COMPARISON OF METHODS FOR PURIFICATION OF BACTERIOPHAGE LYSATES OF GRAM-NEGATIVE BACTERIA FOR PERSONALIZED THERAPY

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Phage therapy is a promising method of treating antibiotic-resistant infections. To obtain a safe therapeutic formulation, bacterial cell components, including endotoxins, must be removed from the phage lysate. This study was aimed at comparing the efficacy of purification methods for phage lysates intended for therapeutic use. Phages vB_KpnM_Seu621 (*Myoviridae*) and vB_KpnP_Dlv622 (*Autographiviridae*) were grown using the KP9068 strain of *Klebsiella pneumoniae* as a host. The obtained lysates were purified using phage precipitation with polyethylene glycol, CsCl density gradient ultracentrifugation, sucrose density gradient ultracentrifugation, precipitation with 100 kDa centrifugal filter units, and phage concentration on 0.22 µm cellulose filters in the presence of MgSO₄. Endotoxin concentrations were determined by LAL testing. The obtained lysates contained $1.25 \times 10^{12} \pm 7.46 \times 10^{10}$ and $2.25 \times 10^{12} \pm 1.34 \times 10^{11}$ PFU/ml of vB_KpnM_Seu621 and vB_KpnP_Dlv622, respectively, and had endotoxin concentrations of $3,806,056 \pm 429,410$ and $189,456 \pm 12,406$ EU/ml, respectively. CsCl gradient ultracentrifugation was found to be the optimal conventional purification method in terms of reducing endotoxin concentrations and maintaining phage titers (303 ± 20 — 313 ± 35 EU/ml, 1.5 – $2.75 \times 10^{12} \pm 1.71 \times 10^{11}$ PFU/ml). Sucrose gradient ultracentrifugation and filtration in the presence of MgSO₄ were found to be the optimal non-traditional purification methods. A method for phage lysate purification should be selected for each phage preparation individually. Sucrose gradient ultracentrifugation and filtration in the presence of MgSO₄ hold promise as purification methods that can produce phage preparations suitable for intravenous administration.

Keywords: bacteriophage, phage therapy, purification methods, bacterial lysate, microbiology, endotoxin, *Klebsiella pneumoniae*

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СРАВНЕНИЕ МЕТОДОВ ОЧИСТКИ ФАГОВЫХ ЛИЗАТОВ ГРАМОТРИЦАТЕЛЬНЫХ БАКТЕРИЙ ДЛЯ ПЕРСОНАЛИЗИРОВАННОЙ ТЕРАПИИ

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Фаготерапия является перспективным методом лечения инфекций, вызванных устойчивыми к антибактериальным препаратам бактериями. Для получения безопасных терапевтических препаратов бактериофагов необходима глубокая очистка лизатов от компонентов бактериальной клетки, в частности эндотоксинов. Целью работы было исследовать применимость различных методов очистки фаголизатов для получения терапевтических препаратов. Фаги vB_KpnM_Seu621 (*Myoviridae*) и vB_KpnP_Dlv622 (*Autographiviridae*) использовали для получения лизата штамма *Klebsiella pneumoniae* KP9068. Очистку лизатов проводили методом осаждения бактериофагов с использованием полиэтиленгликоля, ультрацентрифугированием в градиенте CsCl, ультрацентрифугированием в градиенте сахарозы, с помощью центрифужных концентраторов (100 кДа), концентрированием на целлюлозных фильтрах 0,22 мкм в присутствии MgSO₄. Уровень эндотоксинов определяли ЛАЛ-тестированием. В результате действия vB_KpnM_Seu621 и vB_KpnP_Dlv622 были получены лизаты с титром $1,25 \times 10^{12} \pm 7,46 \times 10^{10}$ и $2,25 \times 10^{12} \pm 1,34 \times 10^{11}$ БОЕ/мл и концентрацией эндотоксина 3806056 ± 429410 и 189456 ± 12406 ЕД/мл соответственно. Из традиционных методов оптимальным по снижению уровня эндотоксина и сохранению концентрации фаговых частиц было ультрацентрифугирование в градиенте CsCl (303 ± 20 — 313 ± 35 ЕД/мл, $1,5$ – $2,75 \times 10^{12} \pm 1,71 \times 10^{11}$ БОЕ/мл); из альтернативных — очистка в градиенте сахарозы и фильтрация в присутствии MgSO₄. Метод очистки лизатов следует подбирать для каждого препарата бактериофагов отдельно. Из способов очистки лизатов, пригодных для внутривенного и интратекального введения, перспективны метод ультрацентрифугирования в градиенте сахарозы и фильтрация в присутствии MgSO₄.

