



IN VITRO EFFECT OF TETRACYCLINE ANTIBIOTICS ON *TRUEPERELLA PYOGENES* ISOLATED FROM COWS WITH METRITIS

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Summary

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Trueperella pyogenes is associated with endometritis and metritis in cows. Traditionally these diseases are treated with antibiotics while new approaches include application of the mucolytic N-acetylcysteine. Therefore the study aimed to evaluate the sensitivity of clinical *Trueperella pyogenes* isolates (n=2) to oxytetracycline, doxycycline, N-acetylcysteine and their combinations. The potential for biofilm formation with/without tested drugs was investigated by the method of crystal violet staining. Minimum inhibitory concentrations (MIC) of oxytetracycline for *T. pyogenes* 1 and 2 were 16 and 64 µg/mL, respectively. MIC of doxycycline for both isolates was 32 µg/mL and for N-acetylcysteine – 8 mg/mL. Both *Trueperella pyogenes* isolates did not form biofilm. The growth of *T. pyogenes* 1 cultured in the presence of either oxytetracycline or doxycycline (0.0078–128 µg/mL) under conditions for biofilm formation was significantly inhibited at concentrations ≥ 1 µg/mL and 8 µg/mL, respectively. The growth of *T. pyogenes* 2 was not affected by the antibiotics. N-acetylcysteine at ≥ 4 mg/mL resulted in significant inhibition of the growth of both isolates and its combinations with the antibiotics did not inhibit their growth. The effect of N-acetylcysteine should be validated in clinical settings but its combinations with tetracyclines were not able to improve the sensitivity of *T. pyogenes*, isolated from cows with clinical metritis.

Key words: cows, doxycycline, metritis, N-acetylcysteine, oxytetracycline, *T. pyogenes*

INTRODUCTION

Trueperella pyogenes (*T. pyogenes*) is a Gram-positive, opportunistic microorganism isolated from many animal species and associated with infections such as abscesses, arthritis, endocarditis, pneumonia and others (Ribeiro *et al.*, 2015). *T.*

pyogenes is characterised as a pathogen causing chronic endometritis and clinical metritis in cows (Ribeiro *et al.*, 2015) and abortion in rare cases (Ponnusamy *et al.*, 2017). The infections with this pathogen lead to significant economic losses at cat-

tle farms due to prolongation of service period before the conception in cows. Additional losses are due to decreased milk production of dairy cows (Sheldon & Owens, 2017).

Biochemical properties of bovine *T. pyogenes* isolates, their identification and molecular characteristics were well described (Abdallah, 2016). Rogovskyy *et al.* (2018) revealed a remarkable diversity among ruminant isolates of *T. pyogenes* by investigating phenotypic and genotypic characteristics of this pathogen. By using *in vitro* susceptibility tests, *T. pyogenes* was shown to be susceptible to many antibiotics (Zastempowska & Lassa, 2012). Failure of antibacterial treatment of endometritis and metritis in cattle suggested a role of biofilm production by the pathogenic strains. Significant number of *T. pyogenes* isolates (88.6%) was capable of biofilm production (Ozturk *et al.*, 2016).

Broad spectrum antibiotics are prescribed to treat clinical cases of genital tract inflammation in cows, especially those with impaired appetite and lactation and fever. Beta lactams, macrolides, fluoroquinolones and tetracyclines are among the drugs of choice in treatment of mixed infections, associated with isolation of aerobic and anaerobic bacteria, including *T. pyogenes*, although the data about the efficacy of the applied drugs are contradictory (Lefebvre & Stock, 2012; LeBlanc, 2014; Haimerl *et al.*, 2017). Oxytetracycline is very often applied for treatment of endometritis and metritis in cows (Armengol & Fraile, 2015). Recent literature data revealed a positive effect of intrauterine administration of N-acetylcysteine as mucolytic drug in treatment of endometritis attributed to inhibition of biofilm production of pathogenic bacteria (Gores-Lindholm *et al.*, 2013; Tras *et al.*, 2014). The interest to the po-

tential of N-acetylcysteine alone or combined with antibiotics for inhibition of biofilm formation is increasing (Dincola *et al.*, 2014; Yang *et al.*, 2016). Absence of published data about the effect of N-acetylcysteine and combinations of tetracyclines and N-acetylcysteine on *T. pyogenes* was the incentive to perform the current experiments.

