Isolation and determination of the therapeutic potential of phages specific to *Acinetobacter baumannii* strains

Bacteria of the species *Acinetobacter baumannii* pose a serious threat to human health and life due to their increasing resistance to currently used antibiotics. Studies have shown that the increase in the use of antibiotics in hospitals translates into an increase in resistance among hospital strains of *A. baumannii* to these chemotherapeutics. Patients who are hospitalized for a long time, especially patients in intensive care units, and people with immunodeficiencies are particularly susceptible to infections caused by *A. baumannii* strains. These bacteria may be etiological factors associated with blood, urinary tract or soft tissue infections. Mortality associated with *A. baumannii* infections ranges from 26 to 60% and depends on factors such as untargeted treatment, resistance to the drugs used, thrombocytopenia and the patient being connected to a ventilator. It is worth noting, however, that the link between higher mortality and multidrug resistance of *A. baumannii* strains has not been confirmed. WHO reports classifying bacteria in terms of the priority of research on new antibiotics have assigned *A. baumannii* strains resistant to carbapenems to the critical priority group. These data show how important it is to search for new therapeutic agents directed against resistant bacterial strains. In addition to antibiotic resistance, these bacteria are characterized by resistance to environmental factors. These strains are capable of producing biofilm on surfaces and hospital equipment, including urological catheters, which can result in the development of difficult-to-treat urinary tract infections.

Bacteriophages - viruses capable of replicating exclusively in bacterial cells, are among the most abundant biological particles on Earth. They differ in morphology, genome organization and size. Studies published in recent years on phages specific to *A. baumannii* have shed new light on phage therapy. The results obtained by scientists are promising and indicate that lytic phages specific to *A. baumannii* are capable of reducing strains resistant to antibiotics. *In vitro* studies have confirmed that bacteriophages are capable of effectively removing biofilm produced by *A. baumannii* strains, while *in vivo* studies on animal models have shown that bacteriophages facilitated the fight against bacterial infections without demonstrating cytotoxic effects on animal cells.

In light of the above, phage therapy is a promising alternative to antibiotic therapy in the treatment of infections caused by multidrug-resistant *A. baumannii* strains.

The aim of the doctoral dissertation was to investigate the therapeutic potential of isolated bacteriophages specific to *A. baumannii* strains in *in vitro* conditions in phage therapy as an alternative or support in the treatment of infections caused by antibiotic-resistant strains. In order to achieve the aim of the doctoral dissertation, the following research hypotheses were formulated:

1. municipal and hospital sewage is a source for the isolation of bacteriophages specific to multidrug-resistant *A. baumannii* strains,
2. comprehensive analysis of biological, morphological and genomic properties enable the selection of phages for therapeutic purposes,

3. bacteriophages demonstrate the potential to eradicate biofilms formed by uropathogenic *A. baumannii* strains.

Research tasks (specific objectives) were also defined, which allowed for the verification of the research hypotheses:

1. isolation of bacteriophages specific for multidrug-resistant *A. baumannii* strains,
2. determination of the most favorable infection coefficient (Multiplicity of Infection, MOI) of isolated phages, in order to determine the amplification parameters individually for each phage,
3. study of the biological properties of isolated phages: including plaque morphology, ultrastructure of phage virions, lytic spectrum, specificity, kinetics of phage adsorption to bacterial cells,
4. isolation of the genetic material of the studied phages along with a comprehensive bioinformatic analysis of the obtained phage genome sequences,
5. study of the stability of isolated phages in various storage conditions: in a wide range of temperatures, pH of the culture medium, as well as assessment of the stability of phages in selected disinfectants, metal ions, essential oils and human urine (from both healthy individuals and patients diagnosed with bacterial urinary tract infection),
6. study of the effect of single phages and a phage cocktail on the degradation of bacterial biofilm formed by uropathogenic strains of *A. baumannii*.

The doctoral dissertation is a series of thematically related works in the form of four scientific publications. The research results were presented in two original publications, while two review publications summarized the current state of knowledge about the growing threat to human health and life associated with infections caused by *A. baumannii* strains. The therapeutic potential of phages specific for these strains was discussed, and in particular the use of phage therapy in the treatment of urinary tract infections caused by uropathogenic *A. baumannii* strains.

