RESEARCH ARTICLE

Impact of antibiotic withdrawal and starvation conditions on plasmid elimination and consequent loss of resistance

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Abstract

Objective: To assess resistance-loss due to plasmid elimination under experimental conditions, including withdrawal of antibiotics and administration of starvation conditions.

Method: The experimental study was conducted at the Department of Pathology, King Edward Medical University, Lahore, Pakistan, from July to December 2019. A single sensitive clinical isolate of *escherichia coli,* showing resistance towards ampicillin was collected and separately sub-cultured in three different culture broths: tryptic soya broth, minimal broth and control broth for a period of one month under standard laboratory conditions. Minimum inhibitory concentrations of the strains were calculated after every seven days to check antibiotic susceptibility.

Results: Minimum inhibitory concentrations of the initial *escherichia coli* strain measured on Day 1 was 6mg/mL and it became sensitive after continual sub-culturing in the absence of antibiotics in 21 days. Due to starvation conditions, the bacterial strain exhibited sensitivity to an even lower antibiotic concentration of 1.5mg/mL on the 28th day. Bacterial growth inhibition zones determined by disc diffusion method using an ampicillin disc of 10µg/mL showed no zone of inhibition.

Conclusion: Provision of starvation conditions and withdrawal of antibiotic allowed the *escherichia coli* strain to exhibit gradual loss of resistance over a period of time.

Keywords: Antibiotic resistance, Plasmid elimination, Antibiotic withdrawal, Resistance loss, Starvation conditions.
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Introduction

Antimicrobial agents have been the most significant elements of clinical medicine since the introduction of penicillin in the human therapeutics in the 1940s. However, over the past 60 years, indiscriminate antibiotic use has imposed a great selective pressure, causing the bacteria to evolve resistance against all commercially available agents.[1,2] These bacterial strains possess complex mechanisms through which the resistant traits are not only maintained in the lineage, but are also transferred to sensitive bacteria regardless of the interceding specie barrier. This rampant spread of antimicrobial resistance has made antibiotic treatment clinically ineffective, making infectious diseases the leading cause of deaths worldwide.[3] Along with the increasing threat to life due to this rapidly increasing microbial resistance, there has also been a severe decline in the development of new antibiotics, mainly against gram-negative bacteria. Hence, acknowledging the situation, there is a desperate need to find a way of combating the increasing bacterial defence against all possible antibacterial treatments.[4]

Plasmid curing and antibiotic stewardship programmes are among the various strategies being used to deal with this problem. They both focus on the relation between the exposure to antibiotics and its resulting influence on resistance selection. According to studies, withdrawal of the drug for a specific period of time can possibly lead to the reversal of resistance.[5] There are complex mechanisms by which a bacterial strain acquires resistance that include de novo mutations and horizontal gene transfer (HGT) of the resistance-causing factors. Plasmid, along with several other genetic and environmental factors, elicits resistance through HGT and can practically be segregated from a bacteria, given the proper conditions and strategy.[6] Acquisition of these resistance determinants place a great biological burden on the bacteria. The fitness cost puts a strain on bacterial growth, giving rise to various possibilities that the resistant bacteria would lose its defensive traits in the absence of antibiotics following the law of natural selection.[7] This reversal of resistance by the bacteria can further be aided by administering starvation conditions, enhancing the rate of plasmid loss.[8]

The current study was planned to assess resistance-loss due to plasmid elimination under experimental conditions, including withdrawal of antibiotics and administration of starvation conditions.

Materials and Methods

The experimental study was conducted at the Department of

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Pathology, King Edward Medical University/Mayo Hospital, Lahore, Pakistan, from July to December 2019. After approval from the institutional ethics review board, a single sensitive clinical isolate of *Escherichia (E.) coli* showing resistance specifically to ampicillin was obtained from the Central Diagnostic Laboratory, Department of Pathology, Mayo Hospital. Bacterial antibiotic susceptibility was determined using the Kirby Bauer agar disc-diffusion method according to the Clinical and Laboratory Standards Institute (CLSI) guidelines 2019.[9] Antibiotic disc of ampicillin 10µg was used to which the test strain exhibited no zone of inhibition. Minimum inhibitory concentration (MIC) of the selected bacterial strain was also measured using the broth dilution method.[10] The zone of growth inhibition and MIC values of E. coli for ampicillin used were based on CLSI, implying that a zone diameter up to 13mm and an MIC value of 32µg/mL or higher were taken as resistant.[9] For MIC determination, serial dilutions of the antimicrobial agent were made using the stock solution of the concentration of 100mg/L. Properly adjusted bacterial suspension was prepared such that it contained organism density of 1×10^6 cfu/ml. Equal volumes of antibacterial solution and bacterial suspension was added to each tube. Results were read as in terms of lack of turbidity in the tube after incubating for 18 hours at 37°C, and the tube with minimum amount of antibiotic with no visible turbidity were considered MIC. A control strain of known MIC value was also assessed along with the test strain to check the reagents and conditions. The MIC values of the bacterial strains from tryptone soya broth (TSB) and minimal media of days 1, 7, 14, 21 and 28 were calculated simultaneously. MIC values of the bacterial strain were noted (Table-1).

Three culture mediums were used; TSB, control broth and minimal broth comprising 1% buffered peptone water. TSB (Soyabean Casein Digest Medium) was enriched with pancreatic digest of casein 17g/L, papaic digest of soyabean meal (3g/L), sodium chloride (5g/L), potassium hydrogen phosphate (2.5g/L) and dextrose (2.5g/L). Minimal media constituted only a small amount of glucose and salts for the implementation of starvation conditions, such as protease peptone, sodium chloride, disodium anhydrous phosphate and monopotassium phosphate. The control broth consisted of TSB supplemented with ampicillin depending on the MIC of the bacterial strain. A single colony of the clinical isolate of *E. coli* resistant to ampicillin was inoculated in TSB, control broth and minimal media simultaneously. The bacterial strain from TSB was sub-cultured after every 24 hours by inoculating 10µl of the previous sample in 3ml of freshly-prepared sterile broth. This procedure was repeated for 30 days. The bacterial strain from minimal media was also sub-cultured in a similar manner for 30 days. MICs of the strains were calculated after every 7 days.

Results

As shown by MIC values of the bacterial strains from TSB

Table-1: Minimum Inhibitory Concentration (MIC) values of *Escherichia (E.) coli* test strain (Day 1).

Antibiotic dilution	1:1	1:2	1:4	1:8	1:16	1:32	1:64	Positive control	Negative control
Antibiotic Concentration	32mg/ml	12mg/ml	6mg/ml	3mg/ml	1.5mg/ml	0.75mg/ml	0.375mg/ml	Broth + Inoculum	Only broth
Bacterial growth	-	-	-	+	+	+	+	+	-

Table-2: Minimum Inhibitory Concentration (MIC) values of *Escherichia (E.) coli* test strain from tryptone soya broth subcultures of days 1, 7, 14, 21 and 28.

Antibiotic dilution	1:1	1:2	1:4	1:8	1:16	1:32	1:64	Positive control	Negative control
Antibiotic Concentration	32mg/ml	12mg/ml	6mg/ml	3mg/ml	1.5mg/ml	0.75mg/ml	0.375mg/ml	Broth + inoculum	Only broth
Bacterial growth (Day 1)	-	-	-	+	+	+	+	+	-
Bacterial growth (Day 7)	-	-	-	+	+	+	+	+	-
Bacterial growth (Day 14)	-	-	-	+	+	+	+	+	-
Bacterial growth (Day 21)	-	-	-	-	+	+	+	+	-
Bacterial growth (Day 28)	-	-	-	-	-	+	+	+	-

Table-3: Minimum Inhibitory Concentration (MIC) values of *Escherichia (E.) coli* test strain from minimal media subcultures of days 1, 7, 14, 21 and 28.

Antibiotic dilution	1:1	1:2	1:4	1:8	1:16	1:32	1:64	Positive control	Negative control
Antibiotic Concentration	32mg/ml	12mg/ml	6mg/ml	3mg/ml	1.5mg/ml	0.75mg/ml	0.375mg/ml	Broth + inoculum	Only broth
Bacterial growth (Day 1)	-	-	-	+	+	+	+	+	-
Bacterial growth (Day 7)	-	-	-	+	+	+	+	+	-
Bacterial growth (Day 14)	-	-	-	+	+	+	+	+	-
Bacterial growth (Day 21)	-	-	-	-	+	+	+	+	-
Bacterial growth (Day 28)	-	-	-	-	+	+	+	+	-

and minimal media for different days, the initial *E. coli* strain measured on Day 1 was 6mg/mL and it became sensitive after continual sub-culturing in the absence of antibiotics in 21 days. Due to starvation conditions, the bacterial strain exhibited sensitivity to an even lower antibiotic concentration of 1.5mg/mL on the 28th day (Tables-2, 3). Bacterial growth showed no zone of inhibition.

Discussion

In the current study, the *E. coli* test strain from TSB exhibited an onset of gradual loss of resistance due to continuous sub-culturing in the absence of ampicillin. The bacterial strain, which was initially resistant and able to grow in an ampicillin concentration of 3mg/mL, was unable to survive this antibiotic concentration on day 21. A similar pattern was observed in the bacterial isolates from subsequent sub-cultures.

Similarly, the bacterial strain sub-cultured in minimal media for 1 month showed a positive correlation between antibiotic withdrawal and resistance-loss. The strain that was initially unaffected by 3mg/mL ampicillin concentration became vulnerable to it after continual sub-culturing in the absence of antibiotics in 21 days. Also, due to starvation conditions, the bacterial strain exhibited susceptibility to an even lower antibiotic concentration of 1.5mg/mL on the 28th day. This illustrated that unavailability of sufficient nutrients for the standard bacterial growth and survival can compel the bacteria to further amplify the process of plasmid removal. It was hypothesised that any loss of resistance by the bacterial strain after continual sub-culturing in the absence of antibiotics was due to segregational loss of the plasmid. These findings complement those of a previous study which investigated the effect of glucose-deficient media on segregational loss of plasmid pACYC184 in E. coli and indicated an overall declining trend of the plasmid content.[11]

The *E. coli* isolate that was inoculated in the control broth exhibited no change in antibiotic susceptibility by the end of the study, demonstrating that the bacterial selection against resistance-inducing plasmid occurred solely due to the withdrawal of antibiotics as both isolates of the same bacterial strain were processed under similar laboratory conditions.

Even though a drastic change in resistance-loss due to the elimination of plasmid was not observed in the current study, an overall declining trend in the antibiotic resistance was detected owing to the implementation of antibiotic withdrawal and starvation conditions. This can be a result of various maintenance factors that the bacteria might develop to compensate for the fitness cost induced by the resistant plasmid on bacterial growth, stalling and hindering the process of negative selection against such defensive traits.[12]

The findings of the current study provide practical evidence to various clinical investigations performed in hospital settings that investigated correlation between restriction of antibiotics and its resulting impact on antibiotic susceptibility.[13] Although such studies showed a decrease in overall resistance to antibiotic, this loss of resistance could not be entirely linked with the absence of antibiotics in a local or clinical setting because of the presence of various intervening factors, such as other pathogenic, colonizing or commensal bacteria present in an individual being monitored or the hospital environment being targeted, ensuing the availability of a larger genetic pool that can alter the resistance pattern of bacterial defensive traits to a specific antibiotic.[14] In a properly-monitored laboratory setting, as was the case in the current study, no other intervening factors are present relative to any contaminating or coexisting bacteria that may interfere with the results, proving that the loss of resistance was in fact a consequence of antibiotic withdrawal.

Conclusion

Withdrawal of antibiotics allowed the bacteria to negatively select against the defensive traits, like resistance-inducing plasmid, over a period of time. This investigation also illustrated that unavailability of sufficient nutrients for standard bacterial growth and survival can further amplify the rate of resistance-loss by the bacteria.

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