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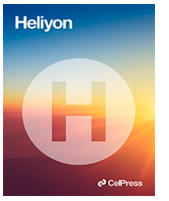

Research article

Biofilm formation *in vitro* by *Leptospira interrogans* strains isolated from naturally infected dogs and their role in antimicrobial resistance

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ABSTRACT

Leptospira interrogans is a biofilm-forming pathogen, however, there are few data involving Brazilian strains isolated from dogs and their antimicrobial sensitivity in planktonic and biofilm forms. The potential for biofilm formation and antimicrobial resistance in naturally infected dogs is a fundamental approach towards disease epidemiology and the establishment of consistent prophylaxis and control measures. The objective of this study was to evaluate *in vitro* biofilm formation of a reference strain (*L. interrogans*, sv. Copenhageni L1 130 - L20) and of *L. interrogans* isolated from dogs (C20, C29, C51, C82), with subsequent evaluation of antimicrobial susceptibility in planktonic and biofilm forms. The semi quantification of biofilm production revealed a dynamic process of development over time, with mature biofilm formation early on the seventh day of incubation. All strains were efficient for *in vitro* biofilm formation and, in this form, they were considerably more resistant compared to their planktonic form, with MIC_{90} of 1600 μg/mL for amoxicillin, 800 μg/mL for ampicillin, and >1600 μg/mL for doxycycline and ciprofloxacin. The strains studies were isolated on naturally infected dogs that might act as reservoirs and sentinels for human infections. The potential to antimicrobial resistance together with the close relation between dogs and humans indicates the need for greater actions on disease control and surveillance. Moreover, biofilm formation may contribute to the persistence of *Leptospira interrogans* in the host and these animals can act as chronic carriers, disseminating the agent in the environment.

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1. Introduction

Leptospirosis is a zoonotic infectious disease of worldwide occurrence, significant impact on public health, and higher prevalence associated with tropical low-income countries [1,2]. Caused by bacteria of the genus *Leptospira*, it affects several mammalian hosts including humans, domestic, synanthropic and wild animals, often implicated as potential sources of infection [3].

Disease severity is variable in dogs, according to the virulence and the concentration of the infecting strain, host immune response, and strain adaptability to the host [4]. Investigation of leptospiral strains and their interactions with the canine population exposes the risk to public health due to the close relationship between humans and dogs, and the possibility of long-term intermittent urinary shedding in chronic infections [5,6].

In the environment and inside the host, the bacterial activity occurs in individualized cells in their planktonic form but also in microorganisms grouped and organized in biofilms [7]. Biofilms consist of a group of bacterial cells organized in different degrees of complexity, adhered to biotic or abiotic surfaces, and surrounded by an organic polymeric matrix, the exopolysaccharide [8]. These structures confer relevant properties to bacteria, including increased protection and resistance to antimicrobials, ultraviolet radiation, dehydration, oxidation, environmental stresses, and host immune responses [7].

In vitro biofilm-formation has been documented in pathogenic and saprophytic leptospires [9]. Studies also reported bacterial aggregates within the lumen of proximal renal tubules in mammalian hosts [10,11]. More recently, Santos et al. [12] documented *Leptospira* biofilm formation in the kidneys of reservoir animals (mice). According to Thibeaux et al. [13], leptospire biofilms may play a key role not only in maintaining infection but also in antimicrobial resistance.

Bacterial resistance to antimicrobials is a relevant public health issue worldwide [14]. The potential for antimicrobial resistance in pathogenic leptospires still represents an underexplored area [15]. Therefore, the isolation of *Leptospira* spp. is a crucial approach to identify the most frequent serogroups and serovars in different regions [16]. The assessment of the potential for biofilm formation and antimicrobial resistance is also fundamental for understanding the epidemiology of the disease and, therefore, establish appropriate prophylaxis and control measures for the local scenario.

The objective of this study was to evaluate *in vitro* biofilm formation of a reference strain (*L. interrogans*, sv. Copenhageni L1 130 - L20) and of *L. interrogans* isolated from dogs (C20, C29, C51, C82), with subsequent evaluation of antimicrobial susceptibility in their planktonic and biofilm forms.

2. Material and methods

2.1. Study design

This experimental research was conducted at the teaching Veterinary Hospital Renato R. de Medeiros Neto - HOSPMEV (Federal University of Bahia – UFBA, Brazil) as part of a multidisciplinary approach on canine leptospirosis. All procedures were approved by the Ethics Committee in the Use of Animals of the Federal University of Bahia (CEUA - EMEVZ/UFBA, process: 06/2021).

Four Brazilian strains of *L. interrogans* (C20, C29, C51, C82) isolated from blood and urine samples of naturally infected dogs with acute leptospirosis were included in this study. *L. interrogans*, Sg. Icterohaemorrhagiae, sv. Copenhageni, (Fiocruz strain L1 130) (L20) was used as positive control. The strains were maintained in BOD incubation at 30 °C with up to 15 passages *in vitro*.

Characterization of the Brazilian strains were carried out through serological and mocelular analysis, as described by Paz et al. [17]. Briefly, BLAST analysis of the amplified 16S rRNA and *secY* sequences from strains C20, C29, C51 and C82 indicated 100% homology with the species *L. interrogans* (GenBank accession numbers KX058888 and MN394905; KX058890 and MN394907; MK330632 and MN394909; OP950583 and OP948067, respectively). Through presumptive serological characterization, the strains showed similarity with *L. interrogans* Sg. Icterohaemorrhagiae.

