



Identification of Anti-staphylococcal and Anti-biofilm Compounds by Repurposing the Medicines for Malaria Venture Pathogen Box

Vasundhra Bhandari*, Shalini Chakraborty, Umarani Brahma and Paresh Sharma

OPEN ACCESS

National Institute of Animal Biotechnology, Hyderabad, India

Edited by:

Maria Tomas,
Complejo Hospitalario Universitario A
Coruña, Spain

Reviewed by:

Taissa Vila,
University of Maryland, Baltimore,
United States

Yan Q. Xiong,
UCLA David Geffen School of
Medicine, United States

*Correspondence:

Vasundhra Bhandari
vasundhra23@gmail.com;
vasundhra@niab.org.in

Specialty section:

This article was submitted to
Molecular Bacterial Pathogenesis,
a section of the journal
Frontiers in Cellular and Infection
Microbiology

Received: 11 May 2018

Accepted: 01 October 2018

Published: 23 October 2018

Citation:

Bhandari V, Chakraborty S, Brahma U
and Sharma P (2018) Identification of
Anti-staphylococcal and Anti-biofilm
Compounds by Repurposing the
Medicines for Malaria Venture
Pathogen Box.
Front. Cell. Infect. Microbiol. 8:365.
doi: 10.3389/fcimb.2018.00365

There has been an alarming increase in infections caused by antimicrobial-resistant pathogens. These infections are responsible for more than half a million deaths globally each year. *Staphylococcus aureus* is one of the deadliest bacterial pathogen responsible for nosocomial and community acquired infections. The open-access Pathogen Box (PBox) provides a potential platform to identify new treatment options against antibiotic-resistant bacteria by repurposing it. In this study, we have screened the PBox library comprised of ~400 compounds to identify novel anti-staphylococcal compounds. *in vitro* antimicrobial screening using *S. aureus* isolates, ATCC 29213 (methicillin-sensitive) and ATCC 700699 (methicillin-resistant) revealed 13 compounds which showed highly potent antibacterial activity against both planktonic and biofilm state. The 13 compounds were not found cytotoxic to mouse macrophage cell line, RAW264.7. Out of the 13 compounds, only MMV687251 and MMV676477 revealed structural similarity with vancomycin by comparing their atomic pair fingerprints using Tanimoto coefficient method. The structural similarities may indicate similar mode of action like vancomycin for the two compounds. Our result showed that PBox compounds offer a promising lead for the development of new anti-staphylococcal treatment options.

Keywords: anti-staphylococcal, anti-biofilm, repurposing, antimicrobial resistance, open source drug discovery

INTRODUCTION

Staphylococcus aureus is an opportunistic pathogen, which contributes to a significant health problem in both the clinical and community settings, especially methicillin-resistant *S. aureus* (MRSA) (Diep et al., 2008). MRSA causes mild to severe infection of various body tissues, and frequently progress to life-threatening diseases (Otto, 2012). MRSA infections represent a mortality rate of 20% and are challenging to treat due to limited treatment options (Klebens et al., 2007). The World Health Organization has kept *S. aureus* under the list of high priority pathogen against

which new treatments options are required (World health Organization (WHO report), 2017). *S. aureus* is also known to cause substantial proportions of infections in patients with indwelling medical devices (catheter, implants etc.), due to its ability to form biofilms (LaPlante and Mermel, 2009). Biofilm provides bacteria with an added benefit to evade immune responses and antibiotic effect resulting in much complicated and challenging treatment.

Reports of isolates with reduced susceptibility and resistance to vancomycin, the traditional choice of treatment against MRSA has further raised the concern about the scarcity of new treatment options (Hiramatsu et al., 1997; Howden et al., 2010). Most traditional antibiotics have significantly reduced antimicrobial activities in biofilm-associated infections (LaPlante and Mermel, 2009). Therefore, we have screened the diverse library of compounds (400 drug-like compounds) assembled by Medicine for Malaria Venture (MMV) referred as Pathogen Box[®] (PBox), to find new treatment option against *S. aureus* (Medicines for Malaria Venture, 2011).

