IN VITRO ANTIBACTERIAL EFFECT OF ENROFLOXACIN DETERMINED BY TIME-KILLING CURVES ANALYSIS

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Summary


Minimal inhibitory concentrations (MIC) are used as in vitro reference values to describe the activity of antibacterial drugs against a given microbial strain. The effect of antibiotics over time could be assessed by time-dependent bactericidal (time-killing) curves. In this study, MIC, mutant prevention concentrations (MPC) and time-killing curves of enrofloxacin were investigated on a pathogenic E. coli O78/H12 strain, isolated from broiler chickens. The tested strain was sensitive with MIC=0.015 µg/mL. MPC value was considerably higher (4 µg/mL). Time-killing curves showed that enrofloxacin activity was better at concentrations higher than 1 µg/mL (16×MIC in serum) whereas at lower concentrations (0.06 µg/mL) bacterial counts increased after a 24-hour incubation. These curves, together with MPC could be used to design a therapeutic schedule for problematic E. coli infections in farms, as they depict the behaviour of pathogenic strains over time and provide information about the possibility for selection or presence of resistant microbial populations.

Key words: enrofloxacin, E. coli, mutant prevention concentrations, time-killing curves

INTRODUCTION

Minimal inhibitory concentrations (MICs) are used as in vitro reference values to describe the activity of antibacterial drugs against a given microorganism (Andrews, 2001). The methods for MIC determination are standardized and reproducible within certain limits (CLSI, 2008). MIC value is thought to be a criterion in the determination of expected outcome of antibacterial therapy: elimination of bacteria or clinical response. In general, the therapy is consistent with MIC values or with pharmacokinetic/pharmacodynamic indices such as the duration of time that plasma/serum drug levels remain above the MIC (T>MIC) and area under the concentration-time curve vs MIC ratio (AUC/MIC) (Drusano, 2000; Toutain et al., 2002; McKellar et al., 2004; Haritova et al., 2004). Although these parameters are related, MIC is not a feature proper to microorganisms. MIC should be regarded as the final result from the growth of a certain number of microorganisms (inoculum) with time (growth rate) and their killing by a given fixed concentration of the antibacterial drug (Hyatt et al., 1994; Mouton & Vinks, 2005).

The in vitro determination of MIC differs from that in vivo, when the concentration is dynamic and changes with time. Nevertheless, MIC values are often used
as criteria of anticipated effects. It is usually considered that the growth of bacterial populations is reinstated when the concentrations of time-dependent antibiotics (such as β-lactams) decrease below MIC. Thus, MIC values are placed at the same footing with concentrations when growth and killing rate are equal (stationary concentration), i.e. the bacterial counts do not change. Antibiotics with time-dependent effect showed a good correlation between MIC values and the stationary concentration. Concentration-dependent antibacterial drugs as quinolones do not exhibit a clear relationship between MIC values and the stationary concentration. By means of time-killing curves it was found out that the microbial killing rate for these drugs was considerably higher at the beginning of incubation than at its end (Hyatt et al., 1994; Mouton & Vinks, 2005). It was shown that bacteria could regrow and replicate following a previously reported lack of growth after shorter incubation time: 6–12 hours (Lister & Sanders, 1999; Schneider et al., 2004; Mouton & Vinks, 2005; Haritova et al., 2006a, b). Differences in killing rates of bacterial strains with equal MIC for quinolones are also reported (Hyatt et al., 1994). All these data demonstrate that time-killing curves are more informative about the effect of antibacterial drugs and would contribute to their more precise use.

The purpose of the present study was to investigate time-killing curves of enrofloxacin on E. coli strains isolated from broiler chickens.

**MATERIALS AND METHODS**

**Antibacterial drug**

Enrofloxacin hydrochloride, ser. No 20020323, provided by Biovet, Peshtera, Bulgaria.

**Isolation and identification of Escherichia coli**

MIC, MBC and MPC were determined for field Escherichia coli isolates from air sacs of broiler chickens and for the reference strain E. coli ATCC 25922. E. coli (one O78/H12 and one non-serotyped) were obtained from farms with previous history of enrofloxacin application and provided by the Epidemiology and Preventive Medicine Unit at the Department of Microbiology, Infectious and Parasitic Diseases, Faculty of Veterinary Medicine.

