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## RESEARCH ARTICLE



# Biofilm formation and its impact on environmental survival and antibiotic resistance of *Mycoplasma anserisalpingitidis* strains

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## ABSTRACT

Several *Mycoplasma* species can form biofilm, facilitating their survival in the environment, and shielding them from therapeutic agents. The aim of this study was to examine the biofilm-forming ability and its potential effects on environmental survival and antibiotic resistance in *Mycoplasma anserisalpingitidis*, the clinically and economically most important waterfowl *Mycoplasma* species. The biofilm-forming ability of 32 *M. anserisalpingitidis* strains was examined by crystal violet assay. Biofilms and planktonic cultures of the selected strains were exposed to a temperature of 50 °C (20 and 30 min), to desiccation at room temperature (16 and 24 h), or to various concentrations of eight different antibiotics. Crystal violet staining revealed great diversity in the biofilm-forming ability of the 32 tested *M. anserisalpingitidis* strains, with positive staining in more than half of them. Biofilms were found to be more resistant to heat and desiccation than planktonic cultures, while no correlation was shown between biofilm formation and antibiotic susceptibility. Our results indicate that *M. anserisalpingitidis* biofilms may contribute to the persistence of the organisms in the environment, which should be taken into account for proper management. Antibiotic susceptibility was not affected by biofilm formation; however, it is important to note that correlations were examined only *in vitro*.

## KEYWORDS

antibiotic resistance, biofilm formation, environmental survival, *Mycoplasma anserisalpingitidis*, waterfowl mycoplasmosis

## INTRODUCTION

*Mycoplasma anserisalpingitidis* is the most important waterfowl-pathogenic *Mycoplasma* species, both clinically and economically (Volkhov et al., 2020). It was first isolated in 1983 from a gander with phallus inflammation in Hungary (Stipkovits et al., 1984; Varga et al., 1986), and since then, it has also been confirmed to be present in other European and Asian countries as well (Stipkovits et al., 1986; Sprygin et al., 2012; Gyuranecz et al., 2020; Gróznér et al., 2021). *Mycoplasma anserisalpingitidis* can cause chronic infections in geese and ducks (Razin and Jacobs, 1992; Gróznér et al., 2019). Clinically manifested mycoplasmosis occurs during excessive stress with cloaca and phallus inflammation, salpingitis and testicular atrophy being the most frequent signs (Stipkovits et al., 1986; Hinz et al., 1994; Stipkovits and Kempf, 1996; Dobos-Kovács et al., 2009).

At present, control of the disease comprises the improvement of housing conditions and antibiotic therapy. Unfortunately, rapid development of multi-drug resistance in *M. anserisalpingitidis* has been described (Gróznér et al., 2016; Gyuranecz et al., 2020). Bacterial biofilm formation is likely to contribute to an increase in the incidence of clinically untreatable infections (Reid, 1999; Daubenspeck et al., 2020).

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Biofilm formation occurs when bacteria switch from a planktonic state to a surface-attached state (Coffey and Anderson, 2014). Bacterial biofilms consist of cells attached to a substratum or to each other, encased within an extracellular matrix composed of an aggregation of polysaccharides, polypeptides, nucleic acids and lipids (McAuliffe et al., 2006; Colvin et al., 2011; Daubenspeck et al., 2020). The physical and chemical properties of the extracellular matrix constituents coupled with their particular interactions allow the matrix to shield the biofilm cells from adverse environmental conditions (e.g. heat, desiccation, chemicals, invasion by other bacteria). The matrix also provides the mechanical properties to protect the cells from external forces and to ensure that the biofilm community remains attached to a surface (Yan and Bassler, 2019).

Compared with planktonic cells, biofilms are commonly found to be 10 to 1,000 times more resistant to antibiotics (Costerton et al., 1999; Mah and O'Toole, 2001). Besides the impeded antibiotic penetration into biofilms, increased antibiotic tolerance likely arises from the altered physiology of the biofilm cells. Cells dwelling inside thick biofilms could be in stationary phase, as penetration of nutrients and oxygen are known to be limited due to consumption by peripherally-located cells. Time-dependent antibiotic killing of a bacterial population shows that actively growing cells are killed first whereas cells in stationary phase are killed at a much lower rate (Yan and Bassler, 2019).

