IN-VITRO STUDY ON POTENTIAL EFFICACY OF ACRIFLAVINE IN THE PREVENTION OF CONJUGATIONAL TRANSFER OF DRUG RESISTANCE IN BACTERIA

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ABSTRACT

BACKGROUND

Acquisition of new resistance in bacteria is brought into effect by a battery of gene transfer systems, among which conjugation accounts for bulk of horizontal gene transfer. Acriflavine is a DNA-intercalating agent with plasmid curing properties. Since it selectively inhibits plasmid replication, it might have a role in interfering with conjugation.

The aim of this study was to observe for any in-vitro inhibitory effect of Acriflavine on horizontal transfer of resistance factors.

MATERIALS AND METHODS

Overnight BHI broth cultures of donor *Escherichia coli* and recipient *Escherichia coli* strains were taken and experiment done simultaneously in following steps. Mating between donor and recipient strain was done by broth mating protocol, and resultant transconjugant colony count (TCC) was calculated and compared between Acriflavine treated and untreated mating mixtures. The bacteriostatic effects were measured and viable colony count (VCC) was performed separately on Acriflavine treated and untreated broth cultures of both donor and recipient. Standard plasmid curing protocol using Acriflavine was performed on donor strain and curing efficacy were calculated. Five repetitions were done for each conjugating pair, and median values were used for further analysis by descriptive statistics.

RESULTS

The % decrease of TCC was greater than % decrease attributed to combined effect of bacteriostasis/curing. This difference hints towards the presence of some inhibitory effects other than bacteriostasis and curing.

CONCLUSION

Acriflavine being a strong DNA-intercalating agent, perhaps has some negative effect on conjugation process which needs to be further investigated. If proven, Acriflavine & other similar compounds will open up the possibility of being used as adjuvant therapy along with antimicrobial agents to slow down the rate of development of new resistance and increased longevity of antibiotic efficacy.

KEYWORDS

Acriflavine, Conjugation, DNA Intercalation, Plasmid, R Factor, Plasmid Curing.

HOW TO CITE THIS ARTICLE: Das T, Chakraborty B, Ray R. In-vitro study on potential efficacy of Acriflavine in the prevention of conjugational transfer of drug resistance in bacteria. J. Evolution Med. Dent. Sci. 2017;6(78):5540-5546, DOI: 10.14260/jemds/2017/1204

BACKGROUND

Bacterial resistance to antimicrobials is an inevitable outcome of relentless antibiotic use and its selection is a naturally occurring and continuous phenomenon. This has become a major threat in control of infectious disease worldwide. One way to tackle this situation is by justified and rational use of antibiotics and creation of newer generation of antibiotics which work via novel mechanisms. However, it is a time consuming and costly process. Another alternative way would be by prevention of dispersal of resistance. If the spread of antimicrobial resistance (AMR) can be appropriately slowed down, then it will enhance the longevity of antibiotic usefulness and decreased dependence on reserve drugs.

'Financial or Other Competing Interest': None.
Submission 22-08-2017, Peer Review 14-09-2017,
Acceptance 21-09-2017, Published 28-09-2017.

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DOI: 10.14260/jemds/2017/1204

Acriflavine is an orange coloured dye, derivative of coal tar & traditionally used as a topical antiseptic. It was introduced in World War II as a remedy for sleeping sickness. It was subsequently used for dressing burn wounds, as bladder wash, etc. It also acts as a strong DNA-intercalating agent and biotechnologists use it widely as a potent plasmid curing agent wherein a bacterium is made free of plasmids. Elimination of plasmid seems to be due to selective interference in replication of plasmid resulting in loss of entire plasmid in the newly replicated bacterium. Due to its nature of selective and specific interaction with extra-chromosomal DNA, we hypothesised that it might also have some effects on conjugal transfer of plasmid-mediated resistance in bacteria. The aim of this study was to see if Acriflavine has any in-vitro inhibitory effect on horizontal transfer of resistance factors and the potential efficacy of such effect.

MATERIALS AND METHODS

Three donor *Escherichia coli* strains and two recipient *Escherichia coli* strains were collected from various sources (Table 1). Biochemical tests, antibiotic susceptibility tests, and Phenotypic Confirmatory Tests for ESBL detection were

done following standard methods. Our experiment comprised of three parts which were performed simultaneously for each conjugating pair.