The studied strains belong to the Bacteriosiss Laboratory's (LABAC, UFBA) collection and were kept frozen in 2.5% dimethylsulfoxide (DMSO) at −196 °C [18], in T80/40LH medium [19]. Strains C20 (CLEP 00127), C29 (CLEP 00129) and C51 (CLEP 00219) were also deposited in the collection of the National Reference Laboratory for *Leptospira* of the Oswaldo Cruz Foundation. An aliquot of each bacterial culture was thawed at room temperature and the contents were transferred to a test tube containing 4.5 mL of T80/40LH medium. To ensure viability and quality of the experiment the cultures were evaluated for: i) growth of the bacterial population, reaching concentrations close to 10^8 leptospires/mL in approximately seven days; ii) active bacterial motion characterized by vigorous rotation; iii) lack of growth of contaminating microorganisms; and iv) evidence of self-agglutination. Growth curves of the strains in T80/40LH medium were enumerated by dark-field microscopy to ensure that mid-exponential-phase bacterial cultures were used for further experimentation [20,21].

2.2. Biofilm formation

Biofilm formation was assessed in 96-well polystyrene plates with low evaporation, specific for cell and tissue culture (Nest Biotechnology®, Jiangsu, China), as described by Thibeaux et al. [21] and Thibeaux et al. [13], with modifications. Briefly, leptospires were grown to mid-exponential phase (approximately 10^8 leptospires/mL), next the cultures used in the experiments were diluted to a bacterial suspension inoculum of 1×10^6 leptospires/mL [13,21]. Subsequently, 150 μL of these cultures were added into wells of polystyrene plates. To minimize evaporation, the peripheral wells were filled with sterile distilled water. For semi-quantitative assays, the plates were incubated at 30 °C under static conditions and evaluated at six different times (1, 3, 5, 7, 14 and 21 days). We evaluated eight replicates by strain. As negative controls, we filled the wells only with medium.

2.3. Semi-quantification of crystal violet-stained biofilm

Following the incubation period, planktonic cells were removed by gentle aspiration of the supernatant. Biofilm fixation was performed by incubation at room temperature with 150 μL of paraformaldehyde (PFA) diluted at 4% in PBS for 30 min. Next, the PFA was removed and the wells washed twice with 200 μL of PBS. To assess biofilm formation, 175 μL of 0.1% crystal violet were pipetted into wells and incubated for 10 min [22]. Excess unadhered dye was removed and the wells were washed twice with 200 μl of PBS. The plates were left to dry at room temperature for approximately 3 h. The remaining stain in the wells were solubilized with the addition of 200 μL of 30% acetic acid. Then, 100 μL of the solution was transferred to a new plate for analysis. Biofilm semi-quantitation reading was performed at 570 nm wavelength in microplate spectrophotometer (MultiskanTM FC Microplate Photometer, Thermo Fisher, USA) [13].

2.4. Scanning electron microscopy

The leptospires were cultured as described in the biofilm formation section, with some modifications [13]. Briefly, *L. interrogans* cultures were observed at four different times (1, 7, 14 and 21 days of incubation) to describe biofilm's formation and architecture over time. 1 mL exponential phase culture inoculum at a concentration of 1×10^6 leptospires/mL were added to a 24-well, flat-bottom, culture-specific plate (JETBIOFIL®, China), each well containing a 12 × 12mm sterile glass coverslip (Fisherbrand® -Fisher Scientific, Pittsburgh PA); wells containing culture medium only were used as controls. At each observation time the supernatant was removed, biofilm was rinsed once with PBS, and fixed in 2.5% glutaraldehyde/4% paraformaldehyde in 0.1 M sodium cacodylate buffer pH 7.4 for 1 h. Subsequently, the biofilm was washed three times with 0.1 M sodium cacodylate buffer pH 7.4, fixed in 1% osmium tetroxide for 30 min, then washed three more times with 0.1 M sodium cacodylate buffer pH 7.4. After fixation, the biofilms were dehydrated through a series of ethanol concentrations (30, 50, 70, 90 and 100%; 10 min each), dissected, the carbon evaporated in a CPD 030 equipment (Leica, Wetzlar, Germany), and subsequently metallized in gold (25 nm). The samples were examinated under a scanning electron microscope JSM-6390LV (Jeol, Tokyo, Japan), operating at 10Kv.

2.5. Antibiotic stock preparation

Four antimicrobial agents commonly used in veterinary medicine were selected [4,6] for leptospirosis treatment: amoxicillina, ampicillin, docyxycline and ciprofloxacin (Sigma-Aldrich™ St. Louis, Missouri, USA). Antibiotic stock solutions were prepared at a concentration of 3.2 mg/mL using solvents and diluents suggested in the Clinical and Laboratory Standards Institute document M100 [23] or according to the manufacturer's recommendations. Antimicrobial stock solutions were stored at – 20 °C until use.

2.6. Antimicrobial susceptibility assay of planktonic Leptospira

Broth microdilution testing was conducted in 96-well round-bottom plates. The assay was performed in triplicate, as described by Murray and Hospenthal [24], with modifications. For each plate we stablished a positive control (leptospires without antimicrobials) and a negative control (culture medium only). Firstly, 100 μl of T80/40LH medium was added to the wells, followed by serial dilutions of the antimicrobial agents in final concentrations raging from 0.02 to 1600 μg/mL. Following the addition of 100 μl of *Leptospira* inoculum containing 1×10^6 organisms/mL, the plates were incubated for 24 h at 30 °C. Each well contained a final volume of 200 μl. To assess viability of leptospires in the planktonic form, 20 μl of AB was added to the wells and incubated for 90 min at 30 °C. For absorbance reading, the contents of each well were transferred to new plates and read at both 570 nm and 600 nm wavelengths. The color change from blue to pink was indicative of cell growth, while unchanged blue wells corresponded to absence of cell metabolism. The absorbance of each well was applied to the calculation procedure indicated by the AB manufacturer (AlamarBlue™ Cell Viability Reagent, Invitrogen by Thermo Fisher). The MIC was defined as the lower concentration that resulted in a reduction in AB to levels equal to or lower than 50%.

