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Extraction of PCR-ready DNA from *Staphylococcus aureus* bacteriophages using carboxyl functionalized magnetic nonporous microspheres

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ABSTRACT

Magnetic microspheres P(HEMA-*co*-EDMA) were used for PCR-ready phage DNA isolation from lysogenic strains of *Staphylococcus aureus*, including two new clinical isolates. The conditions of phage particle lysis were optimized. The quality of eluted phage DNA was evaluated by PCR. It was demonstrated that PCR-ready phage DNA can be isolated from small volumes of phage lysates (150 μl) by magnetic microspheres. The reported method is very expeditious without using toxic compounds such as phenol or chloroform. It can be used for phage identification and phage gene detection.

1. Introduction

Staphylococcus aureus is a major human Gram-positive opportunistic pathogen that causes a wide range of hospital- and community-acquired infections. The invasive potential of *S. aureus* strains depends on the presence of numerous virulence factors [1,2]. Bacteriophages capable of lysogenic life cycle are important mediators of horizontal gene transfer of different virulence factors such as Panton-Valentine leukocidin, staphylokinase, enterotoxins, and immune evasion factors [3,4]. The analysis of staphylococcal prophages is important to understand the emergence and evolution of very dangerous virulent hospital *S. aureus* strains [5]. In addition, DNA-based diagnostics exploiting the latest developments in post-genomics technology is desirable for characterization of bacteriophages used for the differentiation of clinical *S. aureus* strains [6], as well as for staphylococcal phages important in medicine as potential antibacterial agents [7].

Phage DNA isolation is a critical step in analyzing phage samples using PCR. The elimination of bacterial DNA background from phage lysates is necessary due to the prevention of false-positive results because the same gene can be carried both on bacterial and phage genomes. The routinely used method is based on phenol/chloroform extraction [8,9]. A commercial isolation technique based on the precipitation of phage particles by PEG [8] is also available.

Small-scale DNA extraction methods were developed for different bacteriophages. Bae et al. [10] extracted PCR-ready DNA from 1 ml of phage lysate by phenol/chloroform extraction followed by ethanol precipitation. A small-scale procedure, based on the precipitation of phage particles by $ZnCl_2$, was also described [11]. According to another method, phage DNA was extracted from 1 ml of phage lysate using guanidine hydrochloride. This method can be used for phages with high titres (1.10^9 pfu/ml) only [12]. Phage DNA can also be purified by chromatography, microchip sol–gel, or PFGE [13–15]. However, none of these methods is used routinely and most of them are relatively complicated to perform or expensive.

The aim of this study was to develop a quick method for phage PCR-ready DNA extraction from small volumes (150 μl) of different *S. aureus* phage lysates. High-titre (1.10^9 pfu/ml) and low-titre lysates (1.10^3 pfu/ml) induced from lysogenic strains were used for DNA isolation. Newly designed carboxyl-functionalized magnetic poly(2-hydroxyethyl methacrylate-*co*-ethylene dimethacrylate) microspheres (P(HEMA-*co*-EDMA)) were used for phage DNA isolation. The quality of extracted DNA was checked by PCR amplification.

2. Materials and methods

2.1. Bacteriophages and bacterial strains

S. aureus phages of the International Typing Set ϕ77 and ϕ53 were obtained from Professor V. Hájek (Palacký University, Olomouc, Czech Republic). The prophage-less indicator strain of *S. aureus* 1039 was obtained from Dr. Y. Yoshizawa and used as a

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propagating strain of phages [16]. Lysogenic strains *S. aureus* ISP8[53$^+$], *S. aureus* ISP8[77$^+$], and polylysogenic *S. aureus* CCM 7097 were prepared previously [17]. Two toxin-producing clinical isolates of lysogenic *S. aureus* strains designated by NRL in the strain number were isolated in different hospital laboratories in the Czech Republic and obtained from the National Reference Laboratory (NRL) for Staphylococci (National Institute of Public Health, Prague, Czech Republic).

