Strategies to counter transmission of “superbugs” by targeting free-living amoebae

Ruqaiyyah Siddiqui, Naveed Ahmed Khan

Department of Biological Sciences, School of Science and Technology, Sunway University, Malaysia.

Short title: Hyperparasitic superbugs and FLA

*Corresponding address: Department of Biological Sciences, School of Science and Technology, Sunway University, Selangor, 47500, Malaysia. Tel: 60-(0)3-7491-8622. Ext: 7176. Fax: 60-(0)3-5635-8630. E-mail: naveed5438@gmail.com (NAK); ruqaiyyahs@sunway.edu.my (RS).

Summary

Bacterial infections have remained significant despite our advances in the development of a plethora of disinfectants as well as antimicrobial chemotherapy. This is in part due to our incomplete understanding of the prevalence of bacterial pathogens in the environmental and clinical settings. Several lines of evidence suggest that *Acanthamoeba* is one of the most ubiquitous/resilient protists that also acts as a host/reservoir for pathogenic microbes. Thus targeting the hardy host, which harbour microbial pathogens, offer a potential avenue to counter infection transmission, particularly hospital/community-acquired infections. This will complement existing approach of applying disinfectants that are targeted against bacterial pathogens directly.

Keywords: superbugs; infectious diseases; antimicrobial resistance.

Introduction

"Superbugs" or multiple-drug resistant (MDR) microbes, in particular MDR bacteria is a growing public health threat that represents an enormous challenge for healthcare providers, given the few antimicrobials at our disposal. Antibacterial disinfection strategies have had some success but failed to eradicate MDR bacteria completely from healthcare settings. Thus, there is a need to develop alternative strategies to combat MDR bacteria. Several lines of evidence show that *Acanthamoeba* is one of the most widely distributed protists that also acts as a host/reservoir for microbes including potential infectious agents (Khan, 2009). The ability of a unicellular amoeba to host another microbe within itself is an extraordinary trait that require convoluted interactions. In such interactions, if both organisms are pathogenic, they are referred to as hyperparasites, and the relationship is known as hyperparasitic relationship that can benefit one or both organisms. For example, bacterial survival intracellular of amoebae may lead to their evolutionary gain of pathogenicity, immune evasion strategies, or bacterial protection from hostile environments. For latter, the ability of *Acanthamoeba* to form hardy cysts can provide MDR bacteria shelter against common disinfectants within the clinical settings. Cyst is a dormant form, i.e., non-feeding, non-replicating form, during which amoeba encloses itself in a double-walled structure. Cyst can remain viable following exposure to high temperature (~65°C), 50ppm chlorine, 200 mJ per cm^2 and known to resist commonly used disinfectants that include alcohol- or chlorine-based products, even though such disinfectants can prove effective against MDR bacteria (Aksozek et al., 2002; Khan, 2009). This may explain our inability to completely eradicate MDR bacteria from healthcare settings. Thus, it is suggested that targeting the host, i.e., amoeba cysts, that harbour MDR bacteria or possibly other microbial pathogens could

prove to be a pivotal tool to eliminate pathogens within hospitals (Siddiqui and Khan, 2013). This area of research is well-timed and relevant, given a significant rise in hospital-, nursing-, and community-based infections, worldwide (Nordmann et al., 2007; Alanis, 2005; Blondeau, 2013).

MDR bacteria: old enemy presenting new challenges

MDR bacteria are resistant to multiple antimicrobial drugs. Increase in resistance has led to emergence of extensively drug-resistant (XDR) bacteria as well as pan drug-resistant (PDR) bacteria. Infections due to drug resistant bacteria are challenging and expensive. Data from the CDC, USA show that multiple-drug resistant (MDR) microbes affect approximately two million people per year in the United States alone (CDC, 2013). Some examples of MDR bacteria include: *Staphylococcus aureus* derivatives such as MRSA, *Streptococcus pneumonia*, *Klebsiella* spp., *Enterococcus* spp., and *Mycobacterium tuberculosis*, to name a few (Nordmann et al., 2007; Alanis, 2005; Blondeau, 2013). For developing countries MDR bacteria are of increasing concern; as antibiotic-resistant bacteria can be overlooked resulting in increased trend of antibacterial resistance, as witnessed in *K. pneumoniae* (referred as NDM-1) (Yong et al., 2009; Oberoi et al., 2013; Trivedi and Sabnis, 2009). Many developed countries have managed to reduce the antibacterial resistance occurrence and showed reduction, by regulating antibacterial consumption (Barbosa and Levy, 2000), however compliance is poor in developing countries. The wide application of antibacterials, both for clinical applications as well as agriculture/farming/animal feed is exacerbating the emerging trends of antibacterial resistance (Marshall and Levy, 2011; Snitkin et al., 2012).