Ключевые слова: бактериофаги, бактериофаговая терапия, методы очистки, бактериальные лизаты, микробиология, эндотоксины, *Klebsiella pneumoniae*

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Irrational use of antibiotics has driven the emergence and global dissemination of drug-resistant microorganisms. The World Health Organization has prioritized the search for novel antimicrobial agents against *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Klebsiella spp.*, *Escherichia coli*, and *Enterobacter spp.* because the number of multidrug-resistant isolates in this group of microorganisms has been steadily trending upwards [1].

As alternatives to antibiotics, virulent bacteriophages hold promise for the therapy of multidrug-resistant bacterial infections [2]. Phages are naturally occurring antagonists of bacteria, capable of selectively and effectively infecting a bacterial cell. The primary advantage of phage therapy lies in the ability of phages to cause death of the bacterial cell regardless of its sensitivity to antibiotics [3]. Besides, due to remarkable host specificity, phage therapy can eliminate the infectious agent without harming the patient's natural microbiota.

Based on the accumulated clinical experience, the following requirements have been elaborated for therapeutic phages and phage cocktails: phage virulence, the absence of toxin genes and antibiotic resistance determinants in the phage genome, the ability to infect a wide range of hosts, which is determined by the efficiency of plating, high levels of phage particle production per cell, and the effective phage concentration of at least 10^9 PFU/ml [4–7].

However, as typical viruses phages replicate only inside a bacterial cell, usually causing its lysis. So, phage preparations can be contaminated by bacterial cells and their components, including endotoxins [6]. Lipopolysaccharides, which are the main constituent of the outer membrane of gram-negative bacteria, are the most illustrative example of bacterial endotoxins [8]. Endotoxins are highly immunogenic and can provoke septic (endotoxic) shock resulting in intravascular coagulation, organ failure and death [9].

So far, a few approaches to phage cultivation and purification have been proposed; of them, polyethylene glycol (PEG) precipitation and CsCl density gradient ultracentrifugation are the most common [10]. The drawback of these approaches is the presence of harmful compounds (e.g. cesium salts (80 ng/ml) in the end preparation with CsCl centrifugation), which significantly limits its usage as a therapeutic agent [10]. Sucrose density gradient ultracentrifugation exploited by virology studies may offer an alternative [11, 12]. Methods of phage enrichment from environmental sources, too, have been increasingly used for the purification of phage lysates in recent years; among them are 100 kDa filtration and isolation in the presence of salts [10, 12–14]. Several chromatography-based methods for phage purification have been developed, like affinity chromatography-based commercial products for removing endotoxins from phage lysates, for example, EndoTrap® columns (Lionex GmbH; Germany). However, a series of research studies have shown that a single passage of a crude lysate through the column only slightly reduces endotoxin concentrations in the preparation [13, 15, 16]. The aim of this study was to compare the efficacy of purification methods for phage lysates intended for therapeutic use.

METHODS

Bacterial strains, phages, and media

The *K. pneumoniae* strain KP9068 was obtained from the collection of the Laboratory of Molecular Genetics of Microorganisms (Federal Research and Clinical Center of Physical-Chemical Medicine, FMBA; Russia). This strain is

resistant to cephalosporins (cefotaxime, ceftazidime, ceftriaxone, cefixime), fluoroquinolones (ciprofloxacin, ofloxacin), tetracycline, azithromycin, chloramphenicol, gentamicin, and ampicillin and has intermediate sensitivity to meropenem. The strain belongs to sequence type 11. Its capsule type identified by the sequencing of the *wzi* gene is K23 [17]. Bacterial cultures used in our experiment were grown in lysogeny broth (LB, Himedia; India) at 37 °C.

