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Single and combined antimicrobial efficacies for nine metal ion solutions against *Klebsiella pneumoniae*, *Acinetobacter baumannii* and *Enterococcus faecium*

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**ABSTRACT**

Infection caused by *Klebsiella pneumoniae*, *Acinetobacter baumannii* and *Enterococcus faecium* can be difficult to treat. New biocidal products are needed in order to reduce the transmission of such bacteria from surfaces to patients. This fundamental study aimed to investigate the antimicrobial efficacy of nine metal ion solutions (yttrium, indium, niobium, titanium, tantalum, rhodium, ruthenium, zinc and gallium) using zone of inhibition (ZoI), minimum inhibitory concentration (MIC) and minimum bactericidal concentrations (MBC). Fractional inhibitory concentration (FIC) and fractional bactericidal concentration (FBC) assays were used to determine antimicrobial activities of various combinations. The rhodium metal ion solution when applied singly demonstrated the best antimicrobial efficacies against all the bacteria tested. FICs indicated that the rhodium/ruthenium combination was either synergistic or additive against all three tested bacteria. This metal ion combination also exhibited synergistic activity against *E. faecium* following in FBCs. Our data presented indicated the potential of the rhodium and/or ruthenium metal ions for antisepsis, disinfection and for incorporation into hygienic surfaces.

1. Introduction

There has been a rise in the number of multidrug resistant (MDR) bacterial infections in hospital settings leading to increased mortality, morbidity, hospitalization and treatment costs (Olar et al., 2010). It is estimated that 9% of in-patients in England and Wales suffer from hospital-acquired infections, which is reported to result in around 5000 deaths and extra care related costs of over £1 billion per year. These infections are caused in part by transmission across the wards (Smith and Hunter, 2008). There are a number of pathogens that are demonstrating increasing resistance from the biocidal action of antimicrobial agents, producing a new mode of pathogenesis (Pendleton et al., 2013). *Klebsiella pneumoniae*, *Acinetobacter baumannii* and *Enterococcus faecium* are three such bacteria out of the six ‘ESKAPE’ pathogens that are considered to be a leading cause of nosocomial infections (Santajit and Indrawattana, 2016). Such bacteria may persist on hospital and biomaterial surfaces including catheters, stethoscopes and disinfectant soap dispensers (Smith and Hunter, 2008). Ampicillin and vancomycin resistant *E. faecium* has demonstrated a constant threat in the incidence of health-care infections (Pendleton et al., 2013). In recent times, *K. pneumoniae* and *A. baumannii* have acquired the ability to synthesize a variety of beta-lactamase enzymes that can destroy the chemical structure of beta-lactam antibiotics, thus making these bacteria multi-drug resistant (Santajit and Indrawattana, 2016).

The lack of potential antimicrobial agents against such bacteria is a major cause for concern. Thus, there is a need to develop novel approaches to prevent the transmission of bacteria that cause such infections (Santos et al., 2014). Metals such as molybdenum, titanium and tantalum have been used as biomaterials coatings on implants to decrease the bacterial adherence (Ribeiro et al., 2016; Chang et al., 2014; Haenle et al., 2011). Rhodium complexes with tetraaza macrocyclic and ruthenium (II) carbonyl thiosemicarbazone complexes have been shown to have effective antimicrobial efficacy against range of bacteria (Bien et al., 1999; Kannan et al., 2008; Jayabalakrishnan and Natarajan, 2002). Gallium and zinc ions co-ordinated with protoporphyrin and mesoprotoporphyrin respectively have showed up to 90% antibacterial efficacy against *Staphylococcus epidermidis* and *Pseudomonas aeruginosa* (Ma et al., 2013). Titanium and its alloys have been used in various medical implants such as bone screws, dental restorations and artificial joints owing to their biocompatibility and also reputedly due to their
bactericidal properties (He et al., 2017). This fundamental study investigated the antimicrobial efficacy of nine metal ion solutions individually and in combination against K. pneumoniae, A. baumannii and E. faecium to determine if they might provide potential biocidal solutions.