The PBox compounds have been categorized into pathogen-specific subsets, the majority of the compounds have activity against *Plasmodium*, followed by tuberculosis and other Kinetoplastids. Very few compounds with activity against others pathogens: Helminths, *Toxoplasma*, *Dengue* and *Cryptosporidium* are also present in the PBox. The PBox compounds have not been tried against the other pathogens and therefore provides researchers with an opportunity to screen and identify new leads against pathogens with limited treatment options. The PBox compounds were reported to be 5-fold less cytotoxic against the human cell line in comparison to the pathogen, which is within the acceptable levels (Medicines for Malaria Venture, 2011) (<https://www.pathogenbox.org/about-pathogen-box/supporting-information/>).

Thus, in the present study, we have utilized this opportunity to identify antibacterial agent by repurposing the MMV PBox against planktonic and biofilm state of *S. aureus*.

MATERIALS AND METHODS

Bacterial and Cell Culture

ATCC 29213 (methicillin-susceptible *S. aureus* Sensitive and CLSI recommended isolate for broth microdilution assay) and ATCC 700699 (methicillin-resistant *S. aureus* with reduced vancomycin susceptibility) were used for antimicrobial susceptibility and anti-biofilm assays. RAW 264.7 mouse macrophage cell line was used for cytotoxicity assay. In all experiments, vancomycin and oxacillin were used as internal control.

Antimicrobial Susceptibility Assay

The broth microdilution assay was performed in accordance with Clinical and Laboratory Standards Institute (CLSI) with minor modification by the use of resazurin dye as described earlier (Elshikh et al., 2016; Mahato et al., 2017). Resazurin dye (blue) is a redox indicator, which changes color from blue to pink (resorufin dye) by the metabolically active cells. The assay was performed

for all the PBox compounds ranging from (0 to 100 μ M) provided by MMV in a 96 well plate format. After 24 h incubation of the bacterial cells with different dilutions of PBox compounds at 37°C, 30 μ l of resazurin dye (0.015 %) was added to all wells, followed by 2–4 h of incubation. The lowest dilution columns with no color change were reported as the MIC value.

All assays were performed at least twice in triplicates.

Anti-biofilm Activity

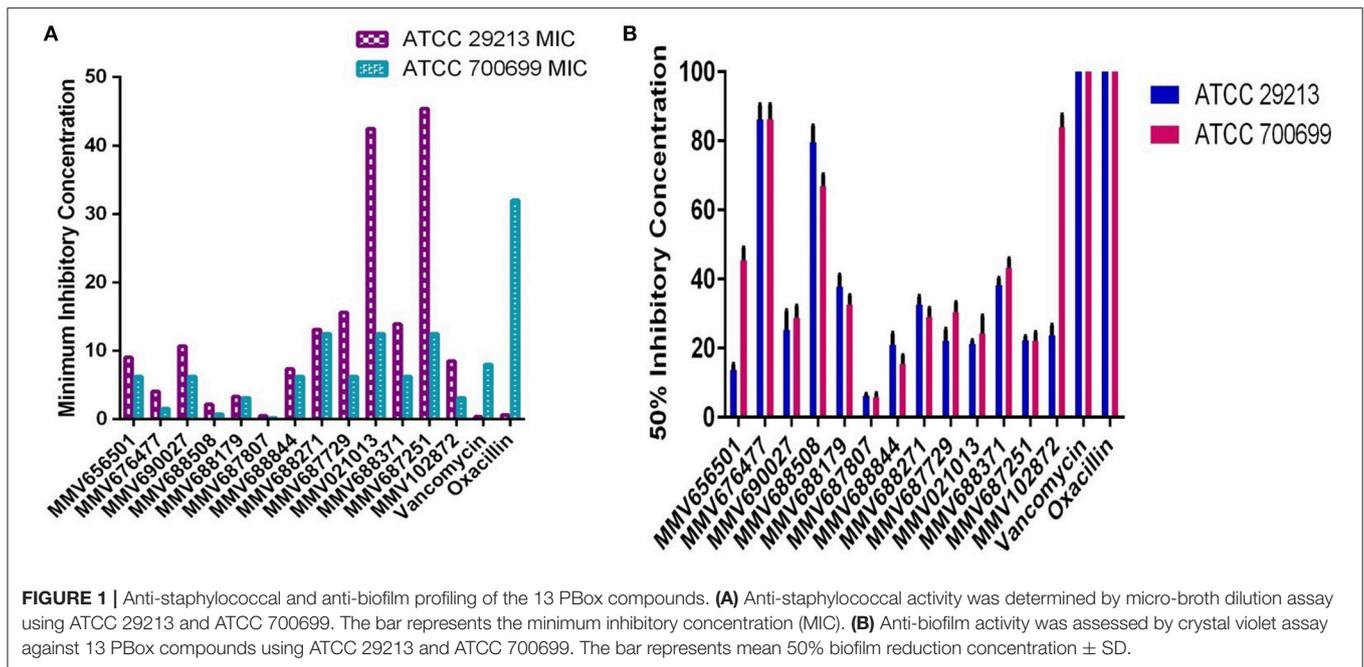
The activity of PBox compounds was tested on *S. aureus* biofilms by Crystal violet (CV) assay in a 96-well plate-format (Coraça-Huber et al., 2012; Masadeh et al., 2013; Ghosh et al., 2015). An overnight cultures of *S. aureus* (ATCC 29213 and ATCC 700699) was diluted 1:200 in Tryptic Soy Broth (TSB) with 0.25 % glucose. Two hundred microliter /well of the diluted culture was dispensed into 96-well plate. The plate was incubated for 24 h at 37°C and washed thrice with PBS. Compounds ranging from 0 to 100 μ M, were added to wells and the plate was further incubated for 24 h at 37°C. The wells were then washed thrice with PBS and methanol fixed for 15 min. The plate was air dried for 30 min and 0.1% CV solution was added to each well and incubated at room temperature for 20 min. After washing with distilled water, 33% acetic acid was added to each well and absorbance was taken at 590 nm using a multimode reader (Enspire, Perkin Elmer). Mean absorbance values of each sample was calculated and compared with the mean value of the control. All assays were performed at least twice in triplicates.