Two review articles (**P1** Bagińska et al., 2019; **P2** Bagińska et al., 2021) included information on the difficulties in combating infections caused by *A. baumannii* strains, due to their increasing resistance to currently used antibiotics and the ability to form bacterial biofilm. The latest information on the use of strain-specific phages targeting *A. baumannii* in the treatment of infections caused by these strains has also been compiled, highlighting the potential of phage therapy as an alternative to current antibiotic treatment.

The first original publication **P3** (Bagińska et al., 2023) described the procedure for isolation, amplification and testing of biological properties of bacteriophages. Samples of environmental, municipal and hospital sewage collected from various locations in Poland were used for the study (Bagińska et al., 2023). The search for phages specific to *A. baumannii* strains was carried out using the phage typing method using raw and concentrated samples or samples previously incubated at 37°C. The conducted studies allowed to determine the biological properties and morphology of the tested phages. As a result of the conducted studies, 12 bacteriophages (of which 6 previously deposited in the Bacteriophage Laboratory and

6 newly isolated) specific for *A. baumannii* strains were characterized - 11 temperate phages and only one lytic. The lytic spectrum values of the phages ranged from 11 to 75%. Most phages produced small and transparent plaques, and one of them produced relatively large plaques with a distinct "halo" effect. Based on phage morphology determined by transmission electron microscopy (TEM), most phages were classified as tailed phages (class *Caudoviricetes*) with a siphovirus morphotype - only one phage was classified as having a podovirus morphotype.

All phages had icosahedral capsid symmetry, and 11 of them had a long tail. The MOI (infection index) was also determined - the ratio of the number of infecting virus particles to the known number of bacterial cells in culture and the adsorption rate constant. The optimal MOI values were different for the tested phages and ranged from 0.001 to 10. Based on genetic studies and bioinformatic analysis of the obtained sequences and similarity to the genomes of previously described bacteriophages, the studied phages specific for *A. baumannii* were originally (date of publication) classified to the subfamily *Beijerinckvirinae* (Acba_6) and *Junivirinae* (all others). Currently, Acba_6 is a representative of the genus *Friunavirus*, phages: Acba_1, Acba_4, Aclw_8, Acba_15 and Acba_16 belong to the class *Caudoviricetes* and the genus *Vieuvirus*. The remaining phages Acba_3, Aclw_9, Acba_11 and Acba_13, Acba_14 and Acba_18 belong to the class *Caudoviricetes*.

The second original publication **P4** (Bagińska et al., 2024) contains stability studies of 12 bacteriophages at different temperatures or pH values of liquid medium, commercially available disinfectants, essential oils and surfactants (Bagińska et al., 2024). Phage stability studies were carried out in urine of healthy individuals and patients diagnosed with urinary tract infection. The last stage of the study was to determine the ability of phages to degrade biofilm produced by uropathogenic *A. baumannii* strains. Based on long-term stability studies, the most optimal method of storing *A. baumannii* phages turned out to be a temperature of 70°C. On the other hand, the temperature of 60°C caused a significant reduction in phage activity after an hour of incubation. The smallest decrease in phage titer was observed after their incubation in a medium with a pH ranging from 7.0 to 9.0, with the greatest reduction in phage titer at low pH values. It is worth noting that ethanol-based disinfectants caused a significant reduction in phage titer even after 30 seconds of incubation. Copper and silver nanoparticle solutions also caused a reduction in phage titer, but to a much lesser extent than disinfectants. On the other hand, bacteriophages incubated for 24 hours in essential oils (cinnamon and eucalyptus) can be considered stable. Phages also remained stable after 60 minutes of incubation in human urine regardless of the origin of urine (healthy donors/people with diagnosed urinary tract infection - obtained the consent of the Bioethics Committee at HIIET PAS, consent number: KB-12/2022). Additionally, studies using bacterial biofilm have shown that single phages degrade the biofilm produced by uropathogenic *A. baumannii* strains to a greater extent than the phage cocktail used.

The above studies expand the state of knowledge about phages specific to *A. baumannii* strains, which currently pose a serious threat to human life and health. The obtained results and further studies, including the search for new lytic phages and studies using isolated phages in a mouse model of urinary tract infection, may in the future contribute to the further development

of targeted phage therapy - aimed at eradicating infections caused by multidrug-resistant *A. baumannii* strains.