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Infectious drug resistance and antibiotic resistance curing in *Salmonella* and *Shigella* isolates from cases of diarrhoea

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RÉSUMÉ

ADETOSOYE (A. I.), ROTILU (I. O.). — Guérison des infections dues à des *Salmonella* et des *Shigella* isolées de cas de diarrhée et résistantes aux antibiotiques et aux anti-infectieux. *Rev. Elev. Méd. vét. Pays trop.*, 1985, **38** (4) : 433-437.

Soixante-trois souches de cas cliniques, à savoir 24 souches de *Salmonella typhimurium* provenant de veaux diarrhéiques, 24 souches de *Shigella* spp. et 15 souches de *Salmonella* spp. provenant d'enfants également atteints de diarrhée, ont été étudiés du point de vue de la résistance aux traitements médicamenteux et aux antibiotiques.

Douze schémas d'antibiorésistance ont été obtenus. Un taux de transfert de résistance élevé a été démontré parmi ces souches bactériennes.

L'acriflavine a été reconnue comme un agent de traitement efficace. Aux basses concentrations, elle élimine les déterminants « r » des bactéries résistantes.

Mots clés : Salmonella typhimurium - *Shigella* - Antibiotique - Acriflavine - Résistance aux médicaments.

SUMMARY

ADETOSOYE (A. I.), ROTILU (I. O.). — Infectious drug resistance and antibiotic resistance curing in *Salmonella* and *Shigella* isolates from cases of diarrhoea. *Rev. Elev. Méd. vét. Pays trop.*, 1985, **38** (4) : 433-437.

Sixty-three clinical isolates including 24 isolates of *Salmonella typhimurium* from diarrhoeic calves, 24 isolates of *Shigella* spp. and 15 isolates of *Salmonella* spp. from diarrhoeic children, were studied for infectious drug resistance and antibiotic resistance curing. Twelve antibiotic resistance patterns were obtained. A high frequency of drug resistance transfer was observed among the bacterial isolates. Acriflavine was found to be an efficient curing agent. At low concentrations it eliminated « r » determinants from the resistant bacteria.

Key words : Salmonella typhimurium - *Shigella* - Antibiotics - Acriflavine - Drug resistance.

INTRODUCTION

Antibiotic resistance in many genera of bacteria may be determined by R-factors which are extrachromosomal genetic material capable of mediating multiple drug resistance. It provides the means for infectious spread of drug resistance among population of bacteria especially under selective pressure of antibiotics (2, 7, 13, 14, 20, 22). Loss of extrachromosomal DNA may arise as a result of mutation in the host or plasmid, or due to replication defect. Plasmid loss may occur spontaneously in all or in one resistance gene because of the physical loss of the R-factor or DNA. The rate of R-factor loss may be increased by exposure to various concentrations of mutagens (4, 10, 12, 15, 21). Included in these groups of mutagens are acriflavine, acridine orange, chloroquine, miracil D, quinine, berbarine, ethidium bromide, urea, sodium dodecylsulphate and atabrine.

This investigation was carried out to see whether multiple drug resistant *Salmonella* and *Shigella* isolates could be cured of their plasmids with acriflavine and acridine orange.

MATERIALS AND METHODS

Bacterial isolates included 24 *Salmonella typhimurium* isolated from calves with diar-

rhoea, 15 *Salmonella* spp. and 24 isolates of *Shigella* spp. isolated diarrhoeic children. The bacterial strains were isolated by standard methods (6) using Selenite F broth (11), Siliker, Delbel and Fagan medium (17) and Tergitol-7-triphenyl tetrazolium (TTC) medium (8).

Antibiotic susceptibility tests

Antibiotic susceptibility testing was performed as described elsewhere (3) using single antibiotic discs (BBL) consisting of the following antibiotics, ampicillin (PN 10 µg), streptomycin (S 10 µg), tetracycline (Te 10 µg), chloramphenicol (C 10 µg), sulpha drugs (S_3 30 µg) and nalidixic acid (Na 30 µg). *E. coli* NCTC 10418 was used as control.

