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High occurrence of heavy metal tolerance genes in bacteria isolated from wastewater: A new concern?

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ABSTRACT

Some heavy metals have antimicrobial activity and are considered as potential alternatives to traditional antibiotic therapy. However, heavy metal tolerance genes (HMTG) have been already detected and coding different tolerance mechanisms. Considering that certain metals are promising for antimicrobial therapy, evaluation of HMTG dissemination in bacteria from sewage is essential to understand the evolution of these bacteria and to predict antimicrobial use and control. The present study aimed to evaluate the occurrence of bacteria carrying HMTG in samples of hospital wastewater and from urban wastewater treatment plant (WWTP). The acquired HMTG were investigated by PCR in bacterial collection previously characterized for antibiotic resistant genes (ARGs). HMTG searched include *arsB* (arsenic efflux pump), *czcA* (cadmium, zinc and cobalt efflux pump), *merA* (mercuric reductase), *pcoD* (copper efflux pump), *silA* (silver efflux pump) and *terF* (tellurite resistance protein). Among 45 isolates, 82% of them carried at last one HMTG, in which the *silA* and *pcoD* tolerance genes were the most prevalent. A very strong positive correlation was found between these genes (r = 0.91, $p < 0.0001$). Tolerance genes *merA*, *arsB*, *czcA* and *terF* were detected in 47%, 13%, 13% and 7% of the isolates, respectively. It was found that 15 isolates co-harbored ARGs (β-lactamase encoding genes). HMTG are probably more dispersed than ARGs in bacteria, representing a new concern for the use of heavy metals as effective antimicrobials. To the best of our knowledge, this is the first study on the HMTG searched in *Hafnia alvei*, *Serratia fonticola* and *Serratia liquefaciens.* Hospital wastewater treatment implementation and additional technologies for treatment in WWTP can reduce the impacts on water resources and HMTG spread, ensureing the environmental and human health safety.

*Keywords***:** metals, antimicrobial resistance, genes, sewage, Gram-negative bacteria.

1. Introduction

In recent decades, the increase of antimicrobial resistance (AMR) has been accelerated due to the incorrect and excessive use of antibiotics in humans, animals and industry (Dadgostar, 2019). In 2017, the World Health Organization (WHO) published a Priority Pathogens List for research and development of new antimicrobial agents as part of efforts to address global resistance (WHO, 2017). A number of researchers have demonstrated the antimicrobial potential of non-antibiotics compounds such as antimicrobial peptides, bacteriolytic enzymes, non-steroidal anti-inflammatory drugs and metals (Afoshin et al., 2020; Burdukiewicz et al., 2020; Lagadinou et al., 2020; Ye et al., 2020).

Heavy metals have antimicrobial potential and, in association with nanomaterials, have been considered as possible alternatives to traditional antibiotic therapy (Cheeseman et al., 2020). Arsenic, cadmium, copper, mercury, silver, tellurium and zinc have shown antimicrobial activity due to their toxicity mechanisms, which involve production of reactive oxygen species (ROS), protein dysfunction and loss of enzymatic activity, membrane potential disturbance, interference in the assimilation of essential nutrients, and genotoxicity (Cheeseman et al., 2020; Lemire et al., 2013). Goss et al. (2018) demonstrated that gallium caused disruption of bacterial iron metabolism, inhibiting the growth of *Pseudomonas aeruginosa* in sputum samples from patients with cystic fibrosis. More recently, the combination of ebselen and silver ions has demonstrated strong bactericidal activity against uropathogenic *Escherichia coli* (UPEC) due to the consumption of intracellular glutathione and inhibition of thioredoxin reductase activity (Wang et al., 2020). On the other hand, heavy metals tolerance mechanisms have been already detected in bacteria as enzymatic detoxification and efflux pump encoded by different heavy metal tolerance genes (HMTG) (Andrade et al., 2018; Hobman and Crossman, 2015).

Wastewater is high selectivity environment for resistant microorganisms due to the presence of chemicals with antimicrobial potential (e.g. antibiotics and heavy metals) (Manaia et al., 2018). Several studies have reported antibiotic resistant genes (ARGs) in bacteria from hospital and urban wastewater, concluding that matrices are reservoir of multiresistant bacteria (Kumar et al., 2020; Lira et al., 2020; Zagui et al., 2020; Zhang et al., 2020). Thus, bacteria surviving in this selective matrix can exchange genetic elements and disperse to the environment if they are not removed in wastewater treatment plants (WWTP) (Manaia et al., 2018). In the context of One Health Perspective multisectoral approach, pollution of water courses by multiresistant bacteria is a threat especially in water reuse, which impacts on human health, biota and food-producing animal (McEwen and Collignon, 2018). Considering that heavy metals are promising for antimicrobial therapy, evaluation of HMTG spread in bacteria from sewage is essential to understand the evolution of these bacteria and to predict antimicrobial use and control. In this context, in the present study we evaluated the occurrence of bacteria carrying HMTG in hospital and urban sewage in a large metropolitan region of São Paulo State, Brazil.

