Final Report

Antivirulence agents against *Pasteurella multocida* in swine

By ANCHANEE KUBERA

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Final Report

Antivirulence agents against *Pasteurella multocida* in swine

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Abstract

Project Code : TRG5780268

Project Title : Antivirulence agents against *Pasteurella multocida* in swine

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Abstract:

Pasteurella multocida is the causative agent of fowl cholera in poultry, hemorrhagic septicemia in ungulates and atrophic rhinitis in swine. This study aims to discover the antivirulence agents against *P. multocida* in order to treat swine. Outer membrane protein A (OmpA), HldE-kinase and Fis transcription factor, are the virulence factors of *P. multocida* which are the targets for this research. Libraries of small molecules were downloaded from the ZINC database and docked against those virulence factors using virtual screening approach. The three dimensional structures of three proteins were constructed by homology modeling. The FDA-approved drug category was selected to perform virtual screening. Deferoxamine mesylate, Mycophenolate mofetil, Famciclovir, Orlistat, Neomycin C, Letrozole, Indocyanine green, Prostaglandin, Vidarabine monohydrate, Ribavirin, Fludarabine phosphate, N-methyl-D-glucamine and Adenosine monohydrate were purchased. The small compounds, Deferoxamine mesylate, Mycophenolate mofetil, Orlistat, Letrozole, Neomycin C, could inhibit the formation of biofilm in the different concentrations with no effect to the growth of *P. multocida* serotype A. Mycophenolate mofetil showed its highest efficacy with the IC_{50} at 7.3 nM. For serotype D, the Indocyanine green showed the highest effect at the IC_{50} value 11.7 nM. The amount of LPS of *P. multocida* serotype A and D were found higher than that of control after adding the compounds, Deferoxamine mesylate, Orlistat, Neomycin C. These results revealed the relationship of LPS and biofilm formation in *P. multocida*. No candidate compound was found to inhibit Fis activity. This study offers an alternative way to combat *Pm*, which could also be applied to combat other pathogens.

Keywords: *Pasteurella multocida*, outer membrane protein A, biofilm formation, FDA-approved drugs, virtual screening

Executive summary

- This project aimed to identify antivirulence agents against 3 proteins, OmpA, HldE and Fis, of *Pateurella multocida* in swine
- Mycophenolate mofetil showed its highest efficacy to inhibit the biofilm formation of *Pm* serotype A with the IC_{50} at 7.3 nM. For *Pm* serotype D, indocyanine green showed the highest effect at the IC_{50} 11.7 nM.
- No candidate compound could reduce lipopolysaccharide (LPS) level.
- No candidate compound could reduce hyaluronic acid (HA) level.

Objective

This study aimed to discover the antivirulence agents against *P. multocida* using computational approach and assay the inhibition activity of top-scoring molecules, *in vitro*.

Research methodology

1. Homology modeling of OmpA, HldE-kinase and Fis

The amino acid sequence of these proteins in *P. multocida* were obtained from NCBI database and submitted to homology modeling web-based tool, www.expasy.org. These structures were used for virtual screening.

2. Virtual screening

In this study, a library of 50,000 drug-like small molecules was retrieved from the ZINC database (http://zinc.docking.org/). These molecules were virtually screened against the virulence factors using Discovery Studio 2.5 software. Twenty small molecules that gave the highest binding score were chosen and purchased to examine their activity against the candidate virulence factors.

3. Growth effect of small molecules to *P. multocida*

P. multocida in swine was obtained from B.F Feed Company. The small molecules were checked their effect to the growth of *P. multocida* by clear zone test. The particular molecules that did not inhibit *P. multocida* growth were used for further experiments.

4. Biofilm formation assay by crystal violet

This experiment characterized the small molecules that could inhibit the function of OmpA. Since OmpA is involved in the biofilm formation, the assay for biofilm was performed. The different concentration of selected small molecules was added into *P. multocida* culture in microtiter plate. The mixture was incubated overnight to let the biofilm formed. After that, the supernatant will discarded and the cell will be washed by medium for one time. The 1% crystal violet was added and left for 15-20 minutes. The supernatant was discarded and the cell was washed by

water for 3 times. The 96% ethanol was added in order to dissolve the crystal violet. The result was recorded at OD 585 nm.

5. Lipopolysaccharide detection

This method examined whether the HldE-kinase is inhibited their activity by small molecules. The HldE-kinase is one of enzymes in the LPS biosynthesis pathway. Once this enzyme is disrupted, the LPS level will be decreased. The different concentrations of selected small molecules was added into *P. multocida* culture. After that, the LPS of the bacteria was extracted by phenol-water method. The amount of LPS was measured at the absorbance 220 nm. [10, 11]

6. Hyaluronic acid (HA) assay

This approach will characterize the function of Fis, transcription factor. Crude capsular material will be extracted. *P. multocida* overnight culture will be collected by centrifugation and washed with PBS buffer. The washed cell will be resuspended in fresh PBS and incubated at 42°C. The mixture will be centrifuged and the supernatant will be collected. HA content will be determined by ELISA technique using the antibody that is specific to HA.

Results

1. Homology modeling of OmpA, HldE-kinase and Fis

The homology three dimensional structures of three proteins, OmpA, HldE and Fis, were constructed. The template structure of OmpA was *Klebsiella pneumoniae* OmpA with 47.55% sequence identity as shown in Figure 1A. The template structure of HldE was *Burkholderia cenocepacia* HldA with 49.35% sequence identity as shown in Figure 1B. The template structure of Fis was *E.coli* Fis with 78.57% sequence identity as shown Figure1C.

2. Virtual screening

As shown in Figure 1, the binding sites of of OmpA were assigned at loop regions. For HldE, the binding site was catalytic site. In Fis protein, the DNA-binding site was selected as the target site. The FDA-approved drug category was selected to perform virtual screening. Five top-scoring compounds of each target proteins were shown in Table 1.

3. Growth effect of small molecules to *P. multocida*

Since some of the listed compounds were not available, the lower scoring compounds were considered to obtain. Thirteen small molecules, Deferoxamine mesylate, Mycophenolate mofetil, Famciclovir, Orlistat, Neomycin C, Letrozole, Indocyanine green, Prostaglandin, Vidarabine monohydrate, Ribavirin, Fludarabine phosphate, N-Methyl-D-Glucamine and Adenosine monohydrate were purchased. These compounds were tested their toxicity against *P. multocida* using clear zone test. Ten microliter of all compounds at the concentration 1mg/ml was loaded

into the wells. In Figure 3A, Deferoxamine mesylate and Neomycin C were toxic against *P. multocida* serotype A. In Figure 3B, only Deferoxamine mesylate showed clear zone against *P. multocida* serotype D. Vidarabine monohydrate, Ribavirin, Fludarabine phosphate, N-Methyl-D-Glucamine and Adenosine monohydrate were not toxic to *P. multocida* serotype D.

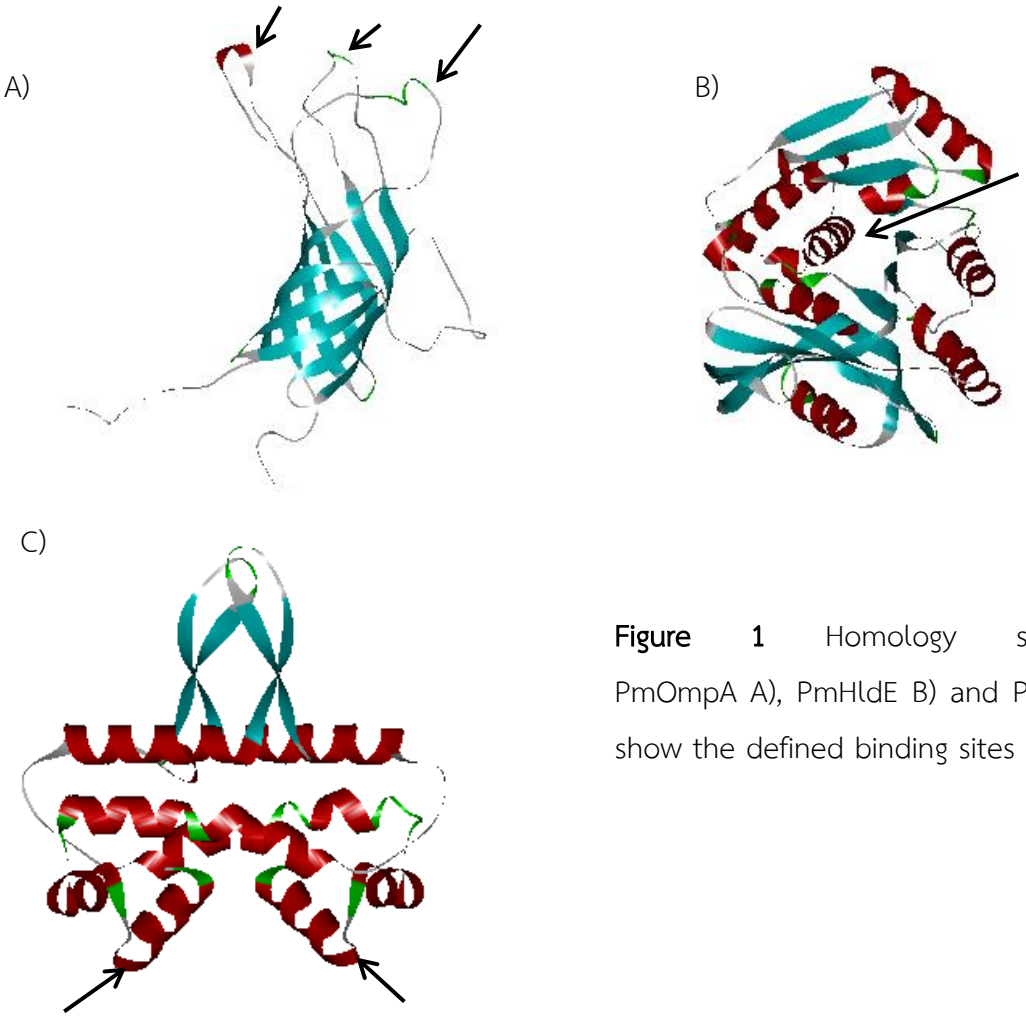

Figure 1 Homology structures of PmOmpA A), PmHldE B) and PmFis C) Arrows show the defined binding sites of each protein

