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FULL PAPER

An alternative molecular approach for rapid and specific detection of clinically relevant bacteria causing prosthetic joint infections with bacteriophage K

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SUMMARY

Prosthetic joint infections (PJI) represent the most serious cause of prosthetic joint loosening, with high impact on patient life and health economics. Although not entirely reliable, the cultivation of intraoperative prosthetic tissue or synovial fluid remains the gold standard for determining the cause of PJI. Therefore, molecular methods are increasingly being introduced. The aim of this study was to optimize and assess an alternative molecular approach with the use of bacteriophage K for more rapid and specific detection of staphylococci in sonicate fluid (SF) of PJI. The best results with the method were obtained after 180 min of sample incubation with 10^4 PFU/mL of bacteriophage K. DNA isolation prior to qPCR analysis was confirmed unnecessary, while chloroform addition to samples after incubation with bacteriophage K improved bacterial detection by 100×. The method had a limit of detection of 6.8×10^2 CFU/mL and was found suitable for the detection of staphylococci in SF of removed prosthetic joints, giving results comparable to standard microbiological methods in just four hours. The optimized method was found fit for the purpose, offering potential advantages over the use of molecular detection methods to detect bacterial DNA.

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INTRODUCTION

Prosthetic joint infection (PJI) represents a high health risk to patients, leading to pain, restricted movement, prosthetic loosening, antibiotic treatment and prolonged hospitalization, as well as re-hospitalization, surgical treatment and, in the most severe cases, amputation or death (Dolinar *et al.*, 2007; Kocjančič *et al.*, 2014; Goswami *et al.*, 2018). Consequently, PJI poses a significant burden on the healthcare economy. Fast and accurate diagnostics are crucial for effective determination of the right treatment to achieve the optimal outcome and to address the problem of emerging microbial resistance due to excessive overuse of antibiotics. So far, the cultivation of intraoperative prosthetic tissue or synovial fluid remains the gold standard for determining the cause of PJI, although results are not entirely reliable and therefore not fit for the purpose (Esteban *et al.*, 2012; Puig-Verdié *et al.*, 2013; Saeed, 2014; Lee and Goodman, 2015; Goswami *et al.*, 2018). In around 7% of cases, these traditional methods produce false negative results (Kocjančič *et al.*, 2014); however, according to the literature this number is underestimated and the real number of PJI cases with false negative results could be much higher (Tande and Patel, 2014). The method of sampling is most likely the cause, especially where biofilm infections are concerned. To address this problem, research has focused primarily on new sampling strategies, that is, sonication of the joint prosthesis immediately after removal and cultivation of the obtained sonicate fluid (SF) (Trampuz *et al.*, 2007). A meta-analysis of 12 studies of SF cultivation showed that sensitivity increased from 81% to 96% compared to the cultivation of prosthetic tissue (Zhai *et al.*, 2014). In addition, various molecular methods, especially polymerase chain reaction (PCR) -based diagnostics, are increasingly being introduced in the diagnosis of PJI. The contamination of samples with bacterial DNA and, in particular, the inability to distinguish between living and dead bacterial cells are the greatest limitations of this method, since the method detects the presence of microbial DNA in the sample regardless of its origin, leading to false positive results (Bjerkan *et al.*, 2012; Mlekuž *et al.*, 2015). The detection of bacteria in mixed culture represents another problem, as detection by broad-range PCR (BR-PCR) is only possible for the dominant bacterial species (Hartley and Harris, 2014). At the same time, the method gives information only on whether the result is positive or negative, while the causative bacterial species is determined only after sequencing, which takes an extra 24 hours (Gomez *et al.*, 2012).

Bacteriophages are bacterial viruses that are interesting for use in both therapeutic and diagnostic purposes (Lu and Koeris, 2011; Schofield *et al.*, 2012). They are easy to