2. Materials and Methods

2.1.Study area and sampling

In Ribeirão Preto city (northeast of São Paulo State, Brazil) is located a large university hospital, considered as reference medical center, which provides care services for approximately 4 million people in the region, as well as patients from other states and countries. This tertiary hospital directs the untreated wastewater to municipal collection system according to the Brazilian legislation (ANVISA, 2002). The urban wastewater treatment has been responsibility of two private WWTP, where activated sludge technology is used for treatment (Zagui et al., 2020).

The wastewater samples were collected in previously sterilized 200 mL glass vials, at five sampling points: ambulatory hospital wastewater, ward hospital wastewater, confluence of hospital wastewater, raw urban wastewater and treated effluent in municipal WWTP. Two sampling campaigns were conducted between March and September 2018, with a total of 10 wastewater samples.

2.2.Heavy metals tolerance genes detection

The bacteria collection was isolated and previously identified, and the results are available in Zagui et al. (2020), when 45 isolates were evaluated for AMR and ARGs. In the present investigation, bacterial DNA for HMTG analysis was extracted for all isolates (*n* = 45) following this protocol: a) dense bacterial suspension in 300 µL of ultrapure water, b) bacterial suspension subjected to boiling for 15 minutes, c) centrifugation at 15,000 rpm, for 15 minutes and d) collection of 200 µL of the supernatant and storage at -20 ºC until analyses. The amount (ng/µL) and purity of extracted DNAs were evaluated in NanoDrop2000 spectrophotometer (Thermo Scientific, Waltham, USA).

Conventional polymerase chain reaction (PCR) was used for HMTG amplification under these conditions: initial DNA denaturation (95 °C, 5 min), followed by 30 cycles of DNA denaturation (95 ºC, 1 min), primers annealing (1 min), DNA polymerase extension (72º C, 1 min) and a final extension (72 ºC, 10 min). HMTG amplification was verified by agarose gel electrophoresis (1.5%) and visualized trough the AlphaImager (Alpha Innotech, San Leandro, USA) photocumenter under UV light, where fragments were compared to HMTG amplification of control bacteria. The acquired HMTG searched includes encoding genes involved in detoxification by enzymes and efflux pump such as *arsB* (arsenic efflux pump), *czcA* (cadmium, zinc and cobalt efflux pump), *merA* (mercuric reductase), *pcoD* (copper efflux pump), *silA* (silver efflux pump) and *terF* (tellurite resistance protein) (Ferreira et al., 2019; Andrade et al., 2018). The used primers for HMTG amplification are shown in Table 1.

2.3.Statistics

Statistical analyses were performed using the GraphPad Prism 8 software. Pearson's Correlation was used to correlate HMTG coexistence. The significance level was established at $p < 0.05$.

3. Results and Discussion

The occurrence of bacteria carrying heavy metal tolerance genes from wastewater samples is depicted in Figure 1. A 82.2% (n = 37) of isolates carried at last one HMTG, in which the silver (*silA*) and copper (*pcoD*) tolerance genes were the most prevalent (53%). Previous studies in which the occurrence of HMTG in bacteria from hospitalized patients and animals was evaluated, also found high occurrences of the *silA* and *pcoD* genes in different species (Ferreira et al., 2019; Andrade et al., 2018), which corroborates the results of the current investigation.

Evidences suggest that *Enterobacter* spp. and *Klebsiella* spp. are important bacteria harboring silver tolerance genes in relation to other bacterial species (Andrade et al., 2018; Sütterlin et al., 2017). However, in the present study, *silA* was found in different species such as *Acinetobacter baumannii*, *Hafnia alvei*, *Pseudomonas aeruginosa*, *Raoultella ornithinolytica*, *Serratia fonticola* and *Serratia liquefaciens*, on which there are few data in the scientific literature. Detection of different species carrying *silA* may be related to horizontal gene transfer and/or exposure to silver used in various medical dispositive in hospitals, such as catheters and heart valves to prevent biofilms or topical creams for treating burn wounds and in preventive eye care solution (Mijnendonckx et al., 2013). Other exposure routes to silver comprise of dental amalgams and its presence in water due to the use as disinfectant for water systems (swimming pool water, hot water systems for hospitals and drinking water) (Mijnendonckx et al., 2013).