**Determination of MIC, MBC and MPC in broth and of MIC and MBC in serum**

MIC and MBC in broth and in blood serum from healthy control broiler chickens were determined by the broth microdilution method according to CLSI (Clinical and Laboratory Standards Institute, 2008), at concentrations between 64 µg/mL and 0.004 µg/mL. Plates were incubated at 35°C for 18 h and read at 650 nm (MultiscanEX, Thermo Scientific). For MBC determination, 100 µL of each well without turbidity were cultivated on tryptic soy agar (TSA, Fluka), Petri dishes were incubated at 35°C for 24 h, and colonies were counted. The limit of detection was 10 colony-forming units (cfu)/mL. MIC in broth and serum were determined as the lowest concentrations at which bacterial growth remained below the original inoculum level. MBC in broth and serum were determined as concentrations at which 99.9% reduction in bacterial counts was achieved.

For MPC determination, 120 mL 24-hour broth culture in Mueller-Hinton broth (MHB, Becton Dickinson) with optical density 0.8 at 540 nm (MultiscanEX, Thermo Scientific) corresponding to ≥10⁷ cells/mL was poured into 10-mL
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Tubes. Tubes were centrifuged at 3000×g for 15 min. The supernatants were discarded and pellets containing ≥10^7 cells were resuspended in the remaining amount of fluid and spread onto Mueller–Hinton agar (MHA, National Centre of Infectious and Parasitic Diseases, Sofia), containing a defined enrofloxacin concentration. The intermediate strain was tested at concentrations from 256 to 1 µg/mL, each level being twice lower than the preceding one. The sensitive strain was tested within 32–0.03 µg/mL, and the reference E. coli ATCC 25922 – at 16–0.015 µg/mL. Petri dishes were incubated at 41 °C for 96 h in closed plastic bags and appearance of colonies was checked every 24 hours. MPC was determined as the lowest antibiotic concentration that prevented the growth of colonies.

MIC and MBC of enrofloxacin against E. coli ATCC 25922 were determined only in broth, whereas against the sensitive field isolate – in both broth and serum. MIC for the intermediate strain was determined in broth and serum. MPC was determined for the three tested strains. All experiments were run in triplicate.

Antimicrobial activity in serum (time-killing curves)

Eight colonies from 24-hour TSA culture of the sensitive E. coli strain were suspended in 9 mL MHB and incubated for 20 h at 35°C. Solutions of enrofloxacin in serum were prepared at the following concentrations: 0.0 µg/mL (control); 0.06 µg/mL; 0.125 µg/mL; 0.25 µg/mL; 0.5 µg/mL; 1 µg/mL; 2 µg/mL; 4 µg/mL and 8 µg/mL. To 1 mL serum containing the respective amount of enrofloxacin, 10 µL broth culture were added so that the final concentration was approximately 2×10^7 cfu/mL. In order to determine colony-forming units, serial dilutions of 10^7 to 10^0 in sterile saline were prepared (for the control, to 10^8) and were incubated for 3, 6 and 24 h at 35 °C. Then, 100 µL of each dilution were inoculated on TSA and colony-forming units were counted after 16 h. The limit of detection was 10 cfu/mL.

Pharmacodynamic analysis

AUC/MIC ratios were determined on the basis of the area under the concentration-time curve over 24 h (AUC, calculated by multiplying the respective serum concentration by the 24-hour period of incubation), divided by serum MIC. The difference of the log10 of the initial bacterial count (cfu/mL) and the bacterial count after 24 h incubation was also determined. To calculate AUC/MIC values, ensuring a bacteriostatic effect and elimination of bacteria, the inhibitory E_{max} model was applied. The effect of the antibiotic (presented though the reduction of initial bacterial counts) was analyzed against AUC/MIC values by the equation:

$$E = E_{\text{max}} - \left(\frac{E_{\text{max}} - E_{0}}{C_e/(C_e + EC_{50})}\right)$$

where: E = antibacterial effect expressed by reduction in initial bacterial counts (in log10 cfu/mL) in serum after 24-hour incubation; E_{max} = log10 of the difference in bacterial counts in control sample between hour 0 and hour 24; E_{0} = log10 of the difference in bacterial counts after 24-hour incubation in serum containing enrofloxacin concentrations when the limit of detection of 10 cfu/mL was attained; EC_{50} = AUC/MIC that produces 50% of the maximum antibacterial effect; C_e = serum AUC/MIC.