Several *Mycoplasma* species are able to form biofilm, including *Mycoplasma agalactiae*, *Mycoplasma bovis* (McAuliffe et al., 2006), *Mycoplasma gallisepticum* (Chen et al., 2012) and *Mycoplasma genitalium* (Daubenspeck et al., 2020). An explanation for the chronicity associated with mycoplasmal diseases is that the bacteria are thought to persist in the host by the formation of an adherent biofilm, shielding the organism from host immune components such as antibodies or phagocytes, and therapeutic agents as well (Hall-Stoodley et al., 2004; McAuliffe et al., 2006; Feng et al., 2020). Furthermore, McAuliffe et al. (2006) provided evidence that biofilm formation facilitates the survival of *Mycoplasma* species in the environment as well. In this study, the biofilm-forming ability of *M. anserisalpinitidis* was examined by the crystal violet assay (McAuliffe et al., 2006). Additionally, the effect of biofilm formation on the resistance of *M. anserisalpinitidis* strains against heat, desiccation and eight different antibiotic agents was also investigated.

## MATERIALS AND METHODS

### Growth conditions and analysis of biofilm-forming ability of *M. anserisalpinitidis*

In total, 32 *M. anserisalpinitidis* strains were used in this study, including the ATCC BAA-2147 type strain, two Polish and 29 Hungarian field isolates (Table 1). All strains were stored at  $-70^{\circ}\text{C}$  until use. The medium used for the propagation of the organism consisted of Mycoplasma broth

medium (pH 7.8) (Thermo Fisher Scientific Inc./Oxoid Inc., Waltham, MA, USA) supplemented with 0.5% (w/v) sodium pyruvate, 0.5% (w/v) glucose, 0.005% (w/v) phenol red and 0.15% (w/v) L-arginine hydrochloride. The number of microorganisms in the cultures used for the tests was determined by broth microdilution method (Hannan, 2000) and standardised ( $10^6$  colour changing units, CCU  $\text{mL}^{-1}$ ) in order to obtain comparable results. The bacterial cultures were inoculated at 1:10 ratio in the broth medium in duplicates (cultures B and P), and were incubated at  $37^{\circ}\text{C}$  for 48 h. Cultures B were left intact to grow biofilm, while cultures P were disrupted by vortexing, suspension and scratching the wall and bottom of the tubes three times daily, in order to prevent biofilm growth and remain in the form of planktonic cells in the broth. Analysis of biofilm growth was performed by crystal violet staining as described previously with minor modifications (McAuliffe et al., 2006). Tubes were emptied by discarding the broth cultures carefully, washed twice in tap water to remove non-adherent cells and stained with 0.5% crystal violet solution for 30 min. Washing steps were repeated before being left to dry at room temperature.

### Investigation of the effect of biofilm formation on heat resistance

To investigate the effect of biofilm formation on environmental survival, three Hungarian *M. anserisalpinitidis* strains with pronounced biofilm-forming ability (high-level staining in the crystal violet assay) were selected randomly (MYCAV70, MYCAV270, MYCAV415). As control, a clinical isolate exhibiting no biofilm formation was used (MYCAV55). After 48-h incubation at  $37^{\circ}\text{C}$ , *M. anserisalpinitidis* biofilms and planktonic cell cultures were exposed to a temperature of  $50^{\circ}\text{C}$  for 20 min or 30 min, then incubated at  $37^{\circ}\text{C}$  for 1 h. After destroying the structure of biofilm by vortexing, suspension and scratching the wall and bottom of the tubes, the broth cultures were mixed well, divided into 500- $\mu\text{L}$  aliquots, and stored at  $-70^{\circ}\text{C}$  until the counting of viable bacteria was performed. The number of colour changing units of the cultures was determined at  $37^{\circ}\text{C}$  on 96-well microtitre plates with broth microdilution method by titrating a 10-fold dilution series of the *Mycoplasma* suspension. The plates were checked daily and the final results were read after 14 days. The highest dilution that showed a colour change was considered to contain  $10^0$  CCU  $\text{mL}^{-1}$  (Hannan, 2000).

### Investigation of the effect of biofilm formation on resistance to desiccation

After 48-h incubation at  $37^{\circ}\text{C}$ , biofilms and planktonic cell cultures of the selected four *M. anserisalpinitidis* strains (MYCAV70, MYCAV270, MYCAV415, and MYCAV55 as control) were harvested by centrifugation at  $9,000\times g$  for 9 min and the supernatant was removed. The pellet was exposed to desiccation at room temperature for 16 h or 24 h, then suspended in fresh broth and incubated at  $37^{\circ}\text{C}$  for 1 h.



