What Antibiotic Threat Do the Heavy Metals Contaminated Sites of Mine Hide?

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Abstract

The environment contaminated by antibiotics and heavy metals as a consequence of human activities is of great concern nowadays. Many pieces of research proved that the environment could act as a reservoir of antibiotic resistance determinants allowing them to spread among different bacterial species via the process called horizontal gene transfer. The result is antibiotic resistance even in pathogen microorganisms. Heavy metals act as important factors in this process because of their potential to select antibiotic resistant bacteria thanks to linkage among antibiotic resistance genes and heavy metals resistance genes.

Thus, this experiment was conducted to screen the antibiotic tolerance profile of bacteria obtained from heavy metal contaminated environment of mine, dump and the contaminated soil near the entry of mine.

Several samples were collected from the only active gold mine in Slovakia in Hodruša – Hámre. The presence of cultivable bacteria was proved via cultivation approaches with subsequent MALDI – TOF MS (Matrix – Assisted Laser Desorption/Ionisation Time of Flight Mass Spectrometry) identification of selected isolates. Representative bacterial isolates were screened for their antibiotic tolerance against chosen antibiotics (ampicillin (AMP), chloramphenicol (CHLOR), tetracycline (TET) and kanamycine (KAN)) with the aim to define their minimal inhibitory concentration (MIC).

The cultivable bacteria from studied environments were dominated by Gram-negative protebacteria of Pseudomonas and Rhizobium genera. Among more than 150 isolates the resistance to ampicillin (MIC>100μg/ml – 49% isolates), kanamycine (MIC>100μg/ml - 18% isolates), and chloramphenicol (MIC>20μg/ml – 16% isolates) dominated. The resistance to tetracycline (MIC>20μg/ml) was detected in less than 1% of isolates. Overall counts of antibiotic resistance and multi-resistance were alarmingly high taking in account that industrial environments with no known antibiotic exposure were analysed.

Our data indicate that heavy metals contaminated environment could influence the occurrence and the spread of antibiotic resistance. Possibly, metal contaminated environment act as a reservoir of antibiotic resistant bacteria.

Keywords: *mines, antibiotic resistance, heavy metals resistance, cross resistance, heavy metals contamination*

Introduction

Increased industrialization and urbanization have led to substantial quantities of toxic pollutants which are continuously released into the environment worldwide. Some of these compounds occur naturally, but many of them are present in the nature due to anthropogenic activities, especially mining activities. Although mining provides irreplaceable economic and social benefits, its long-term unfavorable impact on the environment cannot be underestimated (Fashola et al. 2016).

Manganese, copper, cobalt, zinc or nickel are essential metals inevitable for growth of microbes, but their higher concentrations in the environment have poisonous effects on not only bacteria, but also on human health (Hobman a Crossman 2015).

Microorganisms are the first line organisms affected by heavy metals contamination. It has also been reported that microbial communities are more influenced by high heavy metals concentrations compared to fungal communities (Piotrowska-Seget et al. 2005; Rajapaksha et al. 2004). In order to survive such hostile conditions of the environment, bacteria were forced to evolve various mechanisms to withstand potentially lethal circumstances (Gadd 2010). These mechanisms include the efflux of metal ions outside the cell, accumulation and complexation of the metal ions inside the cell, reduction of the heavy metal ions to a less toxic state etc. (Issazadeh et al. 2013). Resistance genes

coding for these mechanisms are often found on plasmids. But so are the antibiotic resistance genes (Foster 1983; Ghosh et al. 2000; Stepanauskas et al. 2005; Baker-Austin et al. 2006; Stokes a Gillings 2011; Sandegren et al. 2012). Thus, the close co-occurrence of these two different resistance determinants on the same mobile genetic element together with their physical linkage result in the co-selection also in the case, when only one resistance determinant is being selected by the conditions of the environment (Baker-Austin et al. 2006). And thanks to their location on the mobilizable genetic element, they may be transferred together in the process of horizontal gene transfer between different bacterial communities, even pathogenic ones (Suzuki et al. 2012; Andersson a Hughes 2014). Another potential mechanism involved in the selection and proliferation of antibiotic resistance in the conditions of heavy metals contamination, is cross-resistance. This mechanism could manifest itself through efflux of structurally dissimilar compounds using the same mechanism – efflux pumps, which are relatively non-specific and they are able to pump out of the cell antibiotics and heavy metals in order to lower their intracellular concentration (Baker-Austin et al. 2006). Observational studies showed, that antibiotic resistant bacteria are more often found at locations contaminated with metals (Stepanauskas et al. 2005; Hölzel et al. 2012; Zhu et al. 2013) in comparison with non-metal contaminated areas, what suggest, that
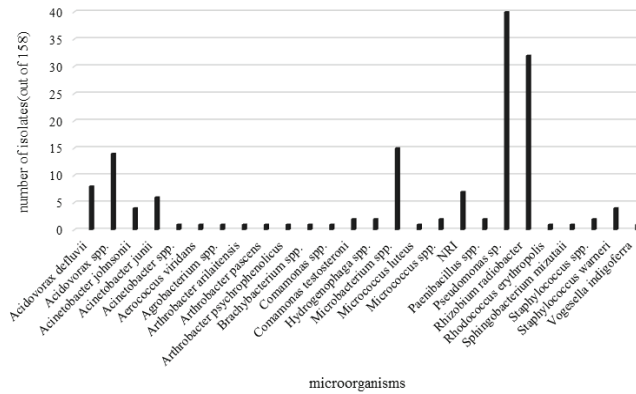
Fig. 1 MALDI-TOF MS identification of bacteria
Rys. 1. Identyfikacja bakterii MALDI – TOF MS