Like silver, copper is also widely used in hospitals especially in surfaces for prevent biofilm formation and health care-associated infections, and obstetrics, where copper-impregnated sanitary pads are used to prevent postpartum infections (Arendsen et al., 2019). In addition, copper is also used in agricultural as wood preservatives, antifungal agents, algicidal, pesticide and in animal food supplementation (Hobman and Crossman, 2015). Although copper is an essential metal for aerobic life, high cell concentrations can become toxic, leading to detoxification mechanisms development (such as copper efflux pump encoded by *pco* operon). Therefore, high concentrations of copper, resulting from various exposure routes, can influence the high occurrence of bacteria carrying *pcoD* here found.

The HMTG *merA, arsB, czcA* and *terF* were detected in 47%, 13%, 13% and 7% of the isolates, respectively (Figure 1). Similarly to silver and copper, these metals are also used in human medicine, as medical devices or as antimicrobial agents, being present in the environment which may reflect the occurrence of these genes in bacteria from wastewater. The *merA* gene has been detected in several Gram-negative species, being known that the *mer* operon is associated with the transposon Tn*21* (Liebert et al., 1999). The *arsB* gene was found in *Hafnia alvei, Klebsiella pneumoniae* and *Acinetobacter baumannii.* Although arsenic is employed for some medical uses and agriculture, which can lead to the tolerance mechanism, it is suggested that the presence of *ars* genes in plasmids and transposons is ancestral (Fekih et al., 2018; Hobman and Crossman, 2015). In turn, the *czcA* gene is frequently found in *Pseudomonas aeruginosa*, which corroborates the current results. However, it was also detected in an isolate of *Serratia liquefaciens*, suggesting the transmission of mobile genetic elements between species. On the other hand, the *terF* gene was detected in three species (*Enterobacter cloacae, Hafnia alvei* and *Klebsiella pneumoniae*). Historically, tellurium has been used as an antimicrobial for the treatment of some infections (e.g., leprosy and tuberculosis) and in different industrial activities (Zare et al., 2017).

Table 2 and Figure 2 show the occurrence and coexistence of HMTG in bacteria by sampling site and Pearson’s Correlation, respectively. It was observed that bacteria from ward wastewater had a diversity of HMTG compared to bacteria from ambulatory wastewater, which may be related to patient's hospitalization period and, consequently, bacteria exposure to metals that are used in different medical areas (e.g., metallic surface and topical cream). Among the positive isolates (*n* = 37), coexistence was observed in 68% of isolates (Table 2). A very strong positive correlation was found between *silA* and *pcoD* genes (*r* = 0.9107, 95% CI [0.84; 0.95], p < 0.0001) (Figure 2). The *sil* operon and *pco* operon are often found adjacent, being known as CHASRI (copper homeostasis and silver resistance island) (Staehlin et al., 2016), which explain the coexistence of *silA* and *pcoD* and the very strong correlation. Although there was no strong correlation in bacteria co-harboring *merA, arsB* and *terF* (Figure 2), we verified the mercury tolerance gene was frequently detected with other HMTG, and the arsenic and tellurium tolerance genes exclusively in coexistence with other HMTG (Table 2). Moreover, the *czcA* gene appeared in coexistence only in two isolates (Table 2), with a negative correlation with other HMTG (Figure 2). It may suggest that this gene is more frequently found without other heavy metals tolerance determinants.

Table 3 shows the coexistence between HMTG and ARGs. Data on ARGs were obtained from Zagui et al. (2020), who evaluated the occurrence of β-lactamase encoding genes in the same bacterial collection, and when crossed of HMTG results, it was found that 15 isolates co-harbored ARGs. Interestingly, 14 isolates that co-harboring ARGs were positive for the *silA* and *pcoD* genes. Although the coexistence between HMTG and ARGs in the same plasmid is rare (Pal et al., 2015), some authors have reported the coexistence of these genes in plasmids (Fang et al., 2016; Mourão et al., 2015). Fang et al. (2016) detected silver and copper tolerance genes in IncHI plasmid, which contained genes for resistance to β-lactams and quinolones in *Escherichia coli* from food-producing animals. Also, Mourão et al. (2015) in *Salmonella* from different sources. According to Pal et al. (2015) only mercury tolerance genes and ARGs are really linked. It may be due to association with Tn*21* that often carries ARGs (Liebert et al., 1999). Notwithstanding, a number of studies have demonstrated the coexistence between HMTG and ARGs (Ferreira et al., 2019; Sütterlin et al., 2014), which would suggest more than one plasmid in bacteria and/or chromosomal integration, as incited by Andrade et al. (2018). Considering that metals are promising for antibiotic-resistant bacteria infection treatment, detection of isolates co-harboring HMTG and ARGs means a concern taking into account that it can lead to therapeutic failure and selection of bacteria resistant to antibiotic and tolerant to metals (Yu et al., 2017).

