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Biofilm Formation and Antimicrobial Tolerance in Clinical Isolates of *Bordetella bronchiseptica*

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Abstract

***Bordetella bronchiseptica* is a major bacterial pathogen causing respiratory infections in small companion animals, with biofilm formation as a key mechanism underlying persistent infection and reduced antimicrobial efficacy. The aim of this study was to investigate the relationship between biofilm formation, metabolic and adhesive activity, and antimicrobial tolerance in clinical isolates of *B. bronchiseptica*. A total of 82 clinical isolates of *B. bronchiseptica* were analysed. Biofilm formation, metabolic activity, and adhesive capacity were assessed using crystal violet, resazurin, and microtiter plate assays, respectively. Antimicrobial susceptibility was assessed by disc diffusion, and minimum inhibitory concentrations were determined by broth microdilution according to Clinical and Laboratory Standards Institute guidelines. Biofilm tolerance was evaluated by a CFU-based killing assay in a representative subgroup of 18 isolates using isolate-specific antibiotic concentrations. The efficacy of enrofloxacin, gentamicin, doxycycline, amoxicillin/clavulanic acid, and trimethoprim/sulfamethoxazole was assessed by log reduction of viable biofilm cells. Isolates from animals with chronic respiratory infection more frequently formed strong biofilms (56.3%), whereas weak biofilm producers predominated in acute cases (49.2 %). Increased biofilm formation was associated with reduced metabolic activity, whereas no significant differences in adhesive activity were observed among biofilm-forming groups. Among planktonic cells, the lowest resistance rates were observed for enrofloxacin (8.5%) and gentamicin (9.8%), whereas multidrug resistance was detected in 23.2% of isolates. Within biofilms, the efficacy of all tested antibiotics decreased, and enrofloxacin showed lower log reduction values in chronic than acute isolates (1.69 vs. 2.49). Biofilm formation intensity was negatively correlated with enrofloxacin efficacy. The**

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findings demonstrate that extensive biofilm formation is associated with increased antimicrobial tolerance in *B. bronchiseptica* and may contribute to persistent infection, highlighting the importance of considering the biofilm phenotype when selecting antimicrobial therapy..

Keywords: ***Bordetella bronchiseptica; biofim; tolerance; antibiotic resistance; log reduction; multidrug resistance***.

Introduction

Diseases of small companion animals remain a widespread issue in modern veterinary medicine, causing significant economic losses and deterioration in animal quality of life (Mavrides et al., 2022; Paliy et al., 2022). One of the key bacterial pathogens of such infections is *Bordetella bronchiseptica*, which is widely distributed among dogs and cats and is capable of causing both acute and chronic disease (Mavrides et al., 2022; Yi et al., 2024).

An important feature of *B. bronchiseptica* is its long-term persistence in the host organism. Recent studies have indicated that a key mechanism underlying this persistence is the formation of biofilm: structured microbial communities encapsulated within an extracellular polymeric matrix (Nicholson et al., 2024; Mugni et al., 2025). Surface properties and the ability of bacteria to perform primary adhesion play a crucial role in this process, as demonstrated for various microbial species in studies of biofilm formation on abiotic surfaces (Dewi et al., 2021; Atiencia-Carrera et al., 2022; Kolchyk et al., 2022). This process is regulated by intracellular signaling systems, in particular by the level of cyclic di-GMP, which influences the expression of virulence genes and bacterial adaptation to environmental conditions (Nicholson et al., 2024; Gutierrez et al., 2022).

In recent years, considerable attention has been focused on the role of biofilms in reducing the effectiveness of antimicrobial therapy. In contrast to planktonic cells, bacteria within biofilms exhibit increased tolerance to antibiotics, which is not always associated with classical resistance (Madduri et al., 2025). This is explained by a number of factors, including limited antibiotic penetration through the biofilm matrix, reduced metabolic activity of cells, and the formation of persistent subpopulations (Peng et al., 2022; Wu et al., 2022). In addition, the effectiveness of antimicrobial agents may depend on environmental conditions and the physicochemical properties of the surrounding medium, as demonstrated for disinfectants and sanitisers (Boucher et al., 2021).

Similar mechanisms of increased tolerance in biofilm-associated cells have also been described for various bacterial species, including *Staphylococcus aureus* and other clinically significant microorganisms, where biofilm formation is associated with reduced effectiveness of antimicrobial agents (Kamble et al., 2022; Manasherob et al., 2021; Parker et al., 2023). Studies conducted on different bacterial species have shown that biofilms can significantly reduce the bactericidal activity of antibiotics even when the susceptibility of planktonic cells is preserved (Brooks et al., 2021; Moore et al., 2023). In addition, the intensity of biofilm formation may correlate directly with a decrease in the effectiveness of antimicrobial agents (Madduri et al., 2025).

B. bronchiseptica is capable of colonising the respiratory tract and persisting there for a prolonged period, forming stable biofilm structures (Yi et al., 2024). It has been shown that the regulation of biofilm formation in this pathogen is associated with virulence and the ability to evade the immune response (Belhart et al., 2023; Nicholson et al., 2024).

Recent studies have indicated the need to distinguish between the concepts of resistance and tolerance. While resistance is defined as the ability of bacteria to grow in the presence of an antibiotic, tolerance is characterised by the survival of cells after exposure to the drug without changes in the minimum inhibitory concentration (Rosca et al., 2022; Madduri et al., 2025). Thus, the study of the biofilm phenotype of *B. bronchiseptica* and its relationship with the effectiveness of antimicrobial agents is a relevant research direction with both fundamental and applied significance for veterinary medicine.

The aim of this study was to determine the the ability to form biofilms, metabolic and adhesive activity of clinical isolates of *B. bronchiseptica* obtained from small companion animals with different clinical courses of respiratory infections and to examine how this affects the antimicrobial tolerance of these isolates.

Materials and methods

The study was conducted in 2023–2025 in specialised laboratories of the National Scientific Center, Institute of Experimental and Clinical Veterinary Medicine in Kharkiv, Ukraine. Clinical material was collected from dogs and cats with suspected respiratory diseases kept in kennels and veterinary clinics. Samples were obtained from the mucous

membrane of the upper respiratory tract of animals using sterile swabs. The samples were placed in a transport medium and delivered to the laboratory for further examination in accordance with generally accepted approaches to the bacteriological diagnosis of respiratory infections (Mavrides et al., 2022; Yi et al., 2024). Bacteriological examination of the clinical material resulted in 82 isolates of *B. bronchiseptica* that were included in further analysis.

