were recovered from In-patients units (IPU) as shown in Table 1., indicating direct dissemination of the organism among hospitalized patients. The General out patients Department (GOPD) recorded the highest number of isolates [Table 1]. Revealing that there could have been easy dissemination of individual multidrug resistant *S. typhi* or from transfers of R-plasmids, as reported [4].

Low molecular weight plasmids were recovered in this study from 9(9%) of the total isolates of which were mainly adult patients, though only 1 patient from the age range of 0-10yrs and 11-20yrs that had plasmids [Table 2]. According to [2], which supports the reports that the gene for antibiotic resistant in *S. typhi* are acquired from *Escherichia coli* and other gram negative bacteria via plasmids. This could be the reason why these organisms with plasmids were seen on these age groups and were resistant to more than three antibiotics in this study [Table 2]. Other authors [2], further stated that the plasmid contains cassettes of resistant genes that are incorporated into a region of the salmonella genomes called an integron and that some of these plasmids also carry multiple cassettes and immediately confer resistance to multiple classes of antibiotics as in [Fig. 1]. Also from the antibiotic reports [Fig. 1] recovered in this study shows that out of the one hundred isolates of *S. enterica* serovars., 87% of the isolates were Amoxicillin/Clavulanic acid resistant (a β-lactamase inhibitor), Amoxicillin had 80% resistant, and 80% were chloramphenicol resistant isolates, thus confirming the sudden phenotypic appearance of MDR strains of *S. typhi* in this part of Nigeria, which were earlier reported in other part of the world [2]. In this study, plasmids were not found in most of the isolates of *S. enterica* but they were resistant to a wide range of antibiotics including the third generation cephalosporin's and floroquinolones [Fig. 1]. These revealed that the resistance could have been mediated through the chromosome or the plasmids.

The results of the effect of the ethidium bromide dye on the multidrug resistant *S. enterica* serovar. Typhi, showed that the strains of *S. enterica* (SO9, SU28, SU40, and SA76) habouring plasmids had increased susceptibility to the antibiotics after they were exposed at concentrations of 1.25 μg/ml and 0.625μg/ml [Tables 4, 5 and 6]. Though, the antibiotics such as ceftriaxone, cefotaxim (cephalosporins- third generation), levofloxacin (3rd generation) and ciprofloxacin (first class of the 2nd generation of floroquinolone) had much effect on the isolates even before exposure, but not as much as that even after the exposure to the dye.

Table 1. Prevalence of *S. enterica* in various departmental unit and hospitals in the South-east region of Nigeria

S/NO	Unit/Department	(Location) Owerri (O)	(Location) Umuahia (U)	(Location) Enugu (E)	(Location) Abia (A)
		S. enterica	***S. enterica***	***S. enterica***	***S. enterica***
		1-25	**26-50**	**51-75**	**76-100**
1.	NHIS	5(20%)	0(0%)	0(0%)	3(12%)
2.	IPU	4(16%)	23(92%)	8(32%)	13(52%)
3.	GOPD	14(56%)	2(8%)	15(60%)	8(32%)
4.	CHOP	1(4%)	0(0%)	1(4%)	0(0%)
5.	EPU	1(4%)	0(0%)	0(0%)	1(4%)
6.	SKIN CLINIC	0(0%)	0(0%)	1(4%)	0(0%)

KEY: NHIS = national health insurance scheme , IPU= In - patient unit , GOPD= general out patient department , CHOP= children out patient , EPU= emergency patient unit, SKIN= skin clinic

Table 2. Distribution of the age range of individual and Sex according to the presence of plasmids and bla$_{ctx-m}$ gene in S. *enterica* serovar. typhi isolates

S/NO	Age Limits	No of patients	Presence of Plasmids		Bla$_{CTX-M}$	
	(yrs)	(%)	(M)	(F)	(M)	(F)
1.	0-10	6	0	1	0	0
2.	11-20	5	0	1	0	0
3.	21-30	11	0	0	1	0
4.	31-40	6	0	0	0	0
5.	41-50	4	0	0	1	0
6.	51-60	6	0	1	0	0
7.	61-70	2	0	0	0	0
8.	71-Above	3	0	0	0	0
9.	Adult(18-Above)	57	3	3	6	5
Total		100	3	6	7	6
Total Percentages			9(9%)		13(13%)	