Based on the results of the present investigation, bacteria in hospital wastewater showed HMTG diversity, a worrying fact, since the Brazilian legislation allows the release of untreated hospital wastewater in municipal sewage collection network, as well as allowed in other countries (Carraro et al., 2016). Although we have identified a few isolates (21.6%) carrying HMTG in the samples from WWTP, possibly due to effluent dilution upon reaching WWTP, it is known that these sites exert selective pressure and offer excellent conditions for genetic exchange due to nutrients and hydraulic retention time wastewater for treatment (Manaia et al., 2018). Therefore, the real occurrence of bacteria carrying HMTG may be being underestimated. Di Cesare et al. (2016) found high abundance of *arsB* and *czcA* in three WWTP in Italy, suggesting an important role of HMTG spread mediated by mobile genetic elements involved in ARGs transfer and by *int1* integrase. Isolates carrying HMTG were detected in treated effluent at WWTP (Tabela 2), indicating that the employed treatment was not totally efficient for bacteria removal. Consequently, bacteria carrying HMTG in treated effluent may be being released on receptor river. In Ganges river, India, a high abundance of HMTG conferring tolerance to copper, iron, cobalt and others metals was detected in water and sediments, being associated with pollution by wastewater and diffuse sources (Reddy and Dubey, 2019). River water pollution by WWTP effluents pose risks to human health since rivers are often used for recreational activities, irrigation of farmland, and drinking water supply after treatment (Bengtsson-Palme et al., 2018). Thus, river pollution by bacteria carrying HMTG can reach to human due to different exposure routes. Therefore, further studies focused on assessing bacteria carrying metal tolerance genes in wastewater and rivers are needed to evaluate environmental and human health risks and improve control measures.

4. Conclusions

A high occurrence of bacteria carrying HMTG was detected in wastewater, especially in samples from hospital, and genes that confer silver (*silA*) and copper (*pcoD*) tolerance were the most prevalent. HMTG are probably more dispersed than ARGs in bacteria, and they represent a new concern to use heavy metals as effective antimicrobials. Consequently, in cases where heavy metals are considered for antimicrobial treatment, it is important to evaluate the HMTG occurrence in bacterial isolates. To the best of our knowledge, this is the first study on the HMTG searched in *Hafnia alvei*, *Serratia fonticola* and *Serratia liquefaciens*. The release of untreated hospital sewage in a municipal collection network is a problem shared by many countries, so it is expected that the results of this study can contribute to future revisions of the legal regulations for implementation of hospital wastewater treatment, especially in large hospitals. Detection of HMTG in treated effluent in

WWTP requires the implementation of additional technologies to reduce environmental impacts on receptor river and HMTG spread.

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Table 1. Primers used for acquired heavy metal tolerance genes detection.

Ta gene	**Primers**	**Nucleotide sequence (5'–3')**	**size (pb)**	**temp. (ºC)**	**Reference**
arsB	arsB_F arsB_R	AGTGAAAGACAGACGAAGCG GGCAGATAGTGTGGAATGCG	1136	60	Mourão et al. 2015
czcA	czcA_F czcA_R	GTTCACCTTGCTCTTCGCCATGTT ACAGGTTGCGGATGAAGGAGATCA	206	63	Bouskill et al. 2007
merA	merA_F merA_R	ACCATCGGCGGCACCTGCGT ACCATCGTCAGGTAGGGGAAC	1238	67	Mourão et al. 2015
pcoD	pcoD_F pcoD_R	CTGGCCACACTTGCCTGGGG CACGCTACGGCGCCCAGAAT	500	55	Mourão et al. 2015
silA	silA_F silA_R	GCAAGACCGGTAAAGCAGAG CCTGCCAGTACAGGAACCAT	936	59	Mourão et al. 2015
terF	terF_F terF_R	ATGCAGGCTCAAGGAATCGC TTCATCGATCCACGGTCTG	990	63	Mourão et al. 2015

Table 2. Occurrence of heavy metal tolerance genes in bacteria isolated from wastewater by sampling site.

