NIGERIAN VETERINARY JOURNAL
ISSN 0331-3026
Nig. Vet. J., September 2018 Vol 39 (3): 199 -208.
https://dx.doi.org/10.4314/nvj.v39i3.3 ORIGINAL ARTICLE

In Vitro Comparative Activity of Ciprofloxacin and Enrofloxacin against Clinical Isolates from Chickens in Benue State, Nigeria

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SUMMARY

This study compares the *in vitro* activities of enrofloxacin and its main metabolite ciprofloxacin against clinical *Escherichia coli* and non-lactose fermenting enterobacteria isolates from chickens. Ten (10) *Escherichia coli* and 8 non lactose fermenting *enterobacteriaceae* species isolated from a pool of clinical cases at the Microbiology Laboratory of the Veterinary Teaching Hospital, University of Agriculture Makurdi were used in this study. Ten-fold serial dilution of 10 varying concentrations (0.1-50µg/mL) of enrofloxacin and ciprofloxacin were tested against the isolates *in vitro* by Bauer's disc-diffusion method to determine and compare their antimicrobial activities against the isolates. The 18 isolates tested were susceptible to both enrofloxacin and ciprofloxacin, and their mean values in the susceptibility of *Escherichia coli* and non-lactose fermenters were significantly different ($p < 0.01$). The study concluded that the clinical isolates are susceptible to both enrofloxacin and ciprofloxacin though ciprofloxacin exhibit higher activity. Comparatively, ciprofloxacin was found to be more potent than enrofloxacin and the difference statistically significant. Ciprofloxacin was recommended as a better choice in the treatment of bacterial infections of chicken in this area compared to enrofloxacin. It was also recommended that proper steps should be taken in the administration of antimicrobials so as to reduce the incidences of bacterial resistance.

Key words: *Escherichia coli,* Non-lactose fermenter, Antimicrobials, Ciprofloxacin. Enrofloxacin, Chicken.

INTRODUCTION

Fluoroquinolones are among the most commonly used antibacterial drugs in general veterinary practice (Escher *et al*., 2011; Kireewan and Suanpairintr, 2017). Enrofloxacin and ciprofloxacin are synthetic fluoroquinolones which acts by inhibiting the DNA gyrase enzyme and commonly indicated in intensive poultry farming for the treatment of chronic respiratory and gastrointestinal infections (Jelena *et al*., 2006; Devreese *et al*., 2014; Vanni *et al*., 2014 Ruennarong *et al*., 2016). They also exhibit rapid bactericidal action against a wide variety of clinically important microorganisms of human and animal origin (Jelena *et al*., 2006; Wayne *et al*., 2011). Though their usage in poultry have been

restricted in some countries to avoid the development of antimicrobial-resistant bacteria, they are still of importance in the treatment of human infections (Ferrari *et al*., 2015). However, ciprofloxacin usage particularly has persisted with arguments for its use in animals intensified on the basis of results of bacterial culture and antimicrobial sensitivity testing demonstrating resistance to enrofloxacin but susceptibility to ciprofloxacin (Sumano and Sunamo, 2001; Boothe *et al*., 2006).

Ciprofloxacin is a major metabolite of enrofloxacin used in human medicine (EMEA, 1998), but is only metabolised to a limited range of 5–10 % in broiler chickens (Redman, 2007; Slana *et al*., 2014). It also has similar spectrum of activity with enrofloxacin but with no reported effect against gram-positive bacteria (Slana *et al*., 2014).

Ciprofloxacin and enrofloxacin are widely used in Nigeria in the management and treatment of poultry diseases. Several reports point to the fact that the (mis)use of fluoroquinolone in chickens have resulted in higher incidences of bacteria resistance (Abu-Basha *et al*., 2012; Devreese *et al*., 2014; Vanni *et al*., 2014). According to the European Food Safety Authority (EFSA), (2014), *Salmonella spp*., *Campylobacter jejuni*, *Campylobacter coli* and *E. coli* derived from domestic chickens' resistance to ciprofloxacin stood at 37.3 %, 44.1 %, 78.4 % and 57.6 %, respectively.