Isolates of *B. bronchiseptica* were identified based on their characteristic morphological, cultural, and biochemical properties (Mavrides et al., 2022; Yi et al., 2024), followed by molecular confirmation of species identity using polymerase chain reaction (PCR). Nucleic acids were extracted using the PathoGene-spin™ DNA/RNA Extraction Kit (iNtRON Biotechnology, Republic of Korea). Detection of *B. bronchiseptica* DNA was performed using conventional PCR, prepared using DreamTaq Green PCR Master Mix (2X) (Thermo Scientific™, USA) and species-specific primers flanking a 298-bp fragment of the *fla* gene at a final concentration of 1 µM each: Fla2 (5′-AGGCTCCCAAGAGAGAAAGGCTT-3′) and Fla3 (5′-CACCTGCCCCATCTCC-3′) (Hozbor et al., 1999). Amplification was carried out using a Biometra TAdvanced thermal cycler (Biometra GmbH, Germany) under the following conditions: initial denaturation at 94°C for 2 min; 35 cycles of 94°C for 1 min, 57°C for 30 s, and 72°C for 40 s; followed by a final extension at 72°C for 7 min. Each PCR run included a positive control (DNA from *B. bronchiseptica* reference strain UNCSM-110, National Center of Microorganism Strains, Ukraine) and a negative control (deionised water). Amplification products were separated by electrophoresis in a 2.5% agarose gel at 140 V for 30 min, stained with ethidium bromide (500 ng/mL), and visualised under ultraviolet light. Amplicon size was determined using a DNA Ladder RTU CSL-M DNA 100 bp molecular weight marker (Cleaver Scientific, UK).

Isolates were divided into three groups depending on the clinical condition of the animals (acute course, chronic course, or absence of clinical signs) for further comparative analysis of their biological properties. This classification was performed based on clinical data, taking into account the duration and nature of symptoms. The acute course group included animals with clinical signs of respiratory pathology (cough, rhinitis, ocular or nasal discharge) with a sudden onset and duration typically up to 2 weeks. The chronic course group included animals with a prolonged (more than 2 weeks) or recurrent course of respiratory symptoms, indicating persistence of the infectious process. This approach corresponds to current concepts of the course of respiratory infections in small companion animals (Mavrides et al., 2022; Yi et al., 2024; Nicholson et al., 2024).

Clinical isolates of *B. bronchiseptica* were cultured on Columbia blood agar (with the addition of 5.0% defibrinated blood) and in liquid nutrient medium Brain Heart Infusion (BHI) broth at a temperature of 37.0 ± 0.5°C for 18–24 hours under aerobic conditions.Bacterial suspensions were prepared in sterile physiological saline (0.85% NaCl) with adjustment of optical density to 0.5 units according to the McFarland standard, corresponding to approximately 1×10^8 CFU/mL. The obtained inoculum was used for further studies of biofilm formation, adhesive activity, and antimicrobial tolerance. Approaches to the cultivation of *B. bronchiseptica* and standardisation of the inoculum have been described elsewhere (Mavrides et al., 2022; Yi et al., 2024; Nicholson et al., 2024).

Biofilm formation intensity was determined using the microtiter plate method with crystal violet (crystal violet assay), one of the most widely used and reproducible methods for quantitative assessment of biofilm biomass. The threshold value (ODc) was defined as the mean optical density (OD570) of the negative control plus three standard deviations. Isolates were classified according to their biofilm-forming ability based on the ratio of OD to ODc as non-biofilm-forming (OD ≤ ODc), weak (ODc < OD ≤ 2×ODc), moderate (2×ODc < OD ≤ 4×ODc), or strong producers (OD > 4×ODc). In the study, ODc was ≈0.095 (2×ODc ≈ 0.190; 4×ODc ≈ 0.380). This approach to the assessment of biofilm formation corresponds to classical methods for quantitative determination of biofilm biomass (Stepanović et al., 2007; Gupta et al., 2021; Pérez-Lavalle et al., 2025).

The metabolic activity of cells within the biofilm was determined using the resazurin assay, which is based on the ability of metabolically active cells to reduce resazurin to the fluorescent product resorufin. The intensity of resazurin reduction was assessed spectrophotometrically (570 nm) in accordance with the standard approach to evaluating resazurin reduction (Gupta et al., 2021; Grimus et al., 2021; Ribeiro et al., 2023). This approach allows for quantitative assessment of the functional state of cells within the biofilm and is widely used to study the metabolic heterogeneity of bacterial populations, including the analysis of cellular responses to antimicrobial agents and stress factors (Wanarska et al., 2022; Mahmoudi-Qashqay et al., 2023). The obtained values were normalised relative to the control and expressed as a percentage of metabolic activity, after which isolates were classified as having low (<30%), moderate (30–70%), or high (>70%) metabolic activity.

Early bacterial adhesion to an abiotic surface was assessed in polystyrene microtiter plates after short-term incubation for 2 hours at 37.0±0.5°C. For quantitative evaluation of adhesive activity, rel-

ative optical density values (OD570) normalised to control wells were used. Depending on the obtained values, isolates were classified as having low (OD ≤ 0.20), moderate (0.21–0.40), or high (> 0.40) adhesive activity. This approach corresponds to modified methods for assessing adhesion and early stages of biofilm formation based on the use of crystal violet and quantitative determination of bound biomaterial, as described previously Rahman et al., 2022; Wan Omar et al., 2023; Pérez-Lavalle et al., 2025).

Antimicrobial susceptibility testing of clinical *B. bronchiseptica* isolates was performed using the disc diffusion and broth microdilution methods in accordance with the recommendations of the Clinical and Laboratory Standards Institute (CLSI) for bacteria isolated from animals (Clinical and Laboratory Standards Institute, 2024a). Interpretation of susceptibility results and quality-control procedures was based on the applicable veterinary standards (Clinical and Laboratory Standards Institute, 2024b). The CLSI VET01 standar provides standardised procedures for disc diffusion and broth dilution antimicrobial susceptibility testing, whereas the VET01S standard provides the corresponding interpretive criteria and quality-control ranges.

For disc diffusion testing, a bacterial suspension equivalent to 0.5 McFarland (approximately 1 × 10^8 CFU/mL) was prepared from a fresh culture and uniformly inoculated onto Mueller–Hinton agar. Antibiotic disks (Liofilchem S.r.l., Roseto degli Abruzzi, Italy) contained enrofloxacin (5 µg), gentamicin (10 µg), doxycycline (30 µg), amoxicillin/clavulanic acid (20/10 µg), and trimethoprim/sulfamethoxazole (1.25/23.75 µg). Plates were incubated at 37.0 ± 0.5°C for 18–24 h under ambient atmospheric conditions. Inhibition-zone diameters were measured to the nearest millimeter.

Because *B. bronchiseptica*-specific clinical breakpoints are not available in the CLSI standards for all antimicrobial agents evaluated in the present study, susceptibility categories for agents lacking species-specific criteria were assigned using predefined surrogate veterinary interpretive criteria derived from breakpoints established for closely related veterinary respiratory pathogens. These criteria were used solely for comparative classification of the isolates and were not interpreted as *B. bronchiseptica*-specific CLSI breakpoints. Isolates were classified as susceptible (S), intermediate (I), or resistant (R) according to the following predefined inhibition-zone diameter criteria: enrofloxacin, S ≥21 mm, I 17–20 mm, R ≤16 mm; gentamicin, S ≥15 mm, I 13–14 mm, R ≤12 mm; doxycycline, S ≥19 mm, I 17–18 mm, R ≤16 mm; amoxicillin/clavulanic acid, S ≥18 mm, I 14–17 mm, R ≤13 mm; and trimethoprim/sulfamethoxazole, S ≥16 mm, I 11–15 mm, R ≤10 mm. These surrogate criteria were applied consistently to all isolates tested in the disc diffusion assay. Multidrug resistance (MDR) was defined as resistance to at least three antimicrobial classes (Mavrides et al., 2022; Yi et al., 2024; Madduri et al., 2025).