Table 1 Five top-scoring compounds that could bind to the target proteins

OmpA		HldE		Fis	
compounds	LibDock score	compounds	LibDock score	compounds	LibDock score
Deferoxamine mesylate	161.38	DCF	170.85	Vidarabine	106.73
Indocyanine green	143.10	Orlistat	159.90	Ribavirin	100.15
Mycophenolate mofetil	124.12	Neomycin C	159.34	Meglumine	94.6381
Famciclovir	113.95	Deferoxamine mesylate	158.03	Oxime	93.11
IsoprostaglandinF2alpha	93.11	Lipotalon	82.14	Acyclovir	97.89

The binding poses of these compounds to the loop regions of *Pm*OmpA are shown in Figure 2. The highest binding score compound was deferoxamine mesylate. This compound could bind to the loop 2 region by hydrogen bonds with nitrogen atoms of main chain of the residues, Arg92, Met93 and Asp102 (Figure 2A). Indocyanine green forms hydrogen bonds with the nitrogen atom of side chain of Lys90 and nitrogen atoms of main chain of Asp97 and Gln98 on loop 2 (Figure 2B). Mycophenolate mofetil binds to the region between loop 2 and 3 by the hydrogen bonds with nitrogen atoms of main chain of Asp85, Phe86 and oxygen atom of side chain Asp144 (Figure 2C). Famciclovir formed the hydrogen bonds with oxygen atom of main chain residue Tyr140 and with nitrogen atom of side chain of Lys141 and the aromatic ring of the compound formed a hydrogen bond to the nitrogen atoms of side chain of Arg156 (Figure 2D). Deferoxamine mesylate could also bind to the loop 1 region by the hydrogen bonds with oxygen atoms of main chain of Glu50, Ala51 and nitrogen atom of main chain of Gln45 (Figure 2E). Isoprostaglandin F2alpha forms hydrogen bonds to oxygen atoms of main chain of Leu43, Glu50 and nitrogen atom of main chain of Glu53 at the loop 1 region (Figure 2F). Letrozole binds to the loop 4 by a hydrogen bond with nitrogen atom of main chain of Asp202 (Figure 2G).

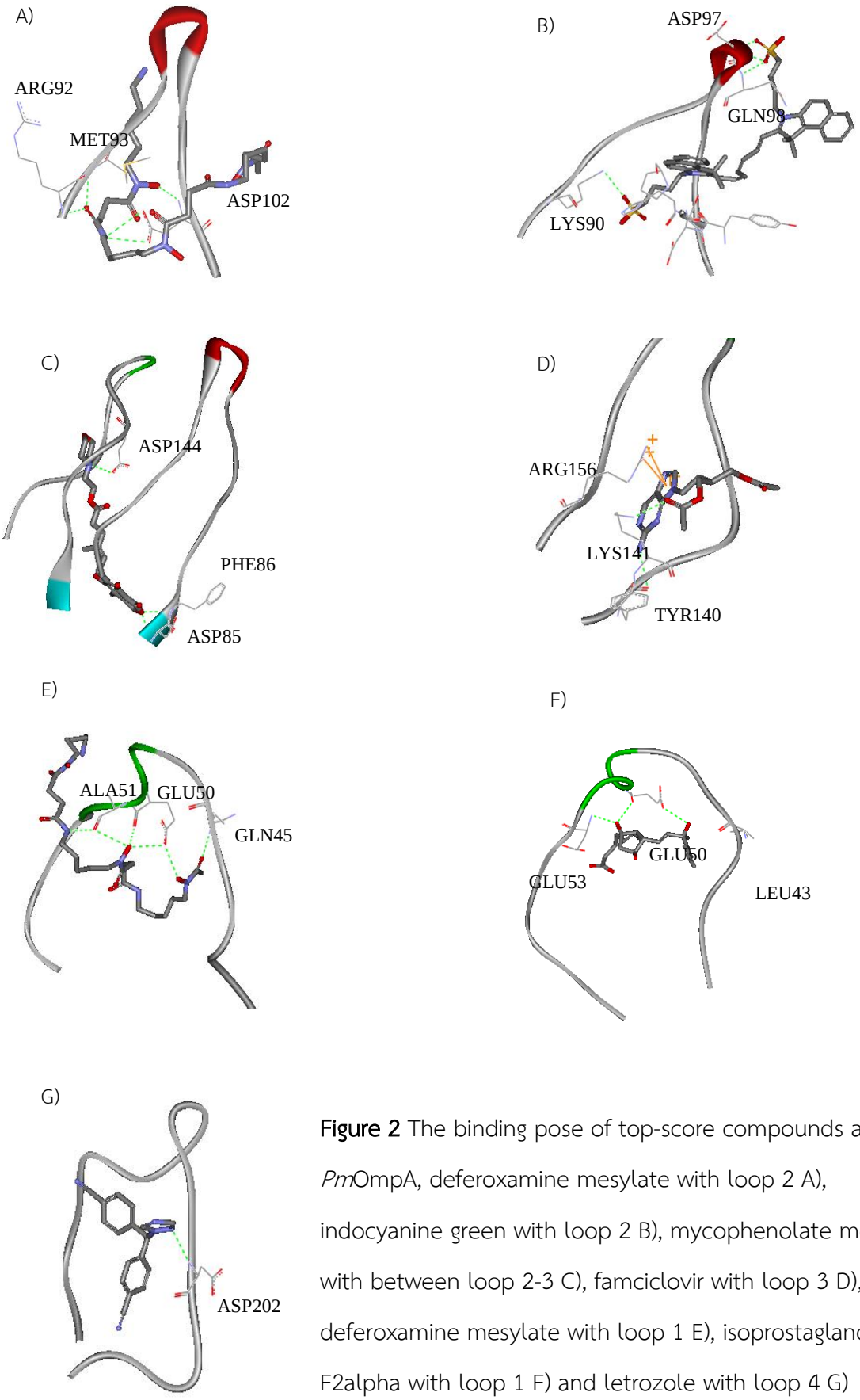

Figure 2 The binding pose of top-score compounds against *Pm*OmpA, deferoxamine mesylate with loop 2 A), indocyanine green with loop 2 B), mycophenolate mofetil with between loop 2-3 C), famciclovir with loop 3 D), deferoxamine mesylate with loop 1 E), isoprostaglandin F2alpha with loop 1 F) and letrozole with loop 4 G)

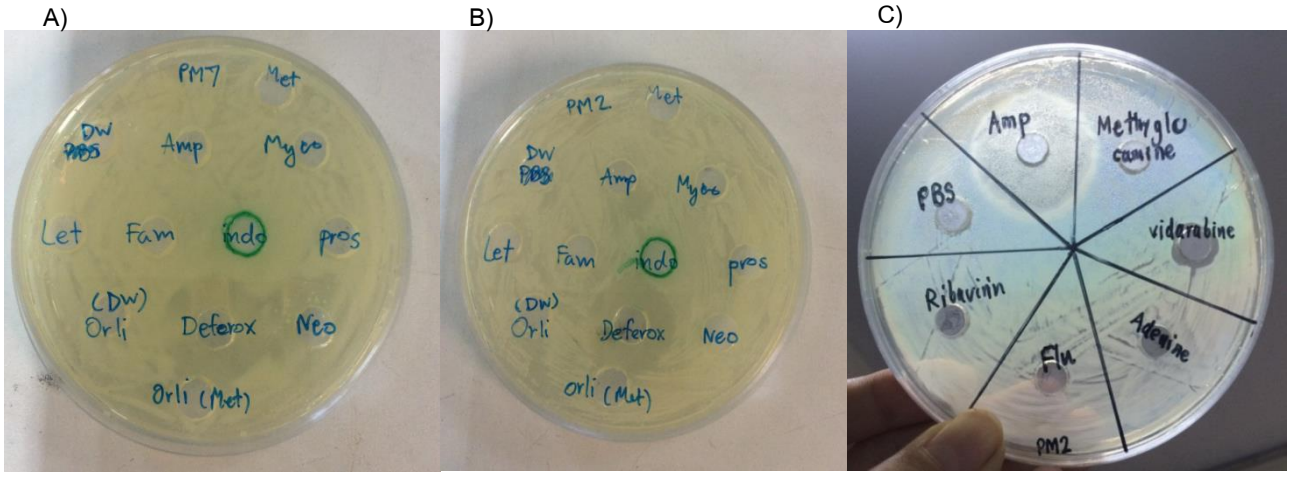

Figure 3 Growth effects of small molecules to *P. multocida*, Met = methanol, DW = distilled water, Amp = ampicillin, Myco = Mycophenolate mofetil, Let = Letrozole, Fam = Famciclovir, indo = Indocyanine green, pros = Prostaglandin, Orli (DW) = Orlistat dissolved in water, Deferox = Deferoxamine mesylate, Neo = Neomycin C and Orli (Met) = Orlistat dissolved in methanol, Flu = Fludarabine phosphate, Deferoxamine mesylate and Neomycin C showed clear zone against *P. multocida* serotype A A) only Deferoxamine mesylate showed clear zone against *P. multocida* serotype D B) Vidarabine monohydrate, Ribavirin, Fludarabine phosphate, N-methyl-D-glucamine and Adenosine monohydrate were not toxic to *P. multocida* serotype D C)