2.2. Chemicals

2.2.1. Magnetic microspheres

Magnetic nonporous P(HEMA-*co*-EDMA) (92/8, w/w) microspheres were prepared by cellulose acetate butyrate-stabilized dispersion copolymerization of 2-hydroxyethyl methacrylate (HEMA) and ethylene dimethacrylate (EDMA) in the presence of maghemite (γ-Fe_2O_3) nanoparticles [18]. Subsequently, the hydroxy groups of the microspheres were oxidized with a 2% (w/v) aqueous solution of potassium permanganate under acidic conditions (2 M sulphuric acid) yielding 0.85 mM COOH/g [19]. Titration of carboxyl groups was carried out using 0.1 M NaOH after ion exchange with a 10% (w/v) aqueous solution of $BaCl_2$ [20]. The microspheres show clearly ferromagnetic behaviour, as reported earlier [21]. The average microsphere diameter D_n was determined from the measurement of at least 500 microspheres on scanning electron micrographs. Microsphere size distribution was characterized by the polydispersity index PDI (PDI = D_w/D_n, where D_w is the average weight of the microspheres and D_n is the average microsphere diameter). The microspheres were of 1.5 μm size with a narrow size distribution characterized by a polydispersity index PDI = 1.1. Magnetic glass particles (5 μm) were purchased from CPG (NY, USA).

2.2.2. Chemicals

Agarose was purchased from Serva (Heidelberg, Germany), ethidium bromide and PEG 6000 from Sigma (St. Louis, Missouri, USA). Iron oxide was needle-like maghemite (γ-Fe_2O_3) from the Société Française d'Electrométallurgie (Marseille, France). Monomers, 2-hydroxyethyl methacrylate (HEMA) and ethylene dimethacrylate (EDMA), both from Röhm (Darmstadt, Germany), were purified by distillation under reduced pressure in nitrogen atmosphere. Cellulose acetate butyrate (CAB) was a kind gift of Eastman (Kingsport, USA). RNase A, DNase I, pronase E from Serva (Heidelberg, Germany), proteinase K and dNTPs were from Roche Diagnostics (Mannheim, Germany). The PCR primers were synthesized by Sigma-Genosys (Steinheim, Germany); *Taq* polymerase was from Invitrogen (Carlsbad, USA), and the DNA marker 100 bp ladder (100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1200, and 1500 bp long DNA fragments) for gel electrophoresis was from New England Biolabs (New England, USA); Phage DNA was isolated from phage lysates (see Section 2.4.1). Nutrient Broth CM1 was from Oxoid (Basingstoke, UK). Other chemicals and solvents were of analytical grade and were taken from common commercial sources.

2.3. Equipment

The carboxyl group content on the microsphere surface was determined by titration on a 799 GPT Titrino (Metrohm, Herrisau, Switzerland). Magnetic particles were separated on an MPC-M magnetic particle concentrator Dynal (Oslo, Norway). DNA was amplified on a DNA thermal cycler T gradient (Biometra, Göttingen, Germany). Agarose gel electrophoreses were carried out using a POWER PAC 300 (Bio-Rad, Richmond, USA). The PCR products were visualized at 302 nm on a UV transilluminator ETX-35.M (Vilber Lourmat, Marne-la-Vallée, France), and photographed with a digital camera KODAK EDAS 290 equipped with 1D Image Analysis Software v3.6 (Eastman Kodak, Rochester, NY, USA).