KEY: M= male patients, F = female patients, adult (18- Above) = patients that are up to 18 years and above and refuse to disclose their actual age., Bla$_{CTX-M}$ = beta lactamase cefotaxime-M class enzyme

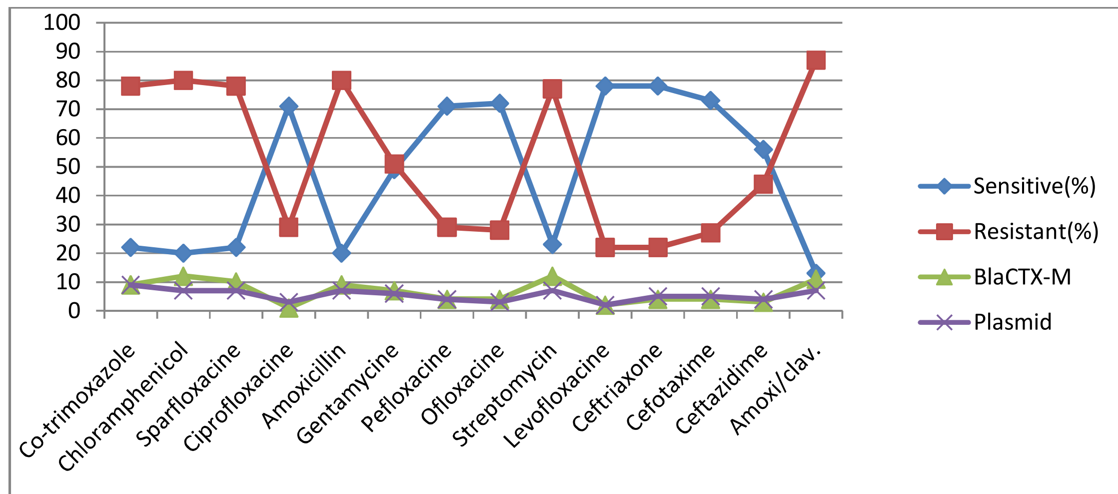

Fig. 1. Percentage prevalence of antibiotic susceptibility pattern of S. *enterica* serovar. typhi in relation to their resistant genes. Bla $_{CTX-M}$ = B-lactamase cefotaxime –M type enzyme, % = percentage

Table 3. Analysis of variance (ANOVA) to determine the treatment effect of antibiotics used *S. enterica* serovar. typhi

Source of variation	Degree of freedom (D.F)	Sum of square	Mean square	F-value
Block	3	209.75	69.9	7.21**
Treatment	4	778.3	194.57	20.07**
Error	12	116.3	9.69	
Total	19	1104.55		

*KEY: ** = highly significant treatment effect*

Table 4. MIC'S of the five selected drugs against resistant microbial strains for curing

	Strain with plasmid				
Treated strain of *S. enterica*	**LEV MIC (µg/ml) before curing**	**CPX MIC (µg/ml) before curing**	**AMC MIC (µg/ml) before curing**	**CTX MIC (µg/ml) before curing**	**CRO MIC (µg/ml) before curing**
O9	3.125	12.5	25.0	3.125	1.56
U28	1.56	1.56	12.5	3.125	1.56
U40	6.25	1.56	25.0	6.25	6.25
A76	12.5	1.56	25.0	12.5	12.5

KEY: S = sensitive, (zones, according to CLSI, (2007), R= resistant, strain O9,O14,24= strain from owerri, strain U28, U40, 46= strain from umuahia, strain A76, A49 = strain from abakaliki, LEV=levofloxacine, CPX= ciprofloxacine, AMC=amoxil/clavulanic , CTX=cefotaxime, CRO = ceftriaxone

Table 5. Effect of the curing of antibiotic resistance on S*almonella enterica* serovar. strains with ethidium bromide (1.25µg/ml)