2.7. Biofilm antimicrobial susceptibility test

For biofilm susceptibility testing we included the same positive and negative controls stablished in the planktonic form. The test was performed in triplicate, in accordance with Murray and Hospenthal [24] and Vinod Kumar et al. [25], with modifications. With complete biofilm formations, in the 21st day of incubation the supernatant was gently aspirated and discarded. Serial dilutions of antibiotics were prepared in T80/40LH medium, with final concentrations ranging from 0.02 to 1600 μg/mL; 150 μl of each antimicrobial concentration was added to the corresponding wells. The plates were then incubated for 24 h at 30 °C. Next, bacterial viability in the biofilm was assessed by adding 20 μl of AB to each well, with subsequent incubation for 6 h at 30 °C. The color change from blue to pink was indicative of cell growth, while unchanged blue wells corresponded to absence of cell metabolism. After exposure to AB, the contents were transferred to a new plate for spectrophotometer reading. The MICs were determined as described for the leptospires in the planktonic form.

2.8. Statistical analyses

The data obtained were analyzed using the Graph Pad Prism 5.0 software (Graph Pad Prism, USA) through descriptive analysis and

Table 1
Origin of *Leptospira interrogans* strains forming *in vitro* biofilm under static conditions.

N° of Strains	Species/Serogroup	Origin of isolation	Origin
L1 130	*L. interrogans*/Icterohaemorrhagiae	Blood	Human
C20	*L. interrogans*/Icterohaemorrhagiae	Blood	Dog
C29	*L. interrogans*/Icterohaemorrhagiae	Urine	Dog
C51	*L. interrogans*/Icterohaemorrhagiae	Blood	Dog
C82	*L. interrogans*/Icterohaemorrhagiae	Blood	Dog

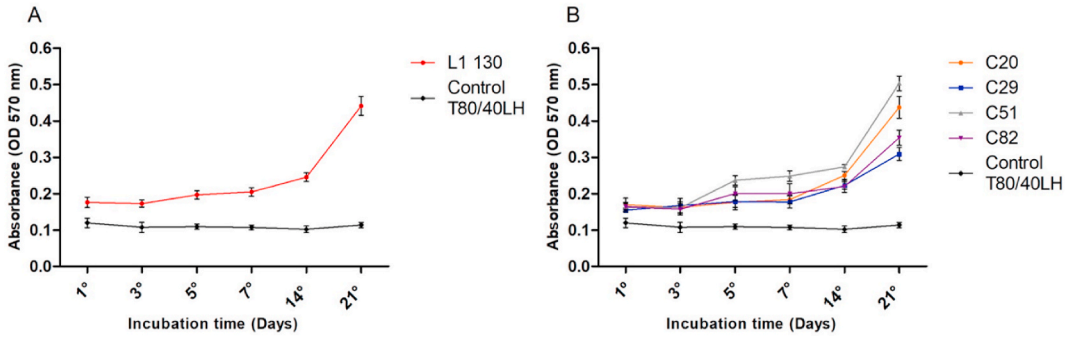

Fig. 1. Semiquantification curve of *Leptospira interrogans in vitro* biofilm evaluated by spectrophotometry. (A) Characterization of *in vitro* biofilm formation of *L. interrogans*, Sg. Icterohaemorrhagiae, sv. Copenhageni, (Fiocruz strain L1130). (B) Characterization of *in vitro* biofilm formation of *L. interrogans* strains isolated from dogs. Data are expressed as mean and error bars represent the standard error of the mean (SEM).

construction of growth curves. All results are represented as mean and standard error of the mean (mean ± SEM). Normality of biofilms ODs data were determined by Shapiro-wilk test. We used One way ANOVA and Tukey tests to assess differences in biofilm production for the studied strains. A value of $p < 0.05$ was considered significant.

3. Results

3.1. Biofilm formation

Timely observation revealed similar behaviours of biofilm formation for the reference strain and the four strains of *L. interrogans* isolated from dogs (Table 1). Interestingly, all of the studied samples were able to form adhesive biofilm *in vitro* up to the twenty-first day of incubation.

3.2. Biofilm semi-quantification

Until the third day of incubation, semi-quantitative evaluation revealed similar ODs (Fig. 1A and B), except for L1 130, which had a significant increase in biofilm production on the first day of incubation ($p = 0.0245$) compared with the C29 strain (Fig. 2 A). On the third day of incubation, there was no significant difference between the strains (Fig. 2B). On days 5 and 7, ODs had a similar behavior, with particular emphasis on the C51 strain, which showed a significant increase in biofilm production (***$p < 0.0001$) (Fig. 1A and B), Fig. 2C and D). On day 14 all strains showed a progressive increase in biofilm mass, with the L1 130 and C20 strains showing a higher production compared to C29 (**$p < 0.001$). On that same date, strains C51 had a higher OD compared with C29 and C82 (***$p < 0.0001$) (Fig. 2E). On day 21, all biofilms registered their maximal ODs [(Fig. 1A and B) and Fig. 2F]. Strains L1 130 and C20 had significantly higher biofilm production compared to C29 and C82 (***$p < 0.0001$). The same behavior was observed in C51 in comparison with all the other strains (***$p < 0.0001$) (Fig. 2F). The mean ODs and Standard Deviation of the eight replicates are summarised in Supplementary Table 1.