Plasmid transfer

Plasmid transfer experiment was performed as previously described (18) using *E. coli* 14 R 525 K_{12} resistant to 200 µg/ml nalidixic acid as selective recipient. All bacterial isolates resistant to ampicillin, tetracycline, streptomycin and chloramphenicol were used as donors. MacConkey agar of 5 formulations was used as selective medium (table n° I).

Antibiotic resistance curing experiments

The mutagens used were acriflavine and acridine orange (Gur's Ltd, London).

a) Each resistant bacterial isolate was inoculated into 3 ml trypticase Soy broth (TSB, Oxoid) to a density of CA 10^5 organisms per millilitre 0.01 ml of each suspension was delivered into 2 sets of 5 ml sterile TSB in bijou bottles. Known concentration of acriflavine and acridine orange namely 10 µg/ml, 20 µg/ml, 30 µg/ml, 40 µg/ml, 50 µg/ml, 60 µg/ml, 80 µg/ml and 160 µg/ml were delivered respectively into each bacterial suspension. The cultures were incubated at 37 °C for 18 h after which the antibiotic sensitivity testing was performed (3). *E. coli* NCTC 10418 was used as control.

b) An isolated colony of each of *Shigella* isolates n^{os} 221, 229, 292, 728 and *Salmonella* isolate n° 294 known to have transferred parts of their « r » determinants to *E. coli* K_{12} were inoculated respectively into 4 ml TSB containing 20 µg/ml of acriflavine. *E. coli* K_{12} 14 R 525 was also inoculated into 4 ml of TSB. The cultures were incubated at 37 °C for 20 h. Plasmid transfer experiment was performed as previously described (18). MacConkey agar of 5 formulations was used as selective medium (table n° I).

TABLE N° I-Concentrations of antibiotics in MacConkey agar 5 formulations

Medium	Antibiotics				
	PN	Te	C	S	Na
1	25				100
2		25			100
3			25		100
4				25	100
5					100

RESULTS

The bacterial isolates showed multiple resistance to ampicillin, streptomycin, chloramphenicol, tetracycline and sulpha drugs or a combi-

Fig. 1-Percentage of cultures cured of their antibiotic resistance patterns after incubation in different concentrations of acriflavine and acridine orange

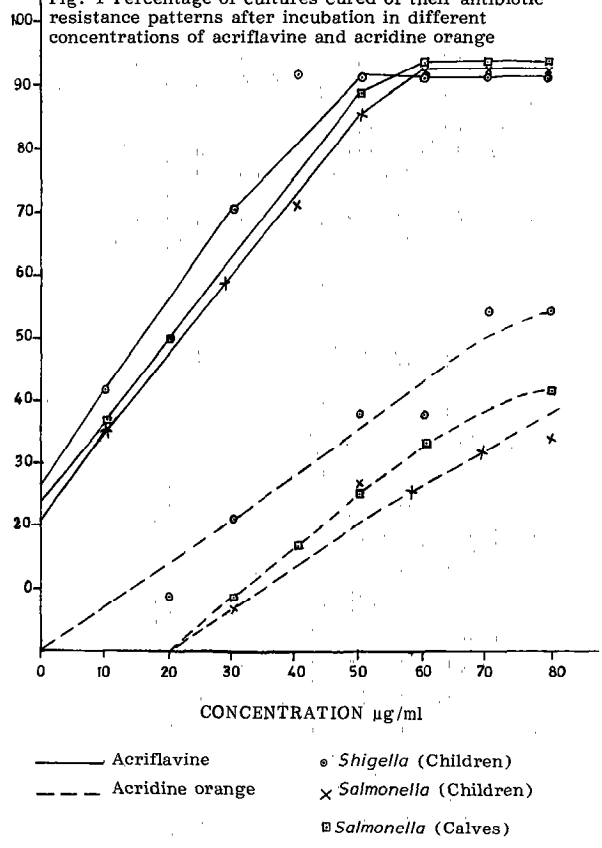

TABLE N° II - Enterobacteriaceae isolated from diarrhoeic children and calves with transfer of resistance patterns to *E. coli* K_{12}