Table 1. Background information and biofilm-forming ability of the 32 tested *Mycoplasma anserisalpinitidis* strains

ID	Host	Sample	Origin	Year of isolation	Biofilm formation <sup>a</sup>
ATCC BAA-2147	goose	phallus lymph	Hungary	1983	–
MYCAV47	duck	lung and air sacs	Tátlár, Hungary	2012	++
MYCAV50	goose	phallus	Cered, Hungary	2013	++
MYCAV55	goose	ovarian follicle	Kiskunmajsa, Hungary	2013	–
MYCAV61	goose	phallus lymph	Tatárszentgyörgy, Hungary	2013	–
MYCAV63	goose	trachea	Sükösd, Hungary	2013	–
MYCAV66	goose	phallus lymph	Tiszaöldvár, Hungary	2014	–
MYCAV67	goose	phallus lymph	Szentes, Hungary	2014	++
MYCAV68	goose	phallus lymph	Érpatak, Hungary	2014	–
MYCAV70	goose	phallus lymph	Cered, Hungary	2014	++
MYCAV75	goose	phallus lymph	Dömsöd, Hungary	2014	–
MYCAV76	goose	phallus lymph	Tiszabábolna, Hungary	2014	++
MYCAV91	goose	phallus	Hajdúsámson, Hungary	2011	++
MYCAV94	goose	cloaca	Tiszabábolna, Hungary	2012	–
MYCAV160	goose	phallus lymph	Érpatak, Hungary	2015	++
MYCAV161	goose	phallus lymph	Szilaspogony, Hungary	2015	++
MYCAV162	goose	phallus lymph	Encsencs, Hungary	2015	++
MYCAV176	goose	phallus	Cered, Hungary	2015	–
MYCAV177	goose	phallus	Cered, Hungary	2015	+
MYCAV178	goose	ovarian follicle	Cered, Hungary	2015	+
MYCAV179	goose	trachea	Apátfalva, Hungary	2015	–
MYCAV180	goose	phallus	Kisbér, Hungary	2015	–
MYCAV270	goose	cloaca	Szentes, Hungary	2016	++
MYCAV271	goose	phallus lymph	Szentes, Hungary	2016	+
MYCAV415	goose	phallus lymph	Hungary	2017	++
MYCAV494	goose	phallus lymph	Hajdúsámson, Hungary	2018	+
MYCAV668	goose	cloaca	Starogard Gdański, Poland	1985	++
MYCAV671	goose	semen	Gödöllő, Hungary	2019	+
MYCAV675	goose	cloaca	Zielona Góra, Poland	1986	+
MYCAV688	goose	cloaca	Rém, Hungary	2019	–
MYCAV929	goose	cloaca	Cered, Hungary	2020	+
MYCAV967	goose	cloaca	Érpatak, Hungary	2021	–

<sup>a</sup> no staining (–), slight staining (+), or strong staining (++) in the crystal violet assay.

After destroying the structure of biofilm by vortexing, suspension and scratching the wall and bottom of the tubes, the broth cultures were mixed well, divided into 500- $\mu$ l aliquots, and stored at  $-70^{\circ}\text{C}$  until the counting of viable bacteria was performed as described above.

### Investigation of the effect of biofilm formation on antibiotic susceptibility

At first, possible correlations between biofilm-forming ability and the initial or final minimal inhibitory concentration (MIC<sub>i</sub> or MIC<sub>f</sub>) values were analysed. MIC values against the 32 examined *M. anserisalpinitidis* strains were previously determined (Gróznér et al., 2016, 2022). The MIC<sub>i</sub> of each strain was defined as the lowest concentration of the antibiotic that completely inhibited the growth at the time of colour change in the growth control (broth culture without antibiotics), while MIC<sub>f</sub> was evaluated at the end of the incubation period (14 days). Additionally, the effect of preventing biofilm formation on antibiotic susceptibility in five selected *M. anserisalpinitidis* strains with biofilm-forming ability and high MIC values of certain antibiotics

