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Mutagenic and Induced Antibiotic Resistance of Acridine Dye on *E. Coli* and *Proteus mirabilis* Isolated from Clinical Samples.

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Abstract

Acridine orange has been associated with chemical mutagenic compound that can cause an increase in the frequency of mutations. Its mutagenic influence varies with its potency because of its ability to penetrate the cell, its reactivity with DNA and its general toxicity. This study evaluated the extent to which acridine orange influence microbial resistance to antibiotic. Of 20 sensitive isolates of *E. coli* and *P. mirabilis* used, 100% of the isolates were resistant to ampicillin-sulbactam, 80% to amoxicillin, 20% to cefalexin and non (0%) were resistant to ciprofloxacin, nitrofurantoin, ofloxacin, chloramphenicol, ceftriazone and gentamicin. A total of 70% of the *E. coli* and 80% of *P. mirabilis* isolated were resistant to cefuroxime, 90% of the *E. coli* and 100% of *P. mirabilis* were resistant to ceftazidime, 50% of the *E. coli* and 80% of *P. mirabilis* to ampicillin, 80% of the *E. coli* and 100% of *P. mirabilis* to amoxicillin-clavulanic acid, 20% of the *E. coli* and 80% of *P. mirabilis* to tetracycline, 30% of the *E. coli* and 50% of *P. mirabilis* to nalidixic acid respectively after incubation at 37°C for 72hrs in acridine dye and overnight broth culture at the ratio of 5:1. This study indicated that acridine orange shows a significant mutagenic antibiotic resistant property to microorganism.

Keywords: *Acridine orange, antibiotic resistance, chemical mutagen*

Introduction

A **Mutation** occurs when a DNA molecule is

damaged or changed in such a way as to alter the genetic message carried by the gene. This can be caused by mutagenes, and **mutagens** are substance that can bring about a permanent alteration to the physical composition of a DNA molecule such that the genetic message is changed. Once the DNA molecule has been damaged or changed the mRNA transcribed from that DNA will now carry an altered message. The polypeptide made by translating the altered mRNA will now contain a different sequence of amino acids. The function of the protein made by folding this polypeptide will probably be changed or lost (**Brown, 2002; John, 2000**). Acridine orange (AO) is an immunologic fluorochrome (fluorescent dye) that non-specifically binds to RNA (red fluorescence). AO differentially stains double-stranded DNA to a green fluorescence (around 520 nm) and single-stranded DNA and RNA to an orange fluorescence (around 610 nm). This metachromatic fluorescence is sensitive to DNA conformation (green fluorescence), proteins, polysaccharides, glycosaminoglycans and in screening cervical smears for abnormal cells, making it a useful probe for detecting apoptotic cells. In mammalian cell culture, apoptotic cells stained by AO show reduced green fluorescence and enhanced orange fluorescence in comparison to normal cells. This is believed to result from the breakdown of DNA to fragments that can more easily denature and bind AO as single-stranded nucleic acid (Darzynkiewicz *et al.*, 1992). Acridine dye can serve as mutagenic compounds that increase the frequency of some types of mutations. In genetics, a **mutagen** is a physical or chemical agent that changes the

genetic material, usually DNA, of an organism and thus increases the frequency of mutations above the natural background level. As many mutations cause cancer, mutagens are therefore also likely to be carcinogens. Not all mutations are caused by mutagens: so-called "spontaneous mutations" occur due to spontaneous hydrolysis, errors in DNA replication, repair and recombination. Genetic analysis reveals that resistance to various dyes is related to the function of different genes.

The toxicology of acridine orange is carcinogenic; causing mutations by intercalating itself within replicating DNA causing insertional or deletional ('frame shift') mutations. This dye has been widely explored for staining, coloring and identification of protein in biotechnology and in the feed of genetics for different purpose; it produces good fluorescent immunostaining that supported induction of enhanced autophagy (Autophagy, 2008). Acridine is a known human carcinogen. It causes frame-shift mutations in incorporating into the DNA, and doing so creating an additional base on the opposite strand, if that mutation occurs in a coding sequence; it almost always leads to inactivation of the protein it encoded in the DNA-replication fork (Herman and Dworkin, 1971; Robert and Bruce, 1984). According to **Giuliano et al. (2002)**, acridine orange can be used for the mutation of the A47 *Bacillus* strain (a plant pathogen antagonist) in order to obtain an improved strain with enhanced capacity to synthesize metabolites with antibiotic activity. The mutant strain M40 was obtained using the mutagenic agent acridine orange. The mutant strain showed a higher antagonistic activity than the wild type A47 against the plant pathogen *Botrytis cinerea* (grey mould), *Ralstonia solanacearum* (bacterial wilt) and *Erwinia carotovora* var. *carotovora* (bacterial soft rot).