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maintain and their production is quick and inexpensive without the need for expensive laboratory equipment. Although phage-based diagnostics are rapid and sensitive, are rarely used in clinical practice (Schofield *et al.*, 2012). Bacteriophage K is a big virulent lytic bacteriophage from the family *Myoviridae* of the order *Caudovirales*. In the literature, it is widely described as a broad host range anti-staphylococcal bacteriophage capable of infecting different staphylococci (O'Flaherty *et al.*, 2005; Cerca *et al.*, 2007; Bean *et al.*, 2014; Šuster *et al.*, 2017). In our previous study (Šuster *et al.,* 2017) we tested the potential of phage K for detection of staphylococcal PJI using spot assay, broth-based test and a developed ATP assay on different bacterial species. Results from 21 bacterial strain cultures and 10 infected sonicate fluids showed that phage K has the ability to specifically distinguish and lyse only staphylococci, and was therefore selected for use in this study. Recently, (Rees and Barr, 2017) published a study on the use of bacteriophage K for the detection of methicillin resistant *Staphylococcus aureus* (MRSA) via bacteriophage amplification and detection of specific bacteriophage peptides by matrix-assisted laser desorption/ionization (MALDI). In the study, they aimed to distinguish between MRSA and methicillin-sensitive *S. aureus* by antibiotic supplementation and subsequent monitoring of multiplication/non-multiplication of bacteriophage K. However, they did not consider bacteriophage K's ability to infect different staphylococci (Cerca *et al.*, 2007; O'Flaherty *et al.*, 2005; Šuster *et al.*, 2017), and thus conducted the study using only different *S. aureus* strains. According to the literature, this and our previous study (Šuster *et al.*, 2017) are the only studies on the use of bacteriophage K in the diagnosis of staphylococcal infections.

Staphylococci are the most frequently isolated etiological agent, causing approximately 50% to 60% of PJI (Zimmerli *et al.*, 2004; Moran *et al.*, 2010; Peel *et al.*, 2011; Tande and Patel, 2014). The purpose of this study, therefore, was to optimize and assess an alternative molecular approach with the use of bacteriophage K for more rapid and specific detection of staphylococci in SF of PJI.

MATERIALS AND METHODS

Bacteria and bacteriophages

Staphylococcus aureus subsp. *aureus* bacteriophage K ATCC 19685-B1™ (phage K) was purchased from the American Type Culture Collection (ATCC, Manassas, Vancouver, Canada). *Staphylococcus aureus* ATCC 25923 was provided by the Institute of Public Health, Koper, Slovenia. *S. aureus* DPC 5246 was kindly provided by *Aidan Coffey, PhD* (Cork, Ireland).

For the maintenance and growth of bacteria, brain heart infusion broth (BHI, Sigma-Aldrich, St. Louis, MO, USA) was used. Soft agar for plaque assays (Ellis and Delbrück, 1939) was BHI supplemented with 0.7% of agar technical (Biolife, Milan, Italy). SM buffer was used for phage storage.

Phage multiplication

Phage K was routinely propagated on *S. aureus* DPC 5246 as described previously (Adams, 1959). Briefly, an overnight *S. aureus* DPC 5246 culture was diluted 1:100 in BHI and grown with shaking until mid-exponential phase (approx. 4 h). 200 µL of the culture and 200 µL of the corresponding phage dilution were mixed and incubated at 37°C for 15 min. The infected bacterial solution was then mixed into 5 mL soft BHI agar cooled to ≈45°C and poured onto a BHI agar plate. Plates prepared in that fashion were incubated overnight at 37°C. The following day, 5 mL of SM buffer (100 mM NaCl, 8 mM $MgSO_4 \cdot 7H_2O$, 50 mM Tris-Cl, 0.01% (w/v) gelatin, H_2O to 1 L, pH 7.5) was poured onto each plate and incubated overnight at room temperature. Afterwards the SM buffer was removed, centrifuged 10 min at 9000× g and filtered through a 0.2 µm Minisart® NML cellulose acetate filter (Sartorius stedim Biotech GmbH, Goettingen, Germany). The phage solution in SM buffer was stored at 4°C until use. Phage stock titer was determined every month by the Adams agar-overlay method (Adams, 1959) and phage numbers in stock solutions were determined as plaque forming units per mL (PFU/mL) from triplicate plate counts.

Bacteriophage genomic DNA isolation

For real-time PCR (qPCR) optimization purposes, DNA of bacteriophage K was extracted and purified using DNA Wizard® Genomic DNA Purification Kit (Promega, Madison, Wisconsin) according to the manufacturer's protocol with modifications. Briefly, 500 µL of bacteriophage K lysate suspension with a concentration of 1×10^{10} PFU/mL was incubated 10 min at 80°C with 300 µL Nuclei lysis solution. After cooling to room temperature, 2 µL of RNase solution was added and incubated 1 h at 37°C. After the addition of 200 µL Protein Precipitation Solution, the suspension was vigorously vortexed at high speed for 20 s and incubated on ice for 5 min. Following a 3 min centrifugation step in a table top Eppendorf® Minispin centrifuge (Eppendorf AG, Hamburg, Germany) at 12045× g, the supernatant was transferred into 800 µL isopropanol, gently mixed and centrifuged again for 2 min. The DNA pellet was then washed with 600 µl of room temperature 70% ethanol by gently inverting the tube several times and centrifuged for 2 min. The ethanol was carefully aspirated and the pellet was allowed to air-dry for 15 min. The DNA pellet was rehydrated by incubating in 50 µL of DNA Rehydration Solution overnight at 4°C. DNA was quantified fluorometrically with a Qubit™ fluorometer using the Qubit® dsDNA BR Assay Kit (Life Technologies, Waltham, Massachusetts) according to the manufacturer's instructions.