2. Materials and methods

2.1. Cultures and media

Enterococcus faecium NCTC 7171 was cultured onto Columbia blood agar (Oxoid, UK) (supplemented with 20 mL defibrinated horse blood), incubated in 5% CO₂ for 24 h at 37 °C in static conditions. K. pneumoniae NCTC 9633 and A. baumannii NCTC 12156 were cultured onto Nutrient agar (NA) (Oxoid, UK) and incubated for 24 h at 37 °C. Brain heart infusion (BHI) agar (Oxoid, UK) and brain heart infusion broth (BHIB) (Oxoid, UK) (E. faecium) and nutrient agar and nutrient broth (NB) (Oxoid, UK) (K. pneumoniae or A. baumannii) were used for all subsequent experiments and were incubated as above (Vaidya et al., 2017).

2.2. Preparation of metal ion solutions

Standard solutions of 1000 mg L⁻¹ of yttrium (Y₂O₃ + HNO₃), titanium (Ti metal + HNO₃), tantalum (Ta metal + HNO₃ (HF traces)), indium (In metal + HNO₃), niobium (Nb metal + HNO₃ (HF traces)), rhodium (RhCl₃ + HCl), ruthenium (Re metal + HNO₃), zinc (Zn metal + HNO₃) and gallium (Ga metal + HNO₃) (Sigma-Aldrich, UK) were used and diluted with sterile water to obtain 500 mg L⁻¹, 100 mg L⁻¹ and 50 mg L⁻¹ metal concentrations.

2.3. Bacterial preparation

The appropriate broth was inoculated with a single colony of bacteria and incubated overnight according to the aforementioned conditions. Cells were harvested at 567 g for 10 min and washed using sterile distilled water three times for the ZoI test and double strength broth for cells to be used in the MIC/MBC tests. Cells were re-harvested by centrifugation for 10 min at 567 g and adjusted using sterile distilled water to an optical density (OD) 1.0 ± 0.1. The colony forming units per mL (CFU mL⁻¹) were calculated and cell concentrations corresponded to K. pneumoniae 2.82 × 10⁸, A. baumannii 1.85 × 10⁸ and E. faecium 3.95 × 10⁸.

2.4. Zone of inhibition (ZoI) assays

One hundred microliters of prepared cell suspension was spread across the agar and three wells (8 mm diameter) were cut from the agar. One hundred microliters of the metal ion solution (at different metal ion solution concentrations, 50 mg L⁻¹, 100 mg L⁻¹, 500 mg L⁻¹ and 1000 mg L⁻¹) was added to the well and the plates were incubated as specified above. The radius of inhibition was measured in mm to determine the average mean value (n = 12).

2.5. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) assays (adapted form Vaidya et al., 2017)

To 9 mL of cell suspension, one mL of triphenyl tetrazolium chloride (TTC) blue metabolic dye (Sigma-Aldrich, UK) was added. One hundred microliters of bacterial dye with the TTC dye and the metal ion solutions was added to a 96 well flat-bottomed micro titre plate. The first column of was mixed, and subsequent 100 μL of the sample/bacterial mix was transferred to sequential wells and repeated until column 10, whereby 100 μL of the mixture was disposed of. A positive control and a negative control was carried out at the same time. The MIC was taken as lowest concentration that inhibited the visible growth of the bacteria, indicated by a change of colour to blue. From the first well that showed no growth and the last well that demonstrated growth, 25 μL of suspension was pipetted onto agar and incubated. The lowest concentration that showed no bacterial growth was determined to be the MBC (n = 3).

