FUDMA Journal of Sciences (FJS)
ISSN online: 2616-1370
ISSN print: 2645 - 2944
Vol. 5 No. 1, March, 2021, pp 309 – 313
DOI: https://doi.org/10.33003/fjs-2021-0501-569

IN VITRO ACTIVITY OF *ENROFLOXACIN* AGAINST CLINICAL *ESCHERICHIA COLI* AND NON-LACTOSE FERMENTING *ENTEROBACTERIA* ISOLATES FROM CHICKENS IN BENUE STATE, NIGERIA

***Jamilu, R. Y. and Asambe, A.**

Department of Animal Science, Federal University Dutsin-Ma, Katsina State Nigeria.

***Corresponding Author:** jamilury@gmail.com

ABSTRACT

Clinical bacterial isolates from chickens were analysed to determine their susceptibilities to antimicrobial agent. Ten (10) *Escherichia coli* and 8 non lactose fermenting *Enterobacteriaceae* species isolated from a pool of clinical cases of chickens from Microbiology Laboratory of the Veterinary Teaching Hospital, University of Agriculture Makurdi were used for the study. Enrofloxacin with 99 % purity obtained from Sigma-Aldriech, USA and prepared in varying concentrations (0.1 - 50µg/mL) was used *in vitro* by Kirby-Bauer's disc-diffusion method. The isolates were susceptible to enrofloxacin at a minimum concentration of 25 (µg/mL) and the mean value in the zones of inhibition exhibited by *Escherichia coli* and non-lactose fermenters were significantly different ($p<0.01$). The enrofloxacin tested also exhibited the concentration dependent effect typical of quinolones in this study. The study concluded that the tested antimicrobial agents can still be applied in the prevention and treatment of bacterial infection of chickens. Usage of these agents by veterinarians in poultry with appropriate clinical judgement and proper dosing principle is recommended. Also, routine assessment of the *in vitro* activities of this agent against common microbial infections in this area is strongly recommended.

Keywords: Antibacterial, Chicken, Enrofloxacin, *Escherichia coli*, Non-lactose fermenter

INTRODUCTION

Enrofloxacin is a fluoroquinolone frequently used in veterinary medicine as a chemotherapeutic agent. This broad spectrum antimicrobial is indicated in poultry for the treatment of respiratory and intestinal tract infections caused by *Mycoplasma gallisepticum*, *Mycoplasma synoviae*, *Avibacterium gallinarum*, *Pasteurella multocida* and *Escherichia coli (E. coli)* (Brown, 1996; Fabrega *et al*., 2008). The drug is a routine choice for the treatment of almost any bacterial disease of poultry (Muller and Hom, 2009).

The (mis)use of fluoroquinolones in chickens has led to an alarming incidence of fluoroquinolone resistance in both pathogenic and commensal bacteria (Devreese *et al*., 2014).The high rate of fluoroquinolone resistance as a result of treatment failure in veterinary medicine is of concern for human medicine as resistant bacteria can be transferred through the food chain (Brown, 1996). Generally, antimicrobials once effective at controlling bacterial infections can be ineffective due to acquired resistance to these compounds. Resistance to two or more classes of antimicrobial agents is now common in veterinary (Gonzalez and Blanco, 1989; Irwin *et al*., 1989; Harnett and Gyles, 1984) and human (Dennesen *et al*, 1998) medicine.

The problem of microbial resistance is growing and the outlook for the use of antimicrobial agents in the near future is uncertain following reports of ciprofloxacin resistance in *Salmonella spp*., *Campylobacter jejuni*, *Campylobacter coli* and the indicator bacteria *E. coli* derived from domestic fowl was 37.3%, 44.1%, 78.4% and 57.6%, respectively (AFSCA, 2013), Considering that ciprofloxacin is used as representative for the fluoroquinolones as it is used in human medicine and it is also the major metabolite of enrofloxacin. Although in broiler chickens the metabolization of enro- to ciprofloxacin is limited, 5 to 10% (FDA, 2005; Anadon *et al*., 1995).

Therefore, the need to continually assess the *in vitro* activities of these frequently used antimicrobial agents informed this study.

MATERIALS AND METHODS

Escherichia coli and non-lactose fermenting *enterobacteria* isolates

Ten Avian *Escherichia coli* and 8 non lactose fermenting *Enterobacteriaceae* species isolates were tested. The isolates were collected from a pool of clinical cases from veterinary microbiology laboratory of the veterinary teaching hospital, University of Agriculture, Makurdi. Proper history of each flock including management practices and previous treatment were noted. Liver, spleen, kidney, lungs and bile samples were collected from either moribund or dead birds during post-mortem examination and labelled individually.

The isolates were identified on the basis of morphological and biochemical characteristics. On the basis of microscopic examination, morphology of bacteria was noted as rod, spiral or filament. It was differentiated by Biochemical

characterization as per Jackie Reynolds (2005). On cultural basis, MacConkey agar and Eosin-methylene blue agar (EMBA) were used to confirm the identity of the *E. coli* isolates.