	Strain with plasmid + (1.25 ug/ml of ethidium bromide)														
Treated strain of *S. enterica*	**LEV MIC (ug/ml) (Referenced)**			**CPX MIC (ug/ml) (Referenced)**			**AMC MIC (ug/ml) (Referenced)**			**CTX MIC (ug/ml) (Referenced)**			**CRO MIC (ug/ml) (Referenced)**		
	1.56,	**3.12,**	**4.0**	**1.56,**	**3.12,**	**4.0**	**1.56,**	**3.12,**	**4.0**	**1.56,**	**3.12,**	**4.0**	**1.56,**	**3.12,**	**4.0**
O9 Strain a	R	S	S	R	R	S	R	R	S	S	S	S	S	S	S
b	R	S	S	R	R	S	R	R	S	S	S	S	S	S	S
c	R	S	S	R	R	S	R	R	S	S	S	S	S	S	S
U28 Strain a	S	S	S	S	S	S	R	S	S	S	S	S	S	S	S
b	S	S	S	S	S	S	R	S	S	S	S	S	S	S	S
c	S	S	S	S	S	S	R	S	S	S	S	S	S	S	S
U40 Strain a	S	S	S	S	S	S	R	R	R	S	S	S	R	S	S
b	S	S	S	S	S	S	R	R	R	S	S	S	R	S	S
c	S	S	S	S	S	S	R	R	R	S	S	S	R	S	S
A76 Strain a	S	S	S	S	S	S	R	R	R	R	S	S	R	S	S
b	S	S	S	S	S	S	R	R	R	R	S	S	R	S	S
c	S	S	S	S	S	S	R	R	R	R	S	S	R	S	S

KEY: S = sensitive, (zones, according to CLSI, (2007) R= resistant, strain O9,O14,24= Strain from owerri, Strain U28, U40, 46= strain from umuahia, strain A76, A49 = strain from abakaliki, LEV=levofloxacine, CPX= ciprofloxacine, AMC=amoxil/clavulanic , CTX=cefotaxime, CRO = ceftriaxone

Table 6. Effect of the curing of antibiotic resistance in *Salmonella enterica* serovar. strains with ethidium bromide (0.625 µg/m)

Strain with Plasmid + (0.625ug/ml of ethidium bromide)																
Treated strain of *S. enterica*		**LEV MIC (ug/ml) (Referenced)**			**CPX MIC (ug/ml) (Referenced)**			**AMC MIC (ug/ml) (Referenced)**			**CTX MIC (ug/ml) (Referenced)**			**CRO MIC (ug/ml) (Referenced)**		
		1.56,	**3.12,**	**4.0**	**1.56,**	**3.12,**	**4.0**	**1.56,**	**3.12,**	**4.0**	**1.56,**	**3.12,**	**4.0**	**1.56,**	**3.12,**	**4.0**
O9 Strain	a	R	S	S	R	R	S	R	R	S	R	S	S	R	S	S
	b	R	S	S	R	R	S	R	R	S	R	S	S	R	S	S
	c	R	S	S	R	R	S	R	R	S	R	S	S	R	S	S
U28 Strain	a	S	S	S	S	S	S	R	R	S	S	S	S	S	S	S
	b	S	S	S	S	S	S	R	R	S	S	S	S	S	S	S
	c	S	S	S	S	S	S	R	R	S	S	S	S	S	S	S
U40 Strain	a	S	S	S	S	S	S	R	R	R	R	S	S	R	S	S
	b	S	S	S	S	S	S	R	R	R	R	S	S	R	S	S
	c	S	S	S	S	S	S	R	R	R	R	S	S	R	S	S
A76 Strain	a	S	S	S	S	S	S	R	R	R	R	S	S	R	S	S
	b	S	S	S	S	S	S	R	R	R	R	S	S	R	S	S
	c	S	S	S	S	S	S	R	R	R	R	S	S	R	S	S