2.6. Fractional inhibitory concentrations (FIC) and fractional bactericidal concentrations (FBC) index

This method was adapted from Vaidya et al. (2017). In brief, the MIC and MBC metal ion solution synergies were determined using FIC and FBC antimicrobial screening respectively. Both metal ion solutions were added in a 1:1 ratio to the wells. The FIC and FBC values were calculated using the following formula; FIC or FBC = FIC or FBC of antimicrobial A + FIC or FBC of antimicrobial B = [1]

\[
\text{MIC or MBC of antimicrobial A in combination} = \frac{\text{MIC or MBC of antimicrobial A alone}}{\text{MIC or MBC of antimicrobial A in combination}} + \frac{\text{MIC or MBC of antimicrobial B in combination}}{\text{MIC or MBC of antimicrobial B alone}}
\]

The antimicrobial interaction was evaluated as ≤0.5 = synergy, > 0.5 ≤ 1 = additivity, > 1 ≤ 4 = autonomy and > 4 = antagonism (Doern, 2014) (n = 3).

2.7. Statistical analysis

The average values were used to compare the results and standard error to determine the distributions of the data. The intervals at 95% confidence were also determined.

3. Results

The nine metal ion solutions showed varying levels of toxicity against the bacteria. The metal ions were in an acidic solution and control assays were carried out to determine the effects of the acids on bacterial viability. It was found that the acids did not significantly affect the results (data not shown).

3.1. Zone of inhibition

The ZoI results displayed a significant increase in antimicrobial activity with an increase in concentration (p < 0.05). At a concentration of 1000 mg L⁻¹ the rhodium metal ion solution was the most antimicrobial (11.5 mm, 12.5 mm and 7 mm K. pneumoniae, A. baumannii and E. faecium respectively) followed by ruthenium for K. pneumonia (10.66 mm) and A. baumannii (8 mm) with antimicrobial efficacy demonstrated for titanium and tantalum against E. faecium (both at 5 mm) (Fig. 1). Overall, at low concentrations, the least antimicrobially effective metal ion solutions were yttrium and zinc. With the exception of titanium ion solution, all the metal ion solutions demonstrated no antimicrobial efficacy at lower concentrations (50 mg L⁻¹ and 100 mg L⁻¹) against E. faecium (Fig. 1) (p > 0.05). Thus, Enterococcus faecium was found to be the most resistant at all the metal ion solutions tested concentrations.

3.2. Minimum inhibitory and bactericidal concentrations

Following the MIC assay, the rhodium and tantalum metal ion solution showed the best antimicrobial efficacy against all the tested bacteria (7.81 mg L⁻¹ against the Gram-negative bacteria and 31.25 mg L⁻¹ against E. faecium). The ruthenium metal ion solution was also inhibitory against A. baumannii and E. faecium (7.81 mg L⁻¹ and 31.25 mg L⁻¹ respectively) and the titanium metal ion solution was also inhibitory against E. faecium (31.25 mg L⁻¹). The zinc ion solution against all three bacteria (31.25 mg L⁻¹ K. pneumoniae and A. baumannii; 125.00 mg L⁻¹ against E. faecium) and the yttrium ion solution...
demonstrated the least antimicrobial inhibition (Table 1).

Following the MBC assays, only the rhodium and the ruthenium demonstrated bactericidal activity against all three bacteria (15.62 mg L\(^{-1}\), 7.81 mg L\(^{-1}\) and 62.50 mg L\(^{-1}\) against \(K.\) pneumo\(n\)iae, \(A.\) baumann\(n\)ii and \(E.\) faecium respectively). Against \(K.\) pneumo\(n\)iae, titanium, tantalum and gallium demonstrated bactericidal activity (15.62 mg L\(^{-1}\)), and against \(E.\) faecium tantalum and gallium also demonstrated bactericidal activity (15.62 mg L\(^{-1}\) and 62.50 mg L\(^{-1}\) respectively). The least bactericidal solutions were niobium (\(K.\) pneumo\(n\)iae 46.87 mg L\(^{-1}\)), zinc, indium and yttrium (\(A.\) baumann\(n\)ii 15.62 mg L\(^{-1}\)) and zinc (\(E.\) faecium 250.00 mg L\(^{-1}\)) (Table 2). In both of the tests, \(E.\) faecium was found to be the most resistant bacteria.

