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Inhibitory effects of reserpine against efflux pump activity of antibiotic resistance bacteria

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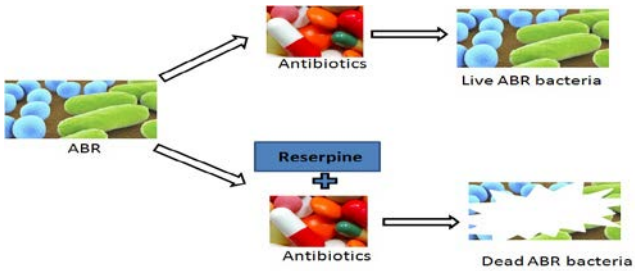
ABSTRACT



Antibiotic resistant bacteria (ABR) is an alarming issue and it has to be eliminated before enter into the environment. The role of efflux pump inhibitor, reserpine – a plant alkaloid in reducing the antibiotic resistance in *Staphylococcus* sp., *Streptococcus* sp. and *Micrococcus* sp. from poultry litter were evaluated. Reserpine increased the activity of antibiotics against bacteria and directly blocks the antibiotic efflux pump. Resistances of bacteria to antibiotics were reduced when they were grown in the presence of reserpine. Furthermore, this compound effectively reduced the Minimum Inhibitory Concentration (MIC) of several antibiotics such as ampicillin, erythromycin, tetracycline, chloramphenicol, streptomycin, kanamycin, rifampicin and tobramycin, suggesting that reserpine are representatives of bacterial efflux inhibitors with the potential application in combination therapy for veterinary medicines. This defensive action will helps us to reduce the existence of ABR in poultry industry as well as in the environment.

Keywords: Antibiotic resistant bacteria, Efflux pump, Minimum Inhibitory Concentration, Poultry, Reserpine

INTRODUCTION

Solid waste usually consists of agricultural waste, livestock waste, commercial, industrial and domestic wastes. Among the livestock waste, the contribution from poultry industry is higher due to the immense poultry consumption. Most of the broiler production operations generate 1460 kg of litter from 1000 poultry during their life cycle. Poultry waste mainly consists of bedding material, removed feathers, waste feed and mixture of excreta. These wastes contain high concentration of antibiotics due to its presence from the livestock feeds (0.2% w/w).[1-2] The application of antibiotics has become integral to the livestock production industry for growth promotion and therapeutic action. But the overuse of antibiotics contributes to the development and spread of resistant organisms as well as transfer of resistant genes among pathogenic and non-pathogenic organisms in the environment.

Over the past few decades the public has become alarmed by the scientific data about the connection between the overuse of antibiotics and the emergence of antibiotic resistant bacteria.

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Hence it becomes necessary to reduce the existence of antibiotic resistant bacteria. Bacteria acquire resistance to antibiotics via three mechanisms: antibiotic activation, target modification and alteration of intracellular antibiotic concentration. The mechanism can occur by either decreasing the permeability to an antibiotic or increasing the activities of a variety of efflux pumps.[3]

Active efflux of toxic compounds out of cells is a general mechanism that bacteria have developed to protect themselves against the adverse effects of their environments. Hence, many academic programs have focused on identifying inhibitors of gram-negative and gram-positive efflux systems that could potentially be used in combination with antibiotics to improve efficacy and suppress resistance.[4] *In vitro*, efflux pump inhibitors (EPI) have been shown to reduce spontaneous resistance frequencies to antibiotics in *Pseudomonas aeruginosa* [5] and *Staphylococcus aureus.*[6]

In gram-negative bacteria, the pumps belong to the resistance-nodulation-cell division family and in gram-positive bacteria, the pumps were of major facilitator super family class. These pumps play greater role in the efflux of antibiotics, contributing to resistance. NorA pumps of *Staphylococcus aureus*,[7] EmeA pump of *Enterococcus faecalis*[8] and pmrA pump of *Streptococcus pneumoniae*[9] are the examples of this class of efflux pumps.

Inhibition of efflux pump is the potential method to control the antibiotic resistance in microorganisms. The role of efflux pump in antibiotic resistance has to be overcome by developing a compound that inhibits the efflux mechanism. Various inhibitors have been used to study these mechanisms. One among them is reserpine, a plant alkaloid which inhibits the active efflux systems that act as multidrug transporters present in gram-positive and gram-negative bacteria.[10]

On the basis of genetic data, it appears that inhibition of efflux pumps in isolated organisms from poultry litter may significantly improve the performance of antibiotics. Inhibition of efflux pumps is expected to decrease the level of intrinsic resistance, significantly reverse the acquired resistance and also to decrease the frequency of emergence of mutant isolates which are highly resistant to antibiotics.

