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Short Communication

Plasmid Curing of *Escherichia coli* Cells with Ethidium Bromide, Sodium Dodecyl Sulfate and Acridine Orange

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The plasmid eliminating abilities of acridine orange, ethidium bromide and sodium dodecyl sulfate were investigated on multi drug resistant *Escherichia coli* from urinary tract infection specimens. Three different concentrations of each curing agent (Et-Br, SDS and AO) were used. The frequencies of cured cells were 5.55 % (with 50 µg/ml) and 11.76 % (with 75 µg/ml) for acridine orange, 14.29 % (with 100 µg/ml), 21.05 % (with 100 µg/ml), 17.65 % (with 125 µg/ml) for ethidium bromide and 7.4 % (with 10 % w/v) & 6.67 % (with 10 % w/v) for sodium dodecyl sulfate. However, no cured cells were obtained from 100 µg/ml acridine orange, 75 µg/ml ethidium bromide and 8 and 12 % SDS. Analysis of profiles of wild type and plasmid cured strains by electrophoresis yielded bands of varying sizes for wild type cells, but none were obtained for Et-Br cured cells. Acridine orange treated cells could eliminate only plasmids of 2.7 MDa and another smaller than 2 MDa.

Key Words: Plasmid curing, *Escherichia coli*, Ethidium Bromide, Sodium Dodecyl Sulfate, Acridine Orange.

Introduction

Plasmid is one of the several environmental and genetic factors that carry the resistance property against a specific drug or a number of drugs in bacteria[1]. R-plasmids from resistant strains of an organism may transfer to a sensitive counterpart that would in due course show the same drug resistance as the donor strain[2]. Plasmids can also be eliminated by curing agents which can be used to display the role of R-plasmid in drug resistance. The techniques used to promote curing include exposing the host strain to elevated temperatures, use of chemical agents such as intercalating dyes (acridine orange, ethidium bromide), treatment with crystal violet, sodium dodecyl sulfate (SDS), thymidine starvation and exposure to UV radiation[1]. Different plasmids vary considerably in their property to be cured, and not necessarily depending upon properties of specific plasmid.

When working with some plasmid-containing bacteria, it is often desirable to obtain a plasmid-cured derivative, allowing a direct comparison to be made between the plasmid-containing and plasmid-cured cells. Some plasmids undergo spontaneous segregation and deletion, but the majority is extremely stable and requires the use of curing agents or other procedures to increase the frequency of spontaneous segregation. The usefulness of curing agents is unpredictable in many bacterial strains, as there are no standard protocols applicable to all plasmids[3]. As no universally effective curing agent has yet been identified, curing experiments are generally conducted on trial and error basis, both with respect to the choice of the curing agent and the culturing conditions used. Some curing agents work in a non-specific way by damaging and stressing out the cells, while some seem to act much selectively[4]. The present study is a preliminary effort to observe the curing efficiencies of acridine orange, ethidium bromide and sodium dodecyl sulphate on multi-drug resistant *Escherichia coli* cells, and also to establish a correlation between plasmid elimination and subsequent loss of drug resistance to determine the involvement of R-plasmids in drug resistance of these strains.

Materials and Methods

Sample collection

Ten clinical isolates of Multi Drug Resistant (MDR) *E. coli* from UTI patients were collected from a local diagnostic centre of Dhaka, Bangladesh.

Antibiotic sensitivity assay of bacterial isolates

Bacterial susceptibilities to antimicrobial agents were determined in vitro by using the Kirby Bauer standardized agar disc-diffusion method[5]. Antibiotic discs of ampicillin (10 µg), amoxicillin (30 µg), cloxacillin (5 µg), ceftazidime (30 µg), ceftriaxon (30 µg), cephalexine (30 µg), gentamicin (10 µg), imipenem (10 µg), nitrofurantoin (10 µg) and tetracyclin (30 µg) were used.

Isolation of plasmid DNA

Plasmid isolation was performed using the alkaline lysis method described by Birnboim and Doly[6]. After isolation of plasmid DNA a horizontal agarose gel electrophoresis was carried out, and the method followed was as described by Meyers *et al.*[7]. *E. coli* PDK-9 strain was used as the DNA molecular weight marker.

Curing of plasmids

Curing of plasmids was performed by the method of Tomoeda *et al.*[8]. Three types of curing agents, each with 3 different

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concentrations [ethidium bromide (75 µg/ml, 100 µg/ml and 125 µg/ml), acridine orange (50 µg/ml, 75 µg/ml, 100 µg/ml), sodium dodecyl sulphate (8 %, 10 %, 12 %)] were used to cure the plasmids. Amoxicillin resistance was used as the selectable marker.