Resistant bacteria from food animals can spread to humans directly or indirectly (Adenipekun *et al*., 2015; Rugumisa *et al*., 2016). This microbial resistance as well as the spread to human population are growing and the outlook for the use of antimicrobial drug in the near future is uncertain. This is a significant public health concern when animal husbandry practices promote resistance to medically important antibiotics (Anderson *et al*., 2003; Rugumisa *et al*., 2016). Several reports of outbreaks of bacterial diseases of poultry are of public health concern and have posed enormous problem to the poultry industry. The indiscriminate use of antimicrobials without recourse to susceptibility testing is often attributed to be the major cause (Ramanan *et al*., 2013).

The aims of this study therefore was to asses and compare the antimicrobial activities of ciprofloxacin and enrofloxacin against clinical isolates to ascertaining the most appropriate and desirable amongst the two thereby limiting the development of resistant strains.

MATERIAL 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 culture, 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 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 inoculum 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 characteristics of *E. coli*, while non-lactose fermenting (LF^-) colourless colonies were isolated and sub cultured on Muller Hinton agar to obtain pure cultures of non-lactose fermenting *enterobacteriaceae*. Pure cultures of both isolates grown in nutrient broth were mixed with sterile glycerol 1:1 and stored at −20°C.

Preparation of antibacterial drug stock solutions and dilution trays

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

Disc diffusion test

The isolates were tested by the Kirby-Bauer's disk-diffusion method as described by Bauer *et al*. (1966). A lawn culture was prepared using the primary inoculums by spreading the inoculums onto the agar surface nicely using a sterile glass spreader (sterilized by 70 % alcohol).

After 15 minutes, ciprofloxacin and enrofloxacin (50 – 0.1 µg) impregnated discs in triplicates were applied onto the agar surface by 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 as judged by an unaided eye were measured to the nearest whole millimetre (mm) using a calibrated scale. The average diameters of the zones of inhibition were calculated and result interpreted for each antibiotic by comparing to the standard chart which represents the National Committee for Clinical Laboratory Standards (NCCLS) subcommittee's recommendation 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

The means were determined standard error of mean (SEM). Mean difference between groups were compared using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test, while t-test was applied to compare the 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

The results from the determination of zones of inhibition by disc diffusion test showed that *Escherichia coli* was susceptible to the two antimicrobials at concentrations of (12.50 - 50.00) µg/mL exhibiting larger zones of inhibition in ciprofloxacin than enrofloxacin. *E. coli* showed resistance to the two antimicrobials at concentrations below 12.50 (µg/mL). The results of *Escherichia coli* isolates susceptibility to ciprofloxacin and enrofloxacin as estimated from growth inhibition zone diameters are presented in TABLE 1.

The non-lactose fermenters exhibited susceptibility to the two antimicrobials within concentrations range of 6.25 - 50.50 (µg/mL) respectively over the entire 24-hour

incubation period. The non-lactose fermenters were however resistant to the two antimicrobials in concentrations below 6.25 (μg/mL). Ciprofloxacin also produced higher zones of inhibition TABLE 2.

The t-test comparing the mean differences in zones of inhibition between *E. coli* and non-lactose fermenting (NLF) *Enterobacteriaceae* measured at varying concentrations of each of the two antimicrobial agents at $p \leq 0.05$ was significantly associated (TABLES 3 and 4).

DISCUSSION

The majority of the 18 tested isolates in this study were susceptible to enrofloxacin and ciprofloxacin. In general, the fluoroquinolones exhibit excellent activity against *Enterobacteriaceae*, fastidious Gram-negative bacteria and some Gram positive bacteria (Wayne *et al*., 2011). Many Gram-negative bacteria that have become resistant to other classes of antibacterial agents remained susceptible to the fluoroquinolones (Sárközy, 2001).

Several reports about *in vitro* activities of the fluoroquinolones against bacterial clinical isolates of animal origin exist (Pohl *et al*., 1991; Cid *et al*., 1994; Šeol, 2005). Ciprofloxacin and enrofloxacin have been used extensively in this area in recent years in the management of poultry diseases generally. Šeol, (2005) reported the proved usefulness of ciprofloxacin and enrofloxacin as potent alternatives for the treatment of

TABLE 1: Average zones of inhibition of *Escherichia coli* against varying concentrations of ciprofloxacin and enrofloxacin

Concentration	Inhibition mean ± SEM (Cipro)	Inhibition mean ± SEM (Enro)
50.000	1.502 ± 0.295	0.367 ± 0.184
25.000	1.204 ± 0.266	0.178 ± 0.120
12.500	0.822 ± 0.212	0.014 ± 0.014
6.250	=	=
3.125	=	=
1.560	=	=