Preparation of Donor and Recipient Cultures

0.5 mL of overnight BHI broth cultures of donor and recipient were added separately to 10 mL (achieved dilution is 1:20) of fresh BHI broth and incubated for up to 3 hours to obtain actively growing phase cultures. Approximate cell concentration to be achieved is 2×10^8 CFU/mL as checked by $OD_{600} = 0.08-0.1$ using spectrophotometer.¹ This preparation was used as the starting source of donor and recipient in all following experiments.

Experiment 1- Conjugation (by broth mating)

0.5 mL of donor and 2.5 mL of recipient culture was added to 10 mL of fresh BHI broth in a 25-mm wide sterile test tube and mixed by gentle pipetting.²⁻⁴ This mating mixture is divided into 4 parts of 2.5 mL each. One would act as control (labelled C) and the others as test (labelled T1, T2, T3). In the tubes "T", Acriflavine solution was added to achieve final concentrations of 50 µg/mL, 25 µg/mL, 12.5 µg/mL respectively. Nothing is added to tube "C". All tubes "C" and "T" are incubated in a slant position² overnight at 35°C in dark & ambient air. Next day, tubes marked "T" were checked for turbidity. If there was an absence of turbidity, that tube was rejected since we needed to study the effects of Acriflavine only at sub-inhibitory levels. 0.1 mL of culture from "C" is taken and diluted⁵ to 10^{-3} . 100 µL of this dilution was streaked onto selective BHI agar plates containing both Streptomycin (Str) 100 µg/mL + Cefotaxime (Ctx) 2 µg/mL.^{4,6} Streaking was done using a glass rod spreader following spread plate technique.⁷ All these plates were incubated for 24-48 hours.

The same process is followed for "T" tubes too.

Successful conjugation would transfer ESBL determining plasmid from donor (Cefotaxime Resistant-Ctx^R) to recipient (Streptomycin Resistant-Str^R) and the resultant product (Transconjugant) would hence be phenotypically Str^R Ctx^R. Thus, a selective antibiotic plate containing Ctx+Str would selectively allow the growth of such transconjugants whereas inhibiting growth of either pure donor (since its Str^S) or the recipient (since its Ctx^S). After incubation, plates were checked for growth of any transconjugants. Colonies were counted by taking a picture with digital camera and then analysing enlarged image on a computer screen. The difference in transconjugant colony count (TCC) between control and test was noted.

To crosscheck for valid plasmid transference, several random transconjugant colonies were subcultured and confirmed for ESBL using Phenotypic Confirmatory Test.

Experiment -2 Plasmid Curing

Alongside conjugation experiment, the donor strains were subjected to curing treatment⁸ to determine plasmid curing efficacy at conditions and concentrations used in test "T". A 10 mL BHI broth containing the same concentration of Acriflavine (as used in conjugation experiment above) was inoculated with 0.5 mL of the donor. This was incubated overnight in dark at 35°C. And 0.1 mL of culture was taken

and made 10^{-3} dilutions, then 100 µL of this dilution was spread on BHI agar using the spread plate technique.⁷ This plate was incubated overnight in dark at 35°C. For patch plating⁹ two BHI agar plates were taken –one containing no additives and other one containing antibiotic (Cefotaxime 2 µg/mL or Tetracycline 30 µg/mL). These plates had a circular grid template attached at the base. Each grid had a serial number. From the spread plate prepared in step 2 above, random isolated colonies were sampled using sterile toothpicks. It was then streaked on both plates at same corresponding grid numbers. About 120 random colonies were patch plated.

After overnight incubation at 37°C, plates were observed for the cured cells. The cured plasmid cells were detected by 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 BHI agar but failed to grow on BHI agar supplemented with antibiotic were the possible cured isolates.¹⁰ Such suspected cured isolates were sampled from the respective grid on a normal BHI plate, and confirmed phenotypically by biochemical and antibiogram studies.¹¹

Experiment -3 Performing Viable Colony Count (VCC)

To measure the bacteriostatic effects, viable colony count (VCC) was performed separately on Acriflavine treated and untreated broth cultures of both donor and recipient. 0.5 mL donor was added to 10 mL BHI broth and divided into 4 parts of 2.5 mL each. Similarly, in a different test tube, 2.5 mL recipient was added to 10 mL BHI broth and then divided into 4 parts. The following steps were done on both.