4. Biofilm formation assay by crystal violet

The biofilm formation assay was performed after applying the small molecules, Deferoxamine mesylate, Mycophenolate mofetil, Famciclovir, Letrozole, Indocyanine green, Prostaglandin, Orlistat, Neomycin C, into the *P. multocida* serotype A and serotype D culture. The two-fold serial dilution concentrations of the small compounds were 0.0016 – 3.33 ug/ml. The percentage of biofilm formation of *P. multocida* serotype A was plotted as shown in Figure 4 and serotype D in Figure 5. In serotype A, the small molecules, Deferoxamine mesylate, Mycophenolate mofetil, Orlistat, Letrozole, Neomycin C could inhibit the formation of biofilm in the different concentration with no effect to the bacterial growth. In serotype D, the small molecules, Deferoxamine mesylate, Mycophenolate mofetil, Famciclovir, Letrozole, Indocyanine green, Orlistat, Neomycin C, could inhibit the formation of biofilm without growth effect. The biofilm formation curve of each compound was fitted with the non-linear regression equation and the IC_{50} of each compound was calculated by Graphpad Prism software as shown in Table 2.

5. Lipopolysaccharide detection

The various concentrations of small compounds, Deferoxamine mesylate, Neomycin C and Orlistat were added into the *P. multocida* serotype A and serotype D culture. The LPS of *P. multocida*

was extracted by LPS extraction kit. The amount of LPS was measured at the absorbance 220 nm. The result showed that those three small compounds could not reduce the amount of LPS. In contrast, the amount of LPS when applied the small molecules into the culture was found higher than that of control.

6. Hyaluronic acid (HA) assay

The various concentrations of Vidarabine monohydrate, Ribavirin, Fludarabine phosphate, N-methyl-D-glucamine and Adenosine monohydrate were added into the *P. multocida* serotype A and serotype D culture. The results showed that the level of HA did not alter after adding these molecules to the culture.

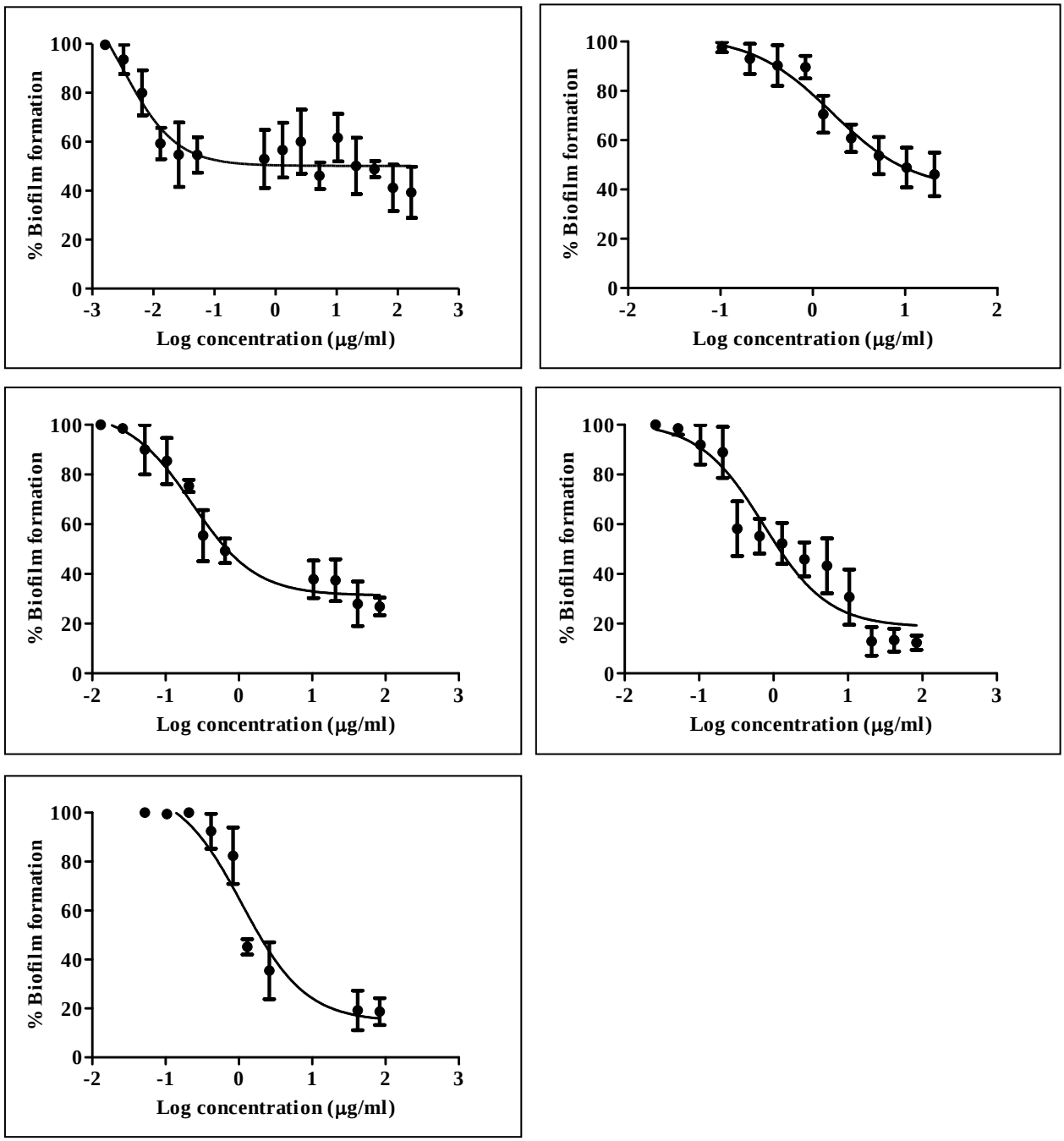

Figure 4 Biofilm formation of *P. multocida* serotype A after applying the small compounds into the culture, Mycophenolate mofetil A), Letrozole B), Orlistat C), Deferoxamine mesylate D) and Neomycin C E)