TABLE 2: Average zones of inhibition of Non-lactose fermenters against varying concentrations of ciprofloxacin and enrofloxacin

Concentration	Inhibition mean ± SEM (Cipro)	Inhibition mean ± SEM (Enro)
50.000	2.530 ± 0.347	1.279 ± 0.260
25.000	1.834 ± 0.456	0.896 ± 0.226
12.500	1.726 ± 0.399	0.546 ± 0.269
6.250	1.626 ± 0.379	0.413 ± 0.226
3.125	=	=
1.560	=	=

TABLE 3: Comparative effects of ciprofloxacin (C) and enrofloxacin (E) against *Escherichia coli*

Concentration	Difference in mean ± SEM	95 % CI/t-test	p-value
C_1 vs E_1	1.135 ± 0.357	0.381 to 1.889/ 3.177	0.005
C_2 vs E_2	1.026 ± 0.303	0.386 to 1.666/ 3.383	0.003
C_3 vs E_3	0.808 ± 0.224	0.334 to 1.281/ 3.601	0.002
C_4 vs E_4	=	=	=
C_5 vs E_5	=	=	=
C_6 vs E_6	=	=	=

TABLE 4: Comparative effects of ciprofloxacin (C) and enrofloxacin (E) against Non-lactose fermenters

Concentration	Difference in mean ± SEM	95 % CI/t-test	p-value
C_1 vs E_1	1.251 ± 0.433	0.322 to 2.180/2.889	0.011
C_2 vs E_2	0.938 ± 0.509	-0.154 to 2.029/1.843	0.086
C_3 vs E_3	1.180 ± 0.481	0.148 to 2.212/ 2.453	0.027
C_4 vs E_4	1.214 ± 0.441	0.269 to 2.159/ 2.755	0.015
C_5 vs E_5	=	=	=
C_6 vs E_6	=	=	=

methicillin-resistant strains. This is an indication that inappropriate use might favour the development of resistant strains *in vivo*. Results of our study are very similar to those discussed above and confirmed the excellent activity of fluoroquinolones particularly enrofloxacin and ciprofloxacin against *Escherichia coli* and *Enterobacteriaceae*.

In this study, ciprofloxacin has shown to be a more potent antimicrobial agent compared to enrofloxacin as evidenced in the larger zones of inhibition. Abu-Basha *et al*. (2012) and Kotilainen *et al*. (2005) similarly reported of better ciprofloxacin activity compared to enrofloxacin against *Escherichia coli* and salmonella infections respectively. This is also similar to previous reports of Hoogkamp-Korstanje, (1984) and Ridgway *et al*. (1984) whose separate studies showed ciprofloxacin of being a more potent fluoroquinolone by exhibiting the broadest spectrum of activity against all Gram-negative bacteria and streptococci tested. Prescott and Yielding, (1990) also reported of a similar activity of ciprofloxacin compared to enrofloxacin though both are reported to have structural similarity and similar antibacterial spectrum. Several other reports of pharmacokinetics of ciprofloxacin in domestic animals (Dowling *et al*., 1995; Ovando *et al*., 2000) showing good pharmacokinetic properties and therapeutic possibilities exist. The present report is expected due to the fact that enrofloxacin has been used in this area for the treatment of animal infections long after the introduction of ciprofloxacin and might have developed acquired resistance.

Cid *et al*. (1994) reported excellent *in vitro* activities of the fluoroquinolones against *E. coli*. The report indicated that 71.0 % of tested strains were sensitive to enrofloxacin, 26.2% were resistant and 2.7% were intermediate in sensitivity, while the majority of tested strains (93.4 %) were susceptible to ciprofloxacin, 3.8% were resistant and 2.7% showed intermediate sensitivity. Among the quinolone antibiotics, ciprofloxacin was still be the most active or potent agent, which also agrees with our findings.

Pohl *et al*. (1991) have reported that relatively high percentages of *E. coli* isolates of bovine origin were resistant to enrofloxacin activity in vitro, whereas our isolates were highly susceptible to enrofloxacin. Hamisi *et al*. (2014) also reported higher resistance (54.5%) among *E. coli* isolates to the fluoroquinolone but with a relatively limited resistance to ciprofloxacin (3.5%). This difference might be explained, in part, by published observations that ciprofloxacin is more active and potent than other fluoroquinolones against most bacteria (Lautzenhiser *et al*., 2001; Rugumisa *et al*., 2016).