KEY: S = sensitive, (zones, according to CLSI, (2007) R= resistant, strain O9,O14,24= strain from owerri, strain U28, U40, 46= strain from umuahia, strain A76, A49 = strain from abakaliki, LEV=levofloxacine, CPX= ciprofloxacine, AMC=amoxil/clavulanic, CTX=cefotaxime, CRO = ceftriaxone

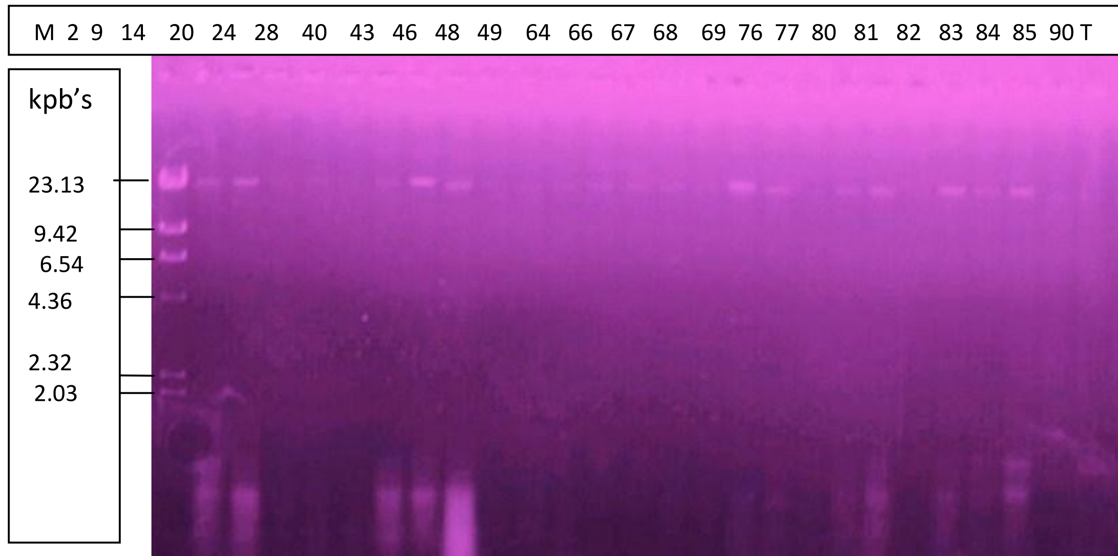

Fig. 2. Agarose gel electrophoresis pattern showing plasmids profiles of *S. enterica* serovar. typhi on lane number 2,9,14,20 and 24 from FMC Owerri, only lane 9 having plasmids with the Mwt. of 1.37 kb and lane 2 with two plasmids of Mwt. of 1.39 kb and 1.37 kb. lane 28, 40,43,46,48 and 49 from FMC umuahia with only lane number 28, 40 and 43 having plasmids of Mwt. of 1.37 kbs. lane number 64,66,67,68 and 69 are from UNTH Enugu without plasmid. while lane number 76,77,80,81,82,83,84,85, and 90 are from FMC abakaliki, with only lane number 81 and 83 having plasmids of the same Mwt. of 1.37kbs respectively, except lane number 85 with two plasmids of 1.39 and 1.37kbs. Lane T (positive control) is the *S. enterica* typed culture ATCC. lane M is the lambda Hind III marker (0.12-23.1kpb)