### 3.3. Fractional inhibitory concentrations and FBC index

The FIC (Table 3) and FBC was used to determine the synergistic antimicrobial efficacy of the metal ion solutions in combination. Following the FIC test, against \(K.\) pneumo\(n\)iae only the rhodium/ruthenium combination demonstrated an additive effect (0.74). All the metal ion solution combinations demonstrated additivity effects against \(A.\) baumann\(n\)ii (0.74–0.99) and \(E.\) faecium (0.99).

Following the FBC synergy assay (Table 4), only the rhodium/ruthenium combination demonstrated a synergistic antimicrobial efficacy against Gram-positive \(E.\) faecium (FBC = 0.48) (Table 4). The titanium/

| Metal ion solutions | \(K.\) pneumo\(n\)iae | \(A.\) baumann\(n\)ii | \(E.\) faecium |
|---------------------|----------------------|----------------------|----------------------|
| Zinc                | 31.25 ± 0            | 31.25 ± 0            | 125.00 ± 0          |
| Titanium            | 15.62 ± 0            | 15.62 ± 0            | 31.25 ± 0           |
| Tantalum            | 7.81 ± 0             | 7.81 ± 0             | 31.25 ± 0           |
| Indium              | 15.62 ± 0            | 15.62 ± 0            | 62.50 ± 0           |
| Yttrium             | 15.62 ± 0            | 31.25 ± 0            | 125.00 ± 0          |
| Rhodium             | 7.81 ± 0             | 7.81 ± 0             | 31.25 ± 0           |
| Ruthenium           | 15.62 ± 0            | 7.81 ± 0             | 31.25 ± 0           |
| Gallium             | 15.62 ± 0            | 11.75 ± 2.76         | 62.50 ± 0           |
| Niobium             | 15.62 ± 0            | 15.62 ± 0            | 62.50 ± 0           |

(31.25 mg L\(^{-1}\) and \(A.\) baumann\(n\)ii; 125.00 mg L\(^{-1}\) against \(E.\) faecium) demonstrated the least antimicrobial inhibition (Table 1).

Following the MBC assays, only the rhodium and the ruthenium demonstrated bactericidal activity against all three bacteria (15.62 mg L\(^{-1}\), 7.81 mg L\(^{-1}\) and 62.50 mg L\(^{-1}\) against \(K.\) pneumo\(n\)iae, \(A.\) baumann\(n\)ii and \(E.\) faecium respectively). Against \(K.\) pneumo\(n\)iae, titanium, tantalum and gallium demonstrated bactericidal activity (15.62 mg L\(^{-1}\)), and against \(E.\) faecium tantalum and gallium also demonstrated bactericidal activity (15.62 mg L\(^{-1}\) and 62.50 mg L\(^{-1}\) respectively). The least bactericidal solutions were niobium (\(K.\) pneumo\(n\)iae 46.87 mg L\(^{-1}\)), zinc, indium and yttrium (\(A.\) baumann\(n\)ii 15.62 mg L\(^{-1}\)) and zinc (\(E.\) faecium 250.00 mg L\(^{-1}\)) (Table 2). In both of the tests, \(E.\) faecium was found to be the most resistant bacteria.

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Following the FBC synergy assay (Table 4), only the rhodium/ruthenium combination demonstrated a synergistic antimicrobial efficacy against Gram-positive \(E.\) faecium (FBC = 0.48) (Table 4). The titanium/
Table 2
Minimum bactericidal concentrations in mg/L for nine metal ion solutions against three tested pathogens demonstrating the best antimicrobial efficacy for Ru and Rh ion solutions (n = 3).

| Metal ion solutions | K. pneumoniae | A. baumanii | E. faecium |
|---------------------|---------------|-------------|------------|
| Zinc                | 31.25 ± 0     | 31.25 ± 0   | 250.00 ± 0 |
| Titanium            | 15.62 ± 0     | 15.62 ± 0   | 125.00 ± 0 |
| Tantalum            | 15.62 ± 0     | 15.62 ± 0   | 62.50 ± 0  |
| Indium              | 31.25 ± 0     | 31.25 ± 0   | 125.00 ± 0 |
| Yttrium             | 23.43 ± 5.52  | 31.25 ± 0   | 125.00 ± 0 |
| Rhodium             | 15.62 ± 0     | 7.81 ± 0    | 62.50 ± 0  |
| Ruthenium           | 15.62 ± 0     | 7.81 ± 0    | 62.50 ± 0  |
| Gallium             | 15.62 ± 0     | 15.62 ± 0   | 62.50 ± 0  |
| Niobium             | 46.87 ± 11.04 | 15.62 ± 0   | 125.00 ± 0 |