was investigated (MYCAV47, MYCAV67, MYCAV70, MYCAV178, MYCAV271). After 48-h incubation at  $37^{\circ}\text{C}$ , *M. anserisalpinitidis* biofilms and planktonic cell cultures were supplemented with fresh broth in order to normalise pH and nutrient content. The cultures were then exposed to various concentrations of the following antibiotics: enrofloxacin (Batch No.: BCBZ6597), oxytetracycline (Batch No.: BCBR8034V), doxycycline (Batch No.: BCBS3626V), tiamulin (Batch No.: BCBW6530), lincomycin (Batch No.: BCBW4661), tylosin (Batch No.: BCBX0715), tilmicosin (Batch No.: BCBT8086), and tylvalosin (Batch No.: TVN1906054). All products originated from VETANAL (Sigma-Aldrich Inc., St. Louis, USA) except for tylvalosin (Aivlosin), which was purchased from ECO Animal Health Ltd. (London, UK). The tested concentrations were selected based on the results of our previous studies:  $0.078\text{--}10\text{ }\mu\text{g mL}^{-1}$  for enrofloxacin, doxycycline and tiamulin, and  $0.125\text{--}64\text{ }\mu\text{g mL}^{-1}$  for the other antibiotics (Gróznér et al., 2016, 2022). Initial MIC values of each strain were determined by broth microdilution method (Hannan, 2000) with a small modification (using Eppendorf tubes instead of microtitre plates). Cultures were incubated



at 37 °C and checked three times daily until acidic colour change or 14 days.

RESULTS

Biofilm-forming ability of *M. anserisalpinitidis*

Crystal violet staining revealed great diversity in the ability of the 32 tested *M. anserisalpinitidis* strains to form biofilm (Table 1). Three categories were distinguished based on the observed strength of staining. Some strains (*n* = 12) showed strong staining below the air/liquid interface. Other strains (*n* = 7) showed only slight staining regarded as weaker biofilm formation, while numerous strains (*n* = 13) including the ATCC BAA-2147 type strain exhibited no staining (i.e. no biofilm formation) at all (Fig. 1).

Effect of biofilm formation on heat resistance

Biofilm-forming *M. anserisalpinitidis* cultures (cultures B) of the strains MYCAV70, MYCAV270 and MYCAV415 were found to be more resistant to heat (50 °C) than their cultures consisting of planktonic cells only (cultures P) (Table 2). At least ten times more viable cells of each strain were detected in cultures B compared to cultures P, except for MYCAV270 in which no difference was observed between cultures B and P after 20 min of heat exposure. In case of the strain MYCAV55, which was found to be unable to form biofilm, no difference could be observed between cultures B and P in the 20-min heat assay, while culture P contained more viable cells in the 30th minute of heating.

Effect of biofilm formation on resistance to desiccation

In agreement with the results of the heat assay, cultures B of the biofilm-forming MYCAV70, MYCAV270 and MYCAV415 strains were more resistant to drying than their

Table 2. Viable count (CCU mL<sup>-1</sup>) of four tested *Mycoplasma anserisalpinitidis* strains after exposure to a temperature of 50 °C for 20 or 30 min, or to desiccation for 16 or 24 h

Strain ID	Culture type	Heat 20 min	Heat 30 min	Dryness 16 h	Dryness 24 h
MYCAV70	culture B	10 <sup>3</sup>	10 <sup>2</sup>	10 <sup>3</sup>	10 <sup>2</sup>
	culture P	10 <sup>2</sup>	10 <sup>1</sup>	10 <sup>1</sup>	10 <sup>1</sup>
MYCAV270	culture B	10 <sup>2</sup>	10 <sup>2</sup>	10 <sup>2</sup>	10 <sup>2</sup>
	culture P	10 <sup>2</sup>	10 <sup>0</sup>	10 <sup>1</sup>	10 <sup>0</sup>
MYCAV415	culture B	10 <sup>3</sup>	10 <sup>2</sup>	10 <sup>2</sup>	10 <sup>1</sup>
	culture P	10 <sup>1</sup>	10 <sup>0</sup>	0	0
MYCAV55	culture B	10 <sup>2</sup>	10 <sup>0</sup>	10 <sup>1</sup>	10 <sup>0</sup>
	culture P	10 <sup>2</sup>	10 <sup>1</sup>	10 <sup>1</sup>	10 <sup>0</sup>

Culture B: biofilm-forming culture; culture P: planktonic cell form culture.

cultures P (Table 2). At least a tenfold difference was detected in the number of viable cells between cultures B and P of each strain. In case of MYCAV415, no surviving planktonic cells could be detected after 16-h drying, whereas biofilm cells were still viable even after 24 h. No difference could be observed between the survival rates of cultures B and P of the MYCAV55 strain that was unable to form biofilm.