In first part nothing was added and in the rest 3 parts, Acriflavine was added to achieve final concentrations of 50 µg/mL, 25 µg/mL, and 12.5 µg/mL respectively. All tubes were incubated overnight at 35°C in dark & ambient air. Next day, 0.1 mL of culture was taken and made 10^{-3} dilution, then 100 µL of this dilution was spread on BHI agar using the spread plate technique. Following overnight incubation, colony count was performed by taking a picture and analysing on a computer screen. VCC thus obtained was compared among Acriflavine and no-Acriflavine tubes of donor and recipient separately.

The tests were performed with 5 repetitions in each experiment set. Descriptive statistics using bar/line diagrams were used to represent median values from each data set.

RESULTS

In our conjugation experiments, there was a clear transfer of the resistance factor among all strains studied. On exposure to Acriflavine, there was a significant decrease in Transconjugant Colony Count (TCC) signifying certain inhibition of conjugation. Viable Colony Count (VCC) performed on Acriflavine treated and untreated cultures of donor and recipient (Bacteriostatic experiment) revealed a decrease in colony counts in Acriflavine treated sets. Plasmid curing was also observed in donor strains, but in insignificant numbers.

However, % decrease of TCC was much greater than % decrease attributed to the effects of bacteriostasis/curing. This difference may hint towards the presence of some other phenomenon which negatively affects resistance transfer.

Donor Strains		Susceptibility to Streptomycin	Susceptibility to Cefotaxime	Susceptibility to Tetracycline
D1	ESBL producing <i>Escherichia coli</i> strains collected from clinical isolates at Dept. of Microbiology, IPGME&R	Sensitive (Str ^S)	Resistant (Ctx ^R)	
D2	ESBL producing <i>Escherichia coli</i> strains collected from clinical isolates at Dept. of Microbiology, IPGME&R	Sensitive (Str ^S)	Resistant (Ctx ^R)	
D3	<i>Escherichia coli</i> from HiMedia X-Pert Bacterial Conjugation Kit HTM004-5PR	Sensitive (Str ^S)		Resistant (Tet ^R)
Recipient Strains				
R1	Genetically engineered <i>Escherichia coli</i> 2443 obtained from Dept. Of Biotechnology, IIT Kharagpur.	Resistant (Str ^R)	Sensitive (Ctx ^S)	Sensitive (Tet ^S)
R2	Recipient <i>Escherichia coli</i> from HiMedia X-Pert Bacterial Conjugation Kit HTM004-5PR	Resistant (Str ^R)	Sensitive (Ctx ^S)	Sensitive (Tet ^S)

Table 1. Strains Used in this Experiment

<i>Escherichia coli</i> Screened Positive for ESBL*	<i>Escherichia coli</i> Confirmed Positive for ESBL by PCT†	No. of ESBL <i>Escherichia coli</i> Sensitive to Streptomycin	<i>Escherichia coli</i> which showed good Conjugation Frequency with Recipient strains.
112	73 out of 112	7 out of 73	2 out of 7

Table 2. Selection of Donor Strains from Clinical Isolates

* ESBL- Extended Spectrum Beta Lactamase, †PCT- Phenotypic Confirmatory Test. All screening and confirmatory tests for ESBL detection was done following CLSI 2017 guidelines.