Swabs collected were directly inoculated onto blood agar and MacConkey agar in duplicates for every sample inoculums and incubated at 37ºC for 24 hours. Similar colonies from growth observed were "gram" stained and examined on the basis of size, morphology and staining characteristics. The gram negative coccobacilli colony types were further characterized.

On MacConkey agar only lactose fermenting (LF^+) pink coloured colonies were isolated and sub cultured for further characterization to check whether the bacteria are *E. coli* (i.e., there are other lactose fermenters like: *Klebsiella* and *Enterobacter*). The LF^+ colonies were reinoculated on EMB agar for presence of metallic sheen which is a characteristics of *E. coli* growth on EMB, while non-lactose fermenting (LF^-) *Enterobacteriaceae* species appeared as colourless colonies were isolated and sub cultured on Muller Hinton agar to obtain pure cultures of non-lactose fermenting *Enterobacteriaceae species.* Pure cultures of both isolates grown in nutrient broth were mixed with sterile glycerol 1:1 and stored at −20°C (Lee and Arp, 1998).

Preparation of Antibacterial drug stock solutions and dilution trays

Standard enrofloxacin with 99% purity was sourced from Sigma-Aldriech, USA. The serial dilutions of the antimicrobial agent were prepared from a stock solution of 10 varying concentrations (50 – 0.1 µg/ml) using appropriate solvents with positive growth control tube without an antimicrobial agent (Andrews, 2001).

Disc diffusion test

Firstly, glass wares and other media were sterilized and kept ready. Muller Hinton agar was sterilized by autoclaving at the rate 121ºC in 15 lbs for 15minutes, poured into Petri plates and checked for sterility. Bacterial inoculums were prepared from each isolates in normal saline and matched with McFarland Standards as mentioned by Kirby and Bauer (1966). The isolates were tested by the Kirby-Bauer's disk-diffusion method for antimicrobial susceptibility against the two antimicrobial agents. A lawn culture was prepared using the primary inoculums by spreading the inoculums onto the agar surface nicely by using a sterile glass spreader (sterilized by 70% alcohol).

After 15 minutes, ciprofloxacin (50 – 0.1 µg) and enrofloxacin (50 – 0.1 µg) antimicrobial discs in triplicates for each concentration were placed on the agar surface by an applicator/ sterile forceps with optimum distance between each antimicrobial discs. All the varying concentrations were prepared on separate plates. The Petri plates embedded with antimicrobial discs were then incubated at 37ºC for 24 hours.

Zones of inhibition indicated by a clear area around the discs were measured to imply the susceptibility to the antimicrobials while growth around the disc implies resistance. The diameters of the zones of inhibition (Andrews, 2008) as judged by an unaided eye were measured to the nearest whole millimetre (mm) using a calibrated scale (Andrews, 2008; NCCLS, 1999).The average diameter of the zones of inhibition were calculated (Andrews, 2008) and result interpreted for each antibiotic by comparing to the standard chart which represents the NCCLS subcommittee's recommendation (NCCLS, 2001) for the particular bacteria of interest. However, as the study was not designed to assess the incidence of resistance to the antimicrobial agents, any isolate that was not sensitive to an antimicrobial in the concentration range tested was deemed resistant and excluded from the analyses.

Data analyses

Analysis of variance (ANOVA) was applied to compare the average effect of the two fluoroquinolones at varying concentrations on all isolates at 5% significant level ($p \leq 0.05$) using the Statistical Package for Social Sciences (SPSS) version 20.0.

RESULTS AND DISCUSSION

The results from the determination of zones of inhibition by disc diffusion test showed that inhibitory effects were observed at concentrations of (6.25 - 50.00) µg/ML for NLF and from 12.5 µg/mL - 50 µg/mL for *E. coli*. The results of the isolates susceptibility to enrofloxacin as estimated from growth inhibition zone diameters are presented in table 1 and table 2 respectively. The greatest zone of inhibition was at 50 µg/ML (0.3300 ± 0.16869) while 6.25 µg/ML (0.0144 ± .01444) recorded the lowest zone of inhibition for *E. coli.* Similarly, NLF greatest zone of inhibition was measured at 50µg/ML (1.2788 ± 0.25956) and it's lowest as 6.25µg/ML (0.4125 ± 0.22554).