Four isolates were selected randomly for plasmid curing which were *E. coli* 212587, *E. coli* 212973, *E. coli* 208366 and *E. coli* 207940. An overnight culture of each test organism in Luria Broth (LB) containing amoxicillin was diluted to 10^4 cells/ml using freshly prepared sterile LB by serial dilution technique. From this diluted culture, 0.5 ml was added with 4.5 ml LB containing different concentrations of curing agents. Thus, the concentration became 10^3 cells/ml. The cultures were then incubated at 37ºC in an orbital shaker at 150 rpm for 48 hours. After incubation, the broth culture was again diluted to 10^3 cells/ml with sterile normal saline. Ten ml of the culture was spread on Luria Agar medium. After a 24-hour incubation at 37°C, the plates were observed for growth. From this plate culture, some well-isolated colonies were randomly selected and simultaneously patched with sterile toothpick on one Luria Agar medium without antibiotic and another Luria Agar containing amoxicillin (25 mg/ml), with a numbered grid line attached on the bottom of each plate. After 24-hour incubation at 37°C, plates were observed for the cured cells. The cured plasmid cells were detected comparing the development of bacterial colonies on antibiotic containing plate with that of the normal (without antibiotic) plate. The samples that showed colonies on normal LB agar but failed to grow on LB agar supplemented with amoxicillin were the possible cured isolates.

Results and Discussion

Cured cells were achieved with 100 µg/ml and 125 µg/ml ethidium bromide (Table 1), 10 % (w/v) SDS (Table 2) and 50 µg/ml and 75 µg/ml acridine orange (Table 3) although the frequencies of cured colonies were quite low. The results from Table 1-3 show that amongst the three curing agents, ethidium bromide was able to cure plasmids successfully at a higher rate than the other two agents. The frequencies of cured cells were 5.55 % (with 50 µg/ml) and 11.76 % (with 75 µg/ml) for acridine orange, 21.05 % (with 100 µg/ml), 17.65 % (with 125 µg/ml) for ethidium bromide and 7.4 % (with 10 % w/v) and 6.67 % (with 10 % w/v) for sodium dodecyl sulfate. On the other hand, no cured cells were obtained from 100 µg/ml acridine orange, 75 µg/ml ethidium bromide and 8 and 12 % SDS.

Table 1. Curing frequencies by treatment with various concentrations of Ethidium Bromide (EtBr)

E. coli isolates	Inoculum size (cells/ml) x 10^4	EtBr conc. (µg/ml)	Time of incubation (hour)	No. of cured colonies / no. of total colonies in Amoxicillin-Luria agar plate	Frequency of cured colonies (%)
E. coli 212587	1.3	75	48	0	0
E. coli 212587	1.7	100	48	0	0
E. coli 212587	0.9	125	48	0	0
E. coli 212973	1.15	75	48	0	0
E. coli 212973	1.4	100	48	0	0
E. coli 212973	0.95	125	48	0	0
E. coli 208366	1.05	75	48	0	0
E. coli 208366	0.85	100	48	0	0
E. coli 208366	0.79	125	48	3/17	17.65
E. coli 207940	0.98	75	48	0	0
E. coli 207940	1.12	100	48	4/19	21.05
E. coli 207940	0.92	125	48	0	0

Table 2. *Curing frequencies by treatment with various concentrations of sodium dodecyl sulfate (SDS)*

E. coli isolates	Inoculum size (cells/ml) x 10^4	SDS conc. (w/v) %	Time ofincubation (hour)	No. of cured colonies / no. of total colonies in Amoxicillin-Luria agar plate	Frequency of cured colonies (%)
E. coli 212587	1.3	8	48	0	0
E. coli 212587	1.7	10	48	2/27	7.4
E. coli 212587	0.9	12	48	0	0
E. coli 212973	1.15	8	48	0	0
E. coli 212973	1.4	10	48	0	0
E. coli 212973	0.95	12	48	0	0
E. coli 208366	1.05	8	48	0	0
E. coli 208366	0.85	10	48	1/15	6.67
E. coli 208366	0.79	12	48	0	0
E. coli 207940	0.98	8	48	0	0
E. coli 207940	1.12	10	48	0	0
E. coli 207940	0.92	12	48	0	0

Table 3. *Curing frequencies by treatment with various concentrations of acridine orange*