Drawing on the literature data on the phylogenetic diversity of bacteriophages in commercial phage cocktails, we picked 2 lytic phages that represented 2 different families and were active against *K. pneumoniae* strains with K23 capsule type [18].

Bacteriophages vB_KpnM_Seu621 (MT939253.1) and vB_KpnP_Dlv622 (MT939252.1) cause lysis of *K. pneumoniae* and belong to the *Myoviridae* and *Autographiviridae* families, respectively [19]. Phage vB_KpnM_Seu621 has the following morphological characteristics: an isometric head of 75 nm in diameter and a 104 nm long contractile tail. Bacteriophage vB_KpnP_Dlv622 has a smaller isometric head (57 nm) and a 12 nm long non-contractile tail. The genomes of the 2 bacteriophages were analyzed in earlier work and did not encode any toxin and integrase genes that could preclude their use in therapy [19].

The phages were grown using the KP9068 strain of *K. pneumoniae* as a host in a thermoshaker at 100 rpm and 37 °C for 18 h. Briefly, the host strain was grown to the mid-log phase ($OD_{600\text{ nm}} = 0.3$) and then inoculated with the phage lysate to achieve the multiplicity of infection of 0.001. The obtained phage lysates were clarified from cell debris by 15 min centrifugation at 3,500 g followed by sequential filtering through 0.45 µm (Merck Millipore; USA) and 0.22 µm (Merck Millipore; USA) membrane filters. Phage concentrations in the phage lysate were measured using the standard double-layer agar technique. Phage titers were expressed as the number of plaque forming units per one ml (PFU/ml) [20].

Phage precipitation with PEG

In this step, phage particles were recovered from the lysate by precipitation with PEG 6000 (Dia-M; Russia) using a previously described but slightly modified technique [21]. Ten ml of the phage lysate was combined with 2.5 ml of a sterile solution containing 20% PEG 6000 and 2.5 M NaCl. The samples were mixed by inverting without vortexing, cooled on ice for 1 h and centrifuged at 20,000 g for 20 min. Most of the supernatant was carefully removed. Then the samples were centrifuged again at 20,000 g for 10 min and the supernatant was again removed. The precipitate containing phage particles was resuspended in 1 ml of a sterile SM buffer (100 mM NaCl, 10 mM $MgSO_4$, 50 mM Tris-HCl, pH 7.5, and 0.01% (w/v) gelatin. The suspension was vortexed, incubated on ice for 1 h, vortexed again to remove PEG, and centrifuged at 13,000 g for 1 min. The supernatant was transferred to a clean testing tube for further experiments.

CsCl density gradient ultracentrifugation

CsCl density gradient ultracentrifugation was performed following the protocol described in [21]. Aliquots (70 ml) of the phage lysates were centrifuged in a Beckman Type 45Ti rotor (Beckman Coulter; USA) at 75,000 g and 20 °C for 1 h. The supernatant was gently removed and the precipitate was resuspended in 800 µl of SM buffer. Next, solutions containing different concentrations of CsCl (1.3–1.4–1.45–1.5–1.6 g/ml)