Experiment Pair	Transconjugant Colony Count (TCC)*				Change in TCC with Respect to Control			
	Control	Acriflavine 12.5 µg/mL	Acriflavine 25 µg/mL	Acriflavine 50 µg/mL	Acriflavine 12.5 µg/mL		Acriflavine 25 µg/mL	
					Absolute Change	% Change	Absolute Change	% Change
D1+R1	659	537	479	×	122	18.51	180	27.31
D1+R2	742	569	510	×	173	23.32	232	31.27
D2+R1	427	338	314	×	89	20.84	113	26.46
D2+R2	478	372	331	×	106	22.18	147	30.75
D3+R1	616	481	434	×	135	21.92	182	29.55
D3+R2	743	574	508	×	169	22.75	235	31.63

Table 3. Results of Conjugation Experiment

* Data is representative of the median value among 5 repetitions for each pair ×; there was no appreciable turbidity, hence this tube was not processed further

Experiment Pair	Donor Viable Colony Count (VCC)*			Change in Donor VCC with respect to Control			
	Control	Acriflavine 12.5 µg/mL	Acriflavine 25 µg/mL	Acriflavine 12.5 µg/mL		Acriflavine 25 µg/mL	
				Absolute change	% Change	Absolute change	% Change
D1+R1	1602	1365	1294	237	14.79	308	19.22
D1+R2	1670	1421	1344	249	14.91	326	19.52
D2+R1	1840	1601	1526	239	12.98	314	17.06
D2+R2	1306	1133	1081	173	13.24	225	17.22
D3+R1	1645	1450	1367	195	11.85	278	16.89
D3+R2	1800	1551	1479	249	13.83	321	17.83

Table 4. Results of Viable Colony Count Experiment on Donor Strains

* Data is representative of the median value among 5 repetitions for each donor strain.

Experiment Pair	Recipient Viable Colony Count (VCC)*			Change in Recipient VCC with respect to Control			
	Control	Acriflavine 12.5 µg/mL	Acriflavine 25 µg/mL	Acriflavine 12.5 µg/mL		Acriflavine 25 µg/mL	
				Absolute Change	% Change	Absolute Change	% Change
D1+R1	1936	1622	1523	314	16.21	413	21.33
D1+R2	1401	1157	1102	244	17.37	299	21.34
D2+R1	1635	1369	1282	266	16.26	353	21.59
D2+R2	1450	1198	1135	252	17.37	315	21.72
D3+R1	1503	1267	1170	236	15.70	333	22.15
D3+R2	1503	1254	1174	249	16.56	329	21.88

Table 5. Results of Viable Colony Count Experiment on Recipient Strains

* Data is representative of the median value among 5 repetitions for each recipient strain.

Acriflavine	No. of Random Colonies Selected for Screening	No. of Colonies which were Sensitive to Cefotaxime (cured)*	Curing % = $\frac{\text{No of CFUs cured}}{\text{Total No of CFU's tested}} \times 100$
Plasmid curing of D1 (on day of conjugating D1+R1)			
Acriflavine 12.5 µg/mL	120	1	0.83%
Acriflavine 25 µg/mL	120	3	2.50%
Plasmid curing of D1 (on day of conjugating D1+R2)			
Acriflavine 12.5 µg/mL	120	1	0.83%
Acriflavine 25 µg/mL	120	4	3.33%
Plasmid curing of D1 (on day of conjugating D2+R1)			
Acriflavine 12.5 µg/mL	120	1	0.83%
Acriflavine 25 µg/mL	120	2	1.66%
Plasmid curing of D1 (on day of conjugating D2+R2)			
Acriflavine 12.5 µg/mL	120	1	0.83%
Acriflavine 25 µg/mL	120	4	3.33%
Plasmid curing of D1 (on day of conjugating D3+R1)			
Acriflavine 12.5 µg/mL	120	1	0.83%
Acriflavine 25 µg/mL	120	2	1.67%
Plasmid curing of D1 (on day of conjugating D3+R2)			
Acriflavine 12.5 µg/mL	120	1	0.83%
Acriflavine 25 µg/mL	120	2	1.67%

Table 6. Results of Plasmid Curing of Donor Strains

* Data is representative of the median value among 5 repetitions for each donor strain.

Figure 1. Representation of Final Results. Decrease in Transconjugant Colony Count (TCC) > Decrease in Viable Colony Count (VCC) and Curing

Figure 2. Spread plate of Transconjugants grown on BHI agar Containing Streptomycin + Cefotaxime. Left Image is of CONTROL PLATE - Mating Mixture Contains no Acriflavine. Right image is of TEST PLATE- Mating Mixture was treated with Acriflavine 25 µg/mL. Number of Colonies on Test Plate is Appreciably less than that of Control Plate

Figure 3.

