



## NOTE

Bacteriology

# Biofilm formation and determination of minimum biofilm eradication concentration of antibiotics in *Mycoplasma hyopneumoniae*

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**ABSTRACT.** The study was aimed to investigate biofilm forming ability of *Mycoplasma hyopneumoniae* and to determine the minimum biofilm eradication concentrations of antibiotics. Biofilm forming ability of six strains of *M. hyopneumoniae* was examined using crystal violet staining on coverslips. The results demonstrated an apparent line of biofilm growth in 3 of the strains isolated from swine with confirmed cases of enzootic pneumonia. BacLight bacterial viability assay revealed that the majority of the cells were viable after 336 hr of incubation. Moreover, *M. hyopneumoniae* persists in the biofilm after being exposed to 10 fold higher concentration of antibiotics than the minimum inhibitory concentrations in planktonic cells. To the best of our knowledge, this is the first report of biofilm formation in *M. hyopneumoniae*. However, comprehensive studies on the mechanisms of biofilm formation are needed to combat swine enzootic pneumonia caused by resistant *M. hyopneumoniae*.

**KEY WORDS:** biofilm, eradication, inhibition, *Mycoplasma hyopneumoniae*

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Enzootic pneumonia is the most significant chronic respiratory disease of swine causing high morbidity, low mortality and contributes to significant economic losses to the commercial swine production industry [17]. Although *Mycoplasma hyopneumoniae* is the primary pathogen responsible for swine enzootic pneumonia, opportunistic bacteria, such as *Pasteurella multocida* are involved in the full development of the disease [27]. Mycoplasmas are a distinct genus of bacteria that are characterized by a single plasma membrane, without a rigid cell wall, and inability to synthesize essential biomolecules [26]. In spite of several studies on the host cell interaction mechanisms in *M. pneumoniae* and related species [2, 21–24], limited information is available on the contributing factors for the pathogenicity of mycoplasmas and mechanisms of survival and/or persistence in the host [15, 16].

Biofilms are adherent bacterial aggregates that are encased in an extrapolymeric matrix composed of DNA, protein, lipid and polysaccharide [7]. Biofilm formation is one of the mechanisms for the survival of a number of bacterial strains in the environment [10]. It is known to provide natural protection which subsequently resulted in increased abilities of bacterial resistance to antibiotics and disinfectants. Biofilms can also cause recalcitrance to antibiotics either due to reducing antibiotic penetration and altering the microenvironment. Phenotypic heterogeneity, adaptive response, and the presence of bacterial persister cells are also mentioned as mechanisms of antibiotic resistance in bacterial biofilms [26]. Studies on *M. pulmonis* showed that the lytic effect of gramicidin, a small antimicrobial peptide, is prevented due to biofilm formation [25].

Despite the use of many antibiotics in pigs, more than 60% of the pig farms in Korea are infected with *M. hyopneumoniae*. We have been studying the pathogenesis of mycoplasmas with the objective of finding an effective treatment [5, 11, 12, 19]. Despite a wealth of information on other species of mycoplasma and bacteria [4, 13–16], biofilm formation and its effect on antimicrobials used for the treatment of enzootic pneumonia caused by *M. hyopneumoniae* in pigs is not studied. Therefore, the current study was designed to investigate biofilm forming ability of *M. hyopneumoniae* and the effect of biofilm formation on the susceptibility of *M. hyopneumoniae* to antibiotics.

*M. hyopneumoniae* was isolated from tracheobronchial swabs of pigs with confirmed cases of enzootic pneumonia from a

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commercial farm in the Republic of Korea according to a previous method [19]. Briefly, samples were cultured in Friis broth medium (FBM) until a color change was observed and then, it was confirmed by PCR analysis of the culture [28]. The titer of *M. hyopneumoniae* culture was expressed as a color changing unit per milliliter (CCU/ml). A semi-quantitative screening assay of biofilm formation in 96-well plates were conducted on 40 strains of *M. hyopneumoniae* identified from the farm, according to a previous method [16]. Accordingly, 3 strong biofilm forming strains (M-IS-1, M-IS-2 and M-IS-3), one non-biofilm strain (M-IS-4) and two reference strains (ATCC) of *M. hyopneumoniae* were included for crystal violet assay on glass coverslips.