Fig. 1. Endotoxin concentrations in the purified vB_KpnM_Seu621 and vB_KpnP_Dlv622 phage lysates. **1** — crude lysate; **2** — precipitation with PEG; **3** — CsCl density gradient ultracentrifugation; **4** — sucrose density gradient ultracentrifugation; **5** — 100 kDa filtration; **6** — filtration through 0.22 µm filters in the presence of MgSO₄

and SM buffer were prepared. The solutions (800 µl) were added to the centrifuge tube layer by layer to create a gradient, starting with the highest CsCl concentration; the top layer solution had the lowest CsCl concentration. A concentrated phage sample (800 µl) containing only one of the studied phages was applied on top of the last layer of the gradient. The samples were centrifuged in a Beckman SW50.1 rotor (Beckman Coulter; USA) at 75,000 g and 20 °C for 1 h. The opalescent phage band was collected, brought to 1 ml by adding SM buffer, and placed into a dialysis tubing (Thermo FS; USA). Dialysis was performed for 18 h against a tenfold volume of SM buffer at 4 °C and constant stirring of the liquid, replacing the buffer every 4 h. After dialysis, the samples were additionally filtered through 0.22 µl filters (Merck Millipore; USA).

Sucrose density gradient ultracentrifugation

Sucrose density gradient ultracentrifugation was performed following a standard but slightly modified protocol [11].

Aliquots (70 ml) of the phage lysates were centrifuged in a Beckman Type 45Ti rotor (Beckman Coulter; USA) at 75,000 g and 20 °C for 1 h. Each of the obtained precipitates was resuspended in 800 µl of SM-buffer. Then sucrose solutions (20, 30, 40, 50 and 60%) were prepared in a buffer containing 50 mM Tris-HCl, 50 mM NaCl, pH 7.5. The solutions (800 µl) were added to the centrifuge tube layer by layer to create a gradient, starting with the solution that had the highest sucrose concentration (60%); the top layer solution had the lowest sucrose concentration (20%). A concentrated phage sample (800 µl) containing only one of the studied phages was applied on top of the last layer of the gradient. The samples were centrifuged in a Beckman Type SW50.1 rotor (Beckman Coulter; USA) at 75,000 g and 20 °C for 1 h. Free phage particles formed a visible opalescent band between the 50% and 60% sucrose layers. The phage layer was collected and brought to 1 ml by adding SM buffer.

Phage purification with 100 kDa centrifugal filters

The phage lysates (1 ml) were placed on 100 kDa Microcon centrifugal filters (Millipore; USA) and centrifuged at 10,000 g for 10 min. To recover trapped phage particles, the filter was coated with 300 µl of sterile SM buffer, placed upside down in a clean sterile centrifuge tube and centrifuged at 1,000 g for 3 min. The purified lysate was brought to 1 ml by adding a sterile SM buffer.

Phage concentration on 0.22 µm cellulose filters in the presence of MgSO₄

Phage concentration on 0.22 µm filters in the presence of MgSO₄ was performed on 50 ml aliquots of the lysates cleared of cell debris [14]. Dry reagent-grade MgSO₄ (Dia-M; Russia) was added to the lysates to reach a final concentration of 50 mM. The samples were mixed by inverting without vortexing until complete dissolution of MgSO₄. The obtained suspensions were slowly filtered through 0.22 µm mixed cellulose ester GSWP membranes (Millipore; USA). The membranes were cut in small pieces (sized ~ 1 mm²) and immersed into 5 ml of a sterile SM buffer. To achieve complete elution of phage particles from the filter surface, the suspension was ultrasonicated treated by ultrasonication for 4 min. Then, large filter fragments were precipitated by 10 min centrifugation at 3,500 g; the supernatant was collected for further experiments.

Measuring endotoxin concentrations in the samples

Endotoxin concentrations were measured using commercial Endosafe® kits for turbidimetric LAL testing (Charles River; USA) following the manufacturer's protocol. Turbidity assays were conducted in a microplate reader (Multiskan Ascent, Thermo; USA) at 405 nm wavelength.

Fig. 2. Concentration of phage particles in purified vB_KpnM_Se621 and vB_KpnP_Dlv622 lysates after purification. 1 — crude lysates; 2 — precipitation with PEG; 3 — CsCl density gradient ultracentrifugation; 4 — sucrose density gradient ultracentrifugation; 5 — 100 kDa filtration; 6 — filtration through 0.22 µm filters in the presence of MgSO₄.

Statistical analysis

Statistical analysis (ANOVA, standard deviations) was carried out in GraphPad Prisma v.8.0.1 (GraphPad Software; USA).