The present study was focused on *in vitro* effect of two tetracycline antibiotics, oxytetracycline and doxycycline, and N-acetylcysteine, applied either alone or in combinations, on clinical isolates of *T. pyogenes*. Additionally, the potential for biofilm formation of *T. pyogenes* was tested.

MATERIALS AND METHODS

Drugs

All tests in the current investigation were performed with substances from Sigma Aldrich, HPLC grade: oxytetracycline hydrochloride ($\geq 95\%$), doxycycline hyclate ($\geq 98\%$) and N-acetyl-L-cysteine ($\geq 99\%$). Tests for biofilm formation were performed by using sterile solution of crystal violet (Crystal violet, general purpose grade $> 99\%$, Sigma), 95% and 70 % ethanol, and 95% methanol.

Isolation and identification of Trueperella pyogenes isolates

The *in vitro* tests were performed with two *T. pyogenes* isolates from uterine secretions of cows with clinical metritis. The uterine secretion was obtained aseptically with a sterile catheter. The cows belonged to the Experimental farm at Trakia University, Stara Zagora, Bulgaria.

Samples from uterine secretion of cows with clinical metritis were cultured on tryptic soy blood agar (TSBA, HiMe-

dia, India) and on MacConkey agar (HiMedia, India) at aerobic conditions for 24–72 h. For initial identification of *T. pyogenes*, the Gram staining test, the presence of haemolytic activity on TSBA and absence of growth on MacConkey agar were taken into consideration. Then, a Löffler's serum slope test (NCIPD, Bulgaria) and a CAMP test with *Staphylococcus aureus* were made according to the manufacturer's instructions and the general rules for aseptic work in the microbiology laboratory (Markey *et al.*, 2013). Additionally, the species affiliation of the isolates was confirmed by using a phenotypic identification system BioLog GenIII (BioLog, USA) following the company's protocol.

Determination of minimum inhibitory concentrations (MIC)

MICs values of the isolates of *T. pyogenes* were determined following the Clinical and Laboratory Standards Institute's Standard Broth Micro-dilution Method (CLSI, 2008). The serial two-fold dilutions of antibiotics were prepared in cation-adjusted Muller-Hinton broth (MHB, HiMedia, India) supplemented with 2% (v/v) lysed horse blood due to specific characteristics of the *T. pyogenes* growth (Pohl *et al.*, 2018). Stock solution of oxytetracycline hydrochloride (1 mg/mL), doxycycline hyclate (1 mg/mL) and N-acetylcysteine (16 mg/mL) were prepared on the day of the experiment. Tetracyclines were tested within the concentration range from 128 to 0.0078 µg/mL and N-acetylcysteine: from 8 to 0.0312 mg/mL. The concentrations of the working solutions were chosen according to literature data and to the results from preliminary tests performed by us. MHB with 2% (v/v) lysed horse blood was used as a negative control and the same me-

dium with *T. pyogenes*, without addition of antibiotics – as a positive control. The plates were incubated at 37 °C for 48 h in a 5% CO₂ atmosphere. Thereafter, optical density (OD) values were measured at 620 nm (Synergy LX Multi-Mode Microplate Reader, BioTek, USA). MIC was defined as the lowest drug concentration resulting in OD value close to blank. Each test was performed in triplicate with three independent repetitions.

Determination of the minimum biofilm inhibitory concentration

Isolates of *T. pyogenes* were subcultured on TSBA twice every 24 h. Thereafter, a suspension containing 10⁷ cfu/mL was prepared. *Staphylococcus aureus* O74, a strain recognised as a strong biofilm producer, was used as positive control to test the culture conditions for biofilm formation. Stock solutions of oxytetracycline and doxycycline were prepared in sterile water at a concentration of 1 mg/mL. N-acetylcysteine was dissolved in sterile TSB (TSB, HiMedia, India) at a concentration of 16 mg/mL.