Cell Cytotoxicity

The cell cytotoxicity assay was performed using RAW 264.7 mouse macrophage adherent cell line as described previously with slight modifications (Hua et al., 2018). Cells at a density of 5000 cells per well were seeded in 96 well tissue culture plate and allowed to adhere in DMEM medium (10% FBS) for 24 h at 37°C, 5% CO₂. The adherent cells were treated with different concentrations of PBox compounds (0–250 μ M) for another 24 h. After incubation, resazurin dye was added to each well, and the plate was incubated for 6–8 h at 37°C, 5% CO₂. Fluorescence was measured (excitation wavelength = 550 nm and emission wavelength = 590 nm) by using a multimode reader (Enspire, Perkin Elmer). The results were expressed as percent cell viability, compared with untreated cells. All assays were performed thrice in triplicates.

Structural Similarity Measurements

Atom Pair fingerprints (APfp) were used for calculating the structural similarity between the PBox compounds showing good antibacterial activity (Bajusz et al., 2015; O'Boyle and Sayle, 2016). The APfp of the 13 PBox compounds and vancomycin was calculated using the sdf files downloaded from the ChemSpider for each compound. The sdf files were there loaded into the ChemMine tools software for calculating APfp of all compounds (Backman et al., 2011). The APfp were submitted to ChemmineR software for hierarchical clustering analysis (Cao et al., 2008). The Tanimoto coefficient (Tc) method was used for the cluster analysis and drawing a phylogenetic tree for calculating the relationship between the compounds.



RESULTS AND DISCUSSION

The rate at which *S. aureus* has developed antibiotic resistance has raised serious concern for the public health. Further, biofilm infections are more dreadful and challenging to treat. An estimated 10 million deaths due to antimicrobial resistance are predicted by 2050 (Review on Antimicrobial Resistance, 2016) (https://amr-review.org/sites/default/files/160518_Final%20paper_with%20cover.pdf). The unregulated use of antibiotic has resulted in the emergence of “Superbug” such as MRSA which have developed resistance against major antibiotics and become extremely difficult to treat. Therefore, it is crucial to identify new treatment options to tackle resistant bacteria. The PBox by MMV has provided us with the platform to screen approximately 400 compounds and move a step ahead in identifying new treatment options.