Biofilm growth on 22 mm<sup>2</sup> glass coverslips and staining with crystal violet was conducted with slight modifications of previous methods [16, 18]. Eight-milliliter pre-warmed FBM was added in 50 ml conical tubes (Corning, CL, U.S.A.) and sterile coverslips were placed vertically, at the center. The medium was inoculated with a 1:100 dilution of a 20 hr planktonic culture and left at 37°C for 336 hr with shaking at 100 RPM. Non-adherent cells were removed by rinsing in Phosphate buffer saline (PBS). The coverslips were then stained with 0.5% crystal violet solution for 30 min. Finally, biofilm growth and structure were examined microscopically.

Confocal laser scanning microscopy was used to determine the architecture of the biofilm. For this purpose, biofilms grown on glass coverslips were stained with BacLight bacterial viability assay kit (Molecular Probes). SYTO9 and propidium iodide stained cells were analyzed using confocal laser scanning microscopy with an excitation wavelength and an emission filter of 488 nm vs 500–550 nm and 568 nm vs 580–650 nm, respectively. Accordingly, green coloration denotes the presence of live cells, however; the red color indicates that cells are dead and/or has damaged membranes. In addition, Calcofluor White (Sigma) stain (excitation of 405 nm and an emission filter of 580–650 nm) was used to examine the presence of extracellular polysaccharide, which stains as blue [16].

Minimal inhibitory concentrations (MICs) of colistin (CL), enrofloxacin (ENR), marbofloxacin (MAR), tylosin (TYL), florfenicol (FF), gentamicin (GM) and oxytetracycline (OTC) (Sigma Chemical Co., St. Louis, MO, U.S.A.) for planktonic cultures of *M. hyopneumoniae* were determined using a broth microdilution method [9]. Whereas, the minimum biofilm eradication concentrations (MBECs) were determined on the Calgary Biofilm Device (CBD), according to the manufacturer's instructions with slight modifications. Briefly, the trough of the CBD was filled with 22 ml fresh FBM and inoculated with approximately 10<sup>6</sup> CCU of *M. hyopneumoniae*. The lid, with 96 pegs for bacterial adherence and uniform biofilm formation, was placed in the trough and incubated at 37°C with 95% relative humidity. Samples were incubated for 336 hr with constant shaking at 10 rpm (Gyro rotary shaker, model C2, New Brunswick Scientific, San Francisco, CA, U.S.A.).