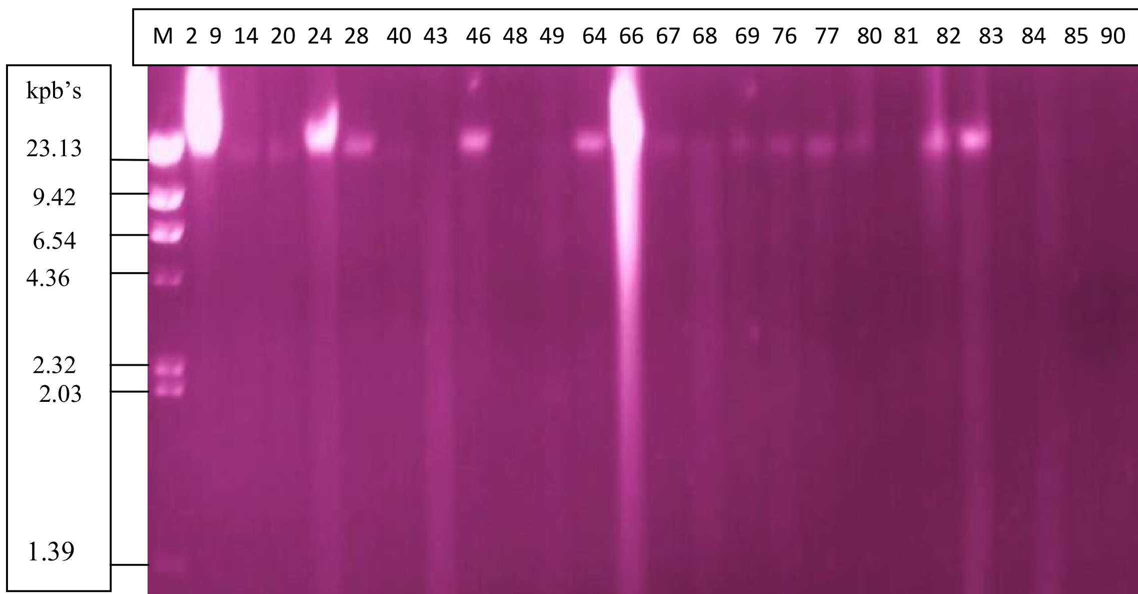

Fig. 3. Agarose gel electrophoresis pattern showing cured plasmids of *S. enterica* serovar. typhi after exposure to ethidium bromide (mutagen) on Lane number 2,9,14,20 and 24 from FMC owerri. lane number 28,40,43,46,48 and 49 from FMC umuahia. lane 64,66,67,68 and 69 are from UNTH enugu. while lane number 76,77,80,81,82,83,84,85, and 90 are from FMC abakaliki. lane M is the lambda hind III marker(0.12-23.1kpb)

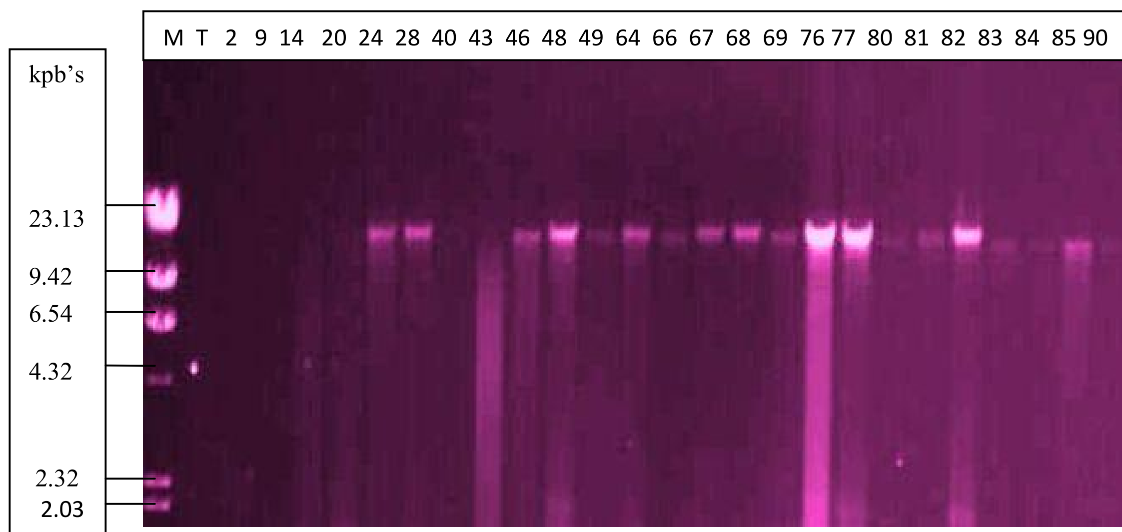

Fig. 4. Agarose gel electrophoresis pattern showing cured plasmids of *S. enterica* serovar. typhi after exposure to acridine orange (mutagen) on lane number 2,9,14,20 and 24 from FMC owerri. lane number 28,40,43,46,48 and 49 from FMC Umuahia. Lane number 64,66,67,68 and 69 are from UNTH enugu. while lane number 76,77,80,81,82,83,84,85, and 90 are from FMC abakaliki. lane T (positive control) is the *S. typhi* typed culture ATCC. lane M is the lambda hind III marker (0.12-23.1 kpb)