RESULTS

Study design

Five different methods of phage purification were compared: precipitation with PEG, CsCl density gradient ultracentrifugation, sucrose density gradient ultracentrifugation, purification with 100 kDa centrifugal filters, and concentration on 0.22 µm cellulose filters in the presence of MgSO₄. Each of the studied purification procedures was conducted in 3 replicates. Their efficacy was assessed by measuring phage titers and endotoxin concentrations in the purified product.

First, phage lysates were obtained with the following phage titers: $1.25 \times 10^{12} \pm 7.46 \times 10^{10}$ PFU/ml and $2.25 \times 10^{12} \pm 1.34 \times 10^{11}$ PFU/ml for vB_KpnM_Se621 and vB_KpnP_Dlv622,

respectively. Bacterial debris and live bacterial cells were removed by filtration through 0.22 µm filters (Millipore; USA). Endotoxin concentrations in the non-purified phage lysates were $3,806,056 \pm 429,410$ EU/ml for vB_KpnM_Se621 and $189,456 \pm 12,406$ EU/ml for B_KpnP_Dlv622 (Fig. 1).

The obtained phage preparations were tested for contamination by seeding their 50 µl aliquots on nutrient agar. Mean values and standard deviations were calculated for the measured phage titers and endotoxin concentrations. We also calculated endotoxin concentrations in a single therapeutic dose of the phage preparation. Drawing on the literature, we assumed the effective therapeutic phage dose to be 10^9 [22–24].

Changes in endotoxin concentrations after applying different purification methods

In the first part of our study, we analyzed the efficacy of conventional laboratory methods for phage purification: precipitation with PEG and CsCl density gradient ultracentrifugation. Phage precipitation with PEG reduced

Table. Number of doses of therapeutic vB_KpnM_Se621 and vB_KpnP_Dlv622 phage cocktails and endotoxin content per dose calculated for 100 ml of the initial phage lysate

Purification method	Bacteriophage			
	vB_KpnM_Se621		vB_KpnP_Dlv622	
	Number of doses	Endotoxin content, EU/dose 10^9 PFU	Number of doses	Endotoxin content, EU/dose 10^9 PFU
Crude lysate	125,000	3045	225,000	84
PEG precipitation	50,000	0.76	10	3000
CsCl density gradient ultracentrifugation	3850	0.11	2100	0.21
Sucrose density gradient ultracentrifugation	7	40	2800	2
100 kDa filtration	15	3743	50	77
Filtration through 0.22 µm filters in the presence of MgSO ₄	150	47	50	128

endotoxin concentrations by 2–3 orders of magnitude ($p < 0.0001$): from $3,000 \pm 324$ to $3,817 \pm 486$ EU/ml. CsCl density gradient ultracentrifugation is the gold standard of phage purification; it resulted in an even greater reduction of endotoxin concentrations: from 303 ± 35 to 313 ± 20 EU/ml ($p < 0.0001$) (see Fig. 1).

Sucrose density gradient ultracentrifugation, 100 kDa filtration and slow filtration through $0.22 \mu\text{m}$ cellulose filters in the presence of MgSO_4 were chosen as alternative purification methods that could produce safe therapeutic formulations suitable for clinical use. Sucrose density gradient ultracentrifugation reduced endotoxin concentrations in the vB_KpnM_Seu621 lysate by 4 orders of magnitude ($p < 0.0001$), to 200 ± 28 EU/ml; for the vB_KpnP_Dlv622 lysate, the reduction was not so pronounced: by only 2 orders of magnitude, to $3,368 \pm 348$ EU/ml ($p < 0.0001$). Using 100 kDa centrifugal filter concentrators, we were able to lower endotoxin concentration by 1.5 orders of magnitude ($p < 0.0001$) in both lysates, bringing them down to $56,148 \pm 7,832$ EU/ml for phage vB_KpnM_Seu621 and $3,850 \pm 593$ EU/ml for phage B_KpnP_Dlv622. The efficacy of phage isolation in the presence of MgSO_4 was similar to that of PEG precipitation ($p > 0.9999$) and resulted in a reduction in endotoxin concentrations by 2–3 orders of magnitude: from $3,187 \pm 368$ to $3,502 \pm 372$ EU/ml ($p < 0.0001$) (see Fig. 1).

Changes in phage titers after applying different purification methods

A phage titer is an important characteristic of a therapeutic phage formulation; therefore, it needs to be either maintained or increased through purification.

Different purification methods produced different changes in phage titers. Precipitation with PEG did not change vB_KpnM_Seu621 titers significantly. By contrast, vB_KpnP_Dlv622 titers dropped by 3 orders of magnitude ($p < 0.0001$) to $1 \times 10^9 \pm 5.7 \times 10^7$ PFU/ml after PEG precipitation (Fig. 2). The gold standard of purification, CsCl density gradient ultracentrifugation, demonstrated a more uniform result: in both phage preparations, phage titers did not change significantly ($p > 0.9999$) (Fig. 2).

Sucrose density gradient ultracentrifugation led to a reduction ($p < 0.0001$) in vB_KpnM_Seu621 titers to $5 \times 10^9 \pm 2.9 \times 10^8$ PFU/ml but did not change vB_KpnP_Dlv622 titers significantly. Filtration with 100 kDa centrifugal filters reduced ($p < 0.0001$) phage titers in both phage preparations down to $1.5\text{--}5 \times 10^{10} \pm 3.1 \times 10^9$ PFU/ml. Filtration in the presence of MgSO_4 reduced ($p < 0.0001$) phage titers by 2–3 orders of magnitude, to $1.5 \times 10^{10} \pm 1.1 \times 10^9$ PFU/ml and $5 \times 10^9 \pm 2.5 \times 10^8$ PFU/ml for vB_KpnM_Seu621 and vB_KpnP_Dlv622, respectively (see Fig. 2).

DISCUSSION

Phage therapy has been used in clinical practice since the beginning of the 20th century. It is effective against infectious diseases of any etiology, especially against purulent and inflammatory wounds, otitis and bowel infections [25–27]. As more bacteria are acquiring multidrug resistance, the need arises to administer phage preparations intravenously or intrathecally. Phage formulations for intravenous administration must satisfy certain purity and efficacy criteria. According to the pharmacopoeial description provided in the Pharmacopoeia Monograph.1.2.4.0006.15 on Bacterial endotoxins, the amount of endotoxin in the formulation cannot exceed 5 EU/kg

per hour for an intravenous formulation and 0.2 EU/kg per hour for an intrathecal formulation [28]. According to the literature, the recommended phage titer ensuring a stable bactericidal effect is at least 10^9 PFU per dose [22–24].

For the purpose of our study, we selected 2 phages from two different families. Both phages vB_KpnM_Seu621 and vB_KpnP_Dlv622 caused lysis of the bacterial host, and the concentrations of phage particles in the lysates were very similar ($1.25\text{--}2.25 \times 10^{12} \pm 1.34 \times 10^{11}$ PFU/ml), but the detected amount of endotoxin in the phage lysates differed twentyfold (see Fig. 1). This finding is consistent with the literature reports: endotoxin concentrations in phage lysates vary considerably ($101\text{--}105$ EU/ 10^9 PFU) depending on the taxonomic position of the phage [10, 12, 13]. Based on the standard guidelines on the minimum phage titer in the therapeutic dose (10^9 PFU/ml) and the maximum allowed endotoxin concentration (325 EU/ml) in an intravenous phage formulation (calculated for a single 1 ml dose administered intravenously to a patient weighing 65 kg), we conclude that the lysate of phage vB_KpnP_Dlv622 can be used for intravenous administration after dilution, unlike the lysate of phage vB_KpnM_Seu621 with endotoxin concentrations being by an order of magnitude higher than the admissible maximum level (Table).

Being the gold standard of phage purification, CsCl density gradient ultracentrifugation turned out to be most effective in comparison with other tested methods (see Table). There are a few reports of successful phage therapy with intravenous phage formulations purified with CsCl density gradient ultracentrifugation [22, 23]. However, this purification method has its own limitations due to the use of cesium salts because the end preparation, even when dialyzed, may contain residual cesium [10]. According to the literature, CsCl density gradient ultracentrifugation can remove up to 99.6% of the total endotoxin amount, but generally its efficacy varies from 18% to 99.6% [13].

Phage precipitation with PEG is a common laboratory method for phage purification. In our study, its efficacy differed between phage families. After PEG purification, the preparation of vB_KpnM_Seu621, the representative of the Myoviridae family, still contained a fairly high vB_KpnM_Seu621 titer. But the titer of phage vB_KpnP_Dlv622 fell significantly after this procedure, showing its low efficacy for the Autographiviridae family. According to the literature, PEG precipitation is not equally effective for morphologically different phages, and it may be necessary to adjust the composition of the salt fraction or the length of the PEG molecule [10, 12, 29]. PEG precipitation removes up to 88% of endotoxin from the lysate of the filamentous *E. coli* phage M13 and reduces the amount of endotoxin twentyfold for the Myoviridae family [12].

Sucrose density gradient ultracentrifugation is widely used for the purification of virus suspension; however, it is rarely exploited to purify phage lysates [11]. In our study, this method generated purified phage preparations suitable for intravenous and even intrathecal administration, as was the case with vB_KpnP_Dlv622. It should be noted that due to the reduction in phage titers, we were able to obtain only 7 doses of vB_KpnM_Seu621 preparations, which may not be sufficient for one complete course of personalized therapy. The problem could be solved by optimizing the protocol by adjusting the concentration of the gradient solution and centrifugation time.

An endotoxin molecule is usually no larger than 10–20 kDa, so 100 kDa filtration may be effective for phage purification. In our study, the efficacy of this method differed between the phages and caused a reduction in phage titers in both preparations. For the *Myoviridae* phage, high endotoxin concentrations might

be explained by the presence of endotoxin micelles that grow to 1,000 kDa and therefore cannot pass the filter [30]. Earlier, tangential flow filtration, a modified version of 100 kDa filtration, was successfully used to effectively eliminate up to 90% of endotoxin from the phage lysate; however, this method was never tested on *Podoviridae* phages [10].

Slow filtration through 0.22 µm cellulose filters in the presence of MgSO₄ is normally used for phage enrichment from natural water sources but can be applied to purify phage lysates, too. Despite the fall in phage titers during purification, this method generated a sufficient number of therapeutic doses suitable for intravenous administration. Importantly, the efficacy of the method was satisfactory for both phages regardless of their taxonomic position and thus was the only alternative for the purification of *Myoviridae* phages.

Slow filtration in the presence of MgSO₄ and 100 kDa filtration turned out to be the least time-consuming, taking 2 and 1 h, respectively. Sucrose density gradient centrifugation and PEG precipitation took an average of 3.5 h. However, phage lysates purified with CsCl density gradient centrifugation and precipitation with PEG required further purification with dialysis, which extended purification time to 18 h. So, the

most rapid methods for producing purified phage preparations suitable for clinical use were 100 kDa filtration, slow filtration in the presence of MgSO₄, and sucrose density gradient ultracentrifugation.

Our findings were consistent with the literature: the amount of endotoxin after purification and the efficacy of the tested purification methods were different for different phage families. Due to the difference in phage size and/or the value of the sedimentation constant, an optimal purification method or a combination of purification methods will depend on the phylogenetic position of the bacteriophage.

CONCLUSIONS

Phage purification methods or their combination should be selected individually depending on the characteristics of a given phage. Nevertheless, sucrose density gradient centrifugation and slow filtration in the presence of MgSO₄ could be considered as the most promising for the purification of *Myoviridae* and *Autographiviridae* phage lysates. These methods yield safe sufficiently purified phage preparations containing permissible amounts of endotoxins.

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