Effect of biofilm formation on antibiotic susceptibility

No correlation was observed between biofilm-forming ability and the MIC values determined previously (Gróznér et al., 2016, 2022) in the 32 examined *M. anserisalpinitidis* strains, as high MIC values of each antibiotic were determined against strains lacking this ability as well (Table 3). Accordingly, the antibiotic susceptibility profiles of B and P cultures of the five tested *M. anserisalpinitidis* strains were highly similar (Table 4), and these strains were mostly inhibited by a concentration close to the previously determined MIC value (no more than one dilution step difference). A fourfold initial MIC difference was observed in

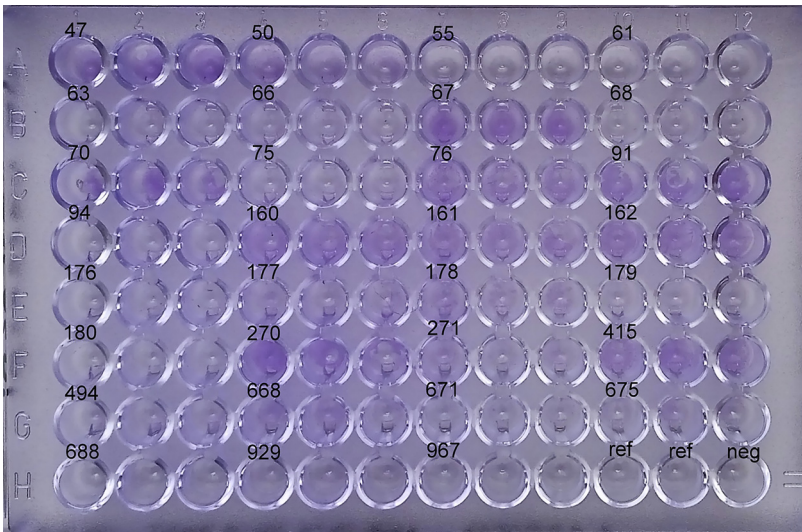


Fig. 1. Biofilm-forming ability of the 32 tested *M. anserisalpinitidis* strains (in triplicate) by crystal violet staining. Abbreviations: numbers: ‘MYCAV’ strain ID numbers; ref: type strain BAA-2147; neg: negative control (no bacteria)





Table 3. Previously determined (Gróznér et al., 2016, 2022) initial (MICi) and final (MICf) minimal inhibitory concentration values ( $\mu\text{g mL}^{-1}$ ) of the 32 tested *Mycoplasma anserisalpinitidis* strains