Escherichia coli ATCC 25922 was used as the quality control strain for disc diffusion testing. Quality control (QC) results were accepted only when the inhibition zone diameters were within the applicable CLSI quality-control ranges. The following quality control zone diameter ranges were used: enrofloxacin (5 µg), 32–40 mm; gentamicin (10 µg), 19–26 mm; doxycycline (30 µg), 18–24 mm; amoxicillin/clavulanic acid (20/10 µg), 18–24 mm; and trimethoprim/sulfamethoxazole (1.25/23.75 µg), 23–29 mm. QC results were considered acceptable only when the inhibition zone diameters obtained for *E. coli* ATCC 25922 fell within the applicable quality control ranges. These QC ranges were used to verify the performance of the disc diffusion procedure and were not used as clinical S/I/R interpretive breakpoints for *B. bronchiseptica*.

Before evaluating biofilm tolerance to antimicrobial agents, the minimum inhibitory concentration (MIC) was determined for each clinical isolate of *B. bronchiseptica* by the broth microdilution method in accordance with the CLSI recommendations for antimicrobial susceptibility testing of bacteria isolated from animals (CLSI VET01, 6th ed., 2024). Interpretation of MIC results and quality control procedures were performed according to the applicable veterinary interpretive and quality control criteria provided in CLSI VET01S (7th ed; 2024). Cation-adjusted Mueller-Hinton broth (CAMHB) was used for MIC determination. The inoculum was prepared from fresh 18–24 h cultures of *B. bronchiseptica* grown on Columbia blood agar. Bacterial suspensions were adjusted to a turbidity equivalent to a 0.5 McFarland standard (approximately 1–2×10^8 CFU/mL) and subsequently diluted in CAMHB to obtain a final inoculum concentration of 5×10^5 CFU/mL in each well. Two-fold serial dilutions of the antimicrobial agents were prepared in sterile 96-well polystyrene microtiter plates. Each well received 100 µL of the appropriate antimicrobial dilution and 100 µL of the standardized bacterial inoculum, resulting in a final reaction volume of 200 µL. Plates were incubated aerobically at 37.0 ± 0.5°C for 18–24 h. The MIC was defined as the lowest concentration of the antimicrobial agent that completely inhibited visible bacterial growth after incubation. Each assay included a growth control (CAMHB supplemented with the bacterial inoculum without antimicrobial agent) and a sterility control (CAMHB without bacterial inoculum). Quality control of the broth microdilution assay was performed using *Escherichia coli* ATCC 25922 and *E. coli* ATCC

35218, and the obtained MIC values were required to fall within the applicable CLSI quality control ranges. All determinations were performed in at least three replicates.

The individually determined MIC values were subsequently used to calculate antimicrobial concentrations for the biofilm tolerance experiments. Biofilm tolerance was evaluated using a CFU-based killing assay. For each clinical isolate–antimicrobial agent combination, biofilms were exposed to antimicrobial concentrations individually calculated as 8×MIC, ensuring a standardised antimicrobial challenge relative to the baseline susceptibility of each isolate. Each experiment included sterility controls (culture medium only) and growth controls without antimicrobial agents. The antimicrobial concentration used in the subsequent biofilm killing assay was calculated individually for each isolate as 8× its corresponding MIC.

A representative subset of isolates was selected for the CFU-based killing assay. From the total collection of 82 clinical *B. bronchiseptica* isolates, three phenotypic groups were previously established according to their biofilm-forming capacity (weak, moderate, and strong). The isolates were selected according to predefined stratification criteria rather than by random sampling: six isolates were selected from each phenotypic group, including three isolates recovered from animals with acute disease and three from animals with chronic disease. Preliminary statistical analysis demonstrated that the selected isolates did not differ significantly from the remaining isolates within their respective phenotypic groups in terms of biofilm-forming capacity ($p > 0.05$), confirming their representativeness. Consequently, a total of 18 clinical isolates were included in the CFU-based killing assay, providing balanced representation of all biofilm-forming phenotypes and clinical disease forms, and enabling comparison of biofilm-associated antibiotic tolerance according to biofilm-forming capacity.

The level of biofilm tolerance was assessed by the reduction in the number of viable cells (log reduction), which was calculated as the difference between the logarithms of CFU counts in control and antibiotic-treated samples. Log reduction values were interpreted as follows: >2 pronounced bactericidal effect; 1–2 partial reduction in viability; <1 high tolerance of biofilm cells. This approach allows quantitative assessment of cell survival within the biofilm independently of antibiotic susceptibility parameters of planktonic forms.

The CFU-based killing assay method and approaches to the assessment of biofilm bacterial tolerance have been described in previous studies (Brooks et al., 2021; Rosca et al., 2022; Moore et al., 2023).

Statistical analysis of the data was performed using standard biostatistical methods. The χ^2 test was used to compare frequencies, the Mann–Whitney U test for the analysis of independent samples, and Spearman's correlation coefficient to assess relationships. For multiple comparisons, p-values were adjusted using the Holm step-down procedure. Adjusted p-values < 0.05 were considered statistically significant. Data were presented as mean ± standard deviation (SD) for replicates and median (IQR) for group analysis. Replicates were averaged for each clinical isolate before statistical analysis, and the individual clinical isolate was considered the experimental unit.

All manipulations with animals during the collection of clinical material were performed in accordance with bioethical principles, in compliance with the Law of Ukraine "On Protection of Animals from Cruel Treatment" and the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (1986).

Results

A total of 82 isolates of *B. bronchiseptica* obtained from small companion animals with suspected respiratory diseases were analyzed in the study. The majority of isolates were obtained from dogs (n = 56), whereas 26 isolates were obtained from cats (Table 1).

Among the 82 clinical *B. bronchiseptica* isolates included in the study, most were recovered from animals with acute respiratory disease (74.4%), whereas isolates from animals with chronic disease and clinically healthy animals accounted for 19.5% and 6.1% of the total, respectively (Table 1).

Table 1. Distribution of clinical isolates of *B. bronchiseptica* by animal species and clinical course of infection, n = 82.

Animal species	Acute n (%)	Chronic n (%)	No clinical signs n (%)	Total
Dogs	44 (78.6)	9 (16.1)	3 (5.3)	56
Cats	17 (65.4)	7 (26.9)	2 (7.7)	26
Total	61 (74.4)	16 (19.5)	5 (6.1)	82

Table 2. Clinical manifestations of respiratory infection in animals from which *B. bronchiseptica* was isolated (n = 82).

Clinical manifestation	n	%
No clinical signs of respiratory tract disease	5	6.1
Isolated cough	44	53.7
Mixed respiratory disease	28	34.1
Rhinitis	3	3.7
Conjunctivitis	2	2.4

Table 3. Biofilm formation intensity of clinical isolates of *B. bronchiseptica* obtained from animals with different clinical courses (n = 82).

Source of isolates	n	Weak n (%)	Moderate n (%)	Strong n (%)
Animals with acute course	61	30 (49.2%) *	18 (29.5%)	13 (21.3%) *
Animals with chronic course	16	3 (18.8%)	4 (25.0%)	9 (56.3%) **
Animals without clinical signs	5	1 (20.0%)	2 (40.0%)	2 (40.0%)

* $p < 0.05$ when comparing weak and strong biofilm producers in the group of isolates from animals with an acute course of infection (χ^2 test)

** $p < 0.05$ when comparing weak and strong biofilm producers in the group of isolates from animals with a chronic course of infection (χ^2 test).