An aliquot of 100 µL of the bacterial suspension of *T. pyogenes* 10⁷ cfu/mL was added to the wells containing 100 µL of drugs solutions in U-bottom 96-well microtiter polystyrene plate to reach final oxytetracycline and doxycycline concentrations of 128, 64, 32, 16, 8, 4, 2, 1, 0.5, 0.25, 0.125, 0.0625, 0.0312, 0.0156 and 0.0078 µg/mL and 10⁶ cfu/mL for bacterial cells. These concentrations for N-acetylcysteine were 8, 4, 2, 1, 0.5, 0.25, 0.125, 0.0625 and 0.0312 mg/mL. Additionally, the effects combinations of N-acetylcysteine and either of tetracyclines on *T. pyogenes* were tested at tetracycline concentrations between 16 and 0.0078 µg/mL and 8 mg/mL N-acetylcysteine, and 10⁶ cfu/mL for bacterial cells. Wells

with sterile TSB alone served as blanks (n=3), TSB with 10^6 cfu/mL bacterial suspension served as positive control (n=3). The plates were incubated at 37 °C, 5% CO₂ for 40 h. *Staphylococcus aureus* O74 in TSB broth without any drugs was cultured at the same conditions to ensure reliability of the test conditions for biofilm formation. Thereafter the culture medium of each well was discarded and the wells were washed three times with 0.8% sterile sodium chloride solution to remove non-attached bacterial cells. The adhered cells were fixed with 200 µL 95% methanol for 15 minutes. Thereafter methanol was discarded and the plates were left to dry. Each well was stained with 150 µL 1% solution of crystal violet for 15 min. Excess dye was rinsed with water and the plates were air-dried. The dye bound to the adherent cells was dissolved in 70% ethanol for 60 minutes. The optical density of each well was measured at 595 nm (Synergy LX Multi-Mode Microplate Reader, BioTek, USA). Each test was performed in triplicate for every concentration with three independent repetitions. The pathogens were determined as biofilm producers at a ratio of OD of bacterial strain/ODc of TSB ≥ 2 . ODc of TSB was calculated as mean OD plus three SD of the negative control (Stepanovic *et al.*, 2000).

Test for metabolic activities of T. pyogenes cultured under conditions for biofilm formation testing

Metabolic activity of both *T. pyogenes* isolates was tested at the end of the biofilm formation assay. A commercial kit (Vybrant Cell Metabolic Assay, Thermo Fisher, USA) was used according to manufacturer instructions. It was applied in order to determine the viability and metabolic activity of bacterial cells,

cultured under conditions for biofilm formation. First the culture medium was carefully removed and 100 µL of culture medium was added into each well containing biofilm and in the negative control. Shortly, 100 µL from the cultured *T. pyogenes* were added to 10 µL of 100 µM C12-resazurin in a 96-well flat bottom plate. A standard curve of resorufin in TSB was prepared at the following concentrations: 0.039, 0.078, 0.156, 0.312, 0.625, 1.25 and 2.5 µM. The plates were incubated for 15 min at 37 °C. The fluorescence of the samples was read at λ_{ex} 563 and λ_{em} 587 nm in order to detect the concentrations of the formed resorufin, which indicated presence of metabolic activity (Synergy LX Multi-Mode Microplate Reader, BioTek, USA).

Data from the bacterial viability test were evaluated by using PD library, model 108 Inhibitory Effect Sigmoid Imax (Phoenix 8.1.0.34 software, Certara®, Cary, NC, USA). The following equation was used:

$$E = E_0 - (I_{max} * C^\gamma) / (C^\gamma + IC_{50}^\gamma),$$

where E_0 : effect at time 0, I_{max} : the maximum inhibitory effect, IC_{50} : the drug concentration that produced 50% of maximum inhibition, C: the drug concentration and γ : the slope. This model was used to analyse the data for the effect of oxytetracycline and N-acetylcysteine, administered alone, on *T. pyogenes* 1. It was used for test of the effect of oxytetracycline on *T. pyogenes* 2.

Model 107 Inhibitory Effect Sigmoid E_0 was used to evaluate the effect of oxytetracycline plus N-acetylcysteine on viability of the bacterial cells of *T. pyogenes* 1 and 2 and of N-acetylcysteine on viability of the bacterial cells of *T. pyogenes* 2:

$$E = E_0 * (1 - (C^\gamma / (C^\gamma + IC_{50}^\gamma))),$$

where E_0 : effect at time 0, IC_{50} : the drug concentration that produced 50% of maximum inhibition, C : the drug concentration and γ : the slope.

The best fit of the models was chosen according to Akaike's Information Criterion.