At a non-permissive temperature for DNA polymerase I, acridine orange inhibits growth of a temperature-sensitive strain and also increases the rate of elimination of the F'-Lac plasmid. DNA isolated from cells treated with acridine orange under conditions that inhibit growth contains material of low molecular weight, which is absent from DNA isolated from cells

treated under conditions in which growth is not impaired. Cells incubated with acridine orange at both pH 7.4 and 7.9 suffer degradation of DNA, as shown by loss of labelled DNA from the acid-insoluble fraction, which is not observed with untreated cells at either pH (Barker and Hardman, 1978).

Materials and Methods

Subjects

Verbal consent of patients that submitted their samples for diarrhea and UTI analysis were obtained at the Medical Microbiological Department of the Hospital during sample submission for the period of 3 months (February, 2014–April, 2014).

Ethical Approval

Ethical approval with number ABUTH/HRECG04/2013 was obtained from the ethical committee of the Ahmadu Bello University Teaching Hospital, Shika, Zaria to conduct this study.

Statistical Analysis

SPSS version 20 was used to analyze the finding of this study, and p-values were said to be significant at ≤ 0.05 confident interval.

Methods

Stool and urine samples of patients were collected with a sterile swab stick into a sterile container and transported to the Department of Pharmaceutical Microbiology Laboratory of Ahmadu Bello University, in an ice pack. Isolation, microscopy, identification and biochemical test of *E. coli* and *P. mirabilis* were carried out using basic microbiological method (Chakraborty and Nishith, 2008; Ellis and Goodacre, 2006; Harley, 2002; De Silva et al., 2001). Antibiotic susceptibility pattern of the isolates was carried out using disk diffusion method as described by Cheesbrough (2000). Interpretation of the susceptibility results were carried out using CLSI (2006). The antibiotics used were: Penicillin's; Ampicillin-sulbactam (SAM) (20 μ g), Amoxicillin (AML) (10 μ g), Amoxicillin-Clavulanic acid (AMC) (30 μ g), Cephalosporin's: Cefalexin (CL) (30 μ g), Ceftriazone (CRO) (30 μ g), Ceftazidime (CAZ) (20 μ g), Cefuroxime (CXM) (30).

Fluoroquinolones: Ciprofloxacin (CIP)(5µg), Nalidixic Acid (NA)(30µg), Ofloxacin (OFX)(5µg), Amino-glycosides: Gentamicin (CN) (10µg). Tetracycline's: Tetracycline (30 µg). Chloramphenicol (C) (30 µg), Nitrofurantoin (F) (300 µg). The isolates were further grown in 5ml nutrient broth overnight to enhance cell mass and actively propagating cells.

Stock solution of acridine orange was prepared (10, 000µg/ml) and 1ml of the overnight culture of each of the isolates were introduced into 5ml acridine orange (5:1) and incubated for 72 hours at 37°C. The result of the antibiotic susceptibility pattern of various isolates before and after were observed and compared to see if there are variations in antibiotic susceptibility pattern.

Results

Table 1: Isolation, Microscopy, Identification and Biochemical Test for *E. coli*

Lab. Code No.	MacConkey Agar (Pink red = +)	EMB Agar Greenish metallic sheen = +	Glucose (pink to colorless = +)	Lactose (pink to colorless = +)	TSI and gas (pink to colorless = +)	Indole (Ring like red = +)	Urease (pink= +)	Methyl red (red)	V.P test (colorless = -)	Citrate (Blue = +)	Microscopy (Rod Shape = +)
S14	+	+	+	+	+	+	-	+	-	-	+
S46	+	+	+	+	+	+	-	+	-	-	+
S16	+	+	+	+	+	+	-	+	-	-	+
S57	+	+	+	+	+	+	-	+	-	-	+
S45	+	+	+	+	+	+	-	+	-	-	+
U18	+	+	+	+	+	+	-	+	-	-	+
U64	+	+	+	+	+	+	-	+	-	-	+
U60	+	+	+	+	+	+	-	+	-	-	+
U58	+	+	+	+	+	+	-	+	-	-	+
U50	+	+	+	+	+	+	-	+	-	-	+

Table 2.0: Isolation, Microscopy, Identification and Biochemical Test for *P. mirabilis*