Sampling site	*n*	Species	Heavy metal tolerance genes
Ambulatory hospital wastewater	1	*Klebsiella pneumoniae*	*silA, pcoD, merA*
	1	*Klebsiella oxytoca*	*pcoD, merA*
	1	*Klebsiella ozaenae*	*silA, pcoD*
	1	*Escherichia coli*	*merA*
	1	*Klebsiella oxytoca*	*merA*
	1	*Pseudomonas aeruginosa*	*czcA*
	1	*Raoultella ornithinolytica*	*merA*
Ward hospital wastewater	1	*Hafnia alvei*	*silA, pcoD, merA, arsB, terF*
	1	*Serratia liquefaciens*	*silA, pcoD, merA, czcA*
	3	*Klebsiella pneumoniae*	*silA, pcoD, merA*
	3	*Klebsiella pneumoniae*	*silA, pcoD*
	1	*Serratia fonticola*	*silA, pcoD*
	3	*Pseudomonas aeruginosa*	*czcA*
Confluence of hospital wastewater	1	*Klebsiella pneumoniae*	*silA, pcoD, merA, arsB, terF*
	3	*Klebsiella pneumoniae*	*silA, pcoD, merA, arsB*
	1	*Pseudomonas aeruginosa*	*silA, pcoD, merA, czcA*
	2	*Klebsiella pneumoniae*	*silA, pcoD*
	1	*Klebsiella pneumoniae*	*merA*
	2	*Pseudomonas aeruginosa*	*merA*
Raw urban wastewater (WWTP)	1	*Klebsiella pneumoniae*	*silA, pcoD*
	1	*Klebsiella pneumoniae*	*silA, merA*
	1	*Raoultella ornithinolytica*	*silA, pcoD*
	1	*Serratia liquefaciens*	*silA, pcoD*
	1	*Klebsiella pneumoniae*	*merA*
Treated urban wastewater (WWTP)	1	*Acinetobacter baumannii*	*silA, pcoD, arsB*
	1	*Enterobacter cloacae*	*silA, pcoD, terF*
	1	*Pseudomonas aeruginosa*	*merA*

Table 3. Coexistence between heavy metal tolerance genes and β-lactamase encoding genes in bacteria isolated from hospital and urban wastewater samples.

Species	**Heavy metal tolerance genes**	***bla* genes***
Klebsiella pneumoniae	*silA, pcoD, merA, arsB, terF*	TEM
	silA, pcoD, merA, arsB	KPC, TEM
	silA, pcoD, merA, arsB	TEM
	silA, pcoD, merA, arsB	TEM
	silA, pcoD, merA	SHV, TEM
	silA, pcoD, merA	KPC
	silA, pcoD	KPC, CTX-M-8, SHV, TEM
	silA, pcoD	CTX-M-1, SHV, TEM
	silA, pcoD	KPC, SHV
	silA, pcoD	KPC
	merA	KPC
Hafnia alvei	*silA, pcoD, merA, arsB, terF*	CTX-M-1, SHV, TEM
Pseudomonas aeruginosa	*silA, pcoD, merA, czcA*	KPC
Serratia liquefaciens	*silA, pcoD, merA, czcA*	KPC
Enterobacter cloacae	*silA, pcoD, terF*	SHV

*Data on β-lactamase encoding genes were obtained from Zagui et al. (2020).