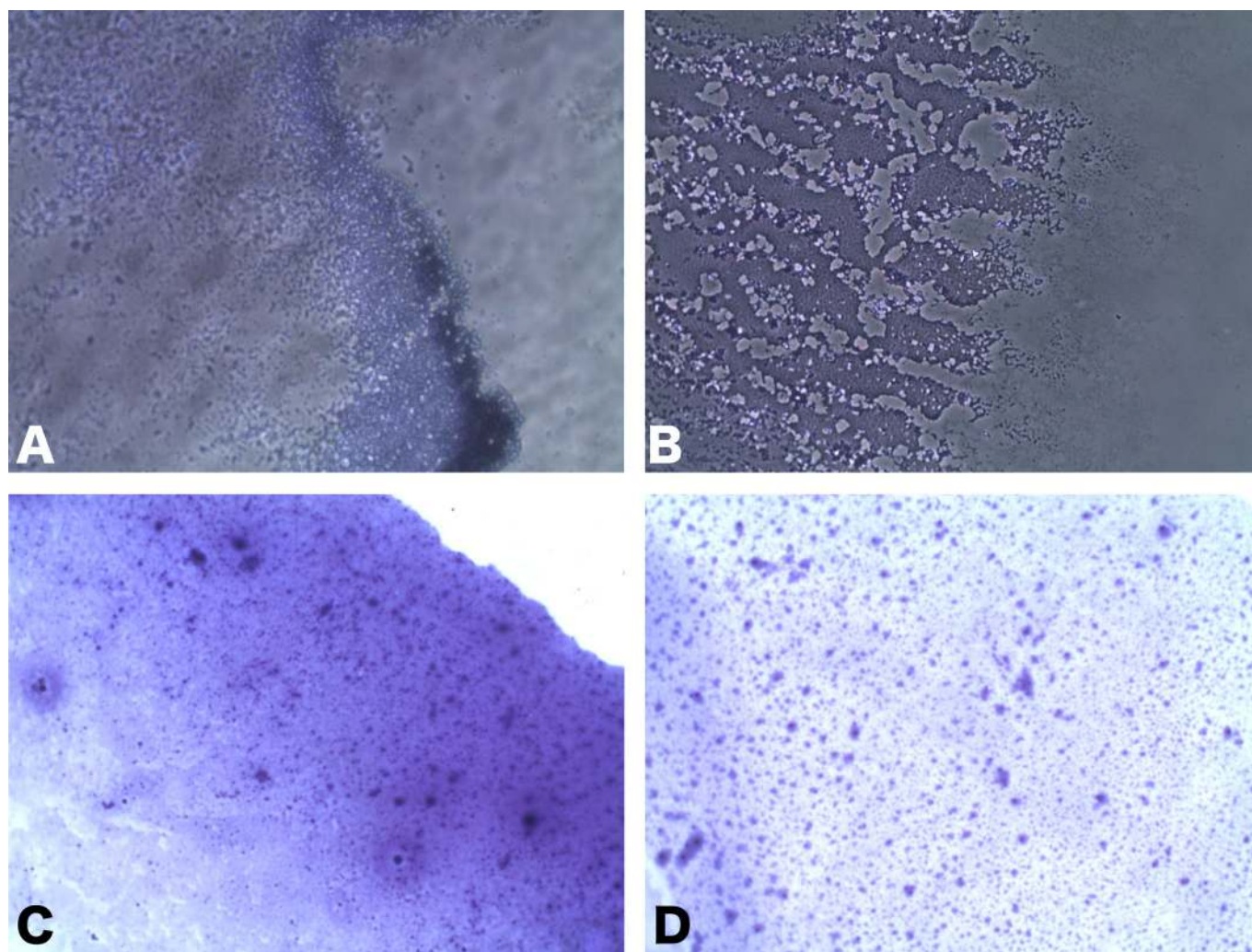
A duplicate, serial twofold dilutions of the antibiotics in FBM used in the determination of MIC were made in 96-well plate. Lid pegs with bacterial biofilm were rinsed in sterile PBS and placed in the 96-well plate containing the antibiotics and incubated at 37°C for 336 hr. The lid was removed, rinsed with PBS, placed in another 96-well plate containing only sterile FBM. The lid was then sonicated for 5 min to remove the biofilm (Aquasonic, model 250; VWR Scientific, Buffalo Grove, IL, U.S.A.). A new plate cover was added and the plates were incubated at 37°C for 336 hr. The presence of viable bacteria was determined by the color change of the broth media. The growth of bacteria in a particular well indicates the regrowth of bacteria as a result of surviving biofilm and the MBEC value was taken as the lowest dilution for the bacteria fail to regrow [3].

Among the six strains assessed, the M-IS-1, M-IS-2 and M-IS-3 showed a clear and consistent biofilm formation on coverslips after 336 hr of incubation. All of the biofilm forming strains (M-IS-1, M-IS-2, and M-IS-3) showed similar biofilm structure. Crystal violet staining on coverslips revealed an apparent line of biofilm growth at the air/liquid interface (Fig. 1). However, the non-biofilm forming strains (for example, strain M-IS-4) could not adhere on the glass coverslips (Fig. 2). Confocal imaging of *M. hyopneumoniae* biofilms stained with backlight viability stain revealed a green fluorescence, indicating that the majority of cells were viable with an intact membrane, after 336 hr of incubation (Fig. 3-Left). The proportion of dead cells or cells with damaged membrane were about 30% of the total. Moreover, Calcofluor white staining demonstrated a clear polysaccharide layer covering the biofilm, stained as blue (Fig. 3-Right).