Real-time PCR primer design and reaction conditions optimization

The genome sequences of bacteriophage K was obtained from GenBank (accession number NC_005880.2). Based on the genome sequence (Gill, 2014), qPCR primers were selected from the major capsid protein gene and were 5'-CGTAGGTCACTCTCGTTTCG-3' (sense) and 5'-CGTCACCGTAGAATGAAGCC-3' (antisense). The primer sequences were compared to known gene databases at NCBI, using BLAST (Basic Local Alignment Search Tool) for *in silico* determination of their specificity. Real-time PCR primers were purchased from IDT**,** Inc. (Integrated DNA Technologies, Coralville, Iowa). To determine the optimal annealing temperature and primer concentration in qPCR, experiments with three different primer final concentrations (250, 450 and 650 nM) were performed at four different annealing temperatures: 55, 58, 59 and 60°C.

Real-time PCR detection assay

Reactions were performed in a total volume of 20 µl containing: 1 µl of DNA template (isolated phage K DNA/

lysate containing live phage K particles), 10 µl of 2× Syber Green PowerUp master mix (Applied Biosystems, Life Technologies, Burlington, Canada), 0.9 µl of each primer (450 nM final concentration), 7.2 µl nuclease free water (Invitrogen, Carlsbad, California) and were run on the QuantStudio 3 Real-Time PCR System (Applied Biosystems, Foster City, California) on a fast mode setting. All qPCR reactions were performed under the following conditions: Uracil-DNA glycosylase activation for 2 minutes at 50°C, DNA polymerase activation for 2 minutes at 95°C followed by 45 amplification cycles consisting of a denaturation step of 1 second at 95°C and a combined primer annealing and extension step of 30 seconds at 60°C. A final dissociation curve step was used to verify the specificity of the qPCR reaction for each amplified product. Threshold cycle (Ct) values were automatically generated by the QuantStudio 3 Real-Time PCR System software. In each qPCR run, a non-template control (using 1 µl nuclease free water) as well as a reference (uninfected) control, containing only the initial amount of added phages/phage DNA, were included.

qPCR performance on intact bacteriophage K

To determine whether the DNA isolation step was necessary or could be discarded, a series of 10-fold purified phage DNA extracted from phage suspension with concentration from 1 to 10^7 PFU/µl and a series of 10-fold phage K lysate dilutions (5.2 to 5.2×10^6 PFU/µl) were prepared in SM buffer and assessed with qPCR. The number of viable phage particles per sample was confirmed by plaque assay prior to DNA isolation for purified phage DNA samples and prior to the qPCR reaction for samples with intact phage particles (phage K lysate suspension), and were 1×10^{10} PFU/mL and 5.2×10^9 PFU/mL, respectively. qPCR results from both sources were analyzed using the linear regression method with a 95% confidence interval.

Chloroform addition

A series of 10-fold *S. aureus* ATCC 25923 dilutions in BHI in the range of 7.8×10^6 CFU/mL to 0 CFU/mL were prepared and 10 µL of bacteriophage K suspension with the final concentration of 10^4 PFU/mL was added to 890 µL of each bacterial dilution and incubated for 180 min at 37°C with shaking on a Sanyo orbital shaker (Sanyo, Osaka, Japonska) at 200 rpm. The experiments were run in parallels, one with the addition of 30 µL chloroform right after the 180 min incubation period and one without. Samples were vortexed vigorously for 2 min and centrifuged for 5 min in a table top Eppendorf® Minispin centrifuge (Eppendorf AG, Hamburg, Germany) at 3287× g. 1 µL of each supernatant was then assessed in qPCR to determine the influence of chloroform addition to qPCR results.