Statistical analysis

The data are presented as mean \pm SD. For the biofilm formation assay, data were evaluated by one-way analysis of variance (ANOVA, Statistica for Windows 10.0, StatSoft, Inc., USA). Dunnett test was used to detect a statistically significant difference in pharmacokinetic parameters between the tested concentrations and the control. The differences were considered statistically significant at $P < 0.05$.

RESULTS

Minimum inhibitory concentrations (MIC) of oxytetracycline, doxycycline and N-acetylcysteine

MIC value of oxytetracycline determined for *T. pyogenes* isolates by using standard broth micro-dilution protocol was 16 $\mu\text{g}/\text{mL}$ for the first isolate and 64 $\mu\text{g}/\text{mL}$ for the second isolate. MIC value of doxycycline against both isolates was 32 $\mu\text{g}/\text{mL}$. MIC value of N-acetylcysteine against both isolates of *T. pyogenes* was 8 mg/mL . Additional tests for determination of MIC value were performed for combinations of either oxytetracycline and doxycycline in the concentration range between 0.078 and 16 $\mu\text{g}/\text{mL}$ with 8 mg/kg N-acetylcysteine. The concentrations of the antibiotics and of N-acetylcysteine were chosen according to pharmacokinetic data and the achievable concentrations in the tissues. The results

showed that MIC value of the combinations oxytetracycline plus N-acetylcysteine and doxycycline plus N-acetylcysteine were $> 16 \mu\text{g}/\text{mL}$.

Effect of oxytetracycline, doxycycline and N-acetylcysteine on *T. pyogenes* cultured under conditions for biofilm production and bacterial viability in these conditions

Both *T. pyogenes* isolates did not form biofilm and the ratio *T. pyogenes*/TSB was < 2 (Fig. 1). *Staphylococcus aureus* O74 used for positive control as a strong biofilm builder showed significant biofilm formation when cultured under the same conditions as *T. pyogenes* isolates. The ratio of OD of *Staphylococcus aureus* O74/TSB was > 4 (Fig. 1).

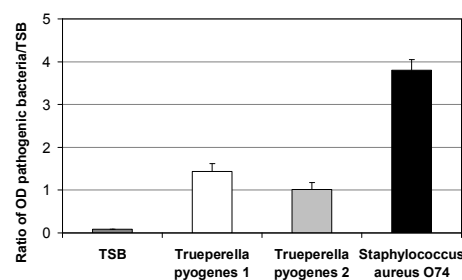


Fig. 1. *Trueperella pyogenes* isolates and *Staphylococcus aureus* O74 biofilm formation within 40 hours.

T. pyogenes isolates were cultured under conditions for biofilm formation in TSB with addition of either oxytetracycline, or doxycycline and of N-acetylcysteine alone and in combination with either antibiotic. No potential for biofilm formation was observed. Oxytetracycline and doxycycline significantly inhibited *T. pyogenes* 1 at concentrations higher than 1 $\mu\text{g}/\text{mL}$ and 8 $\mu\text{g}/\text{mL}$, respectively, although complete inhibition of the growth of the pathogen was not observed (Fig. 2A and 2B). Tetracycline and doxycycline did not inhibit the growth of *T. pyogenes* 2.

In vitro effect of tetracycline antibiotics on *Trueperella pyogenes* isolated from cows with metritis