Lab Code No.	MacConkey Agar (Pink and swarming = +)	EMB Agar (Greenish metallic sheen = +)	Glucose (Pink to colourless = +) and gas	Lactose (Pink to colourless = +)	TSI and gas (Pink to yellow = +) Black = H ₂ S	Indole (Ring like red = +)	Urease (pink= +)	Citrate (Blue = +)	Microscopy (Motile, non - capsulate, pleomorphic bacilli)
S12	+	-	+	-	+	-	+	+	+
S90	+	-	+	-	+	-	+	+	+
S48	+	-	+	-	+	-	+	+	+
S32	+	-	+	-	+	-	+	+	+
S79	+	-	+	-	+	-	+	+	+
U28	+	-	+	-	+	-	+	+	+
U17	+	-	+	-	+	-	+	+	+
U19	+	-	+	-	+	-	+	+	+
U52	+	-	+	-	+	-	+	+	+

Table 3: Antibiotic Susceptibility Profile of Clinical Isolate of *E. coli* and *Proteus mirabilis*

Organism	CAZ	CRO	F	OFX	CXM	NA	TE	AML	CIP	SAM	C	CL	AMC	CN
<i>P. mirabilis</i>	S	S	S	S	S	S	S	S	S	S	S	S	S	S
<i>E. coli</i>	S	S	S	S	S	S	S	S	S	S	S	S	S	S

S = Sensitive R = Resistant Ampicillin-sulbactam (SAM), Amoxicillin (AML), Amoxicillin-Clavulanic acid (AMC), Cefalexin (CL), Ceftriaxone (CRO), Ceftazidime (CAZ), Cefuroxime (CXM), Ciprofloxacin (CIP), Nalidixic Acid (NA), Ofloxacin (OFX), Gentamicin (CN), Tetracycline (TE), Chloramphenicol (C), Nitrofurantoin (F).

Antibiotic Resistance Pattern of Isolates after Introduction into Acridine Orange

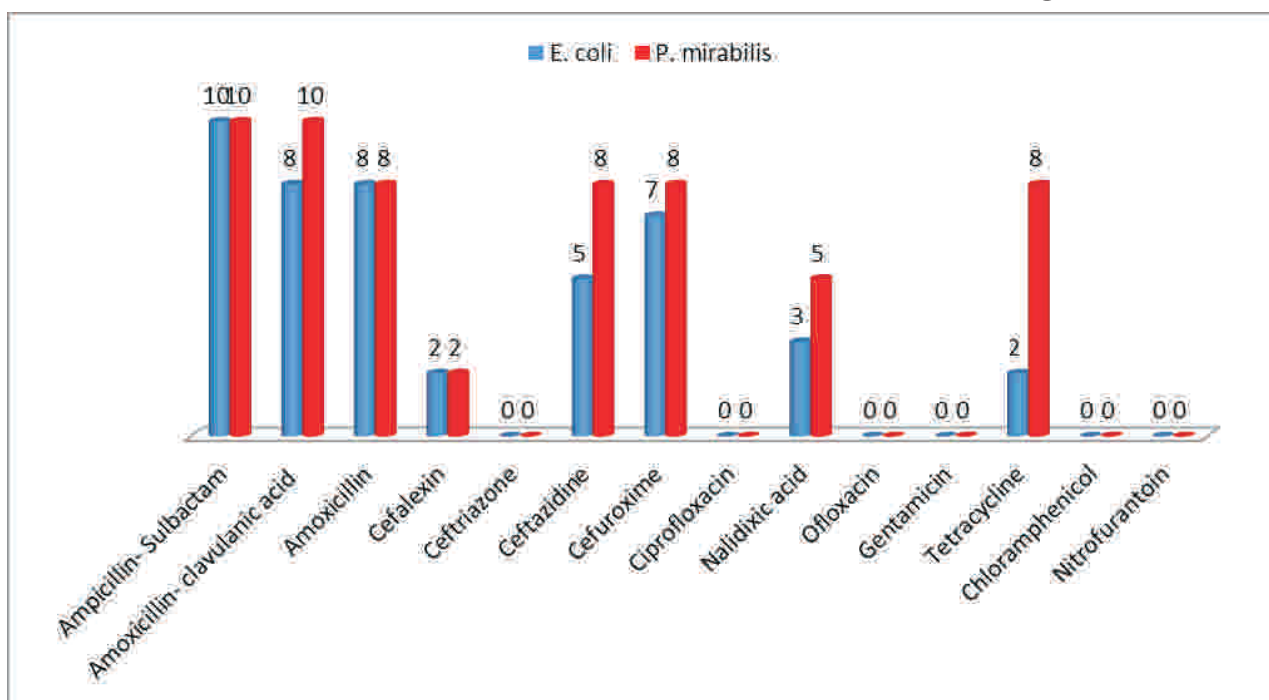


Figure 1: Antibiotic resistance influenced by acridine dye on *E. coli* and *Proteus mirabilis*

Table 4: Justification of the significance of mutagenic properties of acridine dye on *E. coli* and *Proteus mirabilis* using SPSS 19.