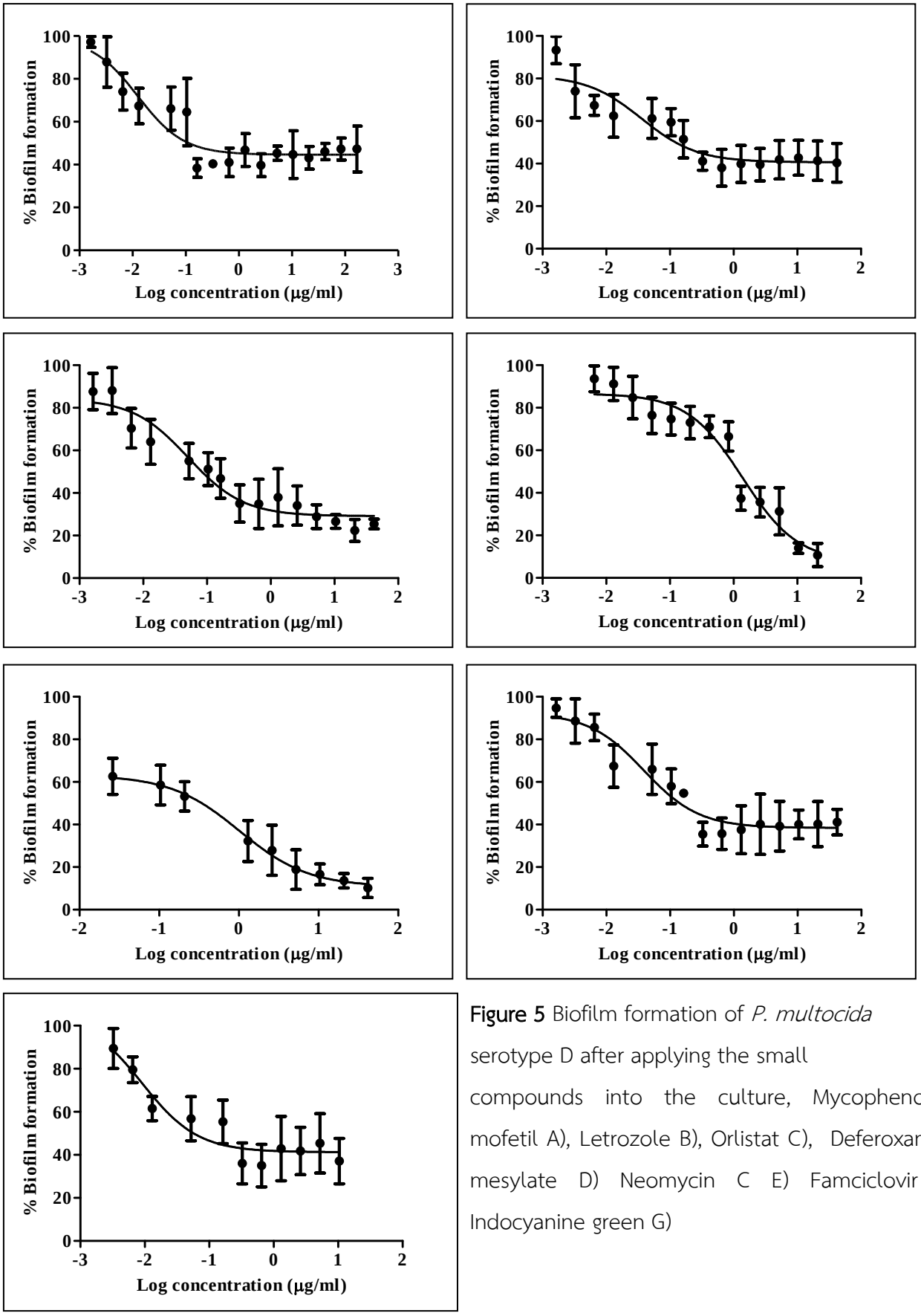

Figure 5 Biofilm formation of *P. multocida* serotype D after applying the small compounds into the culture, Mycophenolate mofetil A), Letrozole B), Orlistat C), Deferoxamine mesylate D) Neomycin C E) Famciclovir F), Indocyanine green G)

Table 2 IC_{50} values of small compounds to inhibit biofilm formation of *P. multocida* serotype A and serotype D

P. multocida serotype A		*P. multocida* serotype D	
Compound	**IC_{50} (uM)**	**Compound**	**IC_{50} (uM)**
Mycophenolate mofetil	0.0073±0.002	Mycophenolate mofetil	0.0289±0.0296
Letrozole	5.6466±0.144	Letrozole	0.1264±0.018
Orlistat	0.4650±0.079	Orlistat	0.1017±0.025
Deferoxamine mesylate	1.1198±0.739	Deferoxamine mesylate	2.1072±0.010
Neomycin C	1.5518±0.317	Neomycin C	1.4283±0.741
		Famciclovir	0.1237±0.009
		Indocyanine green	0.0117±0.008

Discussion and Conclusion

The three dimensional structures of three proteins were constructed by homology modeling. The binding sites of OmpA were defined at the loop regions. These regions are located outside of the bacteria membrane responsible for attachment of host-bacteria interaction and biofilm formation. The catalytic site of HldE was selected as the target site. Any inhibitor that binds to the active site will interrupt the function of this enzyme. The target site of Fis was the DNA-binding site. Once Fis is inhibited, the expression of many genes will be interrupted since Fis is one of the important transcription factors. The FDA-approved drug category was selected to perform virtual screening. Deferoxamine mesylate, mycophenolate mofetil, famciclovir, orlistat, neomycin C, letrozole, indocyanine green, prostaglandin, vidarabine monohydrate, ribavirin, fludarabine phosphate, N-methyl-D-glucamine and adenosine monohydrate were purchased. These small compounds were tested their bactericidal activity against *P. multocida*. The result showed that 1 mg/ml of deferoxamine mesylate was toxic to *P. multocida* serotype A and D. Neomycin C at the concentration 1 mg/ml was toxic to *P. multocida* serotype A. Vidarabine monohydrate, ribavirin, fludarabine phosphate, N-Methyl-D-Glucamine and adenosine monohydrate were not toxic to *P. multocida* serotype D. The small compound, Mycophenolate mofetil, was found its highest efficacy against biofilm formation of *P. multocida* serotype A. The IC_{50} of this compound was 7.3 nM. For serotype D, the Indocyanine green showed the highest effect at the IC_{50} value 11.7 nM.

The amino acid sequences of OmpA in each serotype are different in which resulted the dissimilar of the three dimensional conformations especially at the loop regions [E-kobon T, unpublished data]. This affects the different inhibition properties of compounds against each *Pm* serotype. Mycophenolate mofetil (MMF) docks to the region between loop 2 and 3 of OmpA, which might affect the biofilm formation process of *Pm*. This compound is a derivative of mycophenolic acid produced by *Penicillin stoloniferum* [12]. MMF acts as an inosine-5'-monophosphate dehydrogenase (IMPDH) inhibitor. This compound inhibits recombinant IMDPH of *Barbesia gibsoni* parasite with the IC_{50} at 2.58$\pm$1.32 μM and was shown to inhibit the multiplication of this parasite at the IC_{50} 0.13$\pm$0.05 μM. [13]. MMF has been used for prevention of organ rejection and for treatment of autoimmune diseases i.e. psoriasis [12]. This compound also showed antifungal, antibacterial and antiviral activities including growth inhibition of *Staphylococcus aureus* [14]. In this study MMF was found to inhibit the biofilm formation of *Pm* in the high nanomolar range. This finding illustrates the potency of this compound as a biofilm formation inhibitor. Indocyanine green (ICG) compound is a near infrared fluorescent compound which normally used as an indicator substance for diagnostic in organs and circulatory systems, cardiac circulatory, hepatitis, cerebrum, lymphatic vessels [15, 16]. ICG was reported as antimicrobial agent when it was embedded in polyurethane and irradiated with near-infrared light. This compound showed bactericidal activity against gram-positive bacteria, methicillin-resistant *Staphylococcus aureus* (MRSA) and *Staphylococcus epidermidis* [17]. ICG was also used as an adjuvant for antimicrobial photodynamic therapy in dentistry against Streptococcus salivarius and *Streptococcus pyogenes* [18, 19]. In this study, we discovered that ICG docks to the loop 2 region of OmpA, which might affect the biofilm formation of *Pm* serotype D. In addition, deferoxamine mesylate, famciclovir and letrozole were found to be potent biofilm formation inhibitors in the micromolar range. Deferoxamine mesylate (DM) is a chelating agent isolated from *Streptomyces pilosus* and was used to treat acute iron poisoning, hemochromatosis. DM was found to reduce biofilm formation in *Pseudomonas aeroginosa* [20] and *Streptococcus pneumonia* [21], as an iron chelator. In contrast, this compound was found to enhance the virulence of community-associated MRSA by serving as iron chelating agent for the pathogen [22]. However, this compound had no effect to the growth of *A. baumannii*, *P. aeruginosa*, *S. aureus*, *K. pneumoniae*, and *E. coli* [23] which corresponded to this study. Non-bactericidal antibiofilm compounds have been reported. These compounds were the derivatives of imidazole, phenols, indole, triazole, sulfide, furanone, bromopyrrole and peptides [24]. The compound series of 2-aminoimidazoles together with menthyl carbamate showed their antibiofilm activity in micromolar range against multidrug resistant bacteria, MRSA, *S. aureus* (ATCC#29213), *P.*

aeroginosa and *A. baumannii* [25]. Most of these compounds could inhibit the biofilm formation by disturbing the quorum sensing in bacteria. The potent compounds in this study, MMF, ICG and DM, also contained some of these functional groups such as phenols, indole, furanone and sulfide. These compounds showed antibiofilm activity from nanomolar to micromolar ranges and had no effect to the bacteria survival. One of mechanisms of these compounds could be inhibition of OmpA function which led to the reduction of biofilm formation in *Pm*. These compounds, thus, might be used to treat the infected pigs to reduce the severity of illness symptoms. Moreover, they could be used as food supplement to healthy pigs to maintain the non-virulent condition of the normal flora.

The amount of LPS of *P. multocida* serotype A and D were found higher than that of control after adding the small compounds. The LPS is located in the outer membrane which is the barrier against the drugs. The possibility could be when the biofilm was reduced by the small compounds, the bacteria cells might protect themselves by producing more LPS. Many studies reported the relationship of LPS and biofilm formation. Overproduction of LPS in *E. coli* W3100 showed negative effect to biofilm formation [11]. The LPS-defective mutants in Hep biosynthesis induced the biofilm formation in *E. coli* [26]. Another target of this study was Fis. There was no candidate compound that could reduce the level of HA. Two possibilities could be, first, these compounds could not inhibit the activity of Fis or second, these compounds might be able to inhibit Fis, but there might be other pathways to produce HA. To test these two hypotheses, more experiments should be done in the future.

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- Manuscript submission

Appendix

Title: Biofilm inhibitors targeting the outer membrane protein A of *Pasteurella multocida* in swine

Running title: Biofilm inhibitors against OmpA of *Pasteurella multocida*

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Abstract

Pasteurella multocida (*Pm*) is the causative agent of atrophic rhinitis in swine. This study aims to discover biofilm inhibitors against swine *Pm* to counteract antibiotic resistance and decrease virulence. Outer membrane protein A (OmpA), one of the virulence factors, was used as a target. A library of drugs approved by the Food and Drug Administration (FDA) was used to perform virtual screening against *Pm*OmpA. The top-scoring compounds, deferoxamine mesylate, indocyanine green, mycophenolate mofetil, famciclovir, isoprostaglandinF2alpha and letrozole, had no effect to the growth of *Pm* serotype A and D. Mycophenolate mofetil showed its highest efficacy to inhibit the biofilm formation of *Pm* serotype A with the IC_{50} at 7.3 nM. For *Pm* serotype D, indocyanine green showed the highest effect at the IC_{50} 11.7 nM. This study offers an alternative way to combat *Pm*, which could also be applied to combat other pathogens.