The effect of the ethidium bromide as an antiseptic dye were not so pronounced at the concentration of 0.625 µg/ml (Table 5), when compared to the 1.25 µg/ml of the same dye [Table 6]. So, based on these criteria, the isolates exposed at 1.25 µg/ml of the dye were re-screened for the presence of plasmid DNA. The effect was clearly visible when the plasmid DNA of the isolates were absent (note the results on Fig. 2 (before exposure), Fig. 3 (after exposure of SO9, SU28, SU40, SA76 at 1.25 µg/ml), thus revealing that it has a very good antiseptic property. The result showed that at 0.625µg/ml and 1.25 µg/ml in increasing progression an antiseptic and mutagenic effect was observed. Therefore it is advised that in no condition should the concentration of this dye (ethidium bromide) be increased when used for laboratory investigation, especially in agarose gel electrophoresis and as an antiseptic. However, the isolates showed little increase in susceptibility to the antibiotics screened as shown on the Table 5and 6. Also, Table 7 and 8 shows the antiseptic effect of acridine orange as a dye in this study on *S. enterica* when its concentration was increased to 2.50 ug/ml. The effect of the acridine at higher concentrations of 2.50 ug/ml, showed the sensitivity were less when compared to ethidium bromide at concentrations of 1.25 ug/ml. Nevertheless acridine still showed its antiseptic activity on resistant plasmid DNA of *S. enterica* in this study by displacing the R-plasmids on the isolates as shown on Fig. 2 (before) and Fig. 4 (after exposure to 2.5 ug/ml of acridine orange). Also from Table 8, the effect of acridine orange at 1.25 ug/ml on the *S. enterica* isolates were reduced based on the sensitivity of the isolates, by using the MIC's of the antibiotics selected as shown above. There is a possibility that chromosomal $bla_{CTX\text{-}M}$ type gene isolated from the isolates may be mediating the resistance as most of the *S. enterica* still retained their resistant to the exposed antibiotics, after the dyes revealed complete removal of the plasmids from the isolates. Thus, in this study , this trend of result shows that ethidium bromide dye is more active than acridine orange even at lower concentration if used as antiseptic or germicide regardless the organism been a multi-drug resistant isolates. Therefore, care should be taken when handling these chemical agents (dyes) during laboratory analysis, especially during agarose gel electrophoresis, as ethidium bromide is the dye of choice. Statistically, Analysis of Variation (ANOVA) Table 3, shows that there are high significant differences at ($p<0.05$) based on the individual performance of the five selected antibiotics on the isolates of *S. enterica* serovar. Typhi.

Table 7. Effect of the curing of antibiotic resistance in S*almonella enterica* serovar. strains with acridine orange (2.50µg/ml)

		Strain with Plasmid + ((2.50µg/ml of Acridine orange)														
Treated strain of *S.enterica*		**LEV MIC (ug/ml) (Referenced)**			**CPX MIC (ug/ml) (Referenced)**			**AMC MIC (ug/ml) (Referenced)**			**CTX MIC (ug/ml) (Referenced)**			**CRO MIC (ug/ml) (Referenced)**		
		1.56,	**3.12,**	**4.0**	**1.56,**	**3.12,**	**4.0**	**1.56,**	**3.12,**	**4.0**	**1.56,**	**3.12,**	**4.0**	**1.56,**	**3.12,**	**4.0**
O9 Strain	a	R	S	S	R	R	S	R	R	S	R	S	S	R	S	S
	b	R	S	S	R	R	S	R	R	S	R	S	S	R	S	S
	c	R	S	S	R	R	S	R	R	S	R	S	S	R	S	S
U28 Strain	a	S	S	S	S	S	S	R	R	S	S	S	S	S	S	S
	b	S	S	S	S	S	S	R	R	S	S	S	S	S	S	S
	c	S	S	S	S	S	S	R	R	S	S	S	S	S	S	S
U40 Strain	a	S	S	S	S	S	S	R	R	R	R	S	S	R	S	S
	b	S	S	S	S	S	S	R	R	R	R	S	S	R	S	S
	c	S	S	S	S	S	S	R	R	R	R	S	S	R	S	S
A76 Strain	a	S	S	S	S	S	S	R	R	R	R	S	S	R	S	S
	b	S	S	S	S	S	S	R	R	R	R	S	S	R	S	S
	c	S	S	S	S	S	S	R	R	R	R	S	S	R	S	S