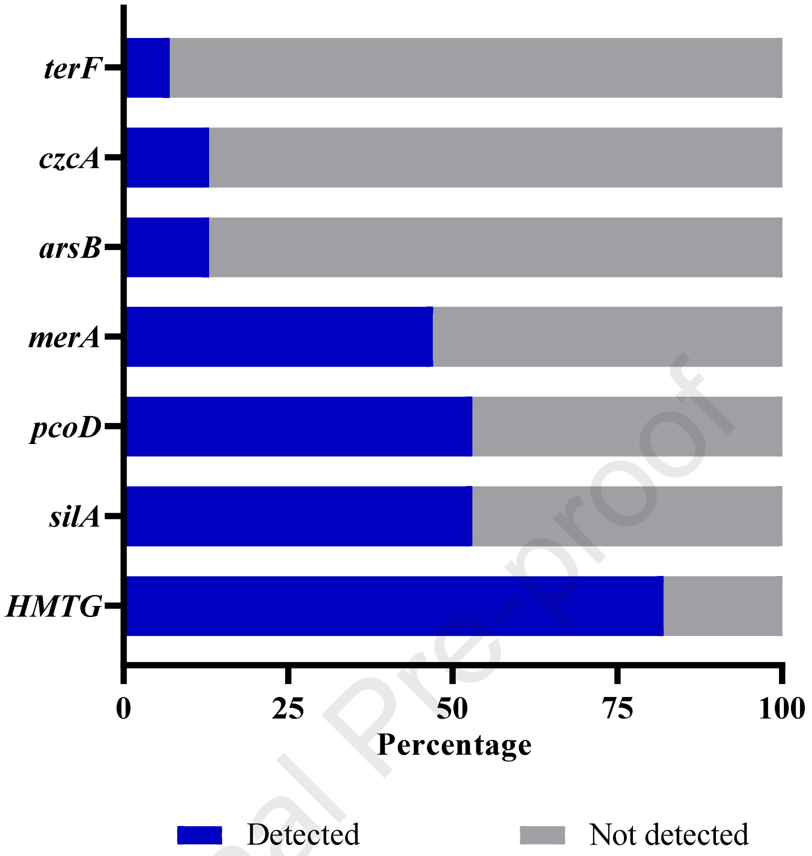

Figure 1. Occurrence (%) of heavy metal tolerance genes (HMTG) in bacteria isolated from wastewater samples.

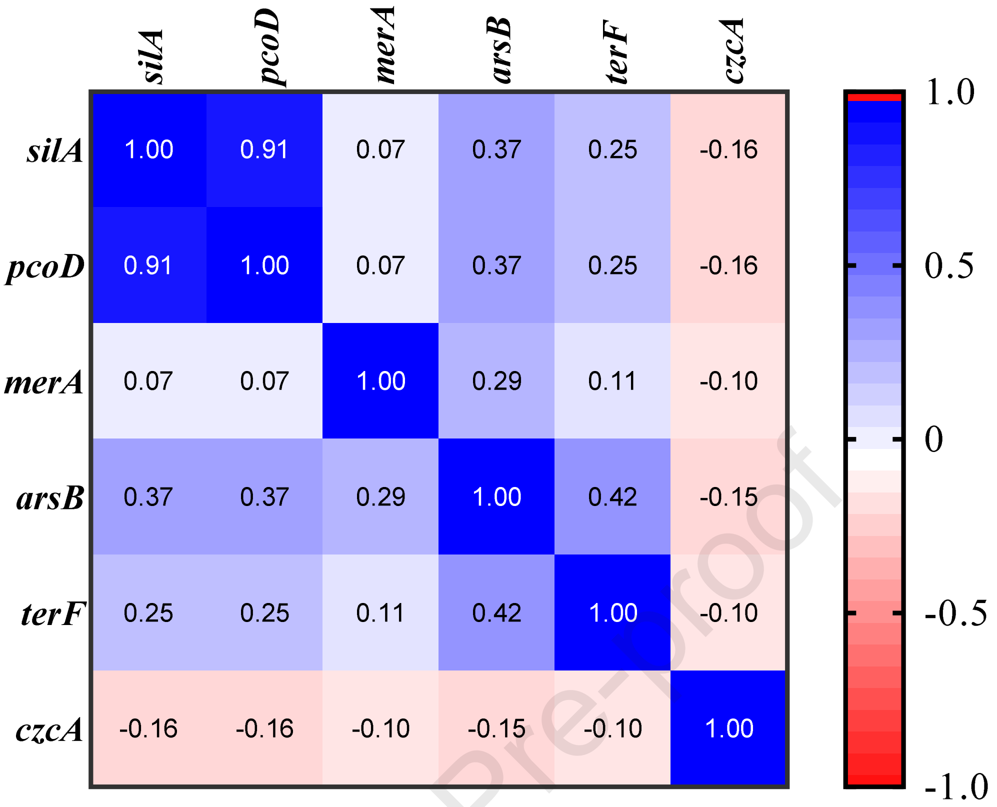

Figure 2. Heat Map showing Pearson's Correlation between heavy metal tolerance genes, where $r > 0.90$ means a very strong correlation.

Highlights

- Metal tolerance genes were detected in several Gram-negative species.
- Genes that confer tolerance to silver and copper were the most prevalent.
- HMTG represent a new concern to metal use as antimicrobials.
- HMTG are probably more dispersed than ARGs in bacteria.
- Improvements in sewage management are needed to control the HMTG spread.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: