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AN *IN VITRO* STUDIES ON EMERGENCE OF RESISTANCE IN FLOROQUINOLONES IN CLINICAL ISOLATES OF *PSEUDOMONAS AERUGINOSA* IN POULTRY

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ABSTRACT

An attempt to induce resistance to enrofloxacin *in vitro* was made against isolates of *Pseudomonas aeruginosa* isolated from poultry birds. This artificial exploitation of strains resulted in the increase of minimum inhibitory concentration from 0.3µg/ml to 1 µg/ml of original strain to 8.0 to 11.5 µg/ml indicating development of resistance to enrofloxacin (Ex) and a major decrease in size of zone of inhibition of ciprofloxacin (Cf) and levofloxacin (Le) indicating cross resistance to these agents.

Results indicate the induced resistance to enrofloxacin can promote development of cross resistance to other flouroquinolones. This indicates that of alertness is required whiles using flouroquinolones for the treatment of *pseudomonas aeruginosa* infections in poultry.

KEYWORD: *P. aeruginosa* enrofloxacin, emergence resistance.

INTRODUCTION

Flouroquinolones are commonly used antibiotics in the treatment of various infections. Resistance to flouroquinolones emerges in to P*seudomonas aeruginosa*. Development of resistance to enrofloxacin and other flouroquinolones during the remedy of CRD, CCRD, septicemia. Devriese and Viaene *et al*. (1975). The emergence of flouroquinolones cross resistance in *p. aeruginosa* is also frequently reported. In the present study on attempt has

been made to induce *in vitro* resistance to enrofloxacin (Ex) in clinical cases of *pseudomonas aeruginosa* in poultry. The same strain were also subjected to study their susceptibility pattern by using the other flouroquinolones like ciprofloxacin (Cf) levofloxacin (Le).

MATERIAL AND METHODS

The eight isolates were recovered from heart, liver, lungs, Airsac, of poultry birds including (day old layer, broiler chicks and breeders that died of septicaemic conditions. Swab samples were Streaked on onto Mac conky agar (Himedia) and incubated aerobically at 37°C for 24 to 48 h. The suspected colonies were further characterized biochemically and identified as *P. aeruginosa* according to accurate procedure (Cowan, S.T. (1974). Collee & Duguid, *et al* (1989). Cruickshank& Duguid, *et al* (1975).

Determination of the Enrofloxacin, ciprofloxacin, and levofloxacin MIC values. Enrofloxacin ciprofloxacin and levofloxacin (all obtained from Himedia). MICs were determined using the two-fold micro-broth dilution method as per CLSI standards (CLSI, 2009). Swiatlo *et al* (2000). In this study subjected to isolates of P. *aeruginosa* to Enrfloxacin, Ciprofloxacin and Levofloxacin which had an MIC range 0.3µg/ml to 1.0µg/ml Enrofloxacin were selected to induce resistance. The strains were inoculated in to peptone water (Pw) and incubated overnight at 37^{O}c. After achieving the final bacterial concentration of $1X10^{5}$CFU/ml approximately 200 µl of peptone water culture was inoculated into 10 ml brain heart infusion (BHI) broth containing 0.05µg enrofloxacin per ml and incubated at 35^{0}c for the 16 hours with intermittent shaking. After incubation 200 µl of culture was transferred serially for 11 times through Brain heart infusion (BHI) broth containing increasing concentration of enrofloxacin. The increasing concentration of enrofloxacin used were 0.1, 0.2, 0.4, 0.8, 01, 0, 1.2, 1.4, 1.6, 1.8, 2.0, 2.2 and 2.5 µg/ml. After serial passage of the strains through the drug containing media MIC to enrofloxacin was determined by agar dilution method. The rate of variant isolation was detected by standard plate count on Mueller hinton agar (MHA) containing 10µg/ml of enrofloxacin. *In-vitro* susceptibility to enrofloxacin, ciprofloxacin levofloxacin was studied in all the 8 isolates by Kirby bauer disc diffusion method (1966). Before and after resistance was induced to enrofloxacin and change in zone of inhibition were noted.

RESULTS

Table-1 shown the MIC and zone of inhibition of the clinical isolates before and after the development of resistance to Enrofloxacin by serial dilution passage it was possible to induce resistance to enrofloxacin in all 8 isolates.

Table 1: Minimum inhibitory concentration of the *Pseudomonas aeruginosa* strains before and after induce resistance to Enrofloxacin.

Source	Strain	MIC of Original and mutant strain	Zone of Inhibition			Frequency
			Enrofloxacin	Ciprofloxacin	Levofloxacin	
Broiler Chicks Liver	1A 1B	0.8 9.0	26 13	24 10	25 15	$2x10^{-7}$
Broiler Chicks heart	2A 2B	0.8 12.0	30 12	26 14	31 16	$1x10^{-7}$
Broiler Chicks lungs	3A 3B	10 12.0	25 14	23 16	27 12	$1x10^{-7}$
Layer chicks liver	4A 4B	0.4 12.5	28 14	26 13	27 14	$2x10^{-7}$
Layer chicks heart	5A 5B	0.8 9.0	23 13	25 14	26 15	$3x10^{-7}$
Breeders liver	6A 6B	0.8 12.0	28 13	27 12	24 15	$4x10^{-7}$
Broiler Airsac	7A 7B	0.7 11.0	22 15	26 14	28 15	$1x10^{-8}$
Broiler Heart	8A 8B	0.6 10.5	25 15	28 12	26 13	$1x10^{-7}$