***E. coli* ANOVA: Single Factor (Summary)**

Groups	Count	Sum	Average	Variance
<i>E. coli</i>	15	54	3.6	14.68571
<i>Proteus mirabilis</i>	15	69	4.6	19.11429

ANOVA

Source of Variation	SS	df	MS	F	P-value	F Crit
Between Groups	7.5	1	7.5	0.443787	0.510752	4.195972
Within Groups	473.2	28	16.9			
Total	480.7	29				

Inference: Since the calculated F (0.443787) is lower than F critical (4.195972) at $p \leq 0.5$ we say that there is a significant different between the mean between the groups and the mean within the groups.

***P. mirabilis* ANOVA: Single Factor (Summary)**

Groups	Count	Sum	Average	Variance
10	13	35	2.692308	10.39744
		49		
10	13		3.769231	16.69231

ANOVA

Source of Variation	SS	Df	MS	F	P-value	F Crit
Between Groups	7.538462	1	7.538462	0.556555	0.462897	4.259677
Within Groups	325.0769	24	13.54487			
Total	332.6154	25				

Inference: Since the calculated F (0.556555) is lower than F critical (4.259677) at $p \leq 0.5$ we say that there is a significant different between the mean between the groups and the mean within the groups.

Discussion

It has been observed that antibiotic susceptibility of bacterial isolates is not constant but dynamic and varies with time and environment (Hassan, 1985) and this influence the rate at which multiple resistant bacteria constitute a global problem (O'Brien, 1997; Shanahan *et al.*, 1994). According to Aibinu *et al.* (2004), *E. coli* is highly resistant to ampicillin, amoxicillin, tetracycline and trimethoprim-sulfamethoxazole. The result obtained from this study showed that of the 20 sensitive isolates of *E. coli* and *P. mirabilis* used respectively, 100% of the isolates were resistant to ampicillin-sulbactam, 80% to amoxicillin, 20% to cefalexin and non (0%) were resistant to ciprofloxacin, nitrofurantoin, ofloxacin, chloramphenicol, ceftriazone and gentamicin. Seventy percent (70%) of the *E. coli* and 80% of *P. mirabilis* were resistant to cefuroxime, 90% of the *E. coli* and 100% of *P. mirabilis* were resistant to ceftazidime, 50% of the *E. coli* and 80% of *P. mirabilis* to ampicillin, 80% of the *E. coli* and 100% of *P. mirabilis* to amoxicillin-clavulanic acid, 20% of the *E. coli* and 80% of *P. mirabilis* to tetracycline, 30% of the *E. coli* and 50% of *P. mirabilis* to nalidixic acid respectively after incubation at 37°C for 72 hours in acridine dye and overnight broth culture at the ratio of 5:1. Acridine orange showed a significant mutagenic antibiotic resistant property to microorganism

($p < 0.01$). This implies that resistant inherent and unexpressed can be induced by mutation and environmental pressure associated with acridine dye. While, bacteria genome has been designed to be adaptable to their surrounding layers, the genetic information for these and other structures associated with a bacterium are capable of alteration (Alberts *et al.*, 2003). Bacteria adapt to changes in temperature, pH, concentrations of ions such as sodium, and the nature of the surrounding support. Some alterations are reversible, disappearing when the particular pressure is lifted. Other alterations are maintained and can even be passed on to succeeding generations of bacteria. This resistance is almost always due to a change in the genetic make-up of the bacterial genome. Acquired resistance can occur because of mutation or as a response by the bacteria to the selective pressure imposed (Dyer, 2003). Bacteria react to a sudden change in their environment by expressing or repressing the expression of a whole lot of genes. This response changes the properties of both the interior of the organism and its surface chemistry. A well-known example of this adaptation is the so-called heat shock response of *Escherichia coli*. One of the adaptations in the surface chemistry of Gram-negative bacteria is the alteration of a molecule called lipopolysaccharide. This lipopolysaccharide chemistry can become more

or less water-repellent (Alberts, *et al.*; 2003). These changes can profoundly affect the ability of antibacterial agents or immune components to kill the bacteria. Also, some certain conditions might influence de-activation of actively expressing genes can occur.

Conclusion

Acquired mutational resistance might be expressed in organisms pre-exposed to chemical mutagenic compounds which when implicated in disease condition might influence a high rate of antibiotic resistance, influence long stay in the hospital and create more economic burden.

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