We have screened antibacterial activity of the PBox compounds against methicillin sensitive and resistant *S. aureus* planktonic cells using broth microdilution assay (Mahato et al., 2017). Vancomycin and oxacillin were included as the internal controls in every plate. All the compounds were screened using ATCC 29213 and ATCC 700699. Out of \sim 400 compounds, we found 13 compounds (Figure 1, Table 1, Supplementary Table 1) with a minimum inhibitory concentration of $\leq 45 \mu\text{M}$. These 13 compounds were grouped under Tuberculosis ($n = 9$) and Kinetoplastids ($n=4$) drugs in the PBox information sheet provided by MMV. Five compounds MMV676477, MMV688179, MMV102872, MMV688508, and MMV687807, showed excellent activity against both methicillin sensitive and resistant *S. aureus* with a MIC value of $\leq 8.50 \mu\text{M}$. Excepting MMV 688179 all the other four compounds belonged to tuberculosis disease set. Therefore, screening of the PBox revealed new compounds with anti-staphylococcal activity

which were not reported earlier. Similarly, the free availability of the PBox compounds to researchers has also resulted in the identification of compounds like MMV688271, MMV688768, MMV687273 and MMV687807 showing an excellent activity against fungal pathogens (Vila and Lopez-Ribot, 2016; Mayer and Kronstad, 2017). More research into the PBox compounds also revealed compounds with anti-plasmodium activity which were not initially reported in PBox and were listed under tuberculosis or schistosomiasis (Duffy et al., 2017).

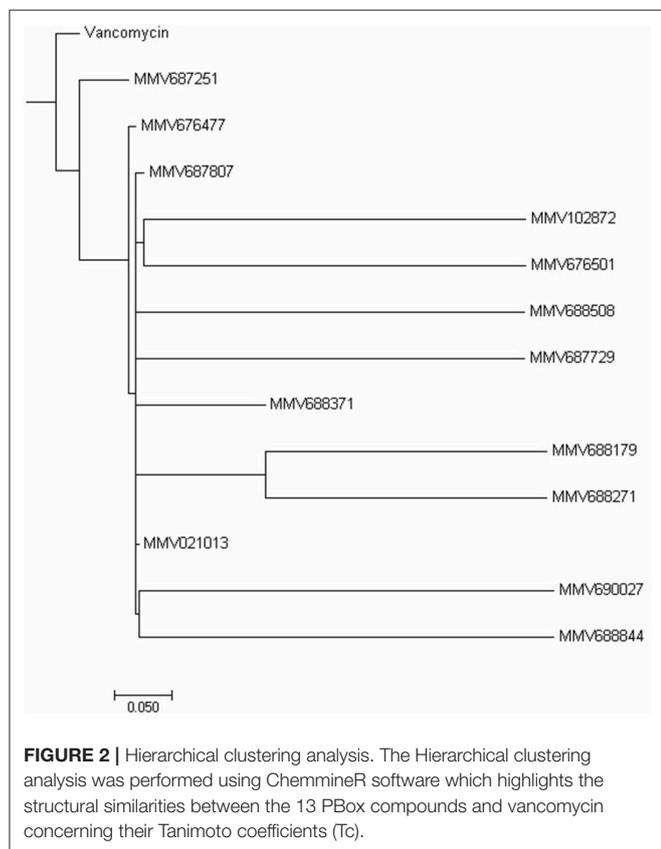
Biofilm infections are more challenging to treat due to increased tolerance to antibiotics and host defense mechanism. Therefore, it is crucial to investigate the anti-biofilm activity of the compounds showing anti-staphylococcal activity for a better treatment option in the future. Hence, the anti-biofilm activity of the 13 compounds was determined by using crystal violet (CV) assay as described previously, using ATCC 29213 and 700699. Out of the 13 compounds tested 6 compounds showed a 50% reduction in biofilm mass at $\leq 30 \mu\text{M}$ (Figure 1, Table 1). Overall, five compounds (MMV656501, MMV687807, MMV688844, MMV687729, and MMV102872) showed good activity against both planktonic and biofilm cells. MMV687807 showed excellent activity against planktonic cells with a MIC of $0.5 \mu\text{M}$ and anti-biofilm activity with an IC_{50} value of $6.20 \pm 0.54 \mu\text{M}$ and $5.73 \pm 1.10 \mu\text{M}$ for methicillin-sensitive and resistant isolate respectively. However, the same compound showed a weaker inhibition of biofilm formed by *Candida albicans* (Mayer and Kronstad, 2017). Interestingly, there were two compounds (MMV021013 and MMV 687251) which were more active against biofilm as compared to planktonic cells.