2.4. Methods

2.4.1. Propagation of bacteriophages and induction of prophages

High-titre lysates (HTL) of 10^9 plaque-forming units per ml (pfu/ml) were prepared from 3 ml of stock phage HTL in 20 ml liquid medium Nutrient Broth CM1 on a propagation strain *S. aureus* 1039 for 2 h at 37 °C. Low-titre lysates (LTL: 1.10^3 pfu/ml) were prepared after UV induction of lysogenic strains carrying ϕ77 and ϕ53 prophages and from lysogenic clinical *S. aureus* strains. Lysogenic bacterial cells were centrifuged at 5000 × *g* for 20 min and resuspended in physiological buffer to optical density $A_{600\,nm}$ = 0.15; then 10 ml of this suspension was irradiated in a Petri dish (diameter 9 cm) by UV light (254 nm, 15 W, 28 cm). After irradiation, 10 ml of 10× concentrated nutrient broth and 30 ml of physiological buffer was added. The resulting mixture was incubated under aeration for 2 h at 37 °C and plated on an *S. aureus* 1039 strain giving low-titre phage lysates. The LTLs were prepared from one plaque in 500 μl of a bacterial culture of the *S. aureus* 1039 strain ($A_{600\,nm}$ = 0.22) for 2 h at 37 °C. The remaining bacteria and bacterial cell debris in lysates were removed by centrifugation at 5000 × *g* for 15 min and by subsequent filtration. Small volumes (150 μl) of phage lysates were used for sample preparation and DNA extraction using the methods tested.

2.4.2. Bacteriophage lysate treatment and DNA isolation using magnetic microspheres

DNA was released from phage virions using different procedures to evaluate optimal phage DNA isolation (see below). Isolation and purification of control DNA was performed by phenol extraction according to authors [8]. The lysis step of phage capsids and phage DNA isolation using magnetic microspheres were optimized. Three different methods of phage lysate treatment were tested to simplify the procedure of phage DNA isolation: (1) phage lysates (150 μl) were treated by RNase A (5 μg/ml) and DNase I (1 μg/ml) for 30 min at 37 °C to remove bacterial RNA and DNA background. Then, RNase A and DNase I were precipitated by chloroform and removed by centrifugation before phage capsid lysis. The sample was subsequently treated with proteinase K (50 μg/ml) activated by Ca^{2+} ions (5 mM) for 1 h at 56 °C; (2) phage lysates were treated by RNase A (5 μg/ml) and DNase I (1 μg/ml) for 30 min at 37 °C to remove bacterial RNA and DNA without their precipitation by chloroform. The sample was subsequently treated as in (1); (3) phage lysates were treated directly with proteinase K (50 μg/ml) activated by Ca^{2+} ions for 1 h at 56 °C. The proteinase K treatment conditions were optimized: $CaCl_2$ concentrations 0, 1, 5 and 10 mM and incubation times 1, 8 and 16 h. In some cases, 10% (w/v) SDS was added to the lysis buffer (without proteinase K) to a final concentration of 0.5%.

A total of 150 μl of the phage lysate sample, 50 μl of the magnetic microsphere suspension (2 mg/ml), 100 μl of 40% (w/v) PEG 6000, and 200 μl of 5 M NaCl was added, mixed, and incubated for 15 min at laboratory temperature. The final PEG and NaCl concentrations of 8.0% (w/v) and 2.0 M, respectively, were used. Carboxyl-functionalized magnetic microspheres carrying adsorbed DNA were separated in a magnetic particle concentrator (5 min), the supernatant was discarded, and the magnetic microspheres were washed with 500 μl of 70% (v/v) ethanol and dried briefly. The DNA adsorbed on the microspheres was eluted in 50 μl of TE buffer at laboratory temperature for 10 min. The DNA in the eluates was used for PCR amplification. DNA concentrations in eluates were determined on Qubit Fluoremeter (Invitrogen, Carlsbad, USA) using Quant-iT dsDNA BR Assay Kit according to the recommendations of producer (Invitrogen, Carlsbad, USA). Experiments were carried out triplicate.