The MICs and MBECs of selected antibiotics against planktonic and biofilm cultures of *M. hyopneumoniae* and the non-biofilm forming standard strains are summarized in Table 1. The planktonic cultures were susceptible to almost all of the antibiotics tested. However, three strains of *M. hyopneumoniae* (M-IS-1, M-IS-2 and M-IS-3) survived in the biofilm at concentrations which are 10 fold higher than the MICs in planktonic cultures. Moreover, we have observed that the antibiotics failed to inhibit polysaccharide production in *M. hyopneumoniae* biofilm cells, even at significantly higher concentrations than the MICs as compared to the planktonic cells.

From a total of 6 strains, three of them (M-IS-1, M-IS-2 and M-IS-3) exhibited biofilm formation with significant variability. Clear biofilm structure with channels and stacks were observed similar to previous studies in other mycoplasma species [15, 16], *Staphylococcus epidermidis* and *Pseudomonas aeruginosa* [6]. Despite variation in passage number, consistent biofilm formation was observed in these three isolates. Two of them had a relatively low passage number compared to the reference strains. Previous studies on *Actinobacillus pleuropneumoniae* showed that increased passage number could contribute to loss of biofilm forming ability and variation in adhering abilities of the pathogen [1]. In addition, a rough colony of *M. hyopneumoniae* with a star shape grown in FB agar plate was observed under light microscope, only in the biofilm forming strains. Earlier studies on other mycoplasma species demonstrated that rough colony types may have been associated with biofilm formation [16]. Therefore, passage number and colony nature could affect the biofilm forming ability of *M. hyopneumoniae*.

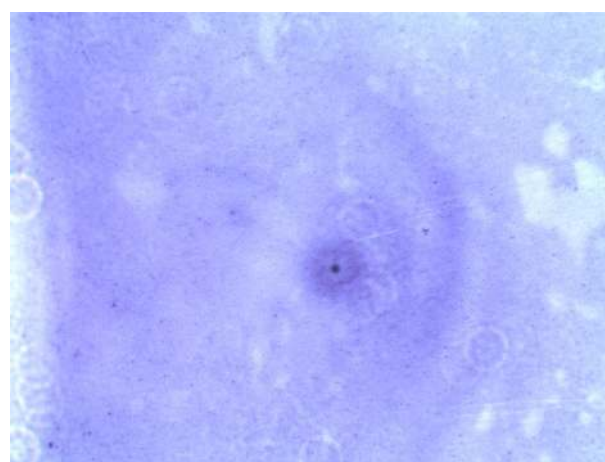
McAuliffe *et al.* [16] demonstrated the absence of a significant difference in the planktonic and biofilm MICs. In the current study, however; the MBECs of the antibiotics in biofilm cells were significantly higher than the MICs in planktonic cells. Cells of *M. hyopneumoniae* in a biofilm were at least 500 times more resistant to the tested antibiotics than their planktonic counterparts.