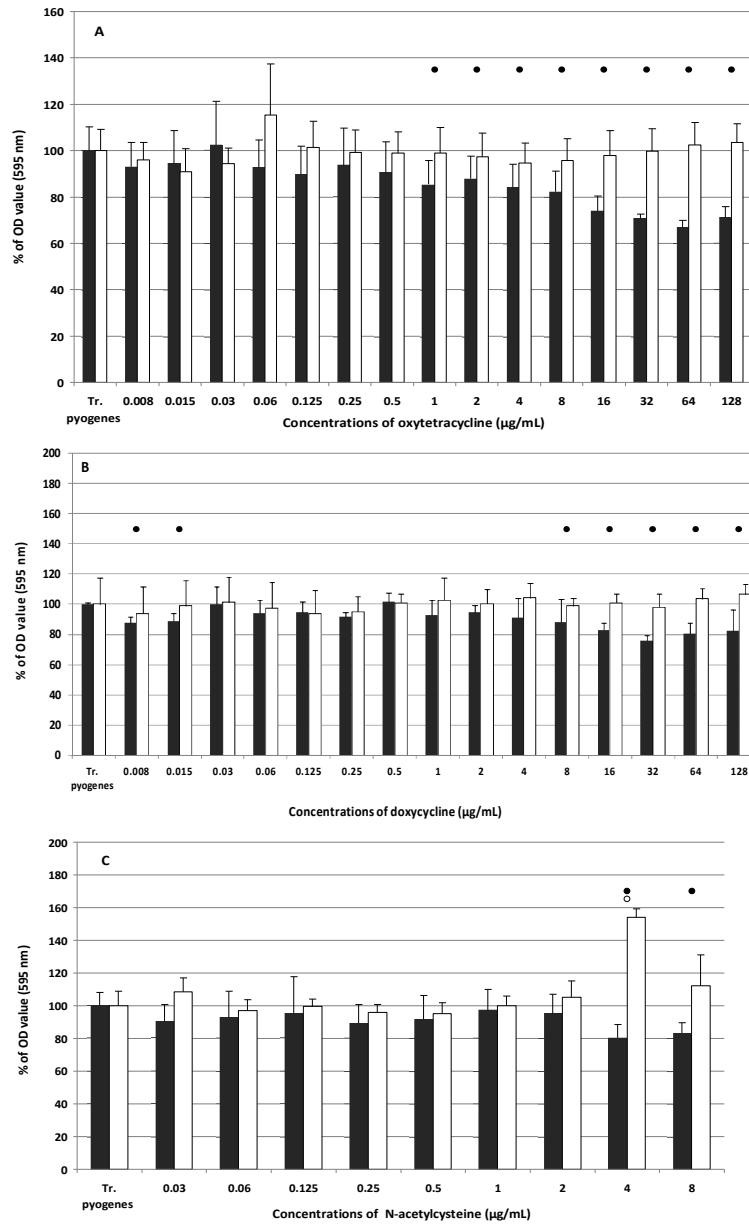


Fig. 2. Effect of oxytetracycline (A), doxycycline (B) and N-acetylcysteine (C) alone on the growth of *T. pyogenes* isolates (*T. pyogenes* 1: black bars; and *T. pyogenes* 2: white bars). cultured at conditions for biofilm formation. Data are expressed as mean \pm SD of OD suppl/OD control ratios. Dots indicate statistically significant differences at $P < 0.05$ between isolate 1 (black dot) or isolate 2 (white dot) vs respective controls.