2.4.3. PCR amplification and detection of PCR products

A multiplex PCR assay with primers designed previously [22] and specific to phage DNAs ϕ53 (primers SGB1 and SGB2) and ϕ77 (SGFa1 and SGFa2) and to the contaminating bacterial *S. aureus* DNA (primers SAU1 and SAU2) were used for the amplification. The genomic DNAs from lysogenic strain of *S. aureus* CCM 7097 and nonlysogenic strain *S. aureus* 1039 were used as the control DNA [22]. The reaction mixtures (25 μl) consisted of 75 mM Tris–HCl (pH 9.0), 50 mM KCl, 2 mM $MgCl_2$, 20 mM $(NH_4)_2SO_4$, 200 μM each of dNTPs, and 0.2 μM each of 6 primers. To each PCR mixture, 1.5 units of *Taq* DNA polymerase and 3 μl of DNA matrix eluate were added. PCR was performed using 30 cycles of amplification consisting of denaturation (1 min, 94 °C), annealing (1 min 30 s, 58 °C), and DNA chain extension (1 min 30 s, 70 °C). The final polymerization step was prolonged to 5 min. PCR products of 548 bp for phage ϕ77, 405 bp for phage ϕ53, and 217 bp for bacterial DNA were detected using gel electrophoresis with 2.0% (w/v) agarose in TAE buffer (0.04 M Tris–acetate; 1 mM EDTA, pH 8). DNA was stained with ethidium bromide (1 μg/ml), and photographed under UV light (see Section 2.3).

3. Results

The PCR-based characterization of virulence genes carried by phage is one of the principal methods used for bacteriophage description. A standard phenol extraction procedure for phage DNA isolation involves degradation of bacterial RNA and DNA in phage lysates to avoid PCR interference. The presence of RNA decreases PCR sensitivity and can lead to false-negative results; bacterial DNA can lead to false-positive results, because the same genes can also be carried on bacterial DNA. As the standard procedure of DNA isolation is time-consuming and laborious, an alternative procedure using a small volume of phage lysates (150 μl) and magnetic microspheres was evaluated. Magnetic hydrophilic P(HEMA-*co*-EDMA) microspheres covered by carboxyl groups were previously used for the isolation of high-molecular weight bacterial PCR-ready DNA with success [23,24]. For this reason, these microspheres were newly applied to phage DNA extraction. Since newly designed P(HEMA-*co*-EDMA) microspheres were used for DNA isolation, their possible interference in PCR was tested [25]. For this reason the PCR sensitivity using DNA eluted from the tested P(HEMA-*co*-EDMA) microspheres and commercially available magnetic glass particles was compared.

Magnetic glass particles were used for the comparison of DNA isolation under the same experimental conditions as were newly designed P(HEMA-*co*-EDMA) microspheres.

The phage DNA yield depends on the number of phage particles (plaque-forming units in phage lysates) and on the efficiency of phage capsid lysis. Bacteriophages ϕ77 and ϕ53 with different capsid composition (classified into different serological groups) were used here for the evaluation of the DNA isolation protocol. To guarantee efficient DNA release from phage capsids both steps – the lysis of bacteriophage capsids and phage DNA isolation using magnetic microspheres – were optimized. At first, the conditions for proteinase K treatment were optimized (see Section 2.4.2 (3)). Proteinase K has two binding sites for Ca^{2+} ions and the removal of Ca^{2+} ions reduces proteinase K activity [25]. Lysates (150 μl) of both phages were treated with proteinase K (final concentration 50 μg/ml) by different incubation times at 56 °C in the presence of different Ca^{2+} ion concentrations (see Section 2.4.2). Increasing incubation time had no statistically significant effect on the efficiency of phage ϕ53 DNA release. If 5 mM Ca^{2+} ion concentration was used for proteinase activation DNA concentrations were 10.37 ± 0.11 μg/ml after 1 h treatment and 10.22 ± 0.07 μg/ml after 16 h treatment. Concentration of Ca^{2+} ions in the range 0–5 mM had no statistically significant effect on the efficiency of DNA release.