Keywords: *Pasteurella multocida*, outer membrane protein A, biofilm formation, FDA-approved drugs, virtual screening

Introduction

Pasteurella multocida is an important pathogen that causes serious diseases in livestock. This gram-negative bacterium is the causative agent of fowl cholera in poultry, hemorrhagic septicemia in ungulates and atrophic rhinitis in swine. *P. multocida* is classified into 5 capsular serogroups, A, B, D, E and F, and 16 serotypes based on lipopolysaccharide antigens**.** (Harper et al. 2006; Hunt et al. 2000; Chung et al. 1998). Different serogroups and serotypes tend to be associated with particular diseases. Fowl cholera is caused by type A:1 or A:3 strain, hemorrhagic septicemia by B:2 or E:5 strain and atrophic rhinitis by type D strain. The pathogenesis of this bacterium is a result of complex interactions between specific host factors, and specific bacterial virulence factors, LPS, capsule, outer membrane proteins (Hatfaludi et al. 2010). The strategy that is used to combat *P. multocida* at present is to administer antibiotics i.e. flofenicol, chlortetracycline, apramycin, cefquinome, chloramphenicol, colistin, doxycycline, enrofloxacin, erythromycin, florfenicol, penicillin, trimethoprim sulphonamide compounds, tetracycline, tulathromycin (Inamoto et al. 1994; Sellyei et al. 2009). Although these antibiotics work well so far, resistance development has been reported against a number of antibiotics [Sellyei et al. 2009; Martin & Rodríguez Ferri 1993; Kehrenberg et al. 2001). An alternative way is to disarm the pathogen of its toxins and other virulence factors rather than killing it. The overall strategy is to inhibit the specific mechanisms that promote the infection cascade, such as binding, invasion, formation of toxins and biofilm in bacteria. This approach will reduce the selection pressure for drug-resistant mutations (Cegelski et al. 2008). There have been several accounts of antivirulence therapy. For example, the response regulator, AgrA, of methicillin-resistant *Staphylococcus aureus* (MRSA) was successfully targeted by small molecules including the FDA-approved non-steroidal anti-inflammatory drug diflunisal. These small molecules inhibited the production of the toxins, α-hemolysin and phenol soluble modulin α without inhibiting bacterial growth (Khodaverdian et al. 2013). In another study the *lac* repressor was discovered to be an antivirulence factor in *Salmonella enterica* (Eswarappa et al. 2009).

Many virulence factors in *P. multocida* have been identified such as capsule, lipopolysaccharide (LPS), fimbriae and adhesins, toxins, outer membrane proteins (Harper et al. 2006). The outer membrane proteins are critical for bacterial cell, nutrient uptake, transport of molecule in and out of the cell and interaction with the environment and host tissues. OmpA is a two-domain protein that provides the linkage between outer membrane and peptidoglycans. OmpA has been reported to function as an adhesin and invasin and to participate in biofilm formation (Hatfaludi et al. 2010). OmpA also serves as a receptor binding to some bacteriophages (Smith et al. 2007). The N-terminal domain of *P. multocida* OmpA is predicted to span the lipid bilayer, while the C-terminus locates in the periplasmic region and interacts with peptidoglycan (Carpenter et al. 2007). In the OmpA of *E. coli*, there are four external loops, eight-membrane spanning antiparallel β-sheets and three short turns in the periplasm (Smith et al. 2007). This structure forms a pore in the bacterial outer membrane that functions as a channel (Arora et al. 2001). The complement-binding protein 4 (C4bp) binds to OmpA of *E. coli* K1 (Prasadarao et al. 2002). This binding promotes the cleavage of complement factors C3b and C4b, which triggers the disruption of the complement cascade (Wooster et al. 2006). Several reports described that OmpA is essential for biofilm formation in *E. coli* (Ma & Wood 2009; Barrios et al. 2006), *Sodalis glossinidius* (Maltz et al. 2012), *Acinetobacter baumannii (*Gaddy et al 2009; Badmasti et al. 2015). A polyclonal antibody against OmpA of *P. multocida* inhibits the proliferation of this bacterium in mouse lungs (Lu et al. 1991). An OmpA-like protein was discovered as potential novel vaccine candidate in leptospirosis (Yan et al. 2010).

In this study, we discovered efficacious biofilm inhibitors against OmpA of *P. multocida*, which might act in a similar way to the antibody against OmpA to reduce the bacterial load in murine lungs. A three-dimensional model of OmpA was built by homology modeling. Loops of OmpA were identified as targets for binding of FDA-approved drugs by virtual screening. The top-scoring small-molecule compounds were tested for their efficacy to inhibit *P. multocida* biofilm formation.

Materials and methods

Homology modeling of OmpA

The amino acid sequence of OmpA of *P. multocida* strain Pm70 with gene name *PM0786* was obtained from Uniprot database. The three-dimensional structure of OmpA was generated by homology modeling method using SWISS-MODEL software (Arnold et al. 2006). The NMR structures of *Klebsiella pneumoniae* OmpA (PDB ID: 2K0L) was used as a template (Renault et al. 2009).

Virtual screening of FDA-approved drug library against *Pm*OmpA

The ligands, FDA-approved drug library, were retrieved from the ZINC database (http://zinc.docking.org/). The Discovery Studio2.5 software (Accelrys, U.S.A.) was employed for performing the virtual screening. Each of four external loops of *Pm*OmpA was assigned as the binding site of the ligands. The binding site volumes of loop 1, 2, 3, 4 were 5363, 10205, 14367 and 13987 $Å^3$, respectively. The top-scoring compounds from virtual screening results were purchased.

Biofilm formation assay by crystal violet and half maximal inhibitory concentration calculation

Pm serotype A and serotype D were kindly provided by Dr. Teerasak E-kobon, Department of Genetics, Faculty of Science, Kasetsart University. The overnight cultures of these two serotypes were diluted to 1% in Brain-Heart Infusion (BHI) medium. The different concentrations of the selected compounds were diluted in two-fold serial dilution. The concentrations of candidate compounds ranged from 9.77 ng/ml to 1 mg/ml. 50 µl aliquots were placed into 96-well microtiter plates. 100 µl of diluted *Pm* culture was added and incubated at 37^oC for 48 hours to let the biofilm formed. The cell density was measured at OD_{600} nm by nanodrop (ThermoScientific, U.S.A.) to observe whether the small-molecule compounds inhibited growth of the culture. Subsequently, the supernatant was discarded and the cell was washed once with BHI medium. 1% crystal violet solution was added and left for 15-20 minutes. The supernatant was discarded and the cell was washed by sterile water for 3 times. 96% ethanol was added in order to dissolve the crystal violet. The intensity of the crystal violet was measured by spectrophotometer (ThermoScientific, U.S.A.) at OD_{585} nm. The percentage of biofilm formation was calculated as following equation

$$\%\text{Biofilm formation} = \frac{\text{A}_{585}\text{ of biofilm} - \text{A}_{585}\text{ of biofilm with compound}}{\text{A}_{585}\text{ of biofilm}} \times 100$$

The percentages of inhibition for each replicate were calculated for a mean value of each experiment. The mean and the standard error of the mean from three experiments were calculated. IC_{50} *was calculated using GraphPad Prism 5 software (San Diego, California, U.S.A.) with nonlinear regression equation between log(inhibitor concentration) vs. %inhibition as following equation*

$$Y = Y_{bottom} + \frac{(Y_{top} - Y_{bottom})}{(1 + 10^{(\text{x} - \log[IC_{50}])})}$$

Y = % Biofilm formation X = log[Concentration]

Y_{bottom} = Y value of the bottom plateau of the predicted curve

Y_{top} = Y value of the top plateau of the predicted curve

Results

Homology modeling of *Pm*OmpA

The *Pm*OmpA strain Pm70 (Uniprot: Q9CMN1) amino acid sequence was used to generate its three-dimensional structure using homology modeling approach. The template structure was the OmpA of *Klebsiella pneumoniae* (*Kb*OmpA, PDB ID: 2K0L) with sequence identity at 47.55%. The homology model of *Pm*OmpA showed structure of β-barrel composed of four external loops, eight-membrane spanning antiparallel β-sheets and three short turns. The signal peptide residues are Met1-Ala21. The four external loops composed of residues Ala37-Val63 (L1), Phe86-Asn108 (L2), Asp139-His158 (L3) and Gln185-Ser209 (L4) as shown in Figure 1.