Figure 3. Patch plates for detecting plasmid curing. Left - marked area shows presence of growth. Right - marked area shows absence of growth. For patch plating, two plates were used in each set. One of the plates had only BHI agar (on left) and the other (on right) was BHI + Cefotaxime (2 µg/mL).

Using sterile toothpick, a random isolated colony was sampled from spread plate of Acriflavine treated donor strain, and streaked in duplicate onto the same corresponding grid numbers on both plates. Following incubation, they were compared for growth at corresponding patches on both plates. Curing of resistance marker would be represented by growth on plain BHI plate only, and absence of growth in a corresponding grid on Cefotaxime added plate. Such suspected cured isolates were sampled from respective grid on normal BHI plate, and confirmed phenotypically by biochemical and antibiogram studies.

Figure 4. Spread Plates of Recipient Strain done for Viable Colony Count. Left- Control plate. Right- Test Plate with Recipient Treated with 25 µg/mL of Acriflavine

DISCUSSION

The explosive increase in prevalence of multidrug resistant bacteria has sparked investigation of new strategies for controlling infection. Conjugative plasmids have recently gained attention as potential targets because of the high frequency of antibiotic resistance arising from conjugation, which can be 10^5 times as frequent as spontaneous chromosomal mutation conferring resistance, and indeed resistance against β -lactams and aminoglycosides has been largely spread by conjugative transfer.¹² In addition, conjugative plasmids readily transmit multiple resistance genes.¹³ Inhibiting conjugation has been suggested as a strategy to prevent or slow the spread of antibiotic resistance genes to dangerous pathogens.¹⁴ Work in this direction has previously focused on inhibiting the apparatus involved in DNA transfer.¹⁵ One such approach is by inhibiting the conjugative DNA relaxase with bisphosphonates like clodronate and etidronate.¹⁵ Targeting similar relaxases,

other compounds too have shown potential like EDTA and Sulbactam.¹⁶ There are also reports of certain Synthetic Fatty Acids¹⁷ having selective inhibition of donors and Phage M13¹⁸ which inhibit conjugation by other means.

Acriflavine is a well-known DNA-intercalating agent which interferes with proper DNA propagation. It has an established role as a plasmid curing agent, wherein a plasmid gets eliminated with prolonged treatment. We hypothesised that since Acriflavine has a negative influence on plasmids, maybe it could have similar negative effects on conjugational transfer too. We devised a very simple setup, wherein effect of Acriflavine was studied by analysing difference in number of transconjugants formed between Acriflavine treated and untreated mating mixture.

In selection of donor strains, we focused on ESBL strains, as studies show that ESBL determining gene is usually plasmid borne,¹⁹⁻²⁰ and successful conjugation could easily be spotted by screening for transference of factors for resistance to Cefotaxime, Ceftazidime, etc. Our study too demonstrated the presence of ESBL genes on plasmids by successful conjugation in 2 out of 7 eligible donor ESBL *Escherichia coli* isolates (Table 2). This establishes a transferability of 28.57% which is almost in concordance with results (30%- 80%) of other similar studies.^{7,21-23} These two strains and another one (from HiMedia Kit) were further experimented to document the effects of Acriflavine.

We performed a pilot study, where Acriflavine was used at serial two-fold dilutions (50 µg/mL, 25 µg/mL, 12.5 µg/mL, 6.25 µg/mL, and 3.125 µg/mL). At 50 µg/mL, there was complete inhibition of growth. Since we wanted to see its effects at sub-inhibitory levels, this concentration was excluded from our study. At concentrations of 6.25 µg/mL and 3.125 µg/mL, there were very minimal changes and clear conclusions could not be drawn from it. Hence, these particular concentrations were also excluded from our study.

In our conjugation experiments, there was successful transfer of resistance factor. With clinical isolates, donor strains transferred their ESBL bearing resistance factor to the recipient cells, thus making the latter resistant to Cefotaxime, as evidenced by antibiogram studies. Similarly, donor strain from HiMedia Kit was also able to transfer its resistance factor to the recipient, eventually making the recipient strain resistant to Tetracycline.