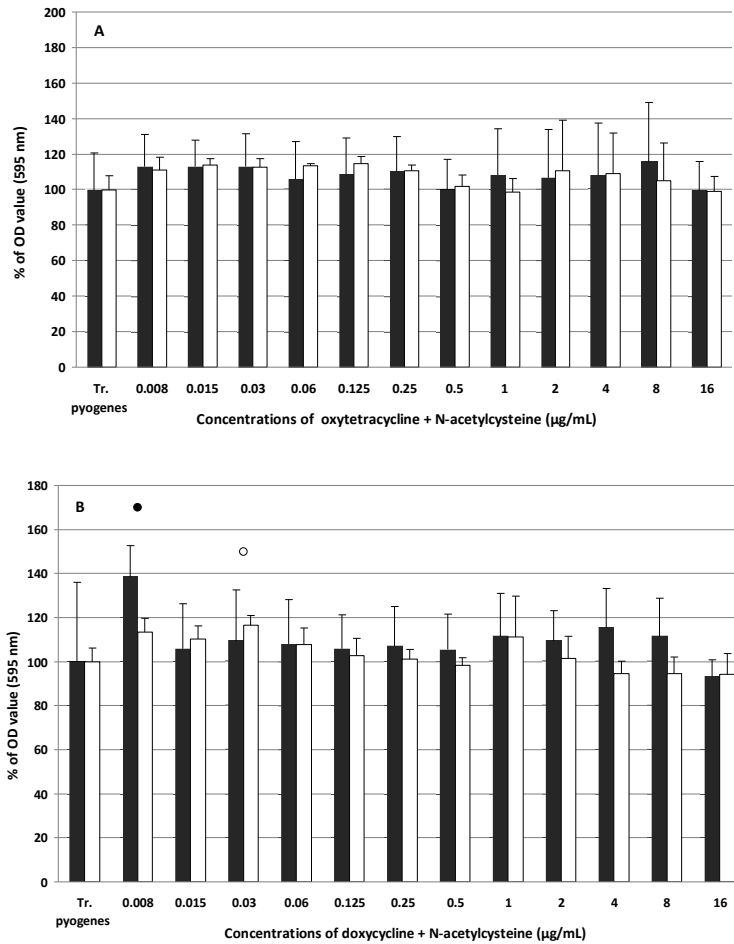


Fig. 3. Effect of oxytetracycline + N-acetylcysteine (A) and doxycycline + N-acetylcysteine (B) on the growth of *T. pyogenes* isolates (*T. pyogenes* 1: black bars; and *T. pyogenes* 2: white bars) cultured at conditions for biofilm formation. Data are expressed as mean ± SD of OD suppl/OD control ratios. Dots indicate statistically significant differences at P<0.05 between isolate 1 (black dot) or isolate 2 (white dot) vs respective controls.

The incubation with N-acetylcysteine at concentrations ≥ 4 mg/mL resulted in significant inhibition of the growth of *T. pyogenes* 1 (Fig. 2C).

A tendency of stimulation of the growth of both *T. pyogenes* isolates was observed when tetracycline and doxycycline were added in the culture medium

in combination with N-acetylcysteine (Fig. 3A and 3B).

Oxytetracycline is registered for use in cattle as injectable solutions for i.m. administration and therefore we further evaluated its effect on the metabolic activity of *T. pyogenes* at concentrations achievable in the uterine tissue. The test for

Table 1. Effect of oxytetracycline, doxycycline and N-acetylcysteine alone and in combination on the metabolic activity of *T. pyogenes* reared at conditions for biofilm formation

Drug	I _{max}	IC ₅₀ (µg/mL)	E ₀	Gamma
<i>Trueperella pyogenes</i> 1				
Oxytetracycline	0.01	15.56	0.017	0.93
N-acetylcysteine	0.04	39.50	0.017	0.70
Oxytetracycline + N-acetylcysteine	–	640.00	0.017	0.20
<i>Trueperella pyogenes</i> 2				
Oxytetracycline	0.007	28.64	0.017	9.99
N-acetylcysteine	–	15.93	0.014	0.72
Oxytetracycline + N-acetylcysteine	–	639.90	0.014	0.46

I_{max}: the maximum inhibitory effect, IC₅₀: the drug concentration that produced 50% of maximum inhibition, E₀: effect at time 0; Gamma: the slope.

metabolic activity of the pathogenic strains showed that inhibitory concentration (IC₅₀) was higher when both isolates were cultured with tetracycline in combination with N-acetylcysteine compared to the effect of either oxytetracycline or N-acetylcysteine alone (Table 1).

DISCUSSION

Clinical metritis in cows is associated with mixed infections, caused by aerobic and anaerobic bacteria. *T. pyogenes* is recognised very often among the causative agents (Ashrafi *et al.*, 2018). Antibiotics are commonly prescribed for treatment of clinical metritis without information about the susceptibility of the bacterial pathogens which provoke the uterine infections, thus the efficacy of the therapy could be compromised (Haimerl *et al.*, 2017). The current study was performed as an extension of the clinical investigation of the pharmacokinetics of intramuscularly administered oxytetracycline in cows with metritis (Mileva *et al.*, 2020). The cited experiment revealed that in some of the cows with metritis associated with *T. pyo-*

genes, complete cure was not achieved (Mileva *et al.*, 2020). Therefore, the present *in vitro* experiments were designed to investigate the susceptibility of *Trueperella pyogenes* to oxytetracycline as an antibiotic registered for use in cattle and to doxycycline as a tetracycline not licensed for ruminants. N-acetylcysteine was also included in *in vitro* experiments with the pathogenic isolates due to its positive effect in treatment of endometritis in cows (Gores-Lindholm *et al.*, 2013; Tras *et al.*, 2014).