heavy metals may be an additional factor indirectly selecting for antibiotic resistance (Alonso et al. 2001). Accordingly, accumulation of antibiotic resistance genes in soil environment and their assumed linkage to metal resistance genes in microbiota are of concern. Hence, the main objective of this study was to screen the antibiotic tolerance profile of bacteria obtained from heavy metals contaminated environment of mine, dump and the contaminated soil near the entry of mine.

Materials and methods

Two soil samples from the mine, one sample from the dump located near the mine and one sample of the contaminated soil near the entry of mine were collected from the only active gold mine in Hodruša – Hámre, Slovakia (GPS N 48°45.658′ E 18°85.166′).

Approx. 50 g of each sample were collected from the top layer (5–10 cm) into the sterile ziplock bag. After the collection, the samples were kept on ice and transported to the laboratory, where the material was processed until 24 hours.

1 g of thoroughly homogenized sample was suspended in 10 ml PBS-T and intensively shaken for 30 minutes. Then, 50μl of appropriate serial 10-fold dilutions of soil suspensions were plated onto the TSA (Tryptic Soy Agar), NA2 (Nutrient Agar no. 2), 100x diluted NA2 agar and R2A (Reasoner´s 2A agar) agar plates, which were cultivated at 25°C for 48 hours. After the cultivation, based on the variable phenotype of bacterial colonies, 40 isolates from each type of cultivation media from one sampling site were selected (i. e. 160 isolates per one sampling site) for MALDI – TOF MS (Matrix – Assisted Laser Desorption/Ionisation Time of Flight Mass Spectrometry) identification. After the MALDI – TOF MS identification, representative isolates from each clade of phylogenetic tree were tested for their antibiotic tolerance. Antibiotic tolerance testing of bacterial isolates was performed on MH (Mueller-Hinton) agar using dilution method with the addition of antibiotics – ampicillin (AMP), chloramphenicol (CHLOR), tetracycline (TET) and kanamycine (KAN). Antibiotics were added to the medium in an appropriate amount to the achievement of final concentrations of antibiotics in the medium. Concentrations of antibiotics were as follows: AMP – 10, 20, 50, 100 μg/ml; CHLOR – 1, 2, 5, 10, 20 μg/ml; TET – 2, 5, 10, 20, 50 μg/ml and KAN – 5, 10, 20, 50, 100, 200 μg/ml. The plates were incubated in the dark at 25°C for 48 hours and then the growth of bacterial isolates was evaluated.

Results and discussion

In our experiments, using MALDI-TOF MS analysis of bacterial isolates, we identified cultivable microflora of studied environments. Our isolates were dominated by Gram-negative bacteria of Pseudomonas and Rhizobium genera followed by Gram-positive Microbacterium sp. microorganisms (Figure 1). Tomova et al. (2015) in their study of bacteria obtained from sediment and soil samples of Antarctic island also determined Pseudomonas genus as the most prevalent one. On the other hand, Safari Sinegani and Younessi (2017) in the research of microbial antibiotic resistance present in different soils samples observed the prevalence of Bacillus spp., Micrococcus spp. and Staphylococcus spp genera.

Furthermore, they detected TET resistance in the majority of the isolates cultivated from mine soil samples (44.33%), whereas in our research, this tolerance was found as the lowest one (less than 1% of overall number of isolates) (Figure 2). However, our findings are in accordance with Berg et al. (2005), who determined AMP resistance as the most abundant one; nearly 100% of their Cu-resistant microorganisms were resistant to AMP. In our experiments, AMP resistance was also observed as the predominant one, but with only 49% of tolerable microorganisms able to withstand antibiotic concentrations higher than 100 μg/ml. Berg et al. also noticed that Cu-resistant bacteria had a significantly higher occurrence of multiple antibiotic resistances, what was confirmed later in 2010 (Berg et al. 2010). This study also defined CHLOR resistance as the second most prevalent with nearly 80% of resistant microorganisms at the concentration 16 μg/ml, what is significantly more than in case of our bacteria, which resembled tolerance to this antibiotic at the concentration > 20 μg/ml in only 16%. Similarly high CHLOR tolerance pattern as in the case Berg et al. was observed in Tomova et al. (2015) – in 58% of the isolates. Inversely, only 8% resistance was observed in their KAN testing (30 μg/ml), whereas our study revealed 18% tolerance to > 100 μg/ml of this antibiotic.