Disturbingly, even the most advanced institutions show limited success in eradicating superbugs (Levy, 1998; CDC). In a recent case of MDR-*K. pneumoniae*

(Snitkin et al., 2012) precautions were taken to halt the spread of infection to other patients. These included patient isolation, hand hygiene practices, restricted traffic and strict use of protective clothing, dedicated equipment, extensive cleaning with bleach, once vacated. Nonetheless, this common bacterial infection spread to other patients within the institution. Consequent intervention measures comprised demolishing drains, extensive use of hydrogen peroxide via a robot. Yet the infection spread to 17 other patients, six of whom deceased, highlighting the ability of bacteria to resist available disinfection practices (Nordmann et al., 2007; Levy, 1998). While the current disinfection strategies are inefficient, a plethora of bacteria have gained resistance to several antibiotics and are now considered MDR bacteria or "superbugs". According to the CDC, MRSA and *C. difficile* alone, contribute to 14,000 and 19,000 deaths annually in the USA alone (CDC). Worryingly, patients are increasingly concerned of visiting hospitals for common diseases or surgeries as the visits may lead to contracting MDR bacteria (Madeo, 2011).

World Health Organisation stresses antimicrobial resistance as one of the most critical threat to the global health (WHO, 2002). Furthermore there is an increasing trend of infections even within the community setting (Klein and Smith, 2009). Centers for Disease Control and Prevention approximates that 12% of MRSA-infected patients are now community-associated (CDC). The aim is to introduce novel strategies/approaches to counter MDR bacteria, as the use of antibacterial disinfectants in healthcare settings is ineffective in eradicating MDR bacteria.

Emerging strategies to tackle MDR bacteria

At present, the main strategy to avoid bacterial infection transmission and spread is isolation and the use of disinfectants (Levy, 1998; Madeo, 2011; Levy, 2000). In general, cleaning solution is chlorine-based such as 5% solution of sodium

hypochlorite (Eckstein et al., 2007). However, this is ineffective against *Acanthamoeba* cysts (Coulon et al., 2010), which could be harbouring and thus protecting MDR bacteria to commonly used antibacterial disinfectnat (Briancesco et al., 2005). Hence, for effective eradication of pathogens, the focus cannot be on one organism. In fact, it is quite the reverse. As diseases often have various and perplexing factors with diverse etiologies and environmental niches that need to be explored to understand the complex subtleties of infection transmission. Much more work is required to understand how pathogens are refuged in the environment/clinical setting despite the use of powerful antibacterial disinfectants, so that preventative measures can be designed appropriately. Other factors that facilitate transmission of microbes to, and between people, must be determined to design innovative interventional measures.

In his landmark observation, Rowbotham witnessed that *Acanthamoeba* is a host and a reservoir for Legionnaires'-causative agent, *L. pneumophila* by showing that *L. pneumophila* survives and multiplies inside the amoeba (Rowbotham, 1980). Now it is well known that *Acanthamoeba* can host viral, bacterial, protists, and yeats (Khan, 2009; La Scola et al., 2003; Greub and Raoult, 2004). *Acanthamoeba* is one of the most omnipresent protist and it can also cause infections (Khan, 2009). It has two stages in its life cycle, an actively growing stage, termed as the trophozoite stage, and a non-dividing stage, termed as the cyst stage. Cysts are non-dividing with little metabolic activity, possess a hardy shell and are air-borne, making them highly resistant to harsh environments, as well as chemicals (Fig. 1) (Aksozek et al., 2002; Lloyd et al., 2001; Turner et al., 2000). The ability of *Acanthamoeba* to differentiate into a dormant cyst form (with negligible metabolic activity) is a remarkable property, as it allows amoeba to evade drugs, which target functional aspect of the parasite such