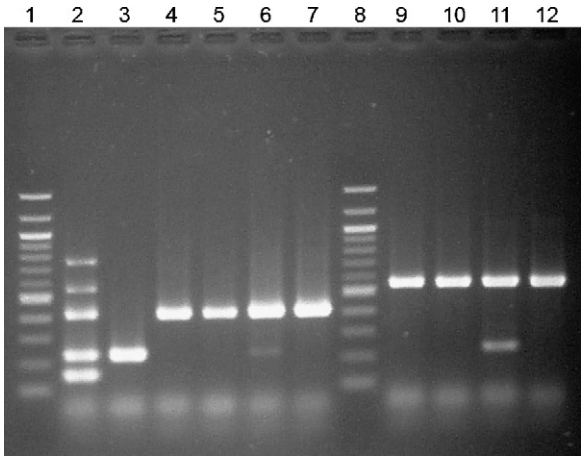

Fig. 1. Agarose gel electrophoresis of PCR products amplified from phage DNA isolated by magnetic P(HEMA-*co*-EDMA) microspheres. Influence of pretreatment procedures on PCR specificity. Conditions: 2.0% (w/v) agarose gel in TAE buffer (0.04 M Tris–acetate; 1 mM EDTA, pH 8). Lanes 1 and 8: DNA standard (100 bp ladder); PCR products obtained by amplification of DNA from—lane 2: lysogenic *S. aureus* CCM 7097 strain containing four prophages (positive control, phenol extraction—A (744 bp), Fa (548 bp), B (405 bp), SAU (217 bp), and Fb (147 bp)); lane 3: nonlysogenic *S. aureus* 1039 strain (bacterial DNA phenol extraction); lanes 4–7: *S. aureus* phage ϕ53; lane 4: DNA isolated with DNase I, RNase A pretreatment, chloroform extraction and capsid treatment by proteinase K; lane 5: DNA isolated with DNase I, RNase A pretreatment and capsid treatment by proteinase K; lane 6: DNA isolated without DNase I, RNase A pretreatment and treated by proteinase K; lane 7: control phage DNA isolated phenol extraction; lanes 9–13: *Staphylococcus aureus* phage ϕ77, samples treated as phage ϕ53 and the results are in the same order as above.

DNA concentration was 10.45 ± 0.09 μg/ml for 0 mM Ca^{2+} ion concentration and 10.22 ± 0.07 μg/ml for 5 mM Ca^{2+} ion concentration after 16 h treatment. For a concentration of 10 mM Ca^{2+} a decrease of DNA yield was observed out and white precipitate containing the bulk of DNA was created. Efficiency of phage capsid lysis was sufficient after 1 h treatment without Ca^{2+} ion addition. Addition of Ca^{2+} ions to proteinase K had no effect on the efficiency of DNA release probably due to the presence of Ca^{2+} ions in external source (medium).

Detergent SDS at the end concentration of 0.5% was added to the lysis mixture with the aim to facilitate DNA release from phage particles. Detergent SDS at a final concentration of 0.5% had a positive effect on DNA yield. However, the amount of DNA was lower (8.12 ± 0.05 μg/ml for 1 h) than in the case of treatment using proteinase K without addition of Ca^{2+} ions (10.47 ± 0.09). There was a slight difference in DNA release between phages ϕ53 and ϕ77, apparently due to different capsid composition or due to presence of defect phages (not forming plaques) in phage stocks.

The UV induction of prophages from lysogenic bacterial strains allows phage amplification and cell lysis. Phage DNA was isolated from phage lysates as given above. The total DNA amount obtained from UV-induced phages (LTL) was much higher than expected probably due to the presence of many defective phage particles that cannot form plaques on the indicator strain.

In the next step, DNA was isolated from 150 μl of lysed capsid phage samples using magnetic P(HEMA-*co*-EDMA) microspheres. DNA macromolecules collapse in aqueous solutions of polyethylene glycol 6000 (PEG 6000) and sodium chloride and adsorb on the surface of hydrophilic magnetic microspheres [19]. The amount of DNA eluted from microspheres in 50 μl TE buffer was approximately 0.5–10.0 ng (10–200 pg/μl). This amount could not be detected on agarose gel; however, it was sufficient for PCR. The amount of eluted DNA was the same for both types of particles and was lower than in experiments with bacterial DNA [19]. However, in previous experiments the recovery of bacterial DNA was determined for higher DNA

