The effect of dimethyl sulfoxide on *Corynebacterium pseudotuberculosis* biofilm: An *in silico* prediction and experimental validation

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**Abstract.** *Corynebacterium pseudotuberculosis* is a Gram-positive pathogen that commonly causes caseous lymphadenitis which occurs in sheep, goats, cattle, buffalo and horses. This disease has long been shown to be a major cause of economic loss on sheep industries. Dimethyl sulfoxide (DMSO) is known to be effective against a wide spectrum of pathogens however, its efficacy against *C. pseudotuberculosis* biofilm remains uncertain. The objective of this study was to predict the antibiofilm potential of DMSO against *C. pseudotuberculosis* using *in silico* protein interaction network analysis and experimentally determine the antibiofilm activity using standard microplate assay system. As compared to the protein interaction network of *S. typhimurium* biofilm that had previously been shown to be inhibited by DMSO, the protein interaction network of *C. pseudotuberculosis* showed similar nodes, hub proteins and functional linkages between glycolytic enzymes. Further experimental validation revealed that the treatment with DMSO significantly (p<0.05) inhibited *C. pseudotuberculosis* biofilm at all tested concentrations (1.56% - 50%). The findings from the present study suggest the potential application of DMSO in controlling caseous lymphadenitis in ruminants.

1. Introduction

It has been established that protein-protein interactions play a crucial role in many cellular functions and responses. They often result from physical and specific contacts between two or more protein molecules in biochemical reactions steered by interactions that include electrostatic forces, hydrogen bonding and the hydrophobic effect. Aberrant protein-protein interactions are known to underlie many abnormal conditions such as autoimmune disorders, cancers and Alzheimer's diseases. Therefore, the use of stabilizer or inhibitor of protein interaction network may offer an advantage in disease control [1].
The antibiotic specific protein interaction network has been reported by Padiadpu \textit{et al.} [2]. Different antibiotics have been shown to produce different effects on gene expression profiles which subsequently alter the functional linkages in protein interaction networks. In line with that, the expression profiles often depend on the mechanism of drug action and changes in gene expression it induces in the cells [3]. Thus, prediction of the antimicrobial effects of new compounds against tested microorganisms can be carried out by comparing them with the previously known drug-specific protein interaction networks.

Treatment with dimethyl sulfoxide (DMSO) has been demonstrated to effectively inhibit a wide range of pathogens. In 2017, Yahya \textit{et al.} [4] revealed that treatment with DMSO significantly (p<0.05) inhibited the formation of \textit{Salmonella typhimurium} biofilm, proteome expression and highly connected protein interaction networks. Some of those proteins were found to be essential to the microorganism and non-homologous to humans [5]. Considering the facts that antimicrobials have specific effects on gene expression profile and protein interaction network pattern [2, 3], the effect of DMSO on other pathogenic microorganisms could possibly be predicted prior to wet lab experiments by using \textit{in silico} approach [6].

\textit{Corynebacterium pseudotuberculosis} is a Gram-positive pathogen that commonly causes bacterial livestock disease known as caseous lymphadenitis which occurs in sheep, goats, cattle, buffalo and horses. It lives in soil and the disease can be transmitted via flies. Caseous lymphadenitis is considered as a major cause of economic loss worldwide because it can decrease profitability of the herds: meat, breeding stock marketing, wool, and reduce overall productivity of the herds. To date, there are two species-specific biotypes of \textit{C. pseudotuberculosis} that have been identified based on differences in nitrate reduction, namely biovar equi for nitrate-positive and biovar ovis for nitrate-negative strains. A previous work on the effect of antibiotics on this veterinary pathogen has previously been reported [7]. However, it has not been integrated with computational prediction dataset. On the other hand, the effect of DMSO on \textit{C. pseudotuberculosis} biofilm remains unknown, thus the present work was carried out to predict the possible inhibitory effects of DMSO against \textit{C. pseudotuberculosis} biofilm based on a comparative study of protein interaction networks using an \textit{in silico} approach. The prediction dataset was further validated by standard microplate biofilm assay.

\section{Methodology}

\subsection{Preparation of protein dataset}
A protein dataset containing 75 protein sequences from \textit{S. typhimurium} was used herein. This protein dataset was retrieved from UniProt KB database in FASTA format and was used as a query in the protein similarity search and construction of protein interaction network. This protein dataset was experimentally validated by Yahya \textit{et al.} [4] whereby the treatment with DMSO significantly (p<0.005) inhibited \textit{S. typhimurium} biofilm formation and completely inhibited expression of those proteins.