Virtual screening of FDA-approved drug library against *Pm*OmpA

Four external loops were selected as the binding sites of ligands. The top-scoring compounds (Table 1) were considered to test their efficacy against OmpA by observing the biofilm inhibition. The binding poses of these compounds to the loop regions of *Pm*OmpA are shown in Figure 2. The highest binding score compound was deferoxamine mesylate. This compound could bind to the loop 2 region by hydrogen bonds with nitrogen atoms of main chain of the residues, Arg92, Met93 and Asp102 (Figure 2A). Indocyanine green forms hydrogen bonds with the nitrogen atom of side chain of Lys90 and nitrogen atoms of main chain of Asp97 and Gln98 on loop 2 (Figure 2B). Mycophenolate mofetil binds to the region between loop 2 and 3 by the hydrogen bonds with nitrogen atoms of main chain of Asp85, Phe86 and oxygen atom of side chain Asp144 (Figure 2C). Famciclovir formed the hydrogen bonds with oxygen atom of main chain residue Tyr140 and with nitrogen atom of side chain of Lys141 and the aromatic ring of the compound formed a hydrogen bond to the nitrogen atoms of side chain of Arg156 (Figure 2D). Deferoxamine mesylate could also bind to the loop 1 region by the hydrogen bonds with oxygen atoms of main chain of Glu50, Ala51 and nitrogen atom of main chain of Gln45 (Figure 2E). Isoprostaglandin F2alpha forms hydrogen bonds to oxygen atoms of main chain of Leu43, Glu50 and nitrogen atom of main chain of Glu53 at the loop 1 region (Figure 2F). Letrozole binds to the loop 4 by a hydrogen bond with nitrogen atom of main chain of Asp202 (Figure 2G).

Biofilm formation assay by crystal violet and half maximal inhibitory concentration calculation

Six compounds, deferoxamine mesylate, indocyanine green, mycophenolate mofetil, famciclovir, isoprostaglandinF2alpha and letrozole, were purchased. The results showed that these compounds had no effect to the growth of *Pm* serotype A and D (data not shown). In *Pm* serotype A, there were three compounds, deferoxamine mesylate, mycophenolate mofetil and letrozole, that inhibited the formation of biofilm as shown in the Figure 3. The most effective compound was mycophenolate mofetil with the lowest IC_{50} at 7.3 nM. For *Pm* serotype D, deferoxamine mesylate, indocyanine green, mycophenolate mofetil, famciclovir and letrozole could affect the biofilm formation as shown in Figure 4. The most effective compound was indocyanine green with

the IC_{50} at 11.7 nM. The IC_{50} values of each compound against the biofilm formation of *Pm* serotype A and D were shown in Table 2.

Discussion

Some FDA-approved drugs showed potent activity to inhibit the biofilm formation of *Pm* by disrupting the function of OmpA. It was found that this protein was essential for biofilm formation in *E. coli, Soldaris glossinidius, Acinetobacter baumannii* (Ma & Wood 2009; Barrios et al. 2006; Maltz et al. 2012; Gaddy et al. 2009; Badmasti et al. 2015). In this report, mycophenolate mofetil and indocyanine green were the most effective compounds to reduce the biofilm formation of *Pm* serotype A and D, respectively. The amino acid sequences of OmpA in each serotype are different in which resulted the dissimilar of the three dimensional conformations especially at the loop regions [E-kobon T, unpublished data]. This affects the different inhibition properties of compounds against each *Pm* serotype. Mycophenolate mofetil (MMF) docks to the region between loop 2 and 3 of OmpA, which might affect the biofilm formation process of *Pm*. This compound is a derivative of mycophenolic acid produced by *Penicillin stoloniferum* (Kitchin et al. 1997). MMF acts as an inosine-5'-monophosphate dehydrogenase (IMPDH) inhibitor. This compound inhibits recombinant IMDPH of *Barbesia gibsoni* parasite with the IC_{50} at 2.58 ± 1.32 µM and was shown to inhibit the multiplication of this parasite at the IC_{50} 0.13 ± 0.05 µM. (Cao et al. 2014). MMF has been used for prevention of organ rejection and for treatment of autoimmune diseases i.e. psoriasis (Kitchin et al. 1997). This compound also showed antifungal, antibacterial and antiviral activities including growth inhibition of *Staphylococcus aureus* (Abraham 1945). In this study MMF was found to inhibit the biofilm formation of *Pm* in the high nanomolar range. This finding illustrates the potency of this compound as a biofilm formation inhibitor. Indocyanine green (ICG) compound is a near infrared fluorescent compound which normally used as an indicator substance for diagnostic in organs and circulatory systems, cardiac circulatory, hepatitis, cerebrum, lymphatic vessels (Marshall et al. 2010; Hardesty et al 2014). ICG was reported as antimicrobial agent when it was embedded in polyurethane and irradiated with near-infrared light.

This compound showed bactericidal activity against gram-positive bacteria, methicillin-resistant *Staphylococcus aureus* (MRSA) and *Staphylococcus epidermidis* (Perni et al, 2011). ICG was also used as an adjuvant for antimicrobial photodynamic therapy in dentistry against Streptococcus salivarius and *Streptococcus pyogenes* (Meister et al. 2014; Hopp & Biffar 2013). In this study, we discovered that ICG docks to the loop 2 region of OmpA, which might affect the biofilm formation of *Pm* serotype D. In addition, deferoxamine mesylate, famciclovir and letrozole were found to be potent biofilm formation inhibitors in the micromolar range. Deferoxamine mesylate (DM) is a chelating agent isolated from *Streptomyces pilosus* and was used to treat acute iron poisoning, hemochromatosis. DM was found to reduce biofilm formation in *Pseudomonas aeroginosa* (O'May et al. 2009) and *Streptococcus pneumonia* (Trappetti et al. 2011), as an iron chelator. In contrast, this compound was found to enhance the virulence of community-associated MRSA by serving as iron chelating agent for the pathogen (Arifin et al. 2014). However, this compound had no effect to the growth of *A. baumannii*, *P. aeruginosa*, *S. aureus*, *K. pneumoniae*, and *E. coli* (Thompson et al. 2012) which corresponded to this study. Non-bactericidal antibiofilm compounds have been reported. These compounds were the derivatives of imidazole, phenols, indole, triazole, sulfide, furanone, bromopyrrole and peptides (Rabin et al. 2015). The compound series of 2-aminoimidazoles together with menthyl carbamate showed their antibiofilm activity in micromolar range against multidrug resistant bacteria, MRSA, *S. aureus* (ATCC#29213), *P. aeroginosa* and *A. baumannii* (Rogers et al. 2010). Most of these compounds could inhibit the biofilm formation by disturbing the quorum sensing in bacteria. The potent compounds in this study, MMF, ICG and DM, also contained some of these functional groups such as phenols, indole, furanone and sulfide. These compounds showed antibiofilm activity from nanomolar to micromolar ranges and had no effect to the bacteria survival. One of mechanisms of these compounds could be inhibition of OmpA function which led to the reduction of biofilm formation in *Pm*. These compounds, thus, might be used to treat the infected pigs to reduce the severity of

illness symptoms. Moreover, they could be used as food supplement to healthy pigs to maintain the non-virulent condition of the normal flora.

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Figure legend

Figure 1 The three dimensional structure of *Kp*OmpA (2K0L) A) used as a template for homology model of *Pm*OmpA B), loops 1-4, N and C termini are indicated.

Figure 2 The binding pose of top-score compounds against *Pm*OmpA, deferoxamine mesylate with loop 2 A), indocyanine green with loop 2 B), mycophenolate mofetil with between loop 2-3 C), famciclovir with loop 3 D), deferoxamine mesylate with loop 1 E), isoprostaglandin F2alpha with loop 1 F) and letrozole with loop 4 G)

Figure 3 The percentage biofilm formation of *Pm* serotype A when treated by deferoxamine mesylate A), mycophenolate mofetil B), and letrozole C)

Figure 4 The percentage biofilm formation of *Pm* serotype D when treated by deferoxamine mesylate A), indocyanine green B), mycophenolate mofetil C), famciclovir D), and letrozole E)

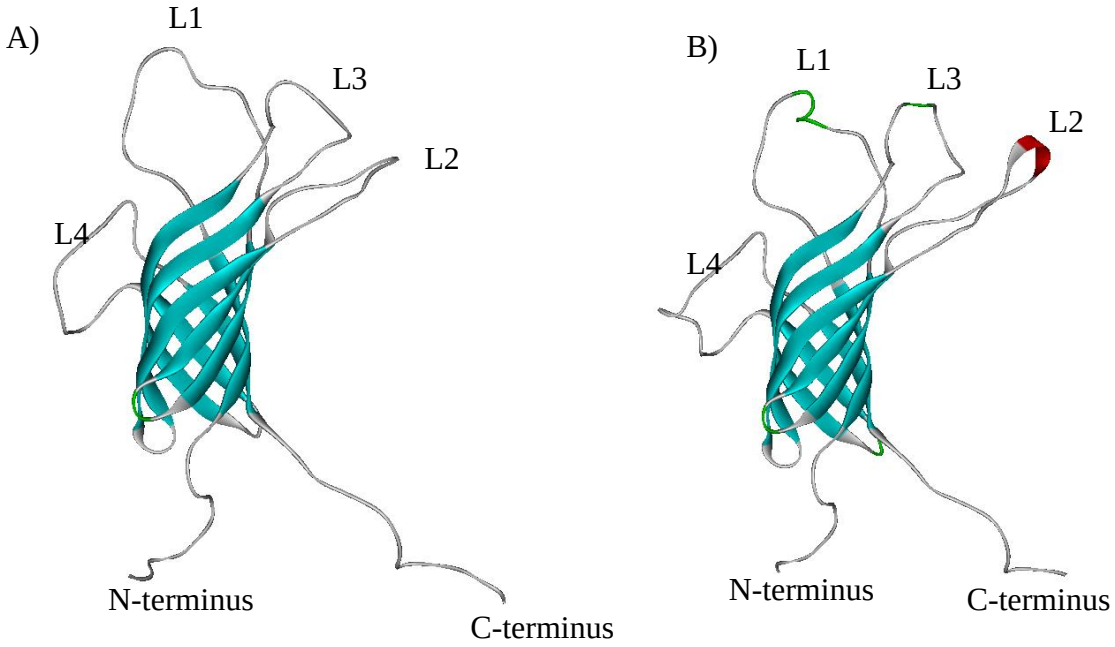
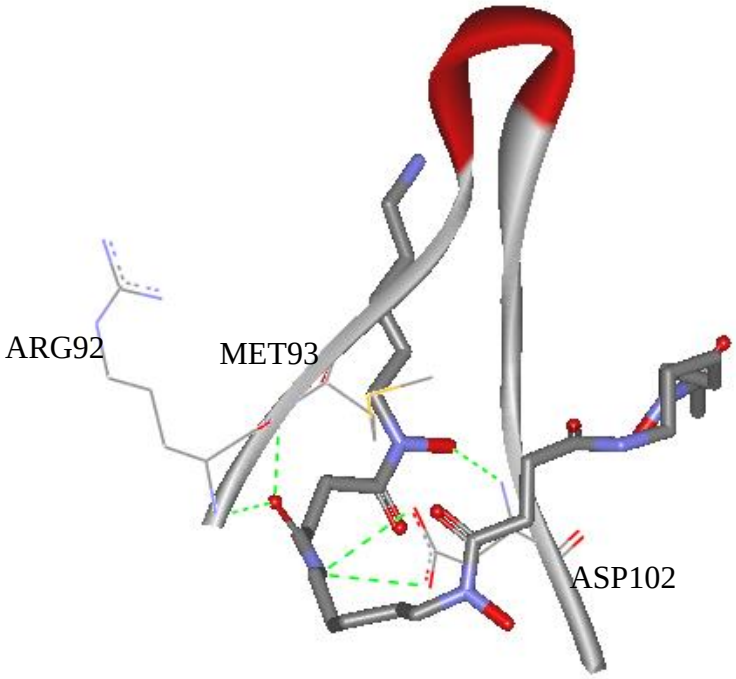

A)
L1
L3
L2
L4
N-terminus
C-terminus
B)
L1
L3
L2
L4
N-terminus
C-terminus

Figure 1

A)



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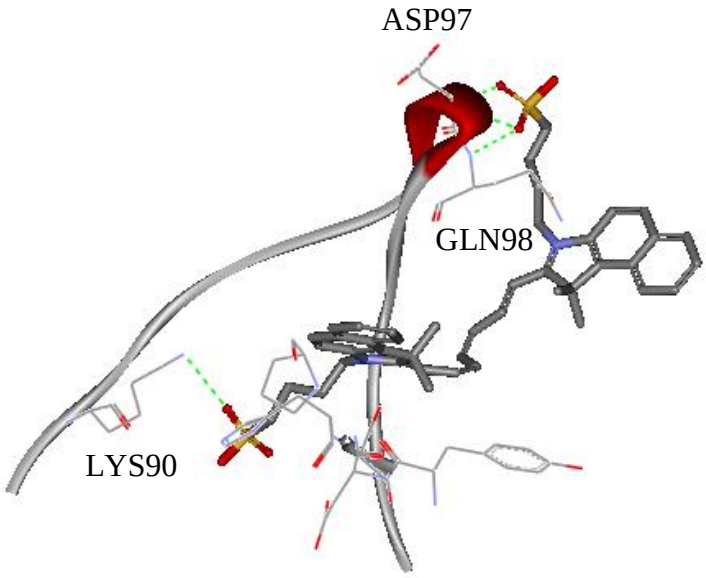
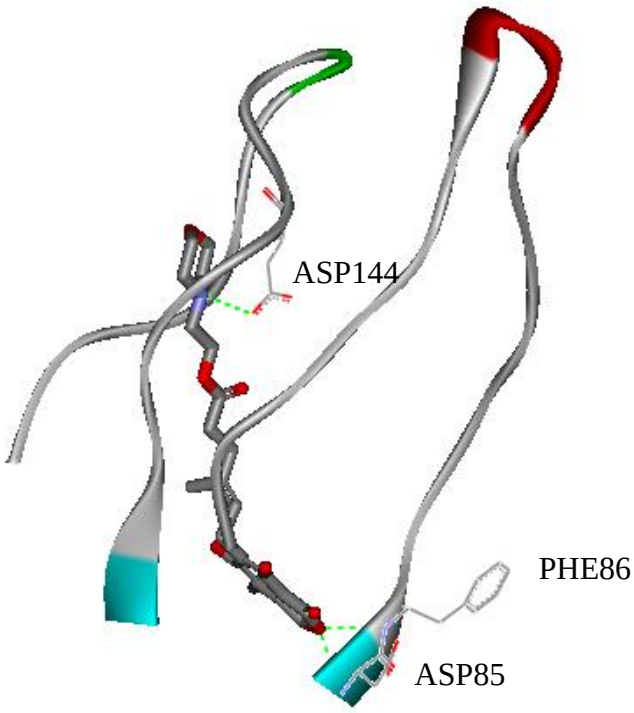


Figure 2

C)



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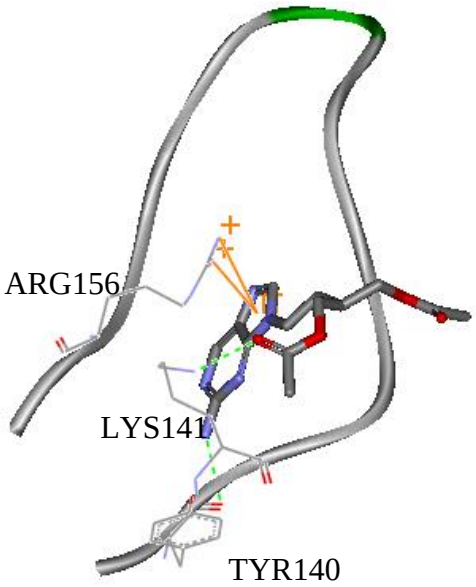
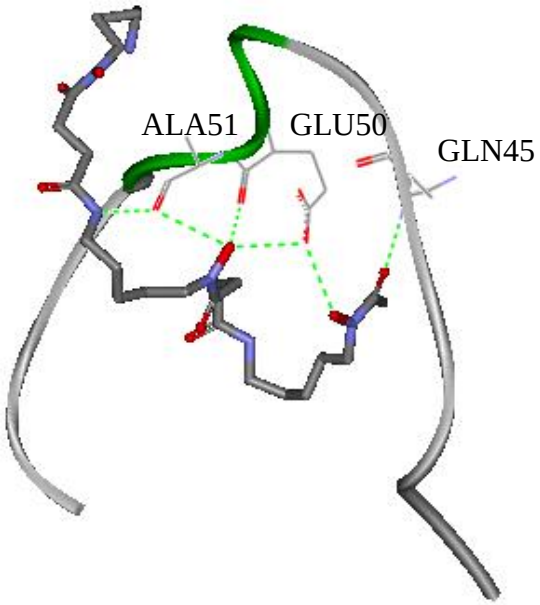


Figure 2

E)



F)

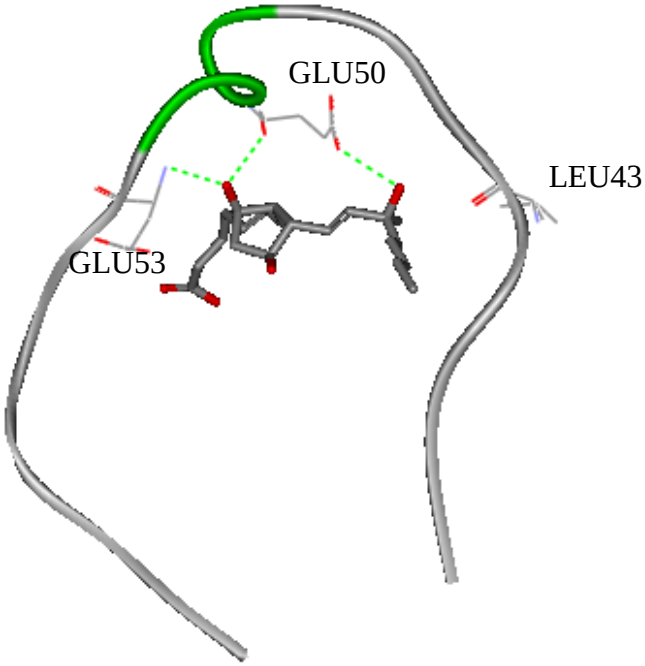


Figure 2

G)