The analyses of the growth curve showed a progressive increase in biofilm formation, with higher production for strains L1 130, C20 and C51 (Fig. 2 E and F). Additionally, biofilm for the reference strain L1 130 was significant difference to at least one of the other 3 strains (C29, C51 and C82) on days 1, 5, 7 and 14 and 21 (Fig. 2A, C - F). Crystal violet staining of *Leptospira* aggregates evidenced a similar growth pattern from days 7–21 of incubation (Fig. 3 A - C).

3.3. Scanning electron microscopy

Scanning electron microscopy imaging of biofilm formation for all five strains are shown in Fig. 4 A-D and Supplementary Figs. 1 and 2. On the first day of incubation, extracellular matrix deposits were visible in all strains. Subsequently, on the seventh day, a mature biofilms were evidenced, characterized by an intrinsic and interconnected network of leptospires attached to the microscope

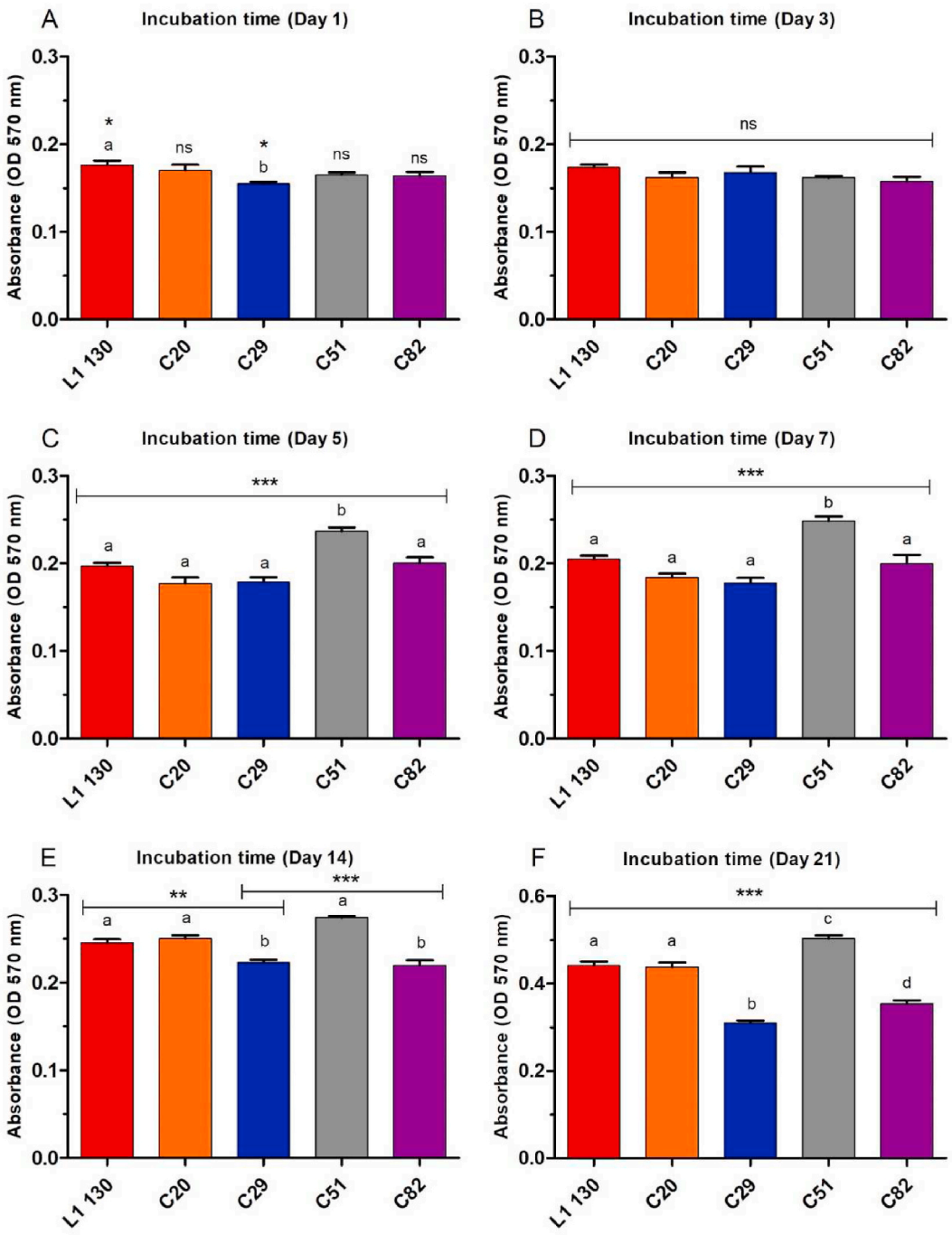

Fig. 2. Semiquantification of *in vitro* biofilm production by *Leptospira interrogans* in different incubation times (1, 3, 5, 7, 14 and 21 days). Fiocruz strain L1 130 is shown in red, C20 in orange, C29 in blue, C51 in grey, and C82 in violet. The letters a, b, c, and d represent statistically significant differences ($p < 0.05$) in the optical density (OD) between strains based on the one-way ANOVA and Tukey tests. *** indicates p value < 0.0001; ** indicates p value < 0.001; * indicates p value < 0.05; ns indicates a non-significant p value between strains (>0.05). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

slide and surrounded by spots of amorphous exopolysaccharide matrix (Fig. 4 A and B and Supplementary Figs. 1 and 2).