The objective of this study is to determine the activity of efflux pump inhibitor (reserpine) for enhancing the activity of antibiotics against the organisms isolated from the poultry litter which transfer the resistance gene to pathogenic and non-pathogenic organisms present in environment.

EXPERIMENTAL PROCEDURES

Compound:

Reserpine, efflux pump inhibitor (EPI) was obtained from Sigma Chemical Co. and stocks were prepared at a concentration of 100 mg/mL in 100% dimethyl sulfoxide (DMSO). A stock solution of 10 mg/mL for each antibiotic (Sigma) was prepared, diluted in concentration range of 10 to 300 µg/mL in 100% DMSO and stored at -20 °C.

Isolation of bacterial strain:

Previous studies focused on the isolation of antibiotic resistant organisms from the poultry litter.[10] The predominant bacterial strains such as *Staphylococcus* sp., *Streptococcus* sp. and *Micrococcus* sp. have been used in this study. The bacterial strains were reactivated in nutrient broth (NB). Nutrient broth and agar plates were prepared using peptic digest of animal tissue - 5 g, beef extract - 3 g, NaCl -5 g and agar - 1.5 g for 1000 mL medium. The standard spread plate method was performed. The inoculated plates were incubated at room temperature (35 – 37 ºC) for 48 h. After 48 h incubation larger identical colonies from each plate were isolated.

Determination of Minimum inhibitory concentration:

Minimum inhibitory concentration (MIC) was determined for *Staphylococcus* sp., *Streptococcus* sp. and *Micrococcus* sp. isolates were reactivated in nutrient broth (peptic digest of animal tissue - 5 g, beef extract - 3 g and NaCl - 5 g for 1000 mL) and incubated at 37 ºC for 18 h and diluted in Muller Hinton broth to a turbidity of 0.5 NTU on the McFarland scale. A stock solution of 10 mg/mL for each antibiotic (Sigma) was prepared and diluted in concentration range of 10 µg - 300 µg/mL. Aliquots containing 50 µL of diluted antibiotics, 100 µL of double strength Muller Hinton broth and 50 µL of isolated cultures at 0.5 NTU on the McFarland concentration (total volume of 200 µL per well) were taken and incubated at 37 ºC for 24 h. Growth was assessed spectrophotometrically in an ELISA plate reader (Biorad 680) at λ 540 nm.[19]

Activity of the active efflux systems inhibitors:

The MIC of the drugs studied was calculated in the presence (100 mg/L) and absence of reserpine.

RESULTS AND DISCUSSION

In our previous study, 120 antibiotic resistant bacteria were isolated from poultry litter.[11] Among them, 75% were occupied by *Streptococcus* sp., *Staphylococcus* sp., and *Micrococcus* sp. and their involvement of an efflux pump for the resistance against ampicillin, erythromycin, tetracycline, streptomycin, tobramycin, chloramphenicol, rifampicin and kanamycin were evaluated. In prokaryotic and eukaryotic cells, the importance of efflux pumps is to remove toxins from the interior of the cell. This protective function enables bacterial cells to survive in hostile environments, including the presence of antibiotics during the treatment of infections. Limited structural homology between bacterial and mammalian efflux systems, create a significant substrate overlap. Because of this overlap, mammalian MDR inhibitors, such as reserpine and verapamil also affect bacterial efflux systems.[10]

In this study, we have shown that reserpine, a novel mammalian MDR inhibitors was active in potentiating the activity of antibiotics against the poultry litter isolates such as *Staphylococcus* sp., *Streptococcus* sp. and *Micrococcus* sp. In the presence of reserpine, the bacterial isolates such as *Staphylococcus* sp., *Streptococcus* sp., and *Micrococcus* sp., partially reverses the antibiotic resistance which may be due to the over expression of different pumps. Furthermore, it is interesting that the activity of reserpine on the MIC of

antibiotics was non-saturable for the bacterial isolates *Staphylococcus* sp., *Streptococcus* sp. and *Micrococcus* sp. which might suggest the targeting of multiple efflux pumps.

A variation of 230 to 15 µg/mL, 240 to 20 µg/mL, 270 to 15 µg/mL, 80 to 1 µg/mL, 260 to 0.25 µg/mL, 110 to 12.5 µg/mL of ampicillin, erythromycin, tetracycline, chloramphenicol, streptomycin and rifampicin, respectively for *Staphylococcus* sp., was obtained in the presence of 100 mg/mL of reserpine. In kanamycin, the variation was 110 to 65 µg/mL but in case of tobramycin, less or no activity was observed (Table 1).

Table 1. Effect of Reserpine on antibiotic susceptibility of *Staphylococcus* sp. isolated from poultry litter.