as respiration, RNA/DNA transcription, translation, protein synthesis and modifications, intracellular trafficking, cell wall/membrane synthesis, motility etc. Notably, the majority of available drugs target function at physiologically-relevant conditions, making them obsolete against the inactive cyst form. In addition, the hardy shell of the cyst form protects *Acanthamoeba* against high levels of radiation, temperatures, disinfectants such as chlorine etc. (Aksozek et al., 2002; Lloyd et al., 2001; Turner et al., 2000). Intriguingly, cysts can remain viable for decades (Mazur et al., 1995) and can be airborne (Rodriguez-Zaragoza and Magana-Becerra, 1997). Surprisingly, a complete understanding of the biochemical profile of the cyst walls of *Acanthamoeba* remains unknown, making it challenging to eradicate them effectively and/or rationally develop disinfectants/biocides to degrade the outer walls and target the masked trophozoite together with any intracellular pathogens. The ability of *Acanthamoeba* to host pathogenic microbes such as MDR bacteria, protect them under harsh conditions (disinfectants), and contribute to pathogen transmission presents a major threat to the public and of great concern. This “hyperparasitism” (parasite within a parasite) is likely contribute indirectly to infections (La Scola et al., 2003; Greub and Raoult, 2004). The details of these interactions are incompletely understood, but it is postulated that the inability of pathogenic microbes (such as non-spore forming bacteria) to resist harsh conditions led to evolutionary need to assist with a protective host, to remain viable when enduring adverse conditions. The hardy cysts can shelter microbes against hostile conditions and allows them to survive harsh conditions such as disinfectants in clinical settings, possibly leading to spread to vulnerable population. Thus, it is rational to hypothesize that amoeba is a potential niche of pathogenic microbes, and plays a role in their spread to the susceptible population. Recently, the prevalence of *Acanthamoeba* and superbugs in a clinical

setting was determined (Siddiqui et al., 2013). Both bacteria and *Acanthamoeba* were found to co-exist in the hospital environment. Furthermore, antibiotic susceptibility showed that all bacterial isolates recovered were MDR. In addition to present practices, interventional strategies targeting amoebae should be investigated to eliminate pathogenic microbes.

The way forward: Translating research to clinical practice

Acanthamoeba cysts can persist as viable cysts for over 20 years while retaining their pathogenicity and can be air-borne (Mazur et al., 1995; Sriram et al., 2008), so it is particularly challenging to target these cysts that may be harbouring MDR bacteria. Previous studies have shown that hydrogen peroxide-based contact lens disinfectants are useful against trophozoites and one-week old cysts as compared to other solutions (Johnston et al., 2009). As per FDA, it is a safe oxidizing agent against microbial pathogens. It can sterilize objects as well as airborne microbes. This may explain why cleaning with vapourised form of hydrogen peroxide-based disinfectants inhibited the chances of a patient developing antibiotic-resistant bacterial colonization by a staggering 80 percent suggesting its effectiveness (Doan et al., 2012; Passaretti et al., 2013; Zoutman et al., 2011; Gatti et al., 1998). We sanction that this effect is due to antiamoebic effects of hydrogen peroxide, in addition to its' anti-bacterial properties. We propose that the use of hydrogen peroxide will target both the "terror cells" i.e. the MDR bacteria and the host harbouring them, i.e., amoeba.

Due to its toxicity and instability, there is a need to find safe, and effective agents targeting both MDR bacteria and amoebal host with long-term shelf-life, cost-effectiveness and practicality, to be appropriate for healthcare settings; particularly in developing countries. Since *Acanthamoeba* is a Trojan horse of the microbial world

including potential pathogens (Huws et al., 2006), we anticipate that disinfectants that are effective in killing both *Acanthamoeba* as well as bacteria will be of enormous worth for applications in clinical settings. Future studies will test such disinfectants with dual action to determine their effectiveness in eradicating superbugs from clinical settings.

In summary, these findings suggest that strategies targeting the hardy host, i.e., *Acanthamoeba* that harbour microbial pathogens offer a potential avenue to counter infectious diseases, particularly hospital/community-acquired infections. The proposed strategy will complement existing approach of applying disinfectants that are targeted against bacterial pathogens directly. These findings should be of interest to public health officials and/or policy makers, medical practitioners, and the scientific community.

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Figure legends

Figure 1. Representative transmission electron micrographs showing a complete double-walled cyst of *Acanthamoeba castellanii* belonging to the T4 genotype (American Type Culture Collection, ATCC 50492). IM is inner membrane; and OM is outer membrane. Bar = 2 μm.