From the second week of incubation, the biofilms presented more developed and cohesive morphological structures, characteristics of a mature biofilm; however, we observed greater deposition of extracellular matrix (Fig. 4C and D and Supplementary Figs. 1 and 2). Notably, on day 21, SEM analysis on a higher magnification revealed extracellular matrix deposition together with interconnecting channel-like structures (Fig. 5 A-D and Fig. 6A–D). The biofilm also contained several branched filaments of matrix surrounding these

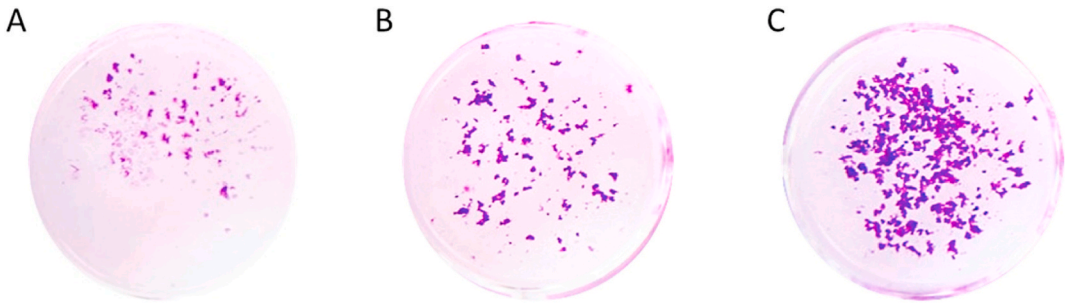

Fig. 3. Crystal violet biofilm staining of *Leptospira interrogans* adhered to the bottom of wells in microtiter plates. Cultures on the (A) 7th day, (B) 14th day, and (C) 21st day of incubation. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

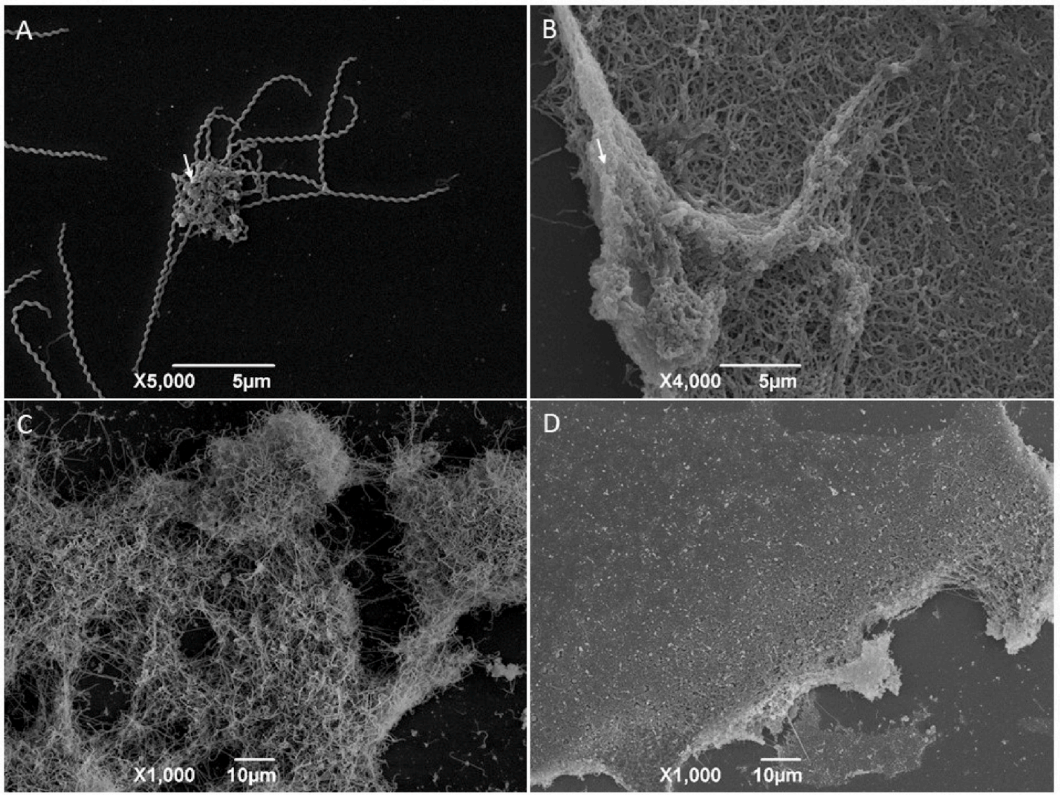

Fig. 4. SEM imaging of C20 strain biofilm formation at different incubation periods. (A) First day, (B) Seventh day, (C) Fourteenth day and (D) Twenty-first day of incubation. White arrows indicate extracellular matrix deposition.

channels (Fig. 5 B-D and Fig. 6B–D).

3.4. Antimicrobial susceptibility in planktonic and biofilm forms

The MIC values for the planktonic and biofilm forms are reported in Table 2. Leptospires in the planktonic form were phenotypically susceptible to all of the antimicrobial agents tested, with MICs equal to or below the minimum limit of detection (MIC_{90}: ≤0.02 μg/mL). Slightly different values were reported for doxycycline and ciprofloxaxin, with higher MIC_{90}: 0.2 μg/mL and of 0.1 μg/mL, respectively.