In these studies, E_{max} is the count of bacteria in the absence of drug (effect), E_{0} is the maximum effect of the antibacterial
drug when bacterial growth is inhibited (Aliabadi & Lees, 2001; Aliabadi et al., 2003b). The antibacterial effect is the variable presenting the reduction of the initial bacterial count. All pharmacodynamic indices were calculated by means of the WinNonlin software (5.0.1, Pharsight Corporation, Mountain View, USA).

The antibacterial effect was determined by AUC/MIC, necessary for bacteriostatic effect (no change in bacterial counts after 24-hour incubation) and for elimination of bacteria (the lowest AUC/MIC value, when bacterial count decreases to 10 cfu/mL) (Aliabadi & Lees, 2001).

RESULTS
The results from the determination of MIC in Mueller-Hinton broth (MHB) showed that one of the two field *E. coli* isolates was intermediate, and the other was sensitive. MIC, MBC and MPC values are given in Table 1.

Table 1. Minimal inhibitory concentrations (MIC), minimal bactericidal concentrations (MBC) and mutant prevention concentrations (MPC) for field *Escherichia coli* isolates and the reference *E. coli* ATCC 25922 strain

<table>
<thead>
<tr>
<th>E. coli strain</th>
<th>MIC (µg/mL)</th>
<th>MBC (µg/mL)</th>
<th>MPC (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MHB</td>
<td>Serum</td>
<td>MHB</td>
</tr>
<tr>
<td><em>E. coli</em> (intermediate)</td>
<td>0.5</td>
<td>1.0</td>
<td>–</td>
</tr>
<tr>
<td><em>E. coli</em> O78/H12</td>
<td>0.015</td>
<td>0.06</td>
<td>0.06</td>
</tr>
<tr>
<td><em>E. coli</em> ATCC 25922</td>
<td>0.008</td>
<td>–</td>
<td>0.015</td>
</tr>
</tbody>
</table>

![Fig. 1. Time-killing curves representing the bacterial growth of the sensitive *E. coli* O78/H12 with time (over 24 hours) in absence or with eight different enrofloxacin concentrations (from 0 to 8 µg/mL) in serum.](image)
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The time course of the antibacterial effect is presented by time-dependent-killing curves (Fig. 1). Inhibitory effect was observed with concentrations from 1 µg/mL to 8 µg/mL. AUC/MIC values necessary to produce a bacteriostatic effect and elimination of bacteria were 257.40 and 2794.40 respectively (Fig. 2).

DISCUSSION

Fluoroquinolones are used for treatment of numerous infections in men and animals. It is thought that the resistance of human enteropathogenic E. coli strains against these drugs is directly related to the improper use in animals (Hopkins et al., 2005). During the recent years various methods are used to determine the activity of antibacterial compounds, including fluoroquinolones, aiming at their more precise application. The commonest method is based upon achievement of an adequate drug concentration at the site of infection, conforming to MIC. Nevertheless, the bacterial population could have subpopulations with lower sensitivity. That is why the contemporary in vitro pharmacodynamic models investigate not only MIC, but also MPC and antibacterial drug activity over time by time-dependent bactericidal curves (Mouton & Vinks, 2005). Other researches demonstrate that MIC, MBC and MPC values in broth and serum were different (Hyatt et al., 1995; Aliabadi & Lees, 2001).

Broth and serum MIC values obtained in this study were comparable to MIC\textsubscript{90} data reported for pathogenic E. coli strains (Smith et al., 2007). MIC serum levels for the intermediate and sensitive E. coli isolates were from 2 to 4 times higher, respectively, from corresponding broth values, evidencing a reduced inhibitory activity of serum. The same trend was observed by MBC. Our results were similar to those published by other researchers.
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(Jacobs et al., 2002; Aliabadi et al., 2003a, b; Wise, 2003). Binding to serum proteins could be responsible for observed variations (Wise, 2003). Other factors such as pH could also contribute to reduction of serum antibacterial activity (Zeitlinger et al., 2004; Mouton & Vinks, 2005).