Cytotoxicity assay using RAW 264.7 mouse macrophage cell line showed all compounds to be less cytotoxic than vancomycin except MMV688371 which has comparable cytotoxicity (Table 1). The low cytotoxicity levels of these compounds reveal

TABLE 1 | Anti-staphylococcal, anti-biofilm and cytotoxicity of the 13 PBox Compounds.

S. No	Compound ID	MMV Disease set	MIC (μM)		Biofilm mass of 50% (μM)		Cytotoxicity (IC_{50} μM)
			ATCC 29213	ATCC 700699	ATCC 29213 *	ATCC 700699*	
1	MMV656501	Tuberculosis	9.02	18.05	13.81 \pm 1.45	45.46 \pm 3.56	>250
2	MMV676477	Tuberculosis	4.06	4.06	98.44 \pm 6.44	86.24 \pm 4.23	>250
3	MMV690027	Kinetoplastids	10.68	10.68	25.26 \pm 5.56	28.68 \pm 3.45	109.32 \pm 2.45
4	MMV688508	Tuberculosis	2.18	2.18	79.53 \pm 4.84	66.89 \pm 3.23	>250
5	MMV688179	Kinetoplastids	3.28	6.56	37.69 \pm 3.49	32.54 \pm 2.69	152 \pm 5.41
6	MMV687807	Tuberculosis	0.50	0.50	6.20 \pm 0.54	5.73 \pm 1.10	>250
7	MMV688844	Tuberculosis	7.35	14.71	20.90 \pm 3.40	15.43 \pm 2.45	>250
8	MMV688271	Kinetoplastids	13.11	26.21	32.50 \pm 2.49	28.91 \pm 2.67	126.94 \pm 5.52
9	MMV687729	Tuberculosis	15.61	15.61	22.15 \pm 3.31	30.34 \pm 2.87	169.32 \pm 12.37
10	MMV021013	Tuberculosis	42.46	42.46	21.23 \pm 0.98	24.27 \pm 4.98	>250
11	MMV688371	Kinetoplastids	13.89	13.89	38.24 \pm 1.94	43.22 \pm 2.65	97.27 \pm 3.07
12	MMV687251	Tuberculosis	45.41	45.41	22.23 \pm 1.18	22.08 \pm 2.48	220.99 \pm 3.77
13	MMV102872	Tuberculosis	8.50	8.50	23.74 \pm 2.90	83.98 \pm 3.45	>250
14	Vancomycin	Internal Control	0.35	5.52	>100	>100	110 \pm 4.66
15	Oxacillin	Internal Control	0.63	> 100	>100	>100	>250

Vancomycin and oxacillin were used as internal controls. *Significant difference was observed in the antibiofilm activity among each MMV compound and the controls (Vancomycin and Oxacillin) with $p < 0.05$.



good potential to be taken up for *in vivo* testing in animal models in future. Additionally, to understand whether the 13 compounds shared any structural similarity, the ChemMine tools software was used, and the reference drug vancomycin

was also included. The hierarchical clustering analysis (HCA) using the T_c method showed levels of similarities between the identified compounds ranging from 0 to 1 with higher values corresponding to greater similarities. The compounds having the T_c values of 1 did not imply identical compounds, however, can be predicted to have similar structural fingerprints. The HCA analysis resulted in the formation of 5 major clusters with vancomycin showing maximum structural similarity with the compounds MMV687251 and MMV676477 with T_c values of 0.993 and 0.960 respectively (Figure 2). The subsequent nearest clusters consist of compounds MMV687807, MMV102872 and MMV676501 (II cluster), MMV688508 and MMV687729 (III Cluster), MMV688371, MMV688179 and MMV688271 (IV Cluster) and MMV021013, MMV690027 and MMV68844 in cluster V based on the analysis. Compounds falling under different clusters indicate structural dissimilarity among them and in comparison, with vancomycin indicating a probable different antibacterial mechanism.