Organisms in the biofilm form were accompanied by a different susceptibility pattern (Table 2). All strains were resistant to amoxicillin and ampicillin, with MIC_{90} of 1600 μg/mL and 800 μg/mL, respectively. Both doxycycline and ciprofloxaxin had MIC_{90} >1600 μg/mL. Overall, based on the ranges of dilutions and antimicrobial concentrations herein applied, leptospires in the *in vitro* biofilm form were 13–16 times more resistant than their planktonic counterparts. Reference strain L1 130 and strains C20, C51 and

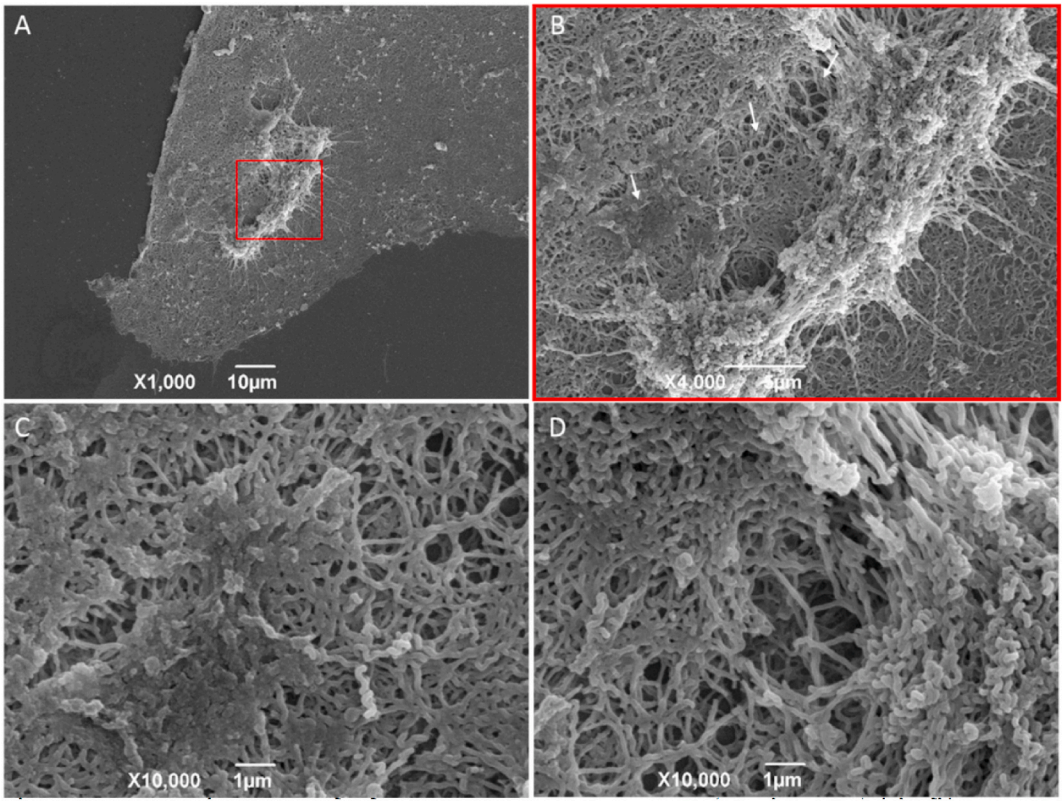

Fig. 5. SEM images of *Leptospira interrogans* (strain C51) biofilm organization and architecture with 21 days of incubation. (A) Strain C51 biofilm. (B) White arrows indicate areas of amorphous exopolysaccharide matrix deposition and interconnected channels. (C) and (D) Higher magnification images of the aforementioned regions and structures.

C82 presented MICs higher than the maximum dilution used in this study (1,600ug/ml) (Table 2).

4. Discussion

Together with its ability to penetrate host tissue, leptospires adapt to several environmental matrices such as water collections, rivers, soil, and abiotic surfaces [26,27]; the skill to form biofilms is regarded as one of the main mechanisms facilitating such adaptations [28]. In the biofilm, cells attach to a surface and form communities within which they multiply through a complex network of intercellular communication, allowing for rapid adjustments in response to changes in the host organism and in the environment [9, 29].

Our investigations showed that all strains formed biofilms up to the 21st day of incubation. Mature biofilms were visible already on the seventh day of incubation, followed by a progressive growth to biomasses with better stablished morphology, consistent with the incubation periods. Bacterial growth and consequent biofilm formation are responsible for the increase in the intensity of crystal violet staining and the optical density of the strains [21]. The greater biofilm production and growth rates observed for the L1 130, C20 and C51 strains could be the consequence of their individual characteristics and/or a possible adaptability to their *in vitro* maintenance [30].

In our study, scanning electron microscopy images highlighted significant structural features in biofilms. Areas where leptospires are strongly interconnected and embedded in amorphous material are structurally compatible with deposition of matrix components, the exopolysaccharide [7]. These components provide mechanical stability for biofilms, mediate their adhesion to surfaces and form a cohesive, three-dimensional polymeric network that interconnects and transiently immobilizes biofilm cells [8].

After three weeks of incubation, SEM images demonstrated structures that resemble interconnected channels and branched filaments of extracellular matrix. These structures provide the morphological characteristics of the biofilm, transport of nutrients, gases and other metabolites required for bacterial survival [31]. Thus, based on our images and as reported by Ref. [13], the architectural aspect of the interconnected channels and filaments resembles a circulatory system that favors the growth of the bacterial community in biofilms, ensuring the inflow of nutrients and oxygen and the exit of toxic and waste products [31].