Table 4. Metabolic activity of bacterial cells in biofilms of different intensities (n = 82).

Biofilm intensity	n	Low n (%)	Moderate n (%)	High n (%)
Weak	21	3 (14.3%)	9 (42.9%)	9 (42.9%)
Moderate	37	11 (29.7%)	18 (48.6%)	8 (21.6%)
Strong	24	13 (54.2%)*	8 (33.3%)	3 (12.5%)*

* $p < 0.05$ for the comparison of the corresponding metabolic activity category between weak and strong biofilm groups (χ^2 test). Overall comparison: $\chi^2 = 10.86$; df = 4; $p = 0.028$.

The clinical manifestations of respiratory disease in animals from which *B. bronchiseptica* isolates were recovered are presented in Table 2.

Analysis of the clinical manifestations in animals from which *B. bronchiseptica* was recovered showed that no clinical signs of respiratory disease were observed in five (6.1%) animals. The most common clinical presentation was isolated cough, recorded in 44 (53.7%) cases. Mixed respiratory disease was observed in 28 (34.1%) animals, while rhinitis and conjunctivitis were detected much less frequently, occurring in three (3.7%) and two (2.4%) cases, respectively (Table 2).

The distribution of biofilm-forming capacity among clinical B. bronchiseptica isolates according to the clinical course of disease in the animals is presented in Table 3

Analysis of biofilm-forming capacity showed that, among the 61 isolates recovered from animals with acute infection, 30 (49.2%) were weak biofilm producers, 18 (29.5%) were moderate producers, and 13 (21.3%) were strong producers. The proportion of weak biofilm producers was significantly higher than that of strong producers ($p < 0.05$). Among the 16 isolates recovered from animals with chronic infection, strong biofilm producers predominated, accounting for nine (56.3%) isolates, whereas four (25.0%) and three (18.8%) isolates were classified as moderate and weak producers, respectively. The difference between the proportions of strong and weak biofilm producers was statistically significant ($p < 0.05$). In the group of isolates recovered from clinically healthy animals (n = 5), weak, moderate, and strong bio-

Table 5. Adhesive activity of clinical isolates of *B. bronchiseptica* depending on the intensity of biofilm formation (n = 82).

Biofilm intensity	n	Low adhesive activity n (%)	Moderate adhesive activity n (%)	High adhesive activity n (%)
Weak	21	7 (33.3%)	9 (42.9%)	5 (23.8%)
Moderate	37	8 (21.6%)	18 (48.6%)	11 (29.7%)
Strong	24	3 (12.5%)	10 (41.7%)	11 (45.8%)

Table 6. Individual MIC values of antimicrobial agents for the 18 *B. bronchiseptica* isolates included in the biofilm killing assay.

Isolate	ENR MIC, µg/mL	GEN MIC, µg/mL	DOX MIC, µg/mL	AMC MIC, µg/mL	TMP/SMX MIC, µg/mL[L
A1	0.25	1	0.5	4/2	1/19
A2	0.5	2	1	8/4	2/38
A3	0.125	1	1	4/2	2/38
A4	0.5	0.5	0.25	16/8	1/19
A5	0.25	1	0.5	2/1	4/76
A6	1	2	2	8/4	1/19
A7	0.25	0.5	1	8/4	0.5/9.5
A8	0.5	2	4	16/8	2/38
A9	0.125	1	0.25	8/4	4/76
C1	0.5	1	1	4/2	2/38
C2	0.25	4	0.5	16/8	0.5/9.5
C3	1	2	4	32/16	4/76
C4	0.5	1	2	8/4	1/19
C5	1	4	1	16/8	8/152
C6	0.25	2	0.5	4/2	2/38
C7	1	4	2	32/16	1/19
C8	0.5	2	1	16/8	4/76
C9	0.25	1	0.25	8/4	0.5/9.5[L

film producers accounted for 20.0%, 40.0%, and 40.0% of the isolates, respectively (Table 3).

The distribution of bacterial metabolic activity according to biofilm-forming capacity is presented in Table 4.

Among the weak biofilm producers (n = 21), high and moderate metabolic activity was observed in nine (42.9%) isolates each, while low metabolic activity was detected in three (14.3%) isolates. In the group of strong biofilm producers (n = 24), isolates with low metabolic activity predominated, accounting for 13 (54.2%), whereas high metabolic activity was detected in only three (12.5%) isolates ($p < 0.05$). Among isolates with moderate biofilm-forming capacity (n = 37), moderate metabolic activity was the most common finding, occurring in 18 (48.6%) isolates, while low and high metabolic activity were observed in 11 (29.7%) and eight (21.6%) isolates, respectively. The overall distribution of metabolic activity differed significantly among the three biofilm-forming groups ($\chi^2 = 10.86$, df = 4, $p = 0.028$).

The distribution of adhesive activity among clinical *B. bronchiseptica* isolates according to biofilm-forming capacity is presented in Table 5.

Among the weak biofilm producers (n = 21), low, moderate, and high adhesive activities were detected in seven (33.3%), nine (42.9%), and five (23.8%) isolates, respectively. Among the moderate biofilm producers (n = 37), moderate adhesive activity was the most common finding, occurring in

Table 7. Assessment of biofilm tolerance of *B. bronchiseptica* (CFU-based killing assay, n = 18).

Isolate	Clinical course	Biofilm (OD570, mean ± SD)	Intensity	Enrofloxacin	Gentamicin	Doxycycline	Amoxicillin/clavulanate	TMP/SMX
A1	acute	0.18 ± 0.02	weak	2.76 ± 0.30	1.51 ± 0.24	1.17 ± 0.18	1.05 ± 0.19	1.62 ± 0.25
A2	acute	0.46 ± 0.03	strong	1.94 ± 0.25	1.53 ± 0.21	0.85 ± 0.14	0.78 ± 0.18	0.71 ± 0.20
A3	acute	0.24 ± 0.02	moderate	2.49 ± 0.29	1.87 ± 0.23	0.91 ± 0.17	0.81 ± 0.17	1.43 ± 0.22
A4	acute	0.12 ± 0.02	weak	3.18 ± 0.36	1.43 ± 0.29	1.44 ± 0.22	1.21 ± 0.22	1.88 ± 0.28
A5	acute	0.43 ± 0.02	strong	2.02 ± 0.27	1.60 ± 0.22	0.85 ± 0.15	0.81 ± 0.16	1.29 ± 0.21
A6	acute	0.27 ± 0.02	moderate	2.31 ± 0.28	1.76 ± 0.23	1.02 ± 0.16	0.77 ± 0.18	1.36 ± 0.23
A7	acute	0.14 ± 0.02	weak	3.09 ± 0.34	1.73 ± 0.28	1.35 ± 0.21	1.18 ± 0.21	1.74 ± 0.26
A8	acute	0.39 ± 0.03	strong	1.86 ± 0.24	1.49 ± 0.20	0.81 ± 0.14	0.69 ± 0.15	1.08 ± 0.19
A9	acute	0.29 ± 0.02	moderate	2.55 ± 0.31	1.92 ± 0.25	1.12 ± 0.19	0.98 ± 0.18	1.51 ± 0.24
C1	chronic	0.32 ± 0.03	moderate	1.58 ± 0.23	1.34 ± 0.19	0.91 ± 0.13	0.78 ± 0.14	1.08 ± 0.18
C2	chronic	0.41 ± 0.02	strong	1.22 ± 0.21	1.15 ± 0.18	0.60 ± 0.12	0.51 ± 0.13	0.94 ± 0.16
C3	chronic	0.16 ± 0.02	weak	2.18 ± 0.27	1.80 ± 0.22	1.01 ± 0.16	0.87 ± 0.17	1.56 ± 0.22
C4	chronic	0.39 ± 0.02	strong	1.44 ± 0.25	1.27 ± 0.20	0.72 ± 0.14	0.58 ± 0.15	1.05 ± 0.18
C5	chronic	0.25 ± 0.02	moderate	1.69 ± 0.24	1.43 ± 0.21	0.85 ± 0.14	0.81 ± 0.16	1.17 ± 0.19
C6	chronic	0.19 ± 0.02	weak	2.03 ± 0.26	1.67 ± 0.22	0.93 ± 0.16	0.84 ± 0.18	1.29 ± 0.21
C7	chronic	0.43 ± 0.03	strong	1.17 ± 0.21	1.11 ± 0.17	0.56 ± 0.11	0.48 ± 0.12	0.89 ± 0.15
C8	chronic	0.28 ± 0.02	moderate	1.72 ± 0.25	1.46 ± 0.21	0.88 ± 0.15	0.69 ± 0.15	1.14 ± 0.18
C9	chronic	0.15 ± 0.02	weak	2.11 ± 0.26	1.74 ± 0.21	1.02 ± 0.15	0.82 ± 0.17	1.31 ± 0.22