KEY: S = sensitive (zones, according to CLSI, (2007) , R= resistant, strain O9,O14,24= strain from owerri, Strain U28, U40, 46= strain from umuahia, strain A76, A49 = strain from abakaliki, LEV=levofloxacine, CPX= ciprofloxacine, AMC=Amoxil/clavulanic , CTX=cefotaxime, CRO = ceftriaxone

Table 8. Effect of the curing of antibiotic resistance in *Salmonella enterica* serovar typhi strains with acridine orange (1.25ug/ml)

Treated strain of *S. enterica*		LEV MIC (ug/ml) (Referenced)			CPX MIC (ug/ml) (Referenced)			AMC MIC (ug/ml) (Referenced)			CTX MIC (ug/ml) (Referenced)			CRO MIC (ug/ml) (Referenced)		
		1.56,	**3.12,**	**4.0**	**1.56,**	**3.12,**	**4.0**	**1.56,**	**3.12,**	**4.0**	**1.56,**	**3.12,**	**4.0**	**1.56,**	**3.12,**	**4.0**
O9 Strain	a	R	R	S	R	R	R	R	R	R	R	R	S	S	S	S
	b	R	R	S	R	R	R	R	R	R	R	R	S	S	S	S
	c	R	R	S	R	R	R	R	R	R	R	R	S	S	S	S
U28 Strain	a	S	S	S	S	S	S	R	R	R	R	S	S	S	S	S
	b	S	S	S	S	S	S	R	R	R	R	S	S	S	S	S
	c	S	S	S	S	S	S	R	R	R	R	S	S	S	S	S
U40 Strain	a	R	R	S	R	R	S	R	R	R	R	R	S	R	R	S
	b	R	R	S	R	R	S	R	R	R	R	R	S	R	R	S
	c	R	R	S	R	R	S	R	R	R	R	R	S	R	R	S
A76 Strain	a	R	R	S	S	S	S	R	R	R	R	R	S	R	R	S
	b	R	R	S	S	S	S	R	R	R	R	R	S	R	R	S
	c	R	R	S	S	S	S	R	R	R	R	R	S	R	R	S

KEY: S = sensitive (zones, according to CLSI, (2007), R= resistant , Strain O9,O14,24= strain from owerri, strain U28, U40, 46= strain from umuahia, Strain A76, A49 = strain from abakaliki, LEV=levofloxacine, CPX= ciprofloxacine, AMC=amoxil/clavulanic , CTX=cefotaxime, CRO = Ceftriaxone

5. CONCLUSION

Based on the study carried out in this Southeast part of Nigeria, analysis revealed that the R-plasmids could be removed or its effect reduced from an organisms with Multi drug resistance especially that of *S. enterica* serovars., when exposed to dyes such as those used in this study. Also, $bla_{\text{CTX-M}}$ type gene detected on some of the isolates may be a major determining factor to multi-drug resistance to cephalosporins among *S. enterica* in this part of Nigeria, and not necessarily plasmids as reported in other studies. Hand washing in the hospital or at home is also recommended as a useful, safe and aseptic technique to patients in order to prevent diseases such as typhoid fever caused by *S. enterica* species. Furthermore, indiscriminate drug prescriptions and intake should be curtailed. Finally, care should be taken in handling these chemicals in the laboratory, especially in plasmid or chromosomal analysis.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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