While MIC allows to determine the susceptibility of most cells of a bacterial population, MPC provides information about the sensitivity of small resistant subpopulations (Zhao & Drlica, 2001; Marcusson et al., 2005). MPC of the reference (1 µg/mL) and the sensitive strain (4 µg/mL) could be attained in the organism by high therapeutic doses whereas the MPC of the intermediate E. coli strain (64 µg/mL) is practically unlikely to be reached in vivo. Assuming that serum concentrations are maintained between 4 and 8 µg/mL, AUC/MPC values (obtained through dividing enrofloxacin concentrations from hour 0 to hour 24 by MPC) would be 24 and 48, respectively. The values for the intermediate E. coli strain would be between 1.5 and 3. Previous studies have shown that a AUC/MPC value of 22 was sufficient to prevent the selection of ciprofloxacin resistant mutants in a population of 10^10 cfu/mL from a sensitive Escherichia coli strain (Olofs-son et al., 2006). According to others, AUC/MPC ratios of 9 to 12 could also prevent the selection of marbofloxacin resistant mutants in Escherichia coli with inoculum sizes of 10^9 and 10^7 cfu/mL, but not 10^5 cfu/mL (Ferran et al., 2007). Ferran et al. (2007) suggest that the value of the PK/PD index AUC/MPC that prevents the selection of resistant mutants may also depend on inoculum size, or the size of the bacterial population at the beginning of the treatment. High MPC for a given strain could be related to resistant subpopulations in the initial inoculum. The necessity of MPC evaluation is determined by the lack of correlation with MIC values (Marcusson et al., 2005). Thus, the MPC determination could provide information as to whether it is reasonable to use a given antibacterial drug, whether it is acceptable to use it alone or there is a need for combined therapy (Drlica, 2003).

Time-dependent bactericidal curves showed that enrofloxacin activity was better at concentrations higher than 1 µg/mL (16×MIC in serum). At lower levels, including at 0.06 µg/mL (MIC in serum), the bacterial counts increased after 24-hour incubation. These data add further evidence to the statement that MIC alone do not provide sufficient information about the effect of therapy. The commonly used dosage (10 mg/kg enrofloxacin) could not yield concentrations over 1 µg/mL (Haritova et al., 2004), that is a prerequisite for increase in bacterial counts by the end of the dosage interval and selection of resistant subpopulations. There was no bacteriostatic effect at concentrations lower than 1 µg/mL over the entire 24-hour incubation. Time-killing curves of danofloxacin and marbofloxacin showed that concentrations over 0.25 µg/mL (MIC in serum 0.25 µg/mL) and over 0.5 µg/mL (MIC in serum 0.5 µg/mL) respectively, resulted in elimination of a turkey E. coli O78/K80 isolate (Haritova et al., 2006a, b). In cited investigations, the initial inoculum size was about 10^7 cfu/mL. The results confirmed that the effect of the antibacterial therapy could not be evaluated on the basis of MIC only. Although MIC of the pathogenic E. coli isolate from broiler chickens against enrofloxacin was 0.06 µg/mL, the behaviour of the microbial population over time showed that bacteria could be eliminated by very high serum concentrations.
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Most probably, this is due to resistant microbial subpopulations that could not be killed by concentrations lower than 16×MIC. Similar results were obtained for moxifloxacin and levofloxacin. Levofoxacin, with MIC=0.25 µg/mL against Streptococcus pneumoniae, applied at standard dosages, could not kill bacteria (DeRyke et al., 2006). Bacteria grown after incubation for 24 hours were with lower susceptibility and up to 16-fold increase in MIC.

The analysis of the relationship between the change in bacterial counts over 24-hour incubation and AUC/MIC by means of the inhibitory $E_{\text{max}}$ model showed that for the studied sensitive pathogenic E. coli strain, a bacteriostatic effect could be achieved at AUC/MIC=257.40 h. Bacteria killing could be observed at AUC/MIC=2794.40 h. Those ratios could not be achieved with the standard enrofloxacin dosage, respectively systemic concentrations and a MIC=0.06 µg/mL. Therefore, a deeper knowledge about the pathogenic agent is needed with regard to the proper choice of antibacterial substance and dose regimen. Time-killing curves could be used to characterize the behaviour of pathogenic strains, that occasionally cause farm outbreaks. These curves, together with MPC, could be used to prepare a therapeutical schedule for problematic infections and to reduce the risk of selection of first-step mutants. The results should be validated in a clinical setting.

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