Successful treatment is based on information about the disposition of the antibiotic at the site of infection and the sensitivity of the pathogens (Toutain *et al.*, 2002). *T. pyogenes* isolates showed a different susceptibility to oxytetracycline. Isolate 1 was more sensitive to oxytetracycline than isolate 2 which correlated with the efficacy of therapy of the cows with clinical metritis (Mileva *et al.*, 2020). There was no difference in the sensitivity of both isolates to doxycycline and the MIC value was close to those, observed for oxytetracycline. Although there are no breakpoint values for MIC of tetracyclines

against *T. pyogenes*, our data were comparable to observations from other authors. Galán-Relaño *et al.* (2020) reported MIC values between 0.06 and 64 µg/mL with bimodal distribution: highly sensitive strains with MIC ≤ 0.12 µg/mL and strains with MIC ≥ 8 µg/mL. The low sensitivity of bovine isolates was explained with extensive tetracycline use (Santos *et al.*, 2010; Galán-Relaño *et al.*, 2020). Another research group found a wide range of MIC values of oxytetracycline: between 0.25 and ≥ 128 µg/mL and determined lower MIC₉₀ of 8 µg/mL (Boer *et al.*, 2015). Altogether, our results and the published data, show that susceptibility of *T. pyogenes* of bovine origin to tetracyclines is highly variable emphasising that MIC value should be determined before the start of the treatment of cows with clinical metritis.

N-acetylcysteine showed antibacterial activity against *T. pyogenes* at concentrations that can be achieved after intrauterine administration which can partly explain the successful treatment of cows with uterine infections (Tras *et al.*, 2014). The bactericidal activity of N-acetylcysteine at a concentration of 5 mg/mL against other pathogens such as *Acinetobacter baumannii* has been described (Oliva *et al.*, 2018). MIC values of N-acetylcysteine against clinical isolates of *T. pyogenes* cannot be compared with other studies because of lack of published data.

Resistance mechanisms of *T. pyogenes* have been investigated by molecular methods (Abdallah, 2016; Ashrafi *et al.*, 2018). Biofilm formation was recognised as an important mechanism determining the resistance of *T. pyogenes* and causing chronification of uterine infections (Markey *et al.*, 2013). Moreover, biofilm producing *T. pyogenes* strains were out-

lined as important contributors to mastitis in dairy cattle (Alkasir *et al.*, 2016). Both isolates of *T. pyogenes* from cows with clinical metritis were not able to produce biofilm as shown from crystal violet staining. In contrary to our results, 45 out of 50 isolates from cows with mastitis were able to form biofilms (Alkasir *et al.*, 2016). Some of these strains demonstrated low-grade biofilm formation while others were classified as highly biofilm positive (Alkasir *et al.*, 2016). The authors found 30% sensitivity of the tested strains to oxytetracycline. The results from our study showed that addition of either oxytetracycline or doxycycline alone at concentrations > 16 µg/mL, inhibited significantly the growth of *T. pyogenes* 1 isolate – by 20% and had no significant effect on the less sensitive *T. pyogenes* 2 isolate when cultured in conditions for biofilm production. The same observation was made when N-acetylcysteine was added in the culture medium at a concentration ≥ 4 mg/mL. Our results for the effect of the mucolytic drug are in agreement with published data for inhibitory activity of N-acetylcysteine on the growth of bacteria in planktonic culture and on biofilm formation (Perez-Giraldo *et al.*, 1997; Dincola *et al.*, 2014). Combinations of N-acetylcysteine with either oxytetracycline or doxycycline increased insignificantly the growth of both *T. pyogenes* isolates. Yang *et al.* (2016) demonstrated decreased susceptibility of Gram-positive and increased sensitivity of Gram-negative pathogenic bacteria which cause mastitis in cattle when tetracycline was combined with N-acetylcysteine, in agreement with our observations. Decreased activity was also suggested by the results from the test for metabolic activity of *T. pyogenes*. Increase in IC₅₀ values was observed when N-acetylcysteine was

combined with each of both tetracyclines. Altogether these data demonstrated that the combinations of N-acetylcysteine with oxytetracycline or doxycycline could be favourable for the growth of *T. pyogenes*. The antioxidant activity of N-acetylcysteine which may help bacteria to survive stress caused by addition of antibiotics, could be an explanation of the observed results (Goswami & Jawali, 2010; Dincola *et al.*, 2014).

In conclusion, the data from the current investigation showed the necessity of susceptibility testing before antibiotic treatment in clinical metritis associated with *T. pyogenes*. Clinical *T. pyogenes* isolates showed low sensitivity to oxytetracycline and doxycycline. The combinations between N-acetylcysteine and investigated tetracyclines stimulated insignificantly the growth of *T. pyogenes*, cultured under conditions for biofilm formation indicating that the combination of these agents was not suitable for treatment of clinical metritis, caused by this pathogen.

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