Differences between isolates obtained from animals with acute and chronic disease were assessed using the Mann-Whitney U test. A statistically significant difference was observed only for enrofloxacin (p = 0.004), whereas no statistically significant differences were found for the other antimicrobial agents (p > 0.05).

18 (48.6%) isolates, whereas low and high adhesive activities were observed in eight (21.6%) and 11 (29.7%) isolates, respectively. Among the strong biofilm producers (n = 24), high adhesive activity was detected in 11 (45.8%) isolates, moderate adhesive activity in ten (41.7%), and low adhesive activity in three (12.5%) isolates. No statistically significant differences were observed among the biofilm-forming groups (p > 0.05).

The tolerance of biofilm structures was evaluated in a representative subset of 18 clinical *B. bronchiseptica* isolates using a CFU-based killing assay. The individual MIC values determined for each isolate–antimicrobial combination are presented in Table 6.

Antimicrobial concentrations used in the biofilm killing assay were calculated individually as 8× the corresponding MIC for each isolate–antimicrobial combination. The results of the individual isolate analysis of biofilm tolerance are presented in Table 7.

Comparison of log reduction values between isolates recovered from animals with acute and chronic infection showed that the median log reduction for enrofloxacin was significantly lower in isolates from animals with chronic disease than in those with acute disease [1.69 (1.44–2.03) vs. 2.49

Figure 1. Comparison of log reduction values for enrofloxacin in isolates obtained from animals with acute and chronic disease courses. Differences between groups were statistically significant (Mann-Whitney U test, *p* = 0.004; Holm-adjusted *p* = 0.020).

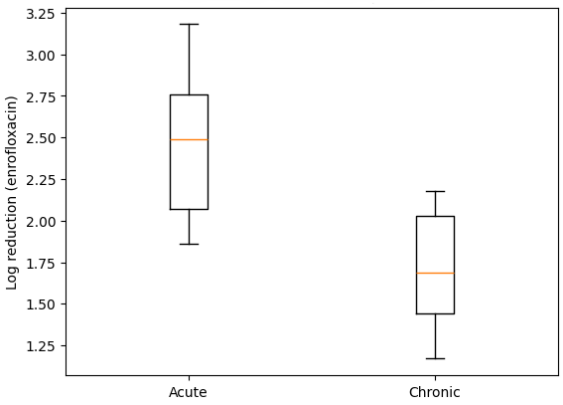


Table 8. Frequency of resistance of clinical isolates of *B. bronchiseptica* (n = 82).

Antibiotic	Class	Resistant isolates (n)	%
Amoxicillin/clavulanic acid	β-lactams	36	43.9
Doxycycline	Tetracyclines	13	15.9
Gentamicin	Aminoglycosides	8	9.8
Enrofloxacin	Fluoroquinolones	7	8.5
Trimethoprim/sulfamethoxazole	Sulfonamides	15	18.3

Table 9. Distribution of multidrug-resistant (MDR) isolates of *B. bronchiseptica* (n = 82).

Indicator	n	%
MDR (≥3 classes)	19	23.2
Non-MDR	63	76.8

Table 10. Comparison of antibiotic susceptibility of planktonic cells and tolerance of biofilm structures of clinical isolates of *B. bronchiseptica*.

Antibiotic	Class[LZ1.1]	Resistance (%, n = 82)	Log reduction (acute course, n = 9, median [IQR])	Log reduction (chronic course, n = 9, median [IQR])	p	Holm-adjusted p
Enrofloxacin	Fluoroquinolones	8.5	2.49 (2.02–2.76)	1.69 (1.44–2.03)	0.004	0.020
Gentamicin	Aminoglycosides	9.8	1.60 (1.51–1.76)	1.43 (1.27–1.67)	0.07	0.280
Doxycycline	Tetracyclines	15.9	1.02 (0.85–1.17)	0.88 (0.72–0.93)	0.09	0.280
Amoxicillin/ clavulanate	β-lactams	43.9	0.81 (0.78–1.05)	0.78 (0.58–0.82)	0.11	0.280
Trimethoprim/ sulfamethoxazole	Sulfonamides	18.3	1.43 (1.29–1.62)	1.14 (1.05–1.29)	0.08	0.280

Comparisons between groups were performed using the Mann-Whitney U test. Data are presented as median (IQR). Raw p-values and Holm-adjusted p-values are presented.

(2.02–2.76), $p = 0.004$]. Median log reduction values for gentamicin, doxycycline, amoxicillin/clavulanic acid, and trimethoprim/sulfamethoxazole were also lower in isolates recovered from animals with chronic infection; however, these differences did not reach statistical significance ($p > 0.05$).

The comparison of log reduction values for enrofloxacin in isolates recovered from animals with acute and chronic infection is presented in Figure 1.

The antimicrobial susceptibility profiles of clinical *B. bronchiseptica* isolates in the planktonic state are presented in Table 8.

The highest frequency of resistance was observed for amoxicillin/clavulanic acid, with 36 of 82 isolates (43.9%) classified as resistant. Resistance to trimethoprim/sulfamethoxazole and doxycycline was detected in 15 (18.3%) and 13 (15.9%) isolates, respectively. The lowest frequencies of resistance were observed for gentamicin and enrofloxacin, with eight (9.8%) and seven (8.5%) resistant isolates, respectively (Table 8).

The distribution of multidrug-resistant *B. bronchiseptica* isolates is presented in Table 9.

A multidrug-resistant phenotype was identified in 19 (23.2%) isolates, whereas 63 (76.8%) isolates were classified as non-multidrug-resistant.