Conclusion

Taking in account industrial habitat with no known antibiotic exposure, the results revealed alarmingly high antibiotic resistance and multi-resistance pattern in bacteria. Microorganisms exposed to elevated concentrations of heavy metals in their natural habitat are forced to develop resistance mechanisms maintaining their fitness even under the stressful conditions of
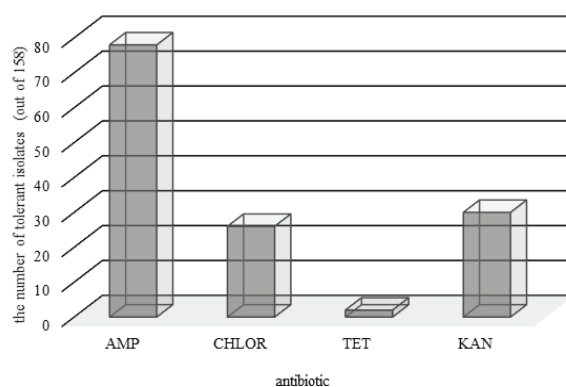
Fig. 2. Antibiotic tolerance of bacteria
Rys. 2. Tolerancja bakterii na antybiotyki

the environment. The widespread presence of metal resistance mechanisms is of concern because of the linkage between metal resistance genes and antibiotic resistance genes, what makes this issue interesting also from the medical point of view. Linkage and subsequent interspecific co-transfer of these two groups of genes via the horizontal transport could become a serious health problem which can cause difficulties with the therapy of bacterial diseases in human. Our data indicate that heavy metals contaminated environment could influence the occurrence and the spread of antibiotic resistance. Possibly, metal contaminated environment act as a reservoir of antibiotic resistant bacteria.

Acknowledgements

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Jakie zagrożenie dla działania antybiotyków stanowią górnicze tereny zanieczyszczone metalami ciężkimi?

Środowisko zanieczyszczone przez antybiotyki i metale ciężkie jako konsekwencja działalności ludzkiej jest obecnie przedmiotem wielu zmartwień. Wiele badań udowodniło, że środowisko może stanowić swoisty zbiornik odporności bakterii na działanie antybiotyków, pozwalając im na swobodne rozprzestrzenianie się wśród różnych bakterii poprzez proces zwany poziomym transferem genów. Wynikiem jest obecność odporności na antybiotyki nawet u mikroorganizmów patogenicznych. Metale ciężkie działają jako ważne czynniki w tym procesie ze względu na swój potencjał wyboru bakterii, która opiera się antybiotykowi ze względu na swego rodzaju połączenie pomiędzy genami opierającymi się antybiotykowi oraz genami opierającymi się metalom ciężkim. Zatem, wykonano eksperyment aby zbadać tolerancję na antybiotyk dla bakterii uzyskanych ze środowiska kopalni, składowiska oraz gleby z pobliża kopalni będących zanieczyszczonymi metalami ciężkimi. Pobrano próbki z jedynej aktywnej kopalni złota w Słowacji, zlokalizowanej w Hodruša – Hámre. Obecność bakterii kultywacyjnych został udowodniony za pomocą badań kultywacji a następnie techniki identyfikacyjnej MALDI – TOF MS (Matrix – Assisted Laser Desorption/Ionisation Time of Flight Spectrometry). Reprezentatywne izolaty bakteryjne zostały zbadane ze względu na ich tolerancję na wybrane antybiotyki (ampicylina (AMP), chloramfenikol (CHLOR), tetracyklina (TET) oraz kanamycyna (KAN) w celu zdefiniowania ich minimalnego stężenia inhibicyjnego (MIC). Bakterie kultywacyjne z badanych środowisk były zdominowane przez Gram-ujemne proteobakterie rodzaju Pseudomonas oraz Rhizobium. Spośród więcej niż 150 izolatów, odporność na ampicylinę (MIC>100 μg/ml- 49% izolatów), kanamycynę (MIC>100 μg/ml – 18% izolatów) oraz chloramfenikol (MIC> 20 μg/ml – 16% izolatów) dominowała. Odporność na tetracyklinę (MIC> 20 μg/ml) został stwierdzony w mniej niż 1% przypadku izolatów. Ogólna liczba odporności na antybiotyki oraz multi-odporności była alarmująco duża, biorąc pod uwagę, że środowiska przemysłowe z nieznanym stopniem wystawienia na antybiotyki była analizowana. Nasze dane wskazały, że środowisko zanieczyszczone metalami ciężkimi może wpływać na obecność i rozwój odporności na antybiotyki. Możliwym jest, że środowisko zanieczyszczone metalami zachowuje się jak zbiornik dla bakterii odpornych na działanie antybiotyków.