On exposure to Acriflavine, there was a significant decrease in Transconjugant Colony Count (TCC) signifying certain inhibition of conjugation (Table 3). At 12.5 µg/mL and 25 µg/mL of Acriflavine, the median %reduction of TCC was 22.05% and 30.15% respectively. This decrease in TCC could have resulted from several factors such as: bacteriostatic effects of Acriflavine, plasmid curing effect on ESBL/Tet^R plasmid, or unknown direct inhibitory effect on conjugation. To document the effects of former two factors, further experiments were conducted in parallel with conjugation experiment.

Acriflavine being an antimicrobial agent has an inherent inhibitory effect on growth of bacteria²⁴⁻²⁵ and might subsequently lead to decreased efficiency in conjugation indirectly. However, such a decrease in conjugation is only a secondary effect and does not really prove any direct action of Acriflavine in relation to conjugation. Hence, such an effect had to be quantified and compared against overall decrease of transconjugants.

To measure growth inhibition by bacteriostasis, a Viable Colony Count (VCC) was performed on Acriflavine treated and untreated cultures of donor and recipient (Table 4,5).

In case of donors, at 12.5 µg/mL and 25 µg/mL of Acriflavine, the median % reduction of VCC was 13.53% and 17.53% respectively. On recipients, at 12.5 µg/mL and 25 µg/mL of Acriflavine, the median %reduction of VCC was 16.41% and 21.65% respectively.

Acriflavine is also a DNA-intercalating agent with plasmid curing action. As studies indicate, ²⁶ plasmid curing could have potentially eliminated the resistance factor from donors and indirectly caused a decrease in conjugation frequency. Hence we needed to estimate the extent of curing action, if any. We performed plasmid curing experiments on each donor (Table 6). Median curing efficacy in our study was 0.83% and 2.08% at 12.5 µg/mL and 25 µg/mL of Acriflavine respectively. Most studies ^{10, 27-28} have indicated that the curing efficacy of Acriflavine is highly variable and range widely between 0-96%. This is because effectiveness of curing methods depends on the nature of the bacterial host and/or plasmids where some may work better than the others. ²⁹ The extent of curing observed in our study was at the lower end of values, and less likely to have significantly affected conjugation to such a great extent.

From our experiment, we found that the %decrease of TCC was greater than %decrease attributed to the effects of bacteriostasis/curing. One way of explaining this imbalance would be due to the possible presence of direct inhibitory action on conjugation in addition to bacteriostatic and curing effects.

Acriflavine being a strong DNA-intercalating agent, perhaps has some negative effect on conjugation process. Studying such direct effects on conjugation requires sophisticated instruments and techniques. Such a facility was not available at our institution, hence we cannot conclusively comment on the definitive inhibitory role of Acriflavine on bacterial conjugation.

CONCLUSION

This simple study shows that Acriflavine has an overall negative effect on conjugational resistance transfer. The real mechanism still remains unexplored and warrants further study. If proved, Acriflavine and other similar compounds could be combined with existing antibiotics to formulate efficacious synergistic antimicrobial cocktails. Given the ubiquitousness of conjugative plasmids within multidrug-resistant bacterial strains, it can conceivably shift the equilibrium towards success in a variety of refractory clinical infections. Superficial skin infections, which incidentally are also breeding sites of nosocomial antibiotic resistance transfer, might be effectively treated using such topical applications. They could also be used in combination with disinfectants to reduce the burden of resistant strains on contaminated surfaces. Overall, development of such compounds will help in the prevention of resistance dispersal and in extending the lifetime of our antibiotic stockpile.

ACKNOWLEDGEMENTS

We must acknowledge the fact that this research work was based upon the basic ideas and thoughts of Prof. Prasanta Kumar Maiti, Professor and Head, Department of Microbiology, IPGME&R. This study would not have

materialised without the help of Prof. Anindya S. Ghosh, Ph.D., Associate Professor, Department of Biotechnology, Indian Institute of Technology, Kharagpur, who provided us with a genetically engineered strain to conduct conjugation experiments. We are grateful to Prof Dr. Manju Banerjee, Ex-Director of IPGMER for providing the facilities and her encouragement in carrying research works.

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