There was no inhibition effect at concentrations below 1.56 and 6.25 (µg/mL) to ciprofloxacin and enrofloxacin respectively, over the entire 24-hour incubation. The mean difference in zones of inhibition between *E.coli* and Non lactose fermenting (NLF) *Enterobacteriaceae* measured at varying concentrations of each of the two antimicrobial agents compared at $p \leq 0.05$ was significantly associated except at 25.00 and 6.25 µg/mL for ciprofloxacin, and 6.25 µg/mL for enrofloxacin (Table 3 and 4)

Table 1: Average inhibition and SEM by Enrofloxacin against *E.coli* and NLF

Concentration (mcg/mL)	*E. coli* growth inhibition ± SEM (mm)	NLF growth inhibition ± SEM (mm)
50	0.3300 ± 0.16869	1.2788 ± 0.25956
25	0.1778 ± 0.11957	0.8963 ± 0.22562
12.5	0.0144 ± .01444	0.5463 ± 0.26924
6.25	0.0000 ± .00000	0.4125 ± 0.22554
3.125	0.0000 ± .00000	0.0000 ± 0.0000
1.56	0.0000 ± .00000	0.0000 ± 0.0000

Table 2: The differences in mean diffusion zones and error between *E.coli* and NLF to enrofloxacin

Concentration (mcg/mL)	Mean diffusion zones (mm)	p-value
50.00	0.752 ± 0.184	0.006
25.00	0.516 ± 0.149	0.011
12.50	0.265 ± 0.139	0.053
6.250	0.183 ± 0.109	0.056
3.125	0.000 ± 0.000	=
1.560	0.000 ± 0.000	=

Significant zones of inhibition were observed at concentrations of 50 and 25 ($P<0.05$) for both *E.coli* and Non Lactose fermenters.

The study assessed the *invitro* activities of enrofloxacin at varying concentrations against clinical isolates using Kirby-Bauer's disc diffusion method.

There was an observed susceptibility effect of the isolates to the tested antimicrobial agent at higher concentration. Also, the tested antimicrobial agents exhibited the typical concentration-dependent bacterial killing effect characteristic of quinolones in which its activity increases with increasing concentrations (EMEA, 1998). This may explain the larger zones of inhibition exhibited in this study at higher concentration compared to lower concentrations. This is similar to the findings of previous studies by Hawkey (2003) and Béraud *et al*., (2008) who reported on quinolones mechanism of actions and microbial response, and faecal *Escherichia coli* susceptibility to nalidixic acid, enrofloxacin and ciprofloxacin respectively.

Avian pathogenic *E. coli* is frequently found to be resistant to commonly used antibacterial agents such as enrofloxacin and a host of others. Though, resistance to one fluoroquinolone confers resistance to the entire class. Susceptibility of the isolates to the tested antimicrobial agentat higher concentration 25 (µg/mL) in the present study is an indication of development of resistance.

Enrofloxacin is reported to decrease mortality rates in poultry flocks with respiratory infections (Jones *et al*., 1998). Published studies indicate that enrofloxacin is effective in the treatment of acute salmonella infections and elimination of the carrier state for *Salmonella* (Brown, 1996) in animals. Clinical field studies conducted with enrofloxacin and difloxacin in swine and poultry colibacillosis, and other poultry bacterial and mycobacterial diseases showed therapeutic success (Brown, 1996).

Piriz *et al*., (1996) reported that ciprofloxacin and enrofloxacin among fluoroquinolones have shown to be useful alternatives in the treatment of methicillin-resistant *S. intermedius* strains. Similarly, Jones and Erwin (1998) findings suggested that ciprofloxacin and enrofloxacin are very active and comparable to sarafloxacin for inhibiting a wide variety of *E. coli* species. Smith *et al*., (2007) demonstrated high sensitivity of *E. coli* broiler chickens strain to enrofloxacin, sulphonamides, oxytetracycline and sarafloxacin, but with quite low susceptibility in the present study.

A study to determine the microbiological activity of enrofloxacin and nine of its metabolites against aerobic bacterial strains of human origin at a single inoculum density of 10^5 cfu/ml also indicate that enrofloxacin and ciprofloxacin were the most active substances while *Escherichia coli* was the most sensitive specie tested giving MIC_{50} value of 0.03 and 0.015 µg/mL against enrofloxacin and ciprofloxacin respectively (Ganière *et al*., 2001).

Studies by Ganière *et al*., 2001, showed that minimal inhibitory concentrations (MIC's) of enrofloxacin against *S. intermedius* range from 0.063 µg/mL in 1995 to 64 µg/mL in 1999. This suggests that inappropriate use might favour the development of resistant strains in vivo.

Although fluoroquinolones are the most effective antibacterial agents used in poultry industry, performing antimicrobial susceptibility tests may be a reliable guide to select a suitable antimicrobial agent. Besides, application of recommended dosage regimens and duration of therapy, as well as elimination of older quinolones from pharmacopoeias may decline the extent of bacterial resistance to fluoroquinolones and enhance the time span that these agents will be used (Jones *et al*., 1998).

CONCLUSION

The study concluded that the tested antimicrobial agent (enrofloxacin) can still be applied in the treatment of bacterial infection of chickens as well as for prophylactic or prevention of bacterial diseases in poultry. The isolates were

susceptible to enrofloxacin at a minimum concentration of 25 (µg/mL) which is considered high and the mean value in the zones of inhibition exhibited by *Escherichia coli* and non-lactose fermenters were markedly different ($p < 0.01$).

CONFLICT OF INTEREST

The authors declared that they have no conflict of interest

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