Original strain= 1A, 2A 3A 4A 5A 6A 7A 8A, Mutant strain= 1B, 2B, 3B, 4B, 5B, 6B, 7B, 8B,

Enrofloxacin MIC range of 0.4 µg/ml to 1 µg/ml of original strain of *p. aeruginosa* were increased to 9 µg/ml . The spontaneous resistant mutant were recovered in MHA containing 9.0 µg/ml of enrofloxacin at frequency $1X10^{-7}$ $1X10^{-8}$. The microbial sensitivity pattern of newly isolated enrofloxacin mutant showed major decrease in the zone of diameter of enrofloxacin, ciprofloxacin, levofloxacin,

DISCUSSION

Flouroquinolone are bactericidal rapidly acting antimicrobial drugs with wide spectrum of activity Swiatalo (2000). They are very effective against many gram negative bacteria

including *P. aeruginosa*. The main mechanism in the development of flouroquinolone is the decrease in the binding of the target quinolones to topoisomerase enzyme Aldred *et al* (2014), Hooper (2000) Piddock L J (1995).

Deguchi &Yasuda *et al* (1997) were studied nucleotide and amino acid sequence of gyrA, gyrB Par C and Par E, genes needed for synthesis of DNA. Gyrase enzyme, mutation occurs in gyrA and par C genes. Hiroshi *et al* (2003). The form of resistance is seen with *p. aeruginosa, E.coli, klebsiella* pneumoniae, *proteus, staphylococcus,* against all floroquinolones.

The second mechanism is the decrease in the amount of flouroquinolone entering the cells of the bacteria due to defective function of porine channels by Hirsch and Tam (2010). The third mechanism is various reflux system localized in the membranes of many bacteria including *p. aeruginosa* which pump the drug out of the bacteria Algun *et al* (2004).

This study reveals that exposure of *P. aeruginosa* to sub inhibitory concentration of enrofloxacin can promote the development of resistance to other quinolones. The rate of development of resistant depends on the bacterial strain the concentration and duration of enrofloxacin exposure. Prolonged exposure to enrofloxacin can lead to enhanced isolation of mutant variants. Li and Nikaido (2004).

Increased rate of development of resistance was observed suggesting that enrofloxacin can increase the rate of frequency of mutation leading to drug resistance. The extended enrofloxacin treatment in our case stimulate the prolonged exposure. That occurs in vivo during enrofloxacin therapy in poultry. Livermore D.M. (2002) said about the *p. aeruginosa* is our worst nightmare.

Repeated exposure of organism to antimicrobial agents is thought to enhance the development and maintenance of resistance. Presence of antimicrobial agents in sublethal concentrations make an environmental suitable for stepwise mutation resulting in the developing resistance of the drug. Wright *et al*, (2013) were studied sub inhibitory concentrations of some antibiotics can drive diversification of *Pseudomonas aeruginosa*. In the present study with exposure to sublethal concentration of enrofloxacin of followed by repeated exposure to increasing concentration it was possible to induce resistance in these 8 isolates and evidenced by increase in MIC.

A major decrease in the zone of diameter of enrofloxacin after exposure to enrofloxacin *in vitro* indicates resistance to enrofloxacin. A major decrease in the zone of diameter in ciprofloxacin, and levofloxacin indicates development of cross resistance to flouroquinolones following exposure to enrofloxacin. These results are similar to Ogle, Reller *et al* (1988) & Haver kron (1988) who studied development of ciprofloxacin resistance in *P. aeruginosa* during therapy and reported the development of cross resistance among the flouroquinolones. The finding in our study were also similar to the results reported by Nagoba *et al* (1998) who studied the development of resistance in floroquinolones *in vitro*. Hirai *et al* (1987) reported type of mutational resistance producing strains of *P. aeruginosa* to norfloxacin. Limb *et al* (1987) studies among the resistance studies in four quinolones. Bruchmann *et al* (2013) studied about the quantitative contributions of drug accumulation regarding with *P. aeruginosa* Fluoroquinolone Resistance. Li, and Nikaido *et al* (2004) studies the efflux mediated mechanism of flouroquinolones resistance in bacteria.

A studies carried out by the Chinese researcher Li, and Bi *et al* (2007) on the *E.coli* isolates from the chicken on development of enrofloxacin resistance sequence the quinolone resistance. They were found several fold greater than the maximal plasma concentration of enrofloxacin in chickens, mutation frequencies were also much lower, compared with frequencies for single-mutation isolates.

Results of the present study indicate that the development of resistance to enrofloxacin simultaneously results in the development of resistance to other flouoroquinolones which is a problem to be considered. These groups of drugs should be used with most carefully and only when it is required.

CONCLUSION

From this study it can be concluded that care should be taken in the use of floroquinolone for *p. aeruginosa* infections as they develop resistance these groups of drugs quickly. *P. aerugionsa* considered as a “charged pistol” that can inhibit flouroquinolone susceptibility. The use of these groups drugs should be limited and avoided in clinical situations where there is possibility of evolution of resistance strains.

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