Strain ID	Biofilm <sup>a</sup>	enrofloxacin		oxytetracycline		doxycycline		tiamulin		lincomycin		tylosin		tilmicosin		tylvalosin	
		MICi	MICf	MICi	MICf	MICi	MICf	MICi	MICf	MICi	MICf	MICi	MICf	MICi	MICf	MICi	MICf
MYCAV47	++	10	>10	n.a.	>64	0.312	5	0.625	2.5	>64	>64	4	16	64	>64	n.a.	1
MYCAV50	++	n.a.	>10	n.a.	>64	1.25	5	0.078	0.625	n.a.	4	0.5	2	0.5	2	0.25	0.25
MYCAV67	++	n.a.	5	>64	>64	0.312	5	0.625	2.5	>64	>64	>64	>64	>64	>64	8	16
MYCAV70	++	n.a.	>10	32	>64	n.a.	>10	n.a.	0.625	2	4	4	16	64	>64	0.25	1
MYCAV76	++	2.5	5	16	64	1.25	5	0.312	1.25	8	8	1	8	16	>64	0.25	0.5
MYCAV91	++	n.a.	10	n.a.	64	0.625	2.5	n.a.	0.625	n.a.	8	0.25	0.25	0.25	0.25	0.25	0.25
MYCAV160	++	5	>10	64	>64	0.625	10	n.a.	0.625	4	4	n.a.	>64	n.a.	>64	1	2
MYCAV161	++	5	>10	64	>64	0.312	>10	0.312	0.625	4	4	4	16	>64	>64	n.a.	0.5
MYCAV162	++	1.25	2.5	32	>64	0.039	5	n.a.	0.625	2	4	8	16	64	>64	n.a.	0.5
MYCAV270	++	n.a.	n.a.	n.a.	n.a.	1.25	10	0.312	0.625	0.5	4	8	64	>64	>64	0.5	2
MYCAV415	++	n.a.	n.a.	n.a.	n.a.	1.25	10	0.625	2.5	n.a.	n.a.	n.a.	n.a.	>64	>64	n.a.	n.a.
MYCAV668	++	0.312	0.625	0.25	2	0.078	0.312	0.156	0.312	0.25	1	0.25	0.25	0.25	0.25	0.25	0.25
MYCAV177	+	2.5	>10	>64	>64	n.a.	10	n.a.	0.625	2	4	n.a.	>64	n.a.	>64	n.a.	4
MYCAV178	+	5	5	>64	>64	1.25	5	n.a.	0.312	n.a.	2	0.25	4	0.25	>64	n.a.	0.5
MYCAV271	+	n.a.	n.a.	n.a.	n.a.	1.25	10	0.625	n.a.	1	4	n.a.	64	>64	>64	0.5	8
MYCAV494	+	5	10	n.a.	n.a.	0.078	0.312	0.312	1.25	0.25	1	2	4	>64	>64	0.25	0.25
MYCAV671	+	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
MYCAV675	+	0.156	1.25	0.25	1	0.039	0.312	0.312	1.25	0.5	4	8	64	>64	>64	0.5	2
MYCAV929	+	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
MYCAV55	–	2.5	10	1	8	0.078	0.312	0.156	0.625	1	4	0.25	0.25	0.25	0.25	0.25	0.25
MYCAV61	–	5	5	0.5	2	0.039	0.078	0.312	0.132	2	2	0.25	0.25	0.25	0.25	0.25	0.25
MYCAV63	–	0.625	1.25	0.5	4	0.039	0.312	n.a.	0.156	0.5	2	n.a.	4	n.a.	64	0.25	0.25
MYCAV66	–	1.25	5	>64	>64	>10	>10	0.156	0.625	1	4	0.25	0.25	0.25	0.25	0.25	0.25
MYCAV68	–	2.5	5	64	>64	0.625	10	n.a.	5	>64	>64	n.a.	>64	n.a.	>64	8	16
MYCAV75	–	n.a.	5	32	>64	n.a.	10	0.156	0.625	2	4	0.25	0.25	0.25	0.25	0.25	0.25
MYCAV94	–	2.5	2.5	>64	>64	5	>10	n.a.	0.625	2	4	0.25	0.25	0.25	0.25	0.25	0.25
MYCAV176	–	n.a.	10	>64	>64	n.a.	5	n.a.	0.625	2	4	n.a.	64	n.a.	>64	n.a.	4
MYCAV179	–	n.a.	10	1	4	0.156	0.312	0.312	1.25	4	4	n.a.	4	1	4	0.25	0.5
MYCAV180	–	5	5	n.a.	4	0.039	0.312	0.625	1.25	n.a.	4	n.a.	32	64	>64	0.25	1
MYCAV688	–	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
MYCAV967	–	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
BAA-2147	–	0.312	1.25	16	>64	5	>10	0.312	1.25	1	2	0.25	0.5	0.25	0.25	0.25	0.25

<sup>a</sup> no staining (–), slight staining (+), or strong staining (++) in the crystal violet assay; n.a.: data not available.



Table 4. Initial minimal inhibitory concentration values (MIC<sub>i</sub>, µg mL<sup>-1</sup>) of different antibiotics against biofilm and planktonic cell cultures of five *Mycoplasma anserisalpinitidis* strains

Strain ID	Culture type	enro	oxy	doxy	tiam	linco	tylo	tilm	tylv
MYCAV47	culture B	5	64	0.312	0.625	>64	2	64	0.25
	culture P	2.5	64	0.312	0.625	>64	4	64	0.25
MYCAV67	culture B	5	>64	0.312	0.312	>64	>64	>64	8
	culture P	5	>64	0.312	0.625	>64	>64	>64	8
MYCAV70	culture B	10	8	0.312	0.312	2	4	64	0.25
	culture P	10	16	0.312	0.312	2	8	64	0.25
MYCAV178	culture B	5	64	1.25	0.156	1	0.25	0.25	0.25
	culture P	5	32	1.25	0.156	1	0.25	0.25	0.5
MYCAV271	culture B	5	64	1.25	0.625	1	32	>64	0.5
	culture P	5	64	1.25	0.312	1	32	>64	0.5

enro: enrofloxacin; oxy: oxytetracycline; doxy: doxycycline; tiam: tiamulin; linco: lincomycin; tylo: tylosin; tilm: tilmicosin; tylv: tylvalosin; culture B: biofilm-forming culture; culture P: planktonic cell form culture.