Spencer (1996) in an 8-year survey of 29 425 hospital *P. aeruginosa* isolates found 95% susceptibility to ciprofloxacin and attributed the higher susceptibility to very limited use of ciprofloxacin in veterinary practice in Croatia. Contrastingly, Mueller-

Premru and Gubina (2000) found 45.7% of the tested strains resistant to ciprofloxacin. Shawar *et al*. (1999), similarly reported a higher resistance of 20.7 % to ciprofloxacin. In contrast, enrofloxacin showed a relatively low activity against *P. aeruginosa* isolates when compared to ciprofloxacin but also higher compared to the results of other authors (Cid *et al*., 1994).

A study by Frazier *et al*. (2000) showed that among marbofloxacin, enrofloxacin, ciprofloxacin and difloxacin; marbofloxacin has greater C_{max} (maximum plasma drug concentration curve lg/ml · h) and ACU0-last (the area under the plasma drug concentration versus time curve lg/ml · h) compared to enrofloxacin, ciprofloxacin, enrofloxacin plus ciprofloxacin combined, or difloxacin. Those results suggest that even though ciprofloxacin showed better activity than enrofloxacin (Ovando *et al*. 1999) and similar activity to marbofloxacin, because of its pharmacokinetic properties, marbofloxacin should be the quinolone of choice.

Grobbel *et al*. (2007) in their studies showed that ciprofloxacin had significantly greater *in vitro* antibacterial activity than enrofloxacin against *M. haemolytica*, *P. multocida* and *E. coli*, whereas enrofloxacin showed greater activity than ciprofloxacin against *S. aureus*. Comparison of the sensitivities of individual pathogen isolates to enrofloxacin and ciprofloxacin highlighted notable differences in the MIC_{50} profiles, in particular when considering *E. coli* and *S. aureus*. Available data showed 54 of 70 *E. coli* isolates to be at least one log_2 step more sensitive to ciprofloxacin than enrofloxacin; conversely 33 of 47 *S. aureus* isolates were more sensitive to enrofloxacin than ciprofloxacin. This difference is reflected in the MIC50 values for each agent in the case of *E. coli* (0.016µg/mL for ciprofloxacin and 0.03µg/mL for enrofloxacin) but not for *S. aureus* (0.12µg/mL for both agents). Higher sensitivity of *E. coli* ATCC 25922 to ciprofloxacin and of *S. aureus* ATCC 29213 to enrofloxacin was also noted both in Grobbel *et al*. (2007) data and in other reports (Riddle *et al*., 2000). Previous studies have shown limited evidence for preferential activity for ciprofloxacin against *E. coli* (Zhao *et al*., 2005) but a more consistent body of evidence indicates that *S. aureus* is more susceptible to enrofloxacin (Watts *et al*., 1997; Jones *et al*., 1998).

Minimum Inhibitory Concentrations (MICs) results also indicates susceptibility to ciprofloxacin at 1 (µg/mL) and 2 (µg/mL) for enrofloxacin (Pohl *et al*., 1991; Cid *et al*., 1994) thereby supporting better antimicrobial activity of ciprofloxacin when compared to enrofloxacin.

The interest of the medical community in fluoroquinolones has not decreased during the last 10 years and many new ones have been developed and are under investigation (Ovando *et al*., 2004). Ciprofloxacin use in human medicine has proved effective in several infections (Grobbel *et al*., 2007). Because of its broad and intense activity against Gram negative bacteria and the fact that no cross-resistance with beta-lactams or aminoglycosides occurs, it was also suggested to be of considerable usefulness in veterinary medicine (Nouws *et al*., 1988; Brown, 1996).

Our *in vitro* data show ciprofloxacin to have greatest potency against *E. coli* and non-lactose fermenting enterobacteria isolates tested in comparison to enrofloxacin. In this case, our *in vitro* fluoroquinolone activity data suggest that treatment with ciprofloxacin is preferential to use of enrofloxacin, as ciprofloxacin has the highest *in vitro* activity against the tested isolates.

CONCLUSION

The study concluded that enrofloxacin and ciprofloxacin are still effective in the management and treatment of bacterial infections of chicken in Benue State. However, ciprofloxacin was reported to have exhibited higher activity compared to enrofloxacin. The mean values in the zones

of inhibition against *Escherichia coli* and non-lactose fermenters were significantly different ($p < 0.01$).

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