Antibiotics	**MIC (µg/mL)**				
	Without EPI	**With EPI(mg/mL)**			
		25	50	75	100
Ampicilin	230	120	60	40	25
Erythromycin	240	160	75	30	20
Tetracycline	270	140	90	40	15
Chloramphenicol	80	20	14	1	1
Streptomycin	260	60	20	0.25	0.25
Tobramycin	10	10	9.5	9.5	8
Rifampicin	110	60	25	12.5	12.5
Kanamycin	110	40	16	8	4

In case of *Streptococcus* sp., MIC variation of 110 to 8 µg/mL, 1 to 0.1 µg/mL, 3 to 0.25 µg/mL, 70 to 1.5 µg/mL, 1 to 0.1 µg/mL, 60 to 3 µg/mL of ampicillin, erythromycin, tetracycline, streptomycin, tobramycin and kanamycin, respectively were obtained in the presence of 100 mg/mL of reserpine. Chloramphenicol showed slight activity in the presence of reserpine but rifampicin showed no activity with reserpine (Table 2).

Table 2. Effect of Reserpine on antibiotic susceptibility of *Streptococcus* sp. isolated from poultry litter.

Antibiotics	**MIC (µg/mL)**				
	Without EPI	With EPI (mg/mL)			
		25	50	75	100
Ampicilin	110	64	16	8	8
Erythromycin	1	0.1	0.1	0.1	0.1
Tetracycline	3	1.5	0.25	0.25	0.25
Chloramphenicol	60	54	36	33	33
Streptomycin	70	40	22	6	1.5
Tobramycin	1	0.5	0.1	0.1	0.1
Rifampicin	3	3	3	2.5	2.5
Kanamycin	60	40	24	6	3

In *Micrococcus* sp., MIC variation of 120 to 10 µg/mL, 10 to 0.25 µg/mL, 90 to 0.625 µg/mL, 20 to 0.1 µg/mL, 70 to 0.25 µg/mL, 80 to 4 µg/mL of ampicillin, chloramphenicol, streptomycin, tobramycin, rifampicin and kanamycin, respectively were obtained in the presence of reserpine which shows that resistance to these antibiotics were strongly reduced by reserpine, whereas this agent has no effect on the degree of susceptibility to tetracycline and low activity against the susceptibility to erythromycin (Table 3).

Table 3. Effect of Reserpine on antibiotic susceptibility of *Micrococcus* sp. isolated from poultry litter.

Antibiotics	**MIC (µg/mL)**				
	Without EPI	With EPI (mg/mL)			
		25	50	75	100
Ampicilin	120	60	15	10	10
Erythromycin	100	85	80	75	75
Tetracycline	50	46	44	44	44
Chloramphenicol	10	3	1	0.25	0.25
Streptomycin	90	15	6.25	1.25	0.625
Tobramycin	20	0.5	0.1	0.1	0.1
Rifampicin	70	30	12.5	0.25	0.25
Kanamycin	80	20	8	8	4

These results demonstrate that an efficient efflux mechanism is involved in antibiotic resistance and this active process is sensitive to reserpine in a dose dependent manner. Reserpine increases the intracellular antibiotic concentration and, therefore, when they are assayed together with certain antibiotics, they increase the *in vitro* activity of the antibiotic and reduce the MIC. These differences are probably associated with the structural characteristics of the molecules. The relationship between the activity of the systems and the chemical structure of the antibiotics has already been reported.[12] The existence of different active efflux systems with varying antibiotic affinities depends upon the bacterial species involved[13] and the intra-specific differences, probably associated with the existence of genes that regulate these systems.[14] Reports suggested that pumps may have specific antibiotic-binding sites and the degree of inhibition of pump protected by inhibitors may be due to competition for these specific binding sites.[15] The existence of multiple-substrate binding sites within single efflux pump has also been reported.[16-18]

The present study suggests that the effect of the active efflux pump inhibitor to decrease the antibiotic susceptibility in isolated bacterial species depends on the characteristics of the antibiotic used, and to lesser extent, on the serotype. Based on

the chemical structure of the compounds, the active efflux systems have different activity.[20] Our data indicates that the reserpine may be a useful tool to study interactions between the isolated bacterial efflux pump and its substrates. In addition, EPI may prove to be useful in the development of new strategies to diagnosis and circumvent antibiotic resistance in bacteria for effective solid waste management.[21]

CONCLUSIONS

The present study concludes that the existence of antibiotic resistance in bacterial species such as *Staphylococcus* sp.,[22] *Streptococcus* sp. and *Micrococcus* sp. has been significantly reduced by the efflux pump inhibitor reserpine. We hope this preliminary data will helps to reduce/destroy the antibiotic resistant bacteria before it enters into the environment and we can also prevent the emergence and spread of antibiotic resistant bacteria and its resistance gene transfer to other microorganisms present in the environment.

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