three cases: enrofloxacin with MYCAV47 P and oxytetracycline with MYCAV70 B and MYCAV178 P. However, no greater than one dilution step difference was observed between cultures B and P of each strain, and this difference could not be consistently attributed to either biofilms or planktonic cultures.

## DISCUSSION

Crystal violet staining revealed great diversity in the biofilm-forming ability of the 32 tested *M. anserisalpinitidis* strains. Exploring the reason for the observed diversity within this species requires further investigations, with special attention to the genetic background. Mycoplasmas lack almost all genes commonly associated with biofilm formation in other bacterial species (McAuliffe et al., 2006). Nevertheless, in addition to participating in various attachment and binding processes, variable surface proteins have been found to be involved in biofilm formation in *M. bovis* (Thomas et al., 2003; Sachse et al., 1996, 2000). Likewise, polysaccharide capsule production may also influence biofilm formation and adhesion, and this phenotype may even differ among closely related mycoplasmas, such as *Mycoplasma mycoides* subsp. *mycoides* SC and LC (McAuliffe et al., 2006).

Biofilm-forming *M. anserisalpinitidis* cultures were found to be more resistant to heat at 50 °C than their cultures consisting of planktonic cells only. In line with the results of the heat assay, cultures B of the examined *M. anserisalpinitidis* strains were more resistant to desiccation than their cultures P. Mycoplasmas are usually considered to be of low resilience due to their reduced metabolic pathways and lack of cell wall. However, in agreement with the findings of McAuliffe et al. (2006) in ruminant-pathogenic *Mycoplasma* species, biofilm cultures of the tested *M. anserisalpinitidis* strains were found to be quite resistant to heat and desiccation. These results indicate that biofilm formation of *M. anserisalpinitidis* may contribute to the persistence of the organisms in the environment. This phenomenon is of great importance in the control of *M. anserisalpinitidis* infection, as increased environmental

survival of their biofilms should be taken into account during the housing management and disinfection procedures.

Increased resistance of biofilms to antibiotics has been described previously in many studies (Costerton et al., 1999; Reid, 1999; Mah and O'Toole, 2001; Stewart, 2002; Sharma et al., 2019; Yan and Bassler, 2019; Daubenspeck et al., 2020). However, no correlation was shown in this study between biofilm-forming ability and the minimal inhibitory concentration values determined previously (Gróznér et al., 2016, 2022) in the 32 examined *M. anserisalpinitidis* strains. In case of other bacteria, the nutrient constitution of the growth media was described to affect inhibitory antibiotic concentrations of sessile bacteria (Chen et al., 2020). This phenomenon may have contributed to the observed lack of correlation between biofilm-forming ability and antibiotic susceptibility in *M. anserisalpinitidis* strains, although propagation and examinations were carried out in the nutrient-rich medium which is also used for isolation from clinical samples. Nevertheless, MIC values are primarily affected by resistance-associated genes and mutations, and other resistance mechanisms, such as extracellular vesicles or efflux pumps may also play a role (Medvedeva et al., 2014; Antunes et al., 2015; Gróznér et al., 2022). Antibiotic susceptibility of the five tested biofilm-forming *M. anserisalpinitidis* strains was not clearly affected by the experimental prevention of biofilm formation, in the same way as in the study of McAuliffe et al. (2006) with ruminant-pathogenic *Mycoplasma* species. However, it is important to note that these studies examined the effect of biofilm formation on the susceptibility to antibiotics under *in vitro* conditions, and the effect of the constitution of growth media was not evaluated.

At present, the control of mycoplasmosis in waterfowl consists of appropriate animal hygiene measures and antibiotic treatment, while elevated MIC values have been detected against this pathogen in many antimicrobials. Therefore, discovering the factors responsible for increased resistance to these actions is essential. This is the first study demonstrating the biofilm-forming ability of *M. anserisalpinitidis* and confirming the protective effect of this characteristic on environmental survival of the pathogen.



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