Antibiotic resistance pattern	Sources and Nb. of isolates with the particular resistance pattern			Total number of cultures with specified resistance pattern	Number of culture transferring particular r determinant			
	Calves *(Salmonella)*	Children *(Shigella)*	Children *(Salmonella)*		PN	S	Te	C
PNSTec	-	17	2	19	18	19	8	5
PNSTe	-	4	2	6	6	6	2	-
PNTec	-	-	4	4	4	4	-	-
TeCSSU	12	-	-	12	-	12	12	12
PNSC	-	-	1	1	1	1	-	1
TeSC	-	2	-	2	-	-	-	-
TeSU	8	-	-	8	-	8	-	-
PNS	-	1	-	1	1	1	-	-
PNC	-	-	3	3	3	-	-	2
PN	-	-	1	1	1	-	-	-
S	-	-	1	1	-	1	-	-
SU	4	-	-	4	ND	ND	ND	ND

ND = not done.

nation thereof. Twelve antibiotic resistance patterns were encountered in this investigation (table n° II). Many of the isolates harboured transmissible R-factor. Acriflavine eliminated the antibiotic resistance patterns from all the isolates except in *Shigella flexneri* 2 (n° 323) and in *Shigella dysenteriae* 2 (n° 362), at 40 µg/ml whereas only a few isolates were cured of their antibiotic resistance patterns when acridine orange was used as mutagen even at 80 µg/ml (fig. 1). However when the resistant bacterial isolates were incubated in TSB containing 160 µg/ml acridine orange for 48 h all the isolates except *Shigella boydii* isolate n° 9 lost their antibiotic resistance patterns.

Shigella isolates n^{os} 221, 229, 292, 728 and *Salmonella* isolate n° 294 after 20 h incubation in TSB containing 20 µg/ml acriflavine transferred ampicillin « r » determinant to *E. coli* K_{12} (table n° III). Thirty-eight bacterial isolates (24 *Shigella* spp. and 14 *Salmonella* spp.) from diarrhoeic children resistant to ampicillin (PN) or more antibiotics transferred 1 (PN, S) or 3 (PNSC, TeSC) « r » determinants of their resistance patterns to the sensitive recipient while each of the 24 *Salmonella typhimurium* strains from diarrhoeic calves transferred their « r » determinants enbloc (table n° II).

DISCUSSION

A high frequency of infectious single and multiple resistant isolates of *Shigella* and *Salmonella* were isolated from diarrhoeic children and calves in this investigation. Isolates of bacteria harbouring R-factors transmissible to *E. coli* K^{12} were recovered from the calves and children covered in this study.

TABLE N° III - Antibiotic resistance patterns of 5 bacterial isolates before curing experiment; r determinants transferred to *E.coli* K_{12} before and after plasmid curing experiments

Strain nb.	Organism	Antibiotic resistance patterns	r determinants transferred before curing experiment	r determinants transferred after curing experiment
221	*Shigella dysenteriae 3*	PNSTec	PNS	PN
229	*Sh. flexneri 6*	PNSTec	PNS	PN
292	*Sh. flexneri 3*	PNSTec	PNS	PN
294	*Salmonella sp.*	PNSTec	PNS	PN
728	*Shigella flexneri 6*	PNSTec	PNS	PN

Chloramphenicol « r » determinant which was transferred at high frequency from 12 *Salmonella* isolates from diarrhoeic calves and from 4 isolates from diarrhoeic children as well as from 3 *Shigella* isolates from diarrhoeic children poses great concern. Published reports have shown that *Salmonella typhi.* harbouring R-factor chloramphenicol resistance have emerged in Vietnam and Mexico (5). The epidemics were controlled with a combination of ampicillin and trimethroprim-sulfamethozole. Public health hazard of chloramphenicol resistant *Salmonella typhimurium* phage type 29 was reported in Britain in 1968 (1). The *Salmonella typhimurium* caused outbreak of infection in intensively reared calves between 1963 and 1965. The public health hazard occurred when transmission occurred directly or indirectly from calves to man. This resulted to the death of 6 people in 1965. The *Salmonella typhimurium* was resistant to antibiotics including chloramphenicol. Should the *Salmonella* and *Shigella* isolates which are resistant to chloramphenicol in this study contaminate food, water meant for man, or infect man in whom there has been a *Salmonella typhi.* infection, the R-factor harbouring chloramphenicol resistance determinant could be passed on, and this might result in serious public health hazard.

Previous curing experiments have shown that mutagens such as ethidium bromide (4), amino acridine dyes (12, 22), were very efficient in eliminating classical F-factors. The R^+ cells became sensitive on exposure to low concentration of the mutagens.

In this investigation it was observed that acriflavine was very efficient in eliminating antibiotic resistance from nearly all of the bacterial isolates. This observation supports the findings of other investigators (12). The higher the concentration of this mutagen the more efficient was its curing activity. On the other hand, acridine orange was only active at higher concentrations (160 µg/ml) and at increased duration of incubation. These observations supported the finding of previous workers (17) that the population of drug susceptible cells increased with the duration of incubation, and the concentration of the mutagen such as sodium dodecylsulphate (SDS).

The mechanism of curing appeared to be the insertion or intercalation of aminoacridine or ethidium bromide molecule between adjacent DNA base pairs causing an extension or unwinding of the phosphodiester backbone (20). The selective effect of mutagens on DNA is not fully known either, however other investigators (10) showed that a significant difference existed between drug fixation by linear and circular DNA. Curing effects appear to be due to the fact that R^+ cells are more sensitive to inhibition by mutagens than preexisting plasmid negative segregants, also the sensitivity of R^+ cells appears to be correlated with the presence of pili. Piliated cells are more sensitive to curing than non-piliated cells. Sex pili are important in conjugation and are essential in transfer of « r » determinants from donors to sensitive recipients. In this investigation a high degree of transfer of R-factors was observed among the *Salmonella* and *Shigella* isolates. Antibiotic resistance patterns of isolates under study were effectively eliminated by acriflavine at concentrations between 10 µg/ml and 60 µg/ml. Since R-factors are more infectious in piliated cells than in non-piliated cells, thus it could be concluded that there is a close relationship between infectiousness of R-factor and plasmid curing. That acridine orange did not eliminate antibiotic resistances in this study disagreed with the findings of others (12, 21). The elimination of tetracycline, chloramphenicol and streptomycin resistances from the resistant bacteria indicated that these « r » determinants were extrachromosomal while ampicillin « r » determinant which was not eliminated from 5 bacterial isolates might be chromosomal (table n° III). It is thought that judicious use of antibiotic in human medicine and in agriculture would reduce the incidence of multiple drug resistance in Nigeria, a nation where antibiotics can be purchased easily without doctors' prescription.

CONCLUSION

High frequency of infective drug resistance transmissible in parts or enbloc was encountered in this study with acriflavine featuring as an efficient mutagenic agent even at low concentrations.

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RESUMEN

ADETOSOYE (A. I.), ROTILU (I. O.). — Curación de las infecciones causadas por *Salmonella* y *Shigella* aisladas a partir de casos de diarrea y resistantes a los antibióticos y a los anti-infecciosos. *Rev. Elev. Méd. vét. Pays trop.*, 1985, **38** (4) : 433-437.

Se estudió la resistencia a los medicamentos y a los antibióticos de 63 cepas de casos clínicos, de las cuales 24 cepas de *Salmonella typhimurium* proviniendo de terneros padeciendo diarrea, 24 cepas de *Shigella* spp. y 15 cepas de *Salmonella* spp. proviniendo de niños padeciendo también diarrea.

Se obtuvieron 12 esquemas de antibioresistencia. Se observó una tasa elevada de transmisión de la resistencia entre estas cepas bacterianas.

La acriflavina fué probada eficaz. A bajas concentraciones, elimina los determinantes « r » de las bacterias resistentes.

Palabras claves : Salmonella typhimurium - Shigella - Antibiótico - Acriflavina - Resistencia a los medicamentos.

REFERENCES

1. ANDERSON (E. S.). Drug resistance in *Salmonella typhimurium* and its implications. *Br. med. J.*, 1968, **3** : 333-339.
2. ANDERSON (E. S.), DATTA (N.). Resistance to penicillin and its transfer in *Enterobacteriaceae*. *Lancet*, 1965, **1** : 407-409.
3. BAUER (A. W.), KIRBY (W. M. M.), SHERRIS (J. C.), TURCK (M.). Antibiotic susceptibility testing by single disc method. *Am. J. clin. Path.*, 1966, **45** : 493-496.
4. BOUACHAUD (D. H.), SCAVIZZI (M. R.), CHABBERT (Y. A.). Elimination by ethidium bromide of antibiotic resistance in enterobacteria and staphylococci. *J. gen. Microbiol.*, 1968, **54** : 417-425.
5. BUTLER (T.), LINK (N. N.), ARNOLD (K.), ADICKMAN (M. D.), CHAU (D. M.), MOOI (M. M.). Therapy of antimicrobial resistant typhoid fever. *Antimicrob. Ag. A.*, 1977, **11** : 645-650.
6. COWAN (S. T.), STEEL (K. J.). Manual for identification of medical bacteria. Cambridge University Press, 1979.
7. DATTA (N.). Transmissible drug resistance in an epidemic strain of *Salmonella typhimurium*. *J. Hyg., Camb.*, 1962, **60** : 301-310.
8. GANGAROSA (E. J.), PERERA (D. R.), MATA (L. T.), MENDZABEL-MORRIS, (C.), GUZMAN (G.), RELLER (L. B.). Epidemic shiga *Bacillus dysenteriae* in Central Africa. *J. infect. Dis.*, 1970, **122** : 181-190.
9. HUDSON (B.), VINOGRAD (J.). Catenated circular DNA molecules in Hela cell mitochondria. *Nature, Lond.*, 1967, **216** : 647-650.
10. JYSSUM (K.). Elimination of genetic elements governing competence on transformation of *Neisserira meningitidis* by treatment with ethidium bromide and acriflavine. *Acta. path. microbiol. scand.*, 1971, **79** : 265-274.
11. LEIFSON (E.). New selenite F enrichment media for isolation of typhoid and paratyphoid bacilli. *Am. J. Hyg.*, 1936, **24** : 423-432.
12. MITSUHASHI (S.), HARADA (K.), KAMEDA (M.). Elimination of transmissible drug resistance by treatment with acriflavine. *Nature*, Lond., 1961, **189** : 947.
13. NASER (A. H. K.). Transmissible drug resistance in *Escherichia coli* isolated from healthy dogs, cattle, sheep and horses. *Vet. Rec.*, 1978, **103** : 587-589.
14. PRIVITERA (G.), DUBLANCHET (A.), SEBALD (M.). Transfer of multiple antibiotic resistance between subspecies of *Bacteroides fragilis*. *J. infect. Dis.*, 1979, **139** : 97-101.
15. ROTIMI (V. O.), DUERDEN (B. I.). Curing antibiotic resistance in bacteriodes species by aminoacridines and ethidium bromide. *Afr. J. med. Sci*, 1981, **10** : 91-96.
16. SILICER (J. H.), DELBEL (R. H.), FAGAN (P. T.). Enhancing effect of faeces on isolation of *Salmonellae* from selenite broth. *Appl. Microbiol.*, 1964, **12** : 100-105.
17. TOMOEDA (M.), TNUZUKA (M.), KUBO (N.), NAKAMURA (S.). Effective elimination of drug resistance and sex factor in *Escherichia coli* by sodium dodecyl sulphate. *J. Bact.*, 1968, **95** : 1079-1089.
18. WALTON (J. R.). Infectious drug resistance in *E. coli* isolated from healthy farm animals. *Lancet*, 1966, **2** : 1300-1302.
19. WARING (M. J.). Complex formation between ethidium bromide and nucleic acids. *J. molec. Biol.*, 1965, **13** : 269-282.
20. WATANABE (T.). Infectious drug resistance in enteric bacteria. *New Engl. J. Med.*, 1966, **275** : 888-896.
21. WATANABE (T.), FUKASAWA (T.). Episome mediated transfer of drug resistance in *Enterobacteriaceae*. 11. Elimination of resistance factor with acridine. *J. Bact.*, 1961, **81** : 679-683.
22. WATSON (C. E.). Infectious drug resistance in *Shigella* in Cape Town. *S. Afr. med. J.*, 1967, **41** : 728-731.