Comparative data on the antimicrobial susceptibility of planktonic cells and the antimicrobial tolerance of biofilm structures are presented in Table 10. For each antimicrobial agent, the frequency of resistance among clinical isolates in the planktonic state and the median log reduction values of biofilm cells formed by isolates recovered from animals with acute and chronic infection are presented.

A statistically significant difference between the groups was observed only for enrofloxacin. The median log reduction was 2.49 (2.02–2.76) for isolates recovered from animals with acute infection

Table 11. Comparison of antibiotic tolerance among *Bordetella bronchiseptica* isolates according to biofilm-forming intensity (n = 18).

Biofilm intensity	Enrofloxacin	Gentamicin	Doxycycline	Amoxicillin/clavulanate	TMP/SMX
Weak (n = 6)	2.47 (2.13–3.00)a	1.70 (1.55–1.74)a	1.10 (1.01–1.31)a	0.96 (0.85–1.15)a	1.59 (1.37–1.71)a
Moderate (n = 6)	2.02 (1.70–2.45)a	1.61 (1.44–1.84)a	0.91 (0.89–0.99)a	0.80 (0.77–0.81)a	1.27 (1.15–1.41)a
Strong (n = 6)	1.65 (1.28–1.92)b	1.38 (1.18–1.52)a	0.77 (0.63–0.84)b	0.64 (0.53–0.76)b	1.00 (0.90–1.07)b
Kruskal-Wallis, p	0.014	0.118	0.0017	0.0033	0.0038
Holm-adjusted p	0.0280	0.1180	0.0085	0.0132	0.0132

Data are presented as median (IQR). Statistical analysis was performed using the Kruskal-Wallis test. Dunn's post hoc test with Holm adjustment was used for pairwise comparisons. Different superscript letters (a, b) within the same column indicate statistically significant differences between groups after Holm adjustment (adjusted $p < 0.05$).

Table 12. Correlation between biofilm formation intensity (OD570) and the effectiveness of antimicrobial agents (Spearman's rank correlation).

Parameter	r	p
Biofilm vs enrofloxacin	-0.722	< 0.001
Biofilm vs gentamicin	-0.412	0.089
Biofilm vs doxycycline	-0.819	< 0.001
Biofilm vs amoxicillin/clavulanate	-0.768	< 0.001
Biofilm vs TMP/SMX	-0.845	< 0.001

and 1.69 (1.44–2.03) for those recovered from animals with chronic infection ($p = 0.004$). Median log reduction values for gentamicin, doxycycline, amoxicillin/clavulanic acid, and trimethoprim/sulfamethoxazole were also lower in isolates recovered from animals with chronic infection; however, the differences between the groups were not statistically significant ($p > 0.05$).

The comparison of log reduction values among weak, moderate, and strong biofilm producers is presented in Table 11.

Statistically significant differences among isolates with different biofilm-forming capacities were observed for enrofloxacin ($p = 0.014$), doxycycline ($p = 0.0017$), amoxicillin/clavulanic acid ($p = 0.0033$), and trimethoprim/sulfamethoxazole ($p = 0.0038$). No statistically significant differences were detected for gentamicin ($p = 0.118$).

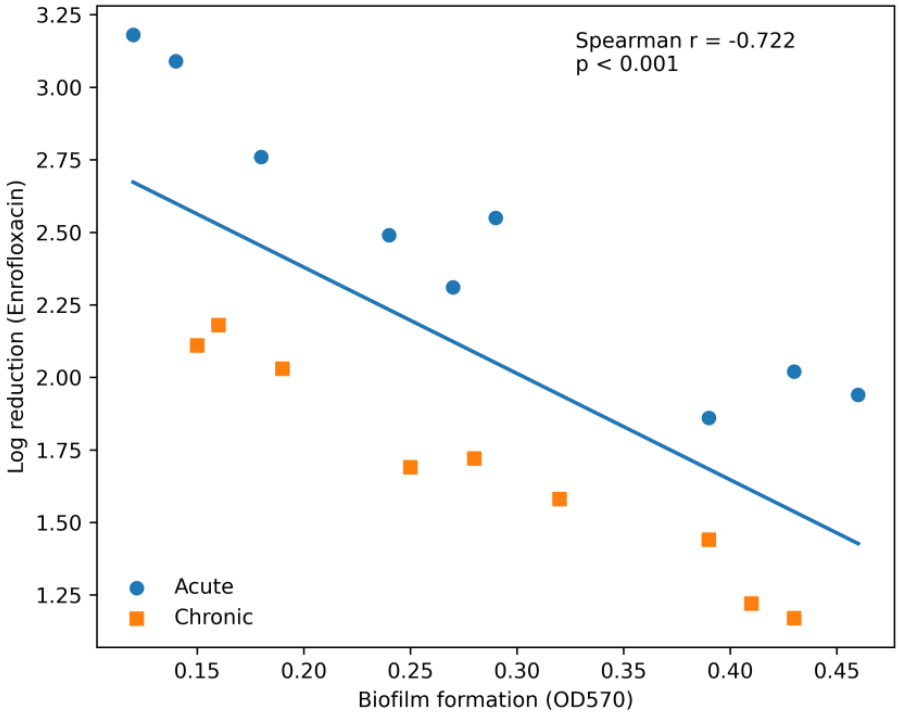
For enrofloxacin, the median log reduction decreased from 2.47 (2.13–3.00) in weak biofilm producers to 2.02 (1.70–2.45) in moderate producers and 1.65 (1.28–1.92) in strong biofilm producers. A similar decrease in median log reduction values was observed for doxycycline, amoxicillin/clavulanic acid, and trimethoprim/sulfamethoxazole.

To evaluate the role of the biofilm phenotype in antimicrobial tolerance, a correlation analysis was performed to assess the association between biofilm-forming capacity and antimicrobial efficacy (Table 12).

A negative correlation was observed between biofilm-forming capacity and the efficacy of all antimicrobial agents tested. The strongest correlations were found for trimethoprim/sulfamethoxazole ($r = -0.845$, $p < 0.001$), doxycycline ($r = -0.819$, $p < 0.001$), amoxicillin/clavulanic acid ($r = -0.768$, $p < 0.001$), and enrofloxacin ($r = -0.722$, $p < 0.001$). A negative correlation was also observed for gentamicin ($r = -0.412$); however, it was not statistically significant ($p = 0.089$).

The negative correlation between biofilm-forming capacity and the efficacy of enrofloxacin is illustrated in Figure 2.

Figure 2. Correlation between biofilm formation (OD570) and enrofloxacin efficacy (log reduction). Spearman's rank correlation showed a significant negative association (r = -0.722, p < 0.001).



Discussion

The present findings demonstrate a clear relationship between the clinical course of respiratory infection and the biofilm-forming capacity of *B. bronchiseptica*. In the present study, strong biofilm producers accounted for 56.3% of isolates recovered from animals with chronic disease, whereas weak biofilm producers predominated among isolates obtained from animals with acute infection (49.2%; $p < 0.05$). This distribution indicates that the ability to form mature biofilms is closely associated with the long-term persistence of the pathogen within the host and is likely one of the key mechanisms underlying the progression of the infectious process to a chronic form.