Table 3
Fractional inhibitory concentration index for six tested metal ion solutions combinations demonstrating combined effects for all metal ion solutions against K. pneumoniae, A. baumanii and E. faecium. FIC index = ≤ 0.50 = synergy, > 0.50 ≤ 1.00 = additivity, > 1.00 ≤ 4.00 = autonomy and > 4.00 = antagonism.

| Metal ion solutions | K. pneumoniae | A. baumanii | E. faecium |
|---------------------|---------------|-------------|------------|
| Titanium/Tantalum   | 1.50          | 1.50        | 1.00       |
| Titanium/Rhodium    | 0.74          | 0.74        | 0.74       |
| Titanium/Ruthenium  | 0.99          | 0.99        | 0.99       |
| Tantalum/Rhodium    | 2.00          | 2.00        | 2.00       |
| Tantalum/Ruthenium  | 3.00          | 3.00        | 3.00       |
| Rhodium/Ruthenium   | 0.74          | 0.99        | 0.99       |

Table 4
Minimum bactericidal concentration synergy index demonstrating combined effects of antimicrobial activity for metal ions against K. pneumoniae, A. baumanii and E. faecium. FBC index = ≤ 0.50 = synergy, > 0.50 ≤ 1.00 = additivity, > 1.00 ≤ 4.00 = autonomy and > 4.00 = antagonism.

| Metal ion solutions | K. pneumoniae | A. baumanii | E. faecium |
|---------------------|---------------|-------------|------------|
| Titanium/Tantalum   | 2.00          | 2.00        | 2.00       |
| Titanium/Rhodium    | 1.00          | 1.50        | 1.50       |
| Titanium/Ruthenium  | 0.75          | 0.75        | 0.56       |
| Tantalum/Rhodium    | 1.00          | 1.00        | 1.00       |
| Tantalum/Ruthenium  | 2.00          | 2.00        | 2.00       |
| Rhodium/Ruthenium   | 0.74          | 0.75        | 0.56       |

4. Discussion

The reoccurrence of MDR infections poses a great risk to public health. The development of novel antimicrobial agents and biocides to control and prevent the transmission of bacteria associated with hospital-acquired infections is in need of exploration (Haenle et al., 2011). This study found that the rhodium ion solution showed the best antimicrobial efficacies when tested alone or in combinations. The differences in the antimicrobial efficacies of the metal ions antimicrobial action may be due to their mechanisms of action. The antimicrobial activity of metals may be due to single or combined mechanisms such as enzyme disruption, cell-membrane/cell-wall degradation, deoxyribonucleic acid denaturation, protein dysfunction or oxidative stress (Lemire et al., 2013; Bruins et al., 2000; Varkey, 2010; Mitchell and Kogure, 2006). It has been suggested that physical contact of metal ions with the bacterial cell wall and internalization in the cell might cause oxidation of the cellular components generating reactive oxygen species and interruption of the transmembrane electron transport chain (Dizaj et al., 2014; Kolmas et al., 2014). The antibacterial activity of the rhodium ion solution may be due to its liposolubility, electro-negativity and initiation of redox reactions (Lemire et al., 2013; Beloglazkina et al., 2016). Rhodium metal possesses liposolubility properties, which is suggested to favour its cell permeability aiding in a greater transport inside a bacterial cell membrane (Bien et al., 1999).