\subsection{Sequence similarity search and functional classification}
The retrieved protein dataset was subjected to sequence similarity search to identify homologues in \textit{C. pseudotuberculosis} proteome using BLASTp programme (E-value < 1e-06; sequence identity > 30\%) in National Center for Biotechnology Information (NCBI) database. Redundant identified homologues were manually removed while functional categories of identified homologues were analysed using SwisProt/TrEMBL database.

\subsection{Construction and comparative analysis of protein interaction network}
Identified homologues in \textit{C. pseudotuberculosis} were used to construct the protein interaction network using the STRING database (max interactions: 100; confidence level: high - 0.7). The same STRING database parameters were used to construct the protein interaction network for \textit{S. typhimurium}. A qualitative comparison of those networks was performed aiming to identify similarities between them.
in terms of nodes, functional linkages and hub proteins [8]. The functional linkages predicted herein were based on neighbourhood, fusion-fission events, occurrence, text mining and data imported from public databases of physical interactions.

2.4 Preparation of test microorganism

*C. pseudotuberculosis* clinical isolate was obtained from Veterinary Laboratory Service Unit (VLSU), Department of Veterinary Pathology and Microbiology, Faculty of Veterinary Medicine, Universiti Putra Malaysia (UPM) while *Salmonella typhimurium* ATCC 14028 was obtained from Microbiology Laboratory, Faculty of Applied Sciences, Universiti Teknologi MARA Shah Alam. This bacterial species was grown in nutrient broth (Difco Laboratories, USA) and incubated at 37°C. Prior to biofilm assay, the bacterial inoculum was adjusted to optical density (OD) of 0.7 at 600 nm.

2.5 Microplate biofilm assay

The susceptibility of *C. pseudotuberculosis* biofilm towards DMSO was evaluated using 96-wells microplate. A stock of 0.02% resazurin was prepared and stored at 4 °C in the dark. DMSO was prepared at 50%, 25%, 12.5%, 6.25%, 3.13% and 1.56%. Overnight inoculum (200 µl) was added into the microplate wells. Then, a volume of 50 µl of test solution (DMSO) was added. Equal volume of fresh broth and intellectual property (IP)-protected antibiofilm cocktail were also added as negative and positive controls, respectively. The microplate was incubated overnight at 37 °C. On the following day, the medium was discarded whilst the biofilm fractions were rinsed with distilled water twice and heat-fixed at 60 °C for 30 minutes. The biofilm fractions were suspended in 220 µl of phosphate-buffered saline and 30 µl of 0.02% resazurin was added to the wells. The microplate was incubated for at least 3 hours at 37 °C and analysed using microplate reader (ThermoFisher Scientific, USA) for measuring absorbance at 570 nm.

2.6 Statistical analysis

All data from antibiofilm screening assay were expressed as mean ± standard deviation with n = 3. Independent T-test was performed to determine the degree of significant difference between control and test groups whereby p < 0.05 was considered significant.

3. Results

3.1 In silico approach

Figure 1 shows the flowchart of workflow performed herein. The present work began with retrieval of *S. typhimurium* protein sequences from the public protein database which were then used in the BLASTp search to identify their homologues in *C. pseudotuberculosis* proteome. Identified *C. pseudotuberculosis* proteins were used to construct protein interaction network. A comparative analysis of protein interaction networks between *S. typhimurium* and *C. pseudotuberculosis* was performed to evaluate similarities between them, which became a basis of the similar biofilm response towards DMSO. This approach offered an advantage over the approach performed by Yahya *et al.* [4] by giving a valuable clue about the potential antibiofilm action of DMSO against *C. pseudotuberculosis* biofilm.
Figure 1. Flowchart of work to predict the potential inhibitory effect of DMSO against *C. pseudotuberculosis* biofilm based on protein interaction network. a) Methodology used in the present study; b) Methodology as reported by Yahya *et al.* [4]. Asterisk indicates a subset of *S. typhimurium* proteins whose expression was inhibited by the treatment with DMSO.

3.2 Identification of homologues
BLASTp search identified a total of 37 *S. typhimurium* homologues in *C. pseudotuberculosis*. Figure 2 shows functional categories of identified homologues in *C. pseudotuberculosis*. Majority of identified homologues were associated with carbohydrate metabolism (29.03%) and transcription (22.58%) pathways. These identified homologues were then used to construct a protein interaction network for *C. pseudotuberculosis*.