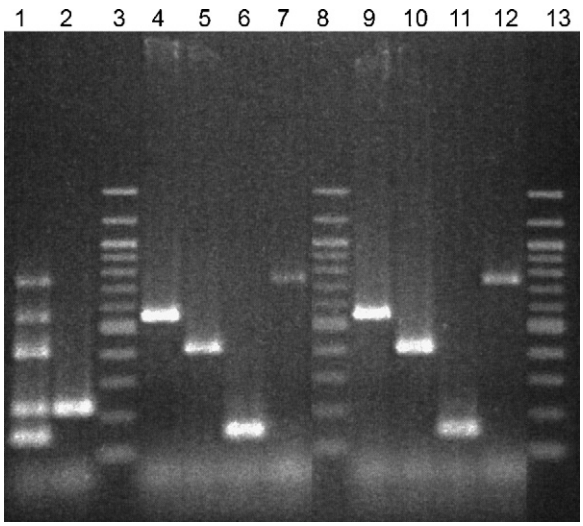

Fig. 2. Agarose gel electrophoresis of PCR products amplified from DNA isolated from phages induced from lysogenic *S. aureus* strains. Conditions: 2.0% (w/v) agarose gel in TAE buffer (0.04 M Tris–acetate; 1 mM EDTA, pH 8). Lanes 3, 8, 13: DNA standard (100 bp ladder); PCR products obtained by amplification of DNA from—lanes 1, 2: control—phenol extraction; 1: lysogenic *S. aureus* CCM 7097 strain containing four prophages (positive control—A (744 bp), Fa (548 bp), B (405 bp), SAU (217 bp), and Fb (147 bp)), 2: nonlysogenic *S. aureus* 1039 strain (bacterial DNA); lanes 4–7: magnetic P(HEMA-*co*-EDMA) microspheres; 4: phage ϕ77 DNA, 5: phage ϕ53 DNA, 6: phage ϕ06/622 DNA, 7: ϕ05/840 DNA; lanes 9–12: magnetic glass; 9: phage ϕ77 DNA, 10: phage ϕ53 DNA, 11: phage ϕ06/622 DNA, 12: phage ϕ05/840 DNA.

concentrations. Phage DNAs (ϕ53–3.1 and ϕ77–2.4 ng/μl) isolated by phenol/chloroform extraction were used as controls.

The quality of isolated DNA was checked by multiplex PCR that specifically detects targeted sequences carried by prophage of the lysogenic strain and/or *S. aureus* chromosomal DNA [22]. The parameters of PCR were optimized to detect femtograms of phage DNAs in the PCR mixture. Agarose gel electrophoresis of PCR products is given in Fig. 1. Using this PCR it was confirmed that DNase I treatment (see Section 2.4.2 (1)) is necessary for degradation of bacterial DNA before phage DNA release from phage heads (compare lanes 3, 6, and 11 in Fig. 1). It was confirmed that the chloroform extraction of DNase I and RNase A before proteinase K is not necessary (see Section 2.4.2 (2)).

The optimized method for phage DNA isolation using magnetic microspheres was applied to 2 hospital lysogenic strains isolated from clinical sources—*S. aureus* NRL 06/622 and *S. aureus* NRL 05/840. The target genes carried by induced phages ϕ05/840 and ϕ06/622 were successfully amplified (Fig. 2). The method proposed is fast and suitable for isolation of PCR-ready DNA from phage particles. The sensitivity of PCR was comparable with that achieved using magnetic glass particles. The advantage of using magnetic P(HEMA-*co*-EDMA) microspheres in contrast to magnetic glass particles was a shorter time of their separation in the presence of an external magnet and a slower sedimentation of the particle suspension.

4. Conclusions

Magnetic microspheres P(HEMA-*co*-EDMA) were used for PCR-ready phage DNA extraction. It was demonstrated that phage DNA can be isolated and amplified from small volumes (150 μl) of high-titre and low-titre phage lysates by tested magnetic microspheres. The reported small-scale DNA extraction method seems to be very effective for DNA-based staphylococcal phage diagnostics. The method is very expeditious without using toxic compounds such as phenol or chloroform.

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