These findings are consistent with the current understanding of the biology of *B. bronchiseptica*. Biofilm formation is recognised as a complex, multistage process regulated by the BvgAS regulatory system and intracellular signaling pathways involving cyclic di-GMP. These regulatory mechanisms facilitate the transition of bacteria from the planktonic to the sessile lifestyle, coordinate the expression of adhesion-related genes and the synthesis of extracellular matrix components, thereby promoting the long-term persistence of bacteria on the mucosal surfaces of the respiratory tract (Gutierrez et al., 2022; Belhart et al., 2023; Nicholson et al., 2024). Consequently, isolates capable of forming robust biofilms are expected to be more frequently associated with the chronic course of respiratory infection.

The predominance of weak biofilm producers among isolates recovered from animals with acute disease may reflect the different biological conditions present during the early stages of infection. During this period, rapid bacterial multiplication, adhesion to the epithelial surface, and expression of virulence factors may predominate, whereas the development of mature biofilm requires time and adaptation to the host environment. As infection progresses, the ability to form a mature biofilm may provide a survival advantage under changing host conditions, thereby contributing to bacterial persistence.

Comparable observations have also been reported for other respiratory bacterial pathogens. According to Yi et al. (2024) and Mugni et al. (2025), intensive biofilm formation is a major factor promoting colonisation of the respiratory mucosa, evasion of the host immune response, and the development of chronic or recurrent infections. Therefore, the findings of the present study not only support the available evidence in the literature but also demonstrate its practical relevance for clinical *B. bronchiseptica* isolates recovered from dogs and cats.

We believe that the relationship identified between the clinical course of disease and the intensity of biofilm formation has important practical implications. The biofilm-forming phenotype may be regarded as an additional marker of the persis-

tence potential of *B. bronchiseptica* and the risk of progression to chronic infection.

Another important finding of the present study was the demonstration of a substantial reduction in the efficacy of antibacterial agents against *B. bronchiseptica* cells residing within biofilms. The data obtained indicate that even when planktonic cells remain susceptible to antimicrobial agents, bacteria embedded within the biofilm structure exhibit a markedly greater ability to survive following antibacterial treatment.

Comparable findings have been widely reported in the literature. In particular, Brooks et al. (2021) and Moore et al. (2023) demonstrated that biofilm formation substantially reduces the bactericidal activity of antibiotics even in cases where planktonic cells remain susceptible according to the results of standard MIC testing. Similar observations were reported by Rosca et al. (2022) and Parker et al. (2023), who showed that bacteria residing within biofilms exhibit significantly greater survival following exposure to bactericidal concentrations of antimicrobial agents than planktonic cultures, even in the absence of phenotypic resistance.

These observations were confirmed experimentally in the present study. Comparison of isolates recovered from animals with acute and chronic disease demonstrated a statistically significant reduction in the bactericidal activity of enrofloxacin against biofilm-associated cells. Specifically, the median log reduction value for enrofloxacin was 2.49 among isolates from animals with acute infection but only 1.69 among isolates from animals with chronic infection (Holm-adjusted $p = 0.020$), suggesting greater biofilm-associated antibiotic tolerance among isolates recovered from animals with chronic infection. A similar trend was observed for the other antimicrobial agents evaluated, although no statistically significant differences between the clinical groups were identified. The biological basis of this phenomenon involves the complex functioning of interconnected mechanisms. The extracellular polymeric matrix of the biofilm restricts the penetration of antibacterial agents into the deeper layers of the bacterial community, resulting in the formation of antibiotic concentration gradients. At the same time, depletion of nutrients and oxygen, together with the development of spatially heterogeneous microenvironmental niches within the biofilm, causes a proportion of bacterial cells to enter a state of low metabolic activity or to form persister subpopulations, which are considerably less susceptible to bactericidal agents (Manasherob et al., 2021). Consequently, antibiotics that are highly effective against actively proliferating planktonic cells often exhibit substantially reduced activity against bacteria residing within mature biofilms.

The present findings are fully consistent with the current understanding of the mechanisms underlying biofilm-associated antibiotic tolerance. Wu et al. (2022), Chavignon et al. (2022), and Peng et al. (2022) demonstrated that the tolerance of biofilm-associated bacteria is a multifactorial phenomenon determined not only by the physical barrier imposed by the biofilm matrix but also by cellular metabolic heterogeneity, activation of stress response pathways, and the formation of persistent subpopulations. These mechanisms provide a biological basis for the increased survival of biofilm-associated cells under antimicrobial exposure.

Particular attention should be paid to the results of the analysis performed after grouping the clinical isolates according to their biofilm-forming capacity. Unlike the comparison between isolates recovered from animals with acute and chronic disease, this approach provided a clearer demonstration of the relationship between the biofilm phenotype and the efficacy of antibacterial agents. As the intensity of biofilm formation increased, the median log reduction values gradually decreased for virtually all antimicrobial agents evaluated, indicating a progressive increase in the antibiotic tolerance of biofilm-associated bacterial communities.

The most pronounced differences were observed for enrofloxacin, doxycycline, amoxicillin/clavulanic acid, and trimethoprim/sulfamethoxazole. For these antimicrobial agents, the Kruskal-Wallis analysis confirmed statistically significant differences among the weak, moderate, and strong biofilm producers ($p = 0.014$, $p = 0.0017$, $p = 0.0033$, and $p = 0.0038$, respectively), while the difference between biofilm production strength was not significant for gentamicin ($p = 0.118$). These findings indicate that the intensity of biofilm formation was more associated with changes in the efficacy of antibacterial agents than with the clinical course of infection.

Further support for this conclusion was provided by the results of the correlation analysis. For enrofloxacin, a significant and strong negative correlation was identified between biofilm-forming capacity and the log reduction value ($r = -0.722$; $p < 0.001$), indicating a consistent decline in the bactericidal efficacy of the drug with increasing biofilm biomass. A similar trend was also observed for doxycycline, amoxicillin/clavulanic acid, and trimethoprim/sulfamethoxazole, although the strength of the correlation varied among the individual antimicrobial agents.

These findings are consistent with literature reports. In a systematic review, Madduri et al. (2025) demonstrated that the intensity of biofilm formation is one of the most important predictors of reduced antimicrobial efficacy, regardless of the bacterial species involved. The authors emphasised

that increasing biofilm biomass is accompanied by the development of a complex three-dimensional architecture, which creates a diffusion barrier that limits antibiotic penetration and promotes the accumulation of bacterial cells with low metabolic activity. Similar findings have also been reported by Wickremasinghe et al. (2021), Kamble et al. (2022), and Thonglao et al. (2022), who confirmed that increasing biofilm maturity is associated with reduced efficacy of antibacterial therapy, whereas disruption of the biofilm matrix or the use of combination antimicrobial therapy can substantially improve the eradication of biofilm-associated infections.

We believe that one of the most important findings of the present study is that the biofilm phenotype proved to be a more informative indicator of antibiotic tolerance than the clinical course of disease. While the comparison of isolates recovered from animals with acute and chronic infection revealed statistically significant differences only for enrofloxacin, grouping the isolates according to their biofilm-forming capacity identified significant differences for several antibacterial agents. This is likely because the clinical course of disease is a complex characteristic influenced by multiple factors, whereas the intensity of biofilm formation directly reflects one of the key biological properties of the pathogen that determines its ability to survive under antimicrobial pressure.