Therefore, our study has highlighted the identification of PBox compounds with bactericidal activity against both planktonic and biofilm cells of *S. aureus*. These compounds have the potential to be developed as a treatment option for biofilm infections, against which vancomycin also has reduced efficacy (LaPlante and Mermel, 2009).

CONCLUSION

The screening of the PBox compounds has opened avenues resulting in the identification of new compounds with antimicrobial activity. These preliminary studies will help us to prioritize the compounds, to be studied at a greater depth for establishing them as a new treatment option for staphylococcal infections.

AUTHOR CONTRIBUTIONS

VB conceived and designed the experiments. VB, SC, and UB performed the experiments. VB and PS analyzed the data. PS and VB contributed reagents, materials, and analysis tools. PS and VB wrote the paper.

ACKNOWLEDGMENTS

We would like to thank Medicines for Malaria Venture (MMV, Switzerland) for support and supply of the Pathogen Box.

REFERENCES

- Backman, T. W., Cao, Y., and Girke, T. (2011). ChemMine tools: an online service for analyzing and clustering small molecules. *Nucleic. Acids. Res.* 39, W486–W491. doi: 10.1093/nar/gkr320
- Bajusz, D., Rácz, A., Héberger, K. (2015). Why Tanimoto index is an appropriate choice for fingerprint based similarity calculations? *J. Cheminformatics.* 7:20. doi: 10.1186/s13321-015-0069-3
- Cao, Y., Charisi, A., Cheng, L. C., Jiang, T., and Girke, T. (2008). ChemmineR: a compound mining framework for R. *Bioinformatics* 24, 1733–1744. doi: 10.1093/bioinformatics/btn307
- Coraça-Huber, D. C., Fille, M., Hausdorfer, J., Pfaller, K., Nogler, M. (2012). *Staphylococcus aureus* biofilm formation and antibiotic susceptibility tests on polystyrene and metal surfaces. *J. Appl. Microbiol.* 112, 1235–1243. doi: 10.1111/j.1365-2672.2012.05288.x
- Diep, B. A., Chambers, H. F., Graber, C. J., Szumowski, J. D., Miller, L. G., Han, L. L., et al. (2008). Emergence of multidrug-resistant, community-associated, methicillin resistant *Staphylococcus aureus* clone USA300 in men who have sex with men. *Ann. Intern. Med.* 148, 249–257. doi: 10.7326/0003-4819-148-4-200802190-00204
- Duffy, S., Sykes, M. L., Jones, A. J., Shelper, T. B., Simpson, M., Lang, R., et al. (2017). Screening the Medicines for Malaria Venture Pathogen Box across multiple pathogens reclassifies starting points for open source drug discovery. *Antimicrob. Agents Chemother.* 61, e00379–e00317. doi: 10.1128/AAC.00379-17
- Elshikh, M., Ahmed, S., Funston, S., Dunlop, P., McGaw, M., Marchant, R., et al. (2016). Resazurin-based 96-well plate microdilution method for the determination of minimum inhibitory concentration of biosurfactants. *Biotechnol. Lett.* 38, 1015–1019. doi: 10.1007/s10529-016-2079-2
- Ghosh, C., Manjunath, G. B., Konai, M. M., Uppu, D. S., Hoque, J., Paramanandham, K., et al. (2015). Aryl-Alkyl Lysines: agents that kill planktonic cells, Persister cells, Biofilms of MRSA and protect mice from skin infections. *PLoS ONE.* 10:e0144094. doi: 10.1371/journal.pone.0144094
- Hiramatsu, K., Hanaki, H., Ino, T., Yabuta, K., Oguri, T., and Tenover, F. C. (1997). Methicillin-resistant *Staphylococcus aureus* clinical strain with reduced vancomycin susceptibility. *J. Antimicrob. Chemother.* 40, 135–136. doi: 10.1093/jac/40.1.135
- Howden, B. P., Davies, J. K., Johnson, P. D., Stinear, T. P., and Grayson, M. L. (2010). Reduced vancomycin susceptibility in *Staphylococcus aureus*, including vancomycin-intermediate and heterogeneous vancomycin-intermediate strains: resistance mechanisms, laboratory detection, and clinical implications. *Clin. Microbiol. Rev.* 23, 99–139. doi: 10.1128/CMR.00042-09
- Hua, X., Yang, Q., Zhang, W., Dou, Z., Yu, S., Schwarz, S., et al. (2018). Antibacterial activity and mechanism of action of Aspidinol against multidrug resistant Methicillin resistant *Staphylococcus aureus*. *Front. Pharmacol.* 9:619. doi: 10.3389/fphar.2018.00619