Inside the bacterial cell, the higher electronegativity of rhodium (2.28) might demonstrate an increase affinity for amine, phosphate or sulf-hydryl groups compared to other tested metal ion solutions (for example Y = 1.22 or In = 1.78) (Beloglazkina et al., 2016; Varkey, 2010). Rhodium being a member of d-block transition metals possesses a tendency to lose electrons and be reduced (Greenwood et al., 1998). This redox reaction between rhodium ions and phosphate/amine/sulf-hydryl groups may possibly affect two vital processes inside the bacterial cell. Firstly, rhodium can bind to the large cavities of the ribosome, such as the peptide-conducting tunnel passing through the ribosomal subunit. Secondly, it might hinder the translation and transcription process required for the RNA and DNA formation. Thus, this two-way redox reaction leads to protein dysfunction and ultimately destruction of bacteria cell (Beloglazkina et al., 2016; Bien et al., 1999).

It is known that by adding antimicrobial substances together, a synergistic, additive, indifferent or antagonistic result might occur (Doern, 2014). In combination, the most effective metal ion solution was demonstrated to be rhodium/ruthenium. In agreement with our results, rhodium complexes with tetraaza macrocyclic and ruthenium (II) carbonyl thiosemicarbazone complexes have been shown to have effective antimicrobial efficacy against range of bacteria (Bien et al., 1999; Kannan et al., 2008; Jayabalakrishnan and Narayanan, 2002). Further, a rhodium (III) ion complex with tetradeionate macrocyclic was shown to demonstrate inhibitory efficacy when compared to platinum (II) and iridium (II) complexes against E. coli and S. aureus (Chandra et al., 2011). Further studies are needed to understand the mode of action of the antimicrobials when used in combination.

This study also demonstrated that tantalum and titanium had some antimicrobial properties. In respect to the other metal ion solutions tested, various values have been previously reported, which seem to be dependent on the formulation of the antimicrobial compounds. In our work, the antimicrobial activity of titanium metal ion solutions was demonstrated. Bis(cyclopentadienyl)titanium (IV) at 1000 ppm has been show to demonstrate greater antimicrobial efficacy using the Zol assay (up to 26 mm) than when used without a titanium (IV) complex (up to 16 mm) against E. coli, S. aureus and Bacillus subtilis (Srivastava et al., 2005; Cai et al., 2012). Surfaces coated with 80% titanium have also been shown to have significant antibacterial properties against E. coli in a bacterial adhesion test (Seddiqi et al., 2014). In work by others, tantalum oxynitride thin films were shown to have little antibacterial efficacy unless combined with silver ions using visible light radiation against E. coli (Hsieh et al., 2010). Gallium and zinc ions co-ordinated.
with protoporphyrin and mesoporphyrin respectively have shown up to 90% antibacterial efficacy against Staphylococcus epidermidis and Pseudomonas aeruginosa (Ma et al., 2013). However, in our work zinc and gallium were not the most antimicrobial metal ion solutions tested. In contrast to our results, indium compounds with curcumin and diacetylcurcumin and ytrrium (III) complex with phenanthroline (at 0.05 mg L⁻¹) have also been shown to have low MICs (187 μg mL⁻¹ to 23 μg mL⁻¹) against E. coli, S. aureus, P. aeruginosa, B. subtilis and S. epidermidis (Tajbakhsh et al., 2008). Our study also showed some antimicrobial efficacies for the tantalum metal ion solutions. The addition of niobium to copper (3.8%) has been shown to decrease the bacterial count by up to 99% against E. coli using viable bacterial count test (Baena et al., 2006). However, in our study, the antimicrobial efficacy of the niobium metal ion solutions were not significant in the MIC and MBC tests. Overall, E. faecium was the most resistant bacteria in the individual ion metal ions, but was the most sensitive bacteria in the FBC assays whereas for K. pneumonia and A. baumannii, the opposite was found.

5. Conclusion

Overall, the rhodium metal ion solution demonstrated the best antimicrobial efficacy against the three tested bacteria. Only the rhodium/ruthenium combination showed synergism antimicrobial efficacies specifically against E. faecium. This fundamental study suggests that specific metal ion combinations used either individually possess the potential to be used as antimicrobials/biocides.

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Declaration of interest

None.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ibiod.2018.06.017.

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