Figure 2. Functional classification of identified homologues in *C. pseudotuberculosis* based on SwissProt/TrEMBL database.
3.3 Protein interaction networks

Figure 3 shows protein interaction networks of *S. typhimurium* and *C. pseudotuberculosis*. Out of 37 homologues in *C. pseudotuberculosis*, only 31 homologues were identified by the STRING database and were used to construct protein interaction networks. It was due to incomplete functional annotation of *C. pseudotuberculosis* proteins in public databases. Proteins in both networks were assigned into five clusters respectively to allow better identification of hub proteins. Hub proteins refer to those showing more than 10 functional linkages. Both networks showed the presence of a similar group of hub proteins consisting of enolase, glucose-6-phosphate isomerase (pgi), triosephosphate isomerase (tpiA), pyruvate kinase (pyk) and phosphoglycerate kinase (pgK). Proteins without any functional interactions such as D-methionine-binding lipoprotein (metQ) and 2,5-diketo-D-gluconic acid reductase A (dkgA) were also identified in both networks.

![Protein interaction networks](image)

**Figure 3.** Protein interaction networks constructed using STRING database. Upper left panel: *S. typhimurium*; upper right panel: *C. pseudotuberculosis*; lower left panel: glycolytic enzymes (red color) in *S. typhimurium*; lower right panel: glycolytic enzymes (red color) in *C. pseudotuberculosis*. Black boxes indicate a group of hub proteins.

3.4 Prediction of inhibitory effect

Table 1 shows a comparison of protein interaction network parameters between *S. typhimurium* biofilm and *C. pseudotuberculosis* biofilm. Number of nodes and number of edges as well as average node degree were largely different between the networks because not all homologues were identified in *C. pseudotuberculosis* proteome during the BLASTp search. On the other hand, several identified homologues were not successfully detected by the STRING database, making them excluded from the network. However, there was a small difference in the average local clustering coefficient between the
networks, suggesting a network similarity. Table 2 shows representative functional linkages between nodes. All functional linkages showed high probability that they are in the same metabolic map in KEGG database. This qualitative comparison showed that *C. pseudotuberculosis* network fulfilled all expected criteria namely i) the presence of selected homologues, ii) the presence of hub proteins associated with carbohydrate metabolism and iii) functional linkages between glycolytic enzymes, giving a clue for further analysis to determine the inhibitory effect of DMSO against *C. pseudotuberculosis* using standard microplate assay system.

**Table 1.** Network parameters of *S. typhimurium* biofilm and *C. pseudotuberculosis* biofilm.

| Parameters                | *S. typhimurium* | *C. pseudotuberculosis* |
|---------------------------|-------------------|-------------------------|
| Number of nodes           | 59                | 31                      |
| Number of edges           | 96                | 89                      |
| Average node degree       | 3.25              | 5.74                    |
| Average local clustering  | 0.403             | 0.452                   |
| coefficient               |                   |                         |
| P value                   | < 1.0e-16         | 6.66e-16                |

**Table 2.** Representative functional linkages between nodes. # indicates hub proteins in *S. typhimurium* while * indicates hub proteins in *C. pseudotuberculosis*.

| Linkages between           | Confidence score |
|---------------------------|------------------|
| pgk# and pgi*             | 0.966            |
| tpiA# and gpmA*           | 0.925            |
| pgk# and pyk*             | 0.847            |
| pyk# and gpmA*            | 0.712            |
| adk# and pgk**            | 0.716            |

3.5 Biofilm Inhibition by DMSO

Figure 4 shows the viability of *C. pseudotuberculosis* biofilm. All test concentrations of DMSO (1.56 %, 3.13 %, 6.25 %, 12.5 %, 25 %, 50 %) significantly (p<0.05) inhibited *C. pseudotuberculosis* biofilm. This finding validated the protein interaction network-based prediction data above.

![Absorbance at 570nm](image)

**Figure 4.** Viability of *C. pseudotuberculosis* biofilm. Each column represents the mean ± standard deviation with n = 3. Asterisks indicate significant difference (p>0.05) as
compared to negative control. Negative control: fresh broth; positive control: intellectual property (IP)-protected antibiofilm cocktail.

4. Discussion
Protein interaction network has become a potential target of existing antimicrobial drugs. The hub proteins that are essential for network integrity and stability should be considered as prospective antimicrobial drug targets [9]. The present study constructed the protein interaction network in *C. pseudotuberculosis* biofilm using identified homologues and compared with that of *S. typhimurium* biofilm, aiming at predicting the potential inhibitory effects of DMSO against *C. pseudotuberculosis* biofilm. In 2019, Folador *et al.* [10] constructed the protein interaction network of *C. pseudotuberculosis* using the complete set of *C. pseudotuberculosis* proteins to identify essential proteins as therapeutic targets.

The protein interaction network in *S. typhimurium* biofilm affected by the treatment with DMSO has previously been reported [4]. Based on that protein interaction network, the biofilm inhibition by DMSO is also expected to occur in *C. pseudotuberculosis* biofilm if the selected nodes (homologous proteins), hub proteins and functional linkages exist in *C. pseudotuberculosis* biofilm [4, 8]. This is due to the fact that the antimicrobial drugs produce specific effects on gene expression profile and protein interaction network [2, 3]. In addition, nodes with similar domain contexts in the protein interaction network may share similar functions and behaviours [11]. Prediction of drug response based on protein interaction network may help to better interpret the patterns observed in high-throughput data of diseases [12]. This approach offers an advantage over existing targeted therapeutic strategies that are based on identification of abnormal molecular expression of a given disease which narrow down the spectrum of putative targets. In the present study, standard microplate assay system was used to validate the result from STRING database analysis. The use of in vitro drug sensitivity assay to validate the computational prediction dataset has also been reported elsewhere [13].

In protein interaction networks, each node corresponds to a protein, and an edge connects two proteins if some experimental or computational procedure suggests that these proteins might share the same function. Thus, these networks would be helpful in annotating a wide spectrum of protein functions, enhancing the current knowledge on biochemical cascades and identifying putative protein targets of therapeutic interest. The present study showed functional linkages between hubs proteins such as enolase, pgi, tpiA, pgK, pgi and adK in *C. pseudotuberculosis* (Figure 3, Table 2). This finding corroborates Yahya *et al.* [4] showing those functional linkages in *S. typhimurium* biofilm. Functional linkages between those glycolytic enzymes have long been reported by Fraenkel [14].

Average clustering coefficient is a measure of the degree of interconnectivity in the neighbourhood of a node. It characterizes the overall tendency of nodes to form clusters. The clustering coefficient values reported herein (Table 1) are much higher than those of metabolic networks of *Mycobacterium tuberculosis*, *Mycobacterium leprae* and *Escherichia coli* [15]. These discrepancies are possibly due to several factors such as number of nodes, number of edges, hub proteins and the overall network size. According to Hao *et al.* [16], topological measures of network density such as clustering coefficient is influenced by hub proteins. Furthermore, the high clustering coefficient values (>0.403) may promote self-organization in the natural networks, which are different from the random networks [17].

DMSO is a polar organic solvent commonly used in preparation of drugs and antibiotics. It has a trigonal pyramidal molecular geometry and an approximately tetrahedral sulfur atom. The sulfur center in DMSO is nucleophilic toward soft electrophiles and the oxygen is nucleophilic toward hard electrophiles. Apart from antimicrobial activity, DMSO also shows antioxidant activity and anti-inflammatory activity. The present study demonstrated the antibiofilm activity of DMSO against *C. pseudotuberculosis* (Figure 4). This result is in agreement with previous works showing antibiofilm action of DMSO against biomass, viability and extracellular matrix of *S. typhimurium* biofilm [4, 18].

Glycolysis is a series of reactions that extract energy from glucose by splitting it into two three-carbon molecules called pyruvates. The free energy released in this process is used to form the high-energy molecules adenosine triphosphate (ATP) and reduced nicotinamide adenine dinucleotide (NADH). This pathway has been shown to be involved in the biofilm formation [19]. It has been
established that the biofilm formation is a biological process that occurs in four main stages: (1) bacterial attachment to a surface, (2) microcolony formation and production of extracellular matrix, (3) biofilm maturation and (4) biofilm dispersal [20]. Bacteria in biofilms often display an exceptional resistance to available antibiotics, making biofilms a major public health problem worldwide [21]. Herein, functional linkages among glycolytic enzymes (enolase, pgi, gpmA, tpiA, pgK and pgi) were identified. It is possible that the treatment with DMSO inhibits functional linkages between these glycolytic enzymes which are also hub proteins, resulting in the inhibition of *C. pseudotuberculosis* biofilm [22, 23]. Inhibition of hub proteins may produce catastrophic consequences since the functions of many other proteins are dependent on hub proteins.

5. Conclusion
The experimental data from microplate biofilm assay successfully validated the *in silico* prediction data on the antibiofilm potential of DMSO against *C. pseudotuberculosis*. The potential application of DMSO in controlling caseous lymphadenitis deserves further attention.

6. References

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