E. coli isolates]	Inoculum size (cells/ml) x 10^4	AO conc. (µg/ml)	Time of incubation (hour)	No. of cured colonies / no. of total colonies in Amoxicillin-Luria agar plate	Frequency of cured colonies (%)
E. coli 212587	1.3	50	48	0	0
E. coli 212587	1.7	75	48	2/17	11.76
E. coli 212587	0.9	100	48	0	0
E. coli 212973	1.15	50	48	1/18	5.55
E. coli 212973	1.4	75	48	0	0
E. coli 212973	0.95	100	48	0	0
E. coli 208366	1.05	50	48	0	0
E. coli 208366	0.85	75	48	0	0
E. coli 208366	0.79	100	48	0	0
E. coli 207940	0.98	50	48	0	0
E. coli 207940	1.12	75	48	0	0
E. coli 207940	0.92	100	48	0	0

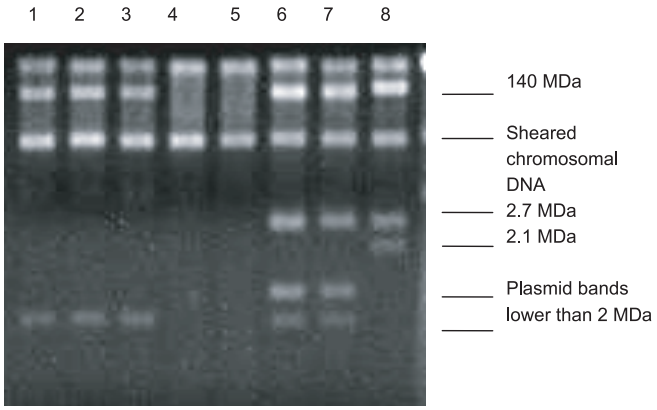

Figure 1. *Plasmid profiles of cured and wild type cells. Lane 1, 2, 3 - E.coli 212587 cured by acridine orange (75µg/ml), Lane 4, 5 - E. coli 208366 cured by ethidium bromide (125 µg/ml), Lane 6 - original E. coli 212587, Lane 7- original E. coli 208366 and Lane 8- E. coli PDK-9 marker.*

Wild type cells contained plasmid bands of various sizes; a band slightly less than 140 MDa, one band at 2.7 MDa and two bands smaller than 2 MDa. Agarose gel electrophoresis of the cured colonies revealed that the cells treated with ethidium bromide were able to eliminate all the plasmid bands. However, acridine orange treated cells could eliminate 2.7 MDa and another plasmid that was smaller than 2 MDa (Figure 1). The cured colonies also lost the amoxicillin resistance. In case of ethidium bromide, the loss of the large plasmid can be correlated with the concommitant loss of amoxicillin resistance. However, although acridine orange could not eliminate the largest plasmid, it still showed the loss of amoxicillin resistance. It can be suggested that acridine orange might have disrupted or changed the larger plasmid in such a way that its amoxicillin resistance gene could not function. Otherwise, the resistance might be borne on the smaller plasmids. Further investigations are necessary to confirm this.

The effectiveness of curing methods depends on the nature of the bacterial host and/or plasmids where some may work better than the others[9]. The plasmid curing of *Escherichia coli* and other bacteria have been previously reported. Several have used ethidium bromide, sodium dodecyl sulfate and acridine orange. A plasmid-containing wild-type *E. coli* strain was treated with sodium dodecyl sulfate and ethidium bromide. Plasmid loss in cured *E. coli* cells resulted in the disappearance of the outer membrane components and a concomitant change in the thickness of the peptidoglycan layer[10]. A rapid, simple, and effective method for curing of a wide range of *Escherichia coli* antibiotic resistance plasmids has been previously described[11], and this involved treatment with acridine orange followed by growth in sub-lethal concentration of antibiotics and penicillin selection. Such bacteriostatic conditions resulted in a curing efficiency of more than 98% in all cases tested. Chin *et al*. subjected five *Lactobacillus* strains with plasmid curing agents, such as novobiocin, acriflavin, SDS, and ethidium bromide. In no cases did the antibiotic resistance of these strains proved to be curable, with the exception of the erythromycin resistance[12].

Conclusion

UTI can be caused by a wide variety of microbes with uropathogenic *E. coli* as the major culprit. They cause this disease either by chromosome mediated or by plasmid mediated mechanism. Plasmid mediated resistance that can be transferred between cells enable rapid spread of the disease. The present study investigated the efficiencies of different curing agents on the *E. coli* isolates from UTI specimen. For this purpose, three different types of curing agents were used and the results obtained was a preliminary indication of association of drug resistance of the clinical isolates of *E. coli* with plasmids. Among the three curing agents used, ethidium bromide displayed greater success rate than the rest.

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