The present findings have important implications for veterinary medicine, as they demonstrate that conventional antimicrobial susceptibility testing of planktonic cells does not always provide an adequate prediction of the therapeutic efficacy against infections caused by *B. bronchiseptica*. In the present study, it was found that even for enrofloxacin, to which only 8.5% of clinical isolates were resistant in the planktonic state, efficacy against biofilm-associated cells was substantially reduced, particularly among isolates associated with chronic infection. In contrast, amoxicillin/clavulanic acid was characterised by both a high level of resistance among planktonic cells (43.9%) and the lowest log reduction values in biofilms.

These findings confirm that antibiotic resistance and antibiotic tolerance are distinct biological phenomena rather than synonymous concepts. Whereas resistance describes the ability of bacteria to grow in the presence of an antibacterial agent, tolerance refers to their ability to survive exposure to bactericidal concentrations without necessarily exhibiting an increase in the minimum inhibitory concentration (MIC). Consequently, relying solely on the results of standard MIC determination or disc diffusion testing may lead to an overestimation of the clinical efficacy of certain antibacterial agents in the treatment of biofilm-associated infections. This distinction between resistance and tolerance is strongly supported by recent studies (Rosca et al., 2022; Madduri et al., 2025) that emphasise the need for a comprehensive assessment of the biological characteristics of the pathogen when selecting antimicrobial therapy.

Another important practical conclusion of the present study is that the biofilm phenotype may be considered an additional laboratory marker for predicting treatment efficacy. Unlike the clinical course of disease, which is shaped by numerous host- and pathogen-related factors, the intensity of biofilm formation directly reflects one of the key biological characteristics of *B. bronchiseptica* that determines its capacity for long-term persistence and survival under antimicrobial pressure. Therefore, incorporating the assessment of biofilm formation into the comprehensive laboratory evaluation of clinical isolates may improve the predictive value of antimicrobial susceptibility testing, particularly in cases of recurrent and chronic respiratory infections.

The present study has several limitations that should be taken into account when interpreting the findings. Antibiotic tolerance was assessed using the CFU-based killing assay in a representative stratified subgroup comprising 18 clinical isolates selected from the overall collection of 82 isolates. This study design was chosen to ensure equal representation of weak, moderate, and strong biofilm producers and allowed an appropriate comparison of the influence of the biofilm phenotype on the efficacy of antibacterial agents. Moreover, a preliminary statistical analysis confirmed that the selected subgroup was representative of the corresponding phenotypic groups ($p > 0.05$), thereby minimising the risk of systematic bias. The relatively small number of isolates obtained from clinically healthy animals (n = 5) limited the statistical power of comparisons involving this subgroup and should be considered when interpreting these findings. In addition, the CFU-based killing assay represents an *in vitro* model of biofilm-associated antimicrobial tolerance, and the observed effects should therefore not be directly extrapolated to clinical treatment outcomes in animals.

Future research should focus on expanding the collection of clinical isolates, investigating the molecular mechanisms underlying biofilm formation, analysing the expression of genes involved in the regulation of biofilm development, and evaluating the efficacy of combination antimicrobial therapy as well as agents capable of disrupting the biofilm matrix. Another promising direction is the development of standardised laboratory methods for assessing biofilm-associated antibiotic tolerance, which could complement conventional anti-

microbial susceptibility testing and support a more personalised approach to the treatment of respiratory infections in companion animals.

Conclusions

Clinical *B. bronchiseptica* isolates exhibited marked variability in their biofilm-forming capacity. The proportion of strong biofilm producers was significantly higher among isolates recovered from animals with chronic infection (56.3%), whereas weak biofilm producers predominated among isolates from acute cases (49.2%). Enhanced biofilm formation was associated with functional changes in bacterial cells. Strong biofilms contained a significantly higher proportion of cells with low metabolic activity than weak biofilms (54.2% vs. 14.3%), whereas the proportion of highly metabolically active cells was significantly lower (12.5% vs. 42.9%05).

Despite the low prevalence of resistance among planktonic cells to enrofloxacin (8.5%) and gentamicin (9.8%), the antimicrobial efficacy of these agents was reduced against biofilm-associated cells. For enrofloxacin, the median log reduction was lower in isolates recovered from animals with chronic infection than in those from animals with acute infection (1.69 vs. 2.49).

A statistically significant negative correlation was identified between biofilm-forming capacity and the efficacy of enrofloxacin (r = -0.722), indicating that the bactericidal activity of the drug decreased as biofilm-forming capacity increased.

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> Stvaranje biofilma i antimikrobna tolerancija u kliničkim izolatima *Bordetella bronchiseptica*

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Bakterija *Bordetella bronchiseptica* je značajan patogen koji uzrokuje respiratorne infekcije u kućnih ljubimaca, pri čemu je tvorba biofilma ključni mehanizam koji uzrokuje perzistirajuće infekcije i smanjenu učinkovitost antimikrobnog liječenja. Cilj ovog rada bio je istražiti odnos između stvaranja biofilma, metaboličke i adhezivne aktivnosti te antimikrobne tolerancije u kliničkim izolatima *B. bronchiseptica*. Analizirana su ukupno 82 klinička izolata vrste *B. bronchiseptica*. Stvaranje biofilma, metabolička aktivnost i adhezivni kapacitet istraženi su postupkom u mikrotitarkoj pločici sa kristal violetom i testom sa resazurinom. Osjetljivost na antimikrobne lijekove određivana je disk difuzijskim postupkom. Minimalne inhibicijske koncentracije određene su mikrodilucijskim postupkom prema smjernicama Clinical and Laboratory Standards Institute (CLSI). Tolerancija biofilma procijenjena je testom ubijanja temeljenim na CFU-u u reprezentativnoj podskupini od 18 izolata korištenjem izolatno-specifičnih koncentracija antibiotika. Učinkovitost enrofloksacina, gentamicina, doksiciklina, amoksicilina s klavulanskom kiselinom i trimetoprima sa sulfametoksazolom procijenjena je logaritamskom redukcijom održivih stanica biofilma. Izolati iz životinja s kroničnom respiratornom infekcijom češće su formirali jake biofilmove (56,3%), dok su u akutnim slučajevima prevladavali (49,2%) izolati sa slabijom tvorbom biofilma. Povećano stvaranje biofilma bilo je povezano sa smanjenom metaboličkom aktivnošću. Nisu uočene značajne razlike u adhezivnoj aktivnosti među skupinama koje stvaraju biofilm. Među planktonskim stanicama, najniže stope rezistencije uočene su prema enrofloksacinu (8,5%) i gentamicinu (9,8%), dok je višestruka otpornost na antimikrobne lijekove otkrivena u 23,2% izolata. Unutar biofilmova, učinkovitost svih testiranih antibiotika smanjila se, a enrofloksacin je pokazao niže logaritamske vrijednosti smanjenja u kroničnim nego u akutnim izolatima (1,69 naspram 2,49). Intenzitet stvaranja biofilma negativno je korelirao s učinkovitošću enrofloksacina. Nalazi pokazuju da je opsežno stvaranje biofilma povezano s povećanom antimikrobnom otpornošću kod bakterije *B. bronchiseptica* i može doprinijeti perzistirajućoj infekciji, što naglašava važnost razmatranja fenotipa biofilma pri odabiru antimikrobnog liječenja.

Ključne riječi: *Bordetella bronchiseptica; biofilm; tolerancija; antimikrobna rezistencija; logaritamska redukcija; višestruka rezistencija*.