Biofilms are described as colonization and virulence factors who partake in the pathogenesis of several infectious diseases [32], including leptospirosis [12]. Some factors such as the genotypic and phenotypic expression inherent to each strain are determinant for the production of bacterial biofilms [33]. Our results are in agreement with Thibeaux et al. [13], who observed progressive growth of

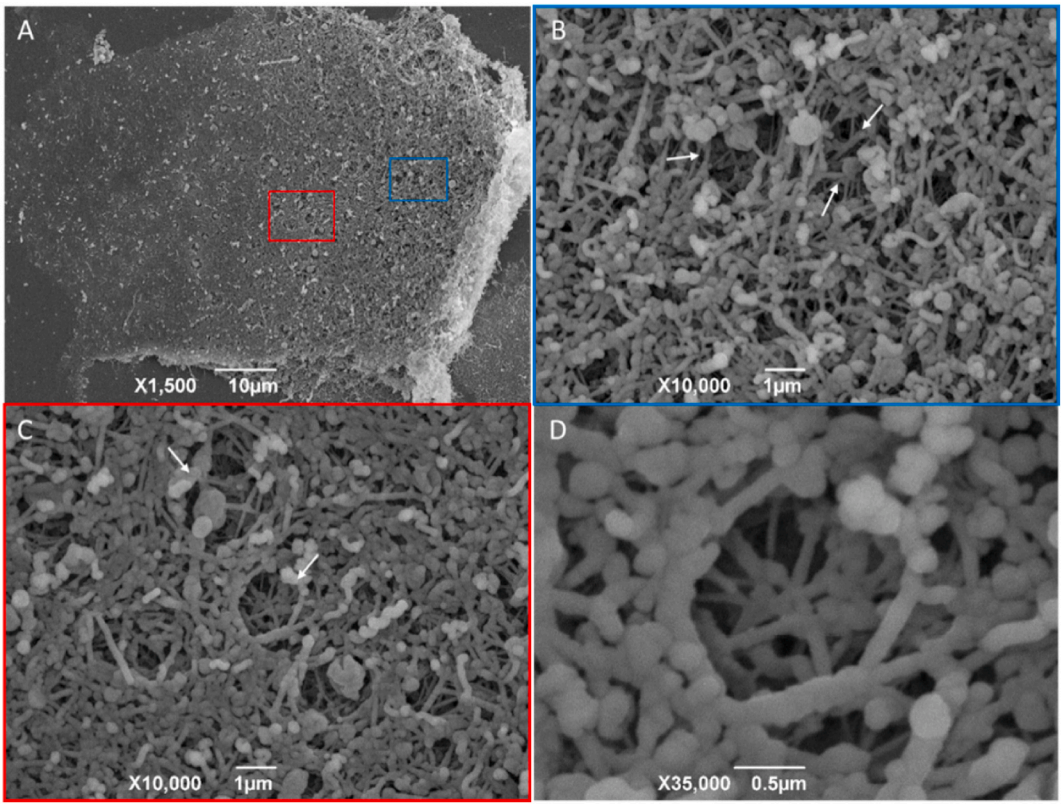

Fig. 6. SEM images of *Leptospira interrogans* (strain C82) biofilm organization and architecture at 21 days of incubation. (A) Strain C82 biofilm. (B) White arrows indicate branched extracellular matrix structures. (C) and (D) Higher magnification images of the interconnected channels and branched filaments surrounding such structures.

biofilms *in vitro* of a wild strain of *Leptospira interrogans*, sv. Manilae. However, our study is the first to document biofilm formation and development for *Leptospira interrogans* strains isolated from naturally infected dogs.

Considering the local scenario from which the studied leptospires were isolated – an endemic area with intense circulation of *Leptospira* in the canine population [17] -, the pathogen and its interactions with the host, and the capacity of *in vitro* biofilm formation, we suggest that these same strains might be capable of *in vivo* tissue biofilm formation in dogs. Eventhough *in vitro* and *in vivo* conditions may influence genotypic and phenotypic expression patterns, biofilm formation was documented for all of the studied strains, making this hypothesis considerable.

Strains in the planktonic form were more sensitive to amoxicillin and ampicillin. In accordance with our findings, *L. interrogans*, sg. Icterohaemorrhagiae, sv. Copenhageni from humans, rats, dogs, and cattle displayed similar results, with low MICs for the same antimicrobial agents [34]. Susceptibility for penicillin and its derivates (amoxicillin and ampicillin) were also previously reported in *Leptospira* spp [35,36].

A contrasting susceptibility profile was reported by Suepaul et al. [37] in Trindad, with resistance patterns to amoxicillin in isolates from dogs and rats. This finding linked to the broad use of amoxicillin in the country, often unaccompanied by medical prescription, with the emergence of resistant isolates in the region. Therefore, we suggest that our phenotypical susceptibility profile could be attributed to the fact that amoxicillin is not commonly used in the treatment of leptospirosis in our region.

The Brazilian strains of *L. interrogans* isolated from dogs in this study were also sensitive to ampicillin, which is one of the recommended options for leptospirosis treatment [4]. This is consistent with other reports that rendered this drug as a suitable treatment option [35,38]. Chakraborty et al. [39], studying the effects of antimicrobial agents on leptospiral cell architecture, documented loss of integrity of the inner and outer cellular membranes with complete lysis of cells after 24 h of ampicillin exposure.

Clinical trials and *in vitro* investigation have shown doxycycline to be an effective drug for leptospirosis treatment [37,38]. This tetracycline, together with the penicillin derivates, are referred as recommended therapeutic choices [4]. In our study and in previous reports [24,35,40], leptospires in the planktonic form displayed higher MICs for doxycycline, still the MICs are considered low and suggest this tetracycline is still an effective inhibitory or bactericidal agent against *Leptospira* [35].

Regarding ciprofloxacin, our results are similar to other *in vitro* findings, which documented susceptibility in rats and humans' isolates, indicating this fluoroquinolone as a viable treatment option [38,41]. In contrast, other studies carried out in Brazil [34,42] displayed resistance profiles to ciprofloxacin, with MICs higher than what is expected for this antimicrobial class.