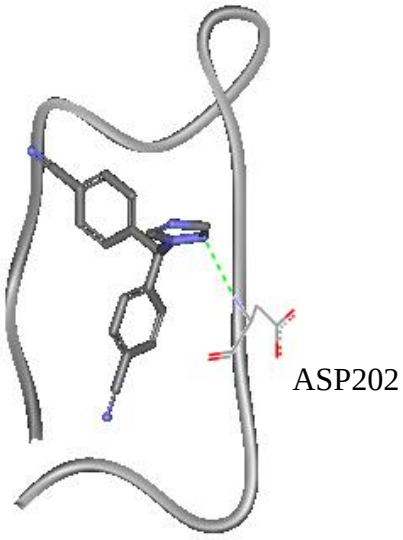
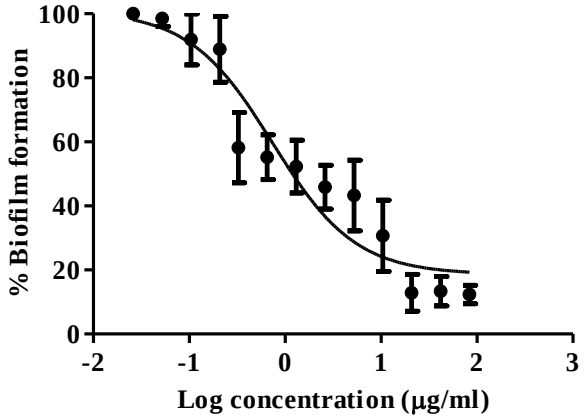
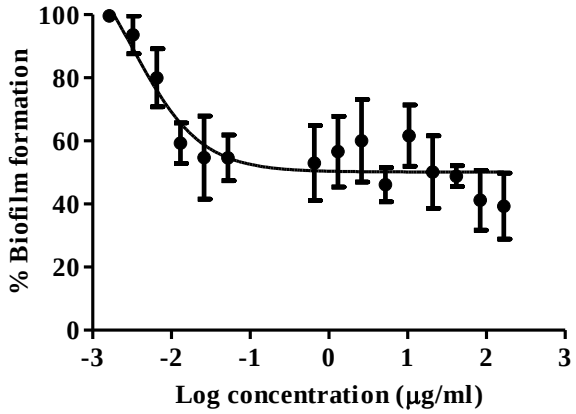


Figure 2

A)


% Biofilm formation
100
80
60
40
20
0
-2
-1
0
1
2
3
Log concentration (μg/ml)

B)


% Biofilm formation
100
80
60
40
20
0
-3
-2
-1
0
1
2
3
Log concentration (μg/ml)

C)

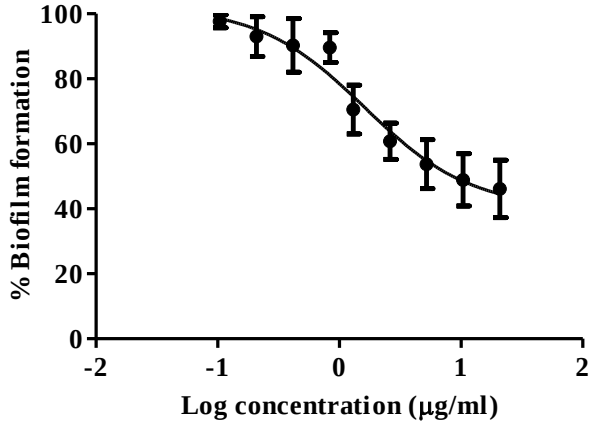
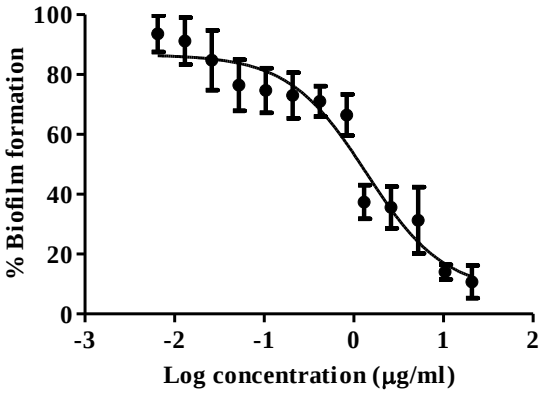
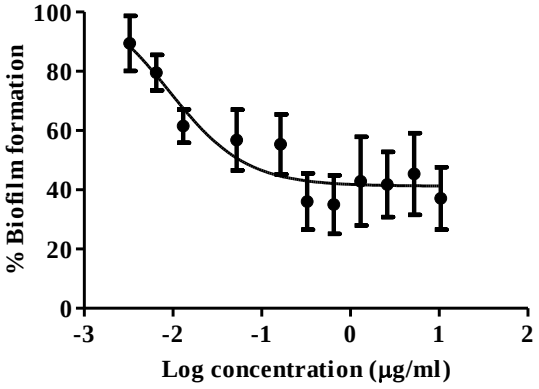

% Biofilm formation
100
80
60
40
20
0
-2
-1
0
1
2
Log concentration (μg/ml)

Figure 3

A)


% Biofilm formation
100
80
60
40
20
0
-3
-2
-1
0
1
2
Log concentration (µg/ml)

B)


% Biofilm formation
100
80
60
40
20
0
-3
-2
-1
0
1
2
Log concentration (µg/ml)

C)

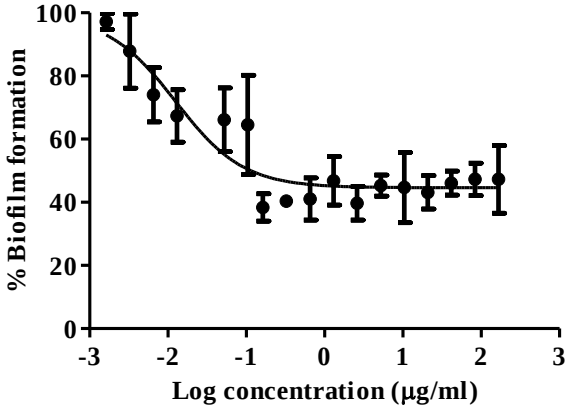
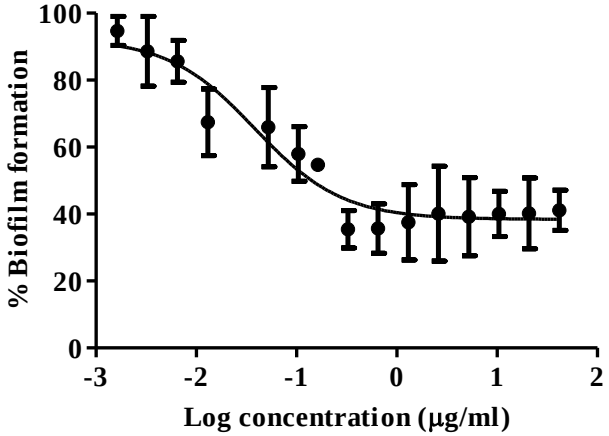

% Biofilm formation
100
80
60
40
20
0
-3
-2
-1
0
1
2
3
Log concentration (µg/ml)

Figure 4

D)


100
80
60
40
20
0
% Biofilm formation
-3
-2
-1
0
1
2
Log concentration (µg/ml)

E)

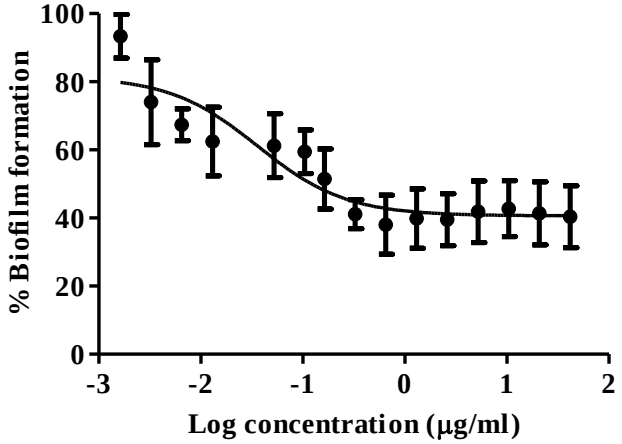

100
80
60
40
20
0
% Biofilm formation
-3
-2
-1
0
1
2
Log concentration (µg/ml)

Figure 4

Table 1 The top-scoring compounds that bind to the loop regions of *Pm*OmpA

ZINC number	Compound	Binding region	Binding score
ZINC03830635	Deferoxamine mesylate	Loop 2	161.38
ZINC08101127	Indocyanine Green	loop 2	143.10
ZINC01530945	Mycophenolate mofetil	Between loop 2-3	124.12
ZINC01530635	Famciclovir	Loop 3	113.95
ZINC03830635	Deferoxamine mesylate	Loop 1	105.86
ZINC15848218	Isoprostaglandin F2alpha	Loop 1	93.11
ZINC03778874	Letrozole	Loop 4	92.27

Table 2 The IC_{50} values of candidate compounds against the biofilm formation of *Pm* serotype A and serotype D

Compound	IC_{50} (nM)	
	***Pm* serotype A**	***Pm* serotype D**
Deferoxamine mesylate	1119.8±739.1	2107.2±10.2
Indocyanine green	> 1 mg/ml	11.7±8.3
Mycophenolate mofetil	7.3±2.2	28.9±30.1
Famciclovir	> 1 mg/ml	123.7±9.5
Isoprostaglandin F2alpha	> 1 mg/ml	> 1 mg/ml
Letrozole	5646.6±144.7	126.4±18.2