VB acknowledges Department of Science and Technology-India for the INSPIRE Faculty Award (INSPIRE/04/2015/000242).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fcimb.2018.00365/full#supplementary-material>

Supplementary Table 1 | Minimum Inhibitory concentration against all MMV Pathogen box compounds.

- Klevens, R. M., Morrison, M. A., Nadle, J., Petit, S., Gershman, K., Ray, S., et al. (2007). Invasive methicillin-resistant *Staphylococcus aureus* infections in the United States. *JAMA* 298, 1763–1771. doi: 10.1001/jama.298.15.1763
- LaPlante, K. L., and Mermel, L. A. (2009). *In vitro* activities of Telavancin and vancomycin against biofilm-producing *Staphylococcus aureus*, *S. epidermidis*, and *Enterococcus faecalis* strains. *Antimicrob Agents Chemother.* 53, 3166–3169. doi: 10.1128/AAC.01642-08
- Mahato, S., Mistry, H. U., Chakraborty, S., Sharma, P., Saravanan, R., and Bhandari, V. (2017). Identification of variable traits among the methicillin resistant and sensitive coagulase negative *Staphylococci* in milk samples from mastitic cows in India. *Front. Microbiol.* 8:1446. doi: 10.3389/fmicb.2017.01446
- Masadeh, M. M., Mhaidat, N. M., Alzoubi, K. H., Hussein, E. I., and Al-Trad, E. I. (2013). *In vitro* determination of the antibiotic susceptibility of biofilm-forming *Pseudomonas aeruginosa* and *Staphylococcus aureus*: possible role of proteolytic activity and membrane lipopolysaccharide. *Infect. Drug Resist.* 6, 27–32. doi: 10.2147/IDR.S41501
- Mayer, F. L., and Kronstad, J. W. (2017). Discovery of a novel antifungal agent in the Pathogen Box. *mSphere.* 2, e00120–e00117. doi: 10.1128/mSphere.00120-17
- Medicines for Malaria Venture (2011). The Pathogen Box. Available online at: <https://www.pathogenbox.org/about-pathogen-box/supporting-information/>
- O’Boyle, N. M., and Sayle, R. A. (2016). Comparing structural fingerprints using a literature based similarity benchmark. *J. Cheminformatics.* 8, 2206–2219. doi: 10.1186/s13321-016-0148-0
- Otto, M. (2012). MRSA virulence and spread. *Cell. Microbiol.* 14, 1513–1521. doi: 10.1111/j.1462-5822.2012.01832.x
- Review on Antimicrobial Resistance (2016). *Tackling Drug-Resistant Infections Globally: Final Report And Recommendations.* Available online at: https://amr-review.org/sites/default/files/160518_Final%20paper_with%20cover.pdf
- Vila, T., and Lopez-Ribot, J. L. (2016). Screening the “Pathogen Box” for the identification of *Candida albicans* biofilm inhibitors. *Antimicrob. Agents Chemother.* 61, e02006–e02016. doi: 10.1128/AAC.02006-16
- World Health Organization (WHO) report. (2017). *Global Priority List of Antibiotic-Resistant Bacteria to Guide Research, Discovery, and Development of New Antibiotics.* Geneva: WHO.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2018 Bhandari, Chakraborty, Brahma and Sharma. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.