Biofilm production influenced the susceptibility of the studied strains; biofilm-producting strains were considerably more resistant

Table 2
Distribution of MICs for amoxicillin, ampicillin, doxycycline, and ciprofloxacin in samples from the reference strain L1 130 and strains of *L. interrogans* isolated from dogs, in the planktonic and biofilm forms.

N° of Strains	Species/Serogroup	Amoxicillin			Ampicillin			Doxycycline			Ciprofloxacin		
		MIC (μg/mL)											
		Plankt	Biofilm	Resist.	Plankt	Biofilm	Resist.	Plankt	Biofilm	Resist.	Plankt	Biofilm	Resist.
L1 130	*L. interrogans*/Icterohaemorrhagiae	≤0.02	1.600	16	≤0.02	800	15	0.2	>1.600	>13	0.1	>1.600	>14
C20	*L. interrogans*/Icterohaemorrhagiae	≤0.02	1.600	16	≤0.02	800	15	0.2	1.600	13	0.39	>1.600	>12
C29	*L. interrogans*/Icterohaemorrhagiae	≤0.02	1.600	16	≤0.02	800	15	≤0.02	1.600	16	0.1	1.600	14
C51	*L. interrogans*/Icterohaemorrhagiae	≤0.02	1.600	16	≤0.02	800	15	0.39	>1.600	>12	0.2	>1.600	>13
C82	*L. interrogans*/Icterohaemorrhagiae	≤0.02	1.600	16	≤0.02	800	15	0.39	>1.600	>12	0.1	1.600	14
MIC_{90}		≤0.02	1.600	16	≤0.02	800	15	0.2	>1.600	>13	0.1	>1.600	>14

MIC - Minimum inhibitory concentration.
Plankt - Planktonic form.
Resist – Biofilm resistance compared to planktonic form.
In cases where the MIC was greater than 1,600, the resistance count was made up to the respective value, added to the symbol of ">".
Cumulative susceptibility results for all strains are expressed as MIC_{90}

when compared to their planktonic form, requiring antimicrobial concentrations 13 to 16 times higher for growth inhibition. This finding is in agreement with Vinod Kumar et al. [25], who also identified higher antimicrobial concentrations for leptospires in biofilm, ranging from 800 to 1; 600 μg/mL, for ampicillin, tetracycline, doxycycline, and penicillin G. Additionally, in *Leptospira interrogans* and *Azospirillum brasilense* associated biofilms, the inhibitory concentrations for penicillin G, ampicillin and tetracycline were higher than those for leptospires in the planktonic form [43]. To date, there is no consensus on the issue of the interpretation of the microdilution method to categorize susceptibility profiles. This may cause variations in data interpretation; therefore, additional studies need to be carried out in an effort to standardize susceptibility results. In this study, the biofilm forms of the strains L1 130, C20, C51 and C82 presented higher MICs than the maximum dilution used (1,600ug/ml). We suggest expanding antimicrobial concentrations in further investigations in order to define the MICs of the evaluated drugs more accurately.

Some characteristics of bacteria in biofilms make them difficult to eradicate [7]. They are phenotypically distinct from their planktonic forms, particularly with respect to growth rates and gene expression [44]. Cells that detach from biofilms are associated with cytotoxicity and mortality [45]; moreover, under these circumstances, the mutation frequency in bacteria is significantly higher when compared to their planktonic growth [46]. Biofilm formation provides an ideal environment for horizontal resistance gene transfer and for cellular chemical communication, allowing for the coordination of their growth activity and to cluster in communities [7]. These physiological conditions explain why bacteria in biofilms easily become multidrug resistant to beta-lactam antibiotics, aminoglycosides, and fluoroquinolones [45].

Bacterial cells in biofilms can simultaneously produce enzymes that degrade antibiotics and alter their target molecules, decreasing their affinity for antibiotics. Additionally, it has been documented that bacterial strains in biofilm tend to increase the expression of efflux systems [45]. Other factors, such as the architecture, organization, and production of extracellular polymeric substance also play key roles in increasing resistance against antimicrobial agents [8]. All these factors could explain our findings, emphasizing the importance of leptospiral biofilm formation in antimicrobial resistance.

Documentation of biofilm formation in strains obtained from naturally infected dogs from an endemic region has substantial relevance within the scope of public health given the close relationship between dogs and humans. Biofilms have been show to influence *L. interrogans* environmental survival [47,48], infection pathogenesis and maintenance in heservoir hosts [12]. Therefore, leptospirosis management need to be accompanied by greater efforts in prevention, diagnosis, assertive treatment protocols, and transition control in order to decrease the infection incidence in endemic areas [49,50]. We also emphasize the need for studies implementing new strategies, which include the identification and evaluation of therapeutic agents with antibacterial and antibiofilm effects (neutralization and disruption of biomass) in animals infected by *Leptospira* spp., specially their effects on chronic carriers.

5. Conclusions

The biofilm-forming leptospires were characterized by increased antimicrobial resistance profiles when compared to their planktonic form. The studied strains were obtained from naturally infected dogs that may act as reservoirs and sentinels for human infections. The potential of antimicrobial resistance in isolates originated from a species in close relation to humans indicates urgency in the control of the disease in these hosts.

Author contribution statement

Melissa Hanzen Pinna: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

Rodrigo Rezende Mires de Carvalho; Carla Silva Dias: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Lucas Nogueira Paz; Thainá Melo de Lima Fires: Performed the experiments.

Cláudio Pereira Figueira; Karine Araújo Damasceno: Performed the experiments; Contributed reagents, materials, analysis tools or data.

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Data availability statement

Data included in article/supplementary material/referenced in article.

Declaration of interest's statement

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e13802.

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