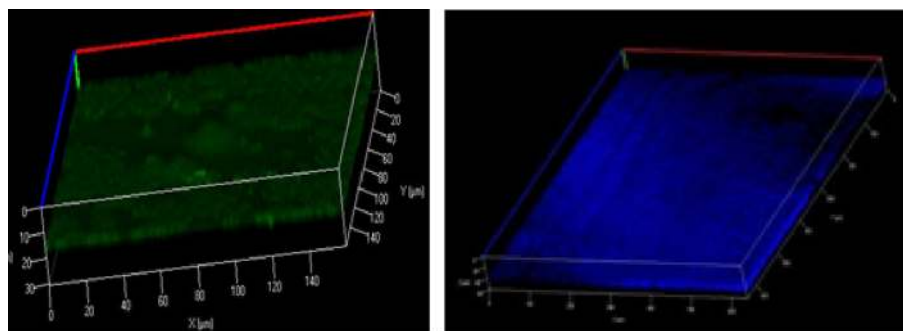
**Fig. 1.** Crystal violet stained *M. hyopneumoniae* biofilms (MI-S-3) on coverslips indicating biofilm growth at the air/liquid interface (10–100 × magnification).

Similar to previous findings in *E. coli*, *Staphylococcus aureus*, *M. gallisepticum* and *P. aeruginosa* [3, 4], *M. hyopneumoniae* grown in biofilm conditions were much less vulnerable to antibiotics than were the planktonic cells suggesting that *M. hyopneumoniae* biofilms are more likely resistant to commonly used antibiotics. This could be due to the protective exopolysaccharide layer in biofilm cultures which may reduce the penetration to antibiotics [15]. Biofilm growth is also indicated in the persistence of mycoplasmas in the environment [16]. This improved resistance suggests that some mycoplasma strains may form biofilms *in vivo*, thus inhibiting the activity of antimicrobial agents and creating a prolonged infection. Our findings showed that higher concentrations of the antibiotics above the MIC failed to inhibit polysaccharide production in biofilm cells, which could contribute to recalcitrance to the antibiotics. On the contrary, studies on *S. epidermidis* demonstrated that subinhibitory concentrations of antibiotics can result in phenotypic variations, especially on adhesion and polysaccharide production [8, 16, 20].

To the best of our knowledge, this is the first report of biofilm formation in *M. hyopneumoniae*. This is a preliminary study on biofilm formation of *M. hyopneumoniae* and the findings



**Fig. 2.** Crystal violet staining of non-biofilm forming *M. hyopneumoniae* (MI-S-4) on coverslips. The *M. hyopneumoniae* could not adhere to the coverslip after washing with PBS (100× magnification).



**Fig. 3.** Confocal images of BacLight stained viable cells (MI-S-3) and green coloration denotes the presence of live cells (Left). Calcofluor White stained MI-S-3 showing blue stained polysaccharide surrounding the biofilm layer (Right).

**Table 1.** MICs (MBECs) ( $\mu\text{g/ml}$ ) of antibiotics against different strains of *M. hyopneumoniae*

Designated strain	ENR	MAR	CL	TYL	FF	GM	OTC
M-ATTC 21774	0.03	0.5	1	0.03	0.12	0.25	0.12
M-ATTC 25156	0.06	0.5	1	0.03	0.25	0.5	0.12
M-IS-1	0.06 (32)	1 (256)	0.5 (512)	0.06 (32)	0.25 (128)	0.25 (512)	0.5 (32)
M-IS-2	0.06 (>2,048)	1 (256)	2 (512)	0.039 (128)	0.5 (512)	0.25 (512)	0.5 (32)
M-IS-3	0.06 (32)	1 (512)	1 (256)	0.03 (256)	0.25 (1,024)	0.5 (256)	0.5 (256)
M-IS-4	0.03	1	1	0.06	0.5	0.5	0.5

ENR: enrofloxacin, MAR: marbofloxacin, CL: colistin, TYL: tylosin, FF: florfenicol, GM: gentamicin and OTC: oxytetracycline.

indicated the ability of *M. hyopneumoniae* to form biofilm under stated conditions. The study also revealed the contribution of biofilms to the persistence of *M. hyopneumoniae* in the presence of higher concentrations of selected antibiotics. This will be an important consideration in antibacterial therapy against *M. hyopneumoniae* infection. However, comprehensive characterization of *M. hyopneumoniae* biofilm, how it adheres to the surface and initiates biofilm formation is needed to combat swine enzootic pneumonia caused by resistant *M. hyopneumoniae*.

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