Bacteriophage concentration and incubation period

Samples were prepared by diluting bacterial cultures overnight in fresh BHI. A series of 10-fold *S. aureus* ATCC 25923 dilutions in the range of 6.8×10^6 CFU/mL to 0 CFU/mL were prepared and 10 µL of bacteriophage K suspension was added to 890 µL of each bacterial dilution and incubated at 37°C with shaking on a Sanyo orbital shaker (Sanyo, Osaka, Japan) at 0.45× g. The experiment was run in four parallels, testing two different bacteriophage concentrations (10^3 PFU/mL and 10^4 PFU/mL) at two different incubation periods (120 min and 180 min). Each experiment was conducted three times and included a bacterial suspension without the addition of bacteriophage as an NTC. Bacterial numbers in sample dilutions were determined as colony forming units per mL (CFU/mL) from triplicate plate counts. After incubation, 30 µL of chloroform was added and samples were vortexed vigorously for 2 min. Following centrifugation in a table top Eppendorf® Minispin centrifuge (Eppendorf AG, Hamburg, Germany) at 3287× g, 1 µL of each supernatant were assessed in qPCR for bacteriophage K DNA detection in triplicates. The limit of positivity was determined as 3 times the sample's standard deviation (SD) below the mean of the reference control sample's Ct. The detection was considered positive in samples with a Ct value below the limit of positivity of a sample and LOD was set at the lowest bacterial concentration that still gave positive results.

Evaluation of the method for testing of clinical samples

SF samples were obtained from sonication of explanted prosthetic joints of patients with hip prostheses loosening, as previously reported (Šuster *et al.*, 2017). Briefly, the prosthesis was subjected to vortexing and sonication at a frequency of 40 kHz and power density of 0.22 W/cm² in a BactoSonic® ultrasonic bath (Bandelin GmbH, Berlin, Germany). Obtained SF samples were then processed by centrifugation (Heraeus Multifuge 1 SR, Heraeus, Hanau, Germany) for 15 min at 5000× g. After the first centrifugation, supernatants were discarded and pellets were resuspended with 2 mL of BHI. Subsequently, samples were centrifuged again (Eppendorf® Minispin, Eppendorf AG, Hamburg, Germany) for 5 min at 3287× g and pellets were resuspended in 2 mL of BHI. Afterwards, 10 µL of bacteriophage K suspension was added to 990 µL of sample to a final concentration of 10^4 PFU/mL. For each clinical sample tested, a sonicate control without bacteriophage K was prepared by adding 10 µL of BHI to 990 µL sample and assessed along with a non-template control (NTC). Likewise, each experiment included a reference control consisting of 990 µL BHI with 10 µL of bacteriophage K suspension with a final concentration of 10^4 PFU/mL. Following 180 min of incubation at 37°C with shaking on a Sanyo orbital shaker (Sanyo, Osaka, Japan) at 200 rpm, 30 µL of chloroform was added to each sample and to its corresponding controls and processed as described above. 1 µL of each obtained supernatant was assessed in qPCR for bacteriophage K DNA detection in triplicates. The detection of staphylococci was considered positive in samples with a Ct value below the lower limit of positivity of a sample.

RESULTS

The designed primers were found specific and suited to obtain the predicted 221 bp amplicon, whereas they did not match to any bacterial or human DNA, even if up to five mismatches were tolerated. Initial experiments were performed with purified phage K DNA to determine optimal qPCR parameters. The optimal annealing temperature and primer concentration were determined to be 60°C and 450 nM, respectively. The same results were achieved with phage lysates.

Results obtained from the incubation of bacteriophage K at a final concentration of 10^3 PFU/mL for 120 min and 180 min are compared in *Figure 1*. Bacterial detection improved with longer incubation, with the limit of detection

lowering from 6.8×10^4 CFU/mL to 6.8×10^3 CFU/mL. When incubating bacterial culture samples with bacteriophage K at a higher final concentration -10^4 PFU/mL (*Figure 2*), the correlation between longer incubation period and improved detection remained the same. In this case, the limit of detection lowered from 6.8×10^3 CFU/mL to 6.8×10^2 CFU/mL. The best results with the method of qPCR were thus obtained after 180 min of sample incubation with bacteriophage K at the final concentration of 10^4 PFU/mL. The method had a limit of detection of 6.8×10^2 CFU/mL, which represents less than one bacteria per µL of sample used in a single qPCR reaction.

The indirect detection of staphylococci is possible from the suspension of newly released bacteriophages without prior DNA isolation. qPCR results of purified phage DNA dilutions and qPCR results of phage lysate dilutions are expressed as Ct value compared to the number of plaque-forming units of bacteriophage K per µL of sample with the method of linear regression (*Figure 3*). Results show a good correlation between the use of purified phage DNA and the use of phage lysate as source of template DNA, for which the step of DNA isolation prior to qPCR analysis is unnecessary and could be omitted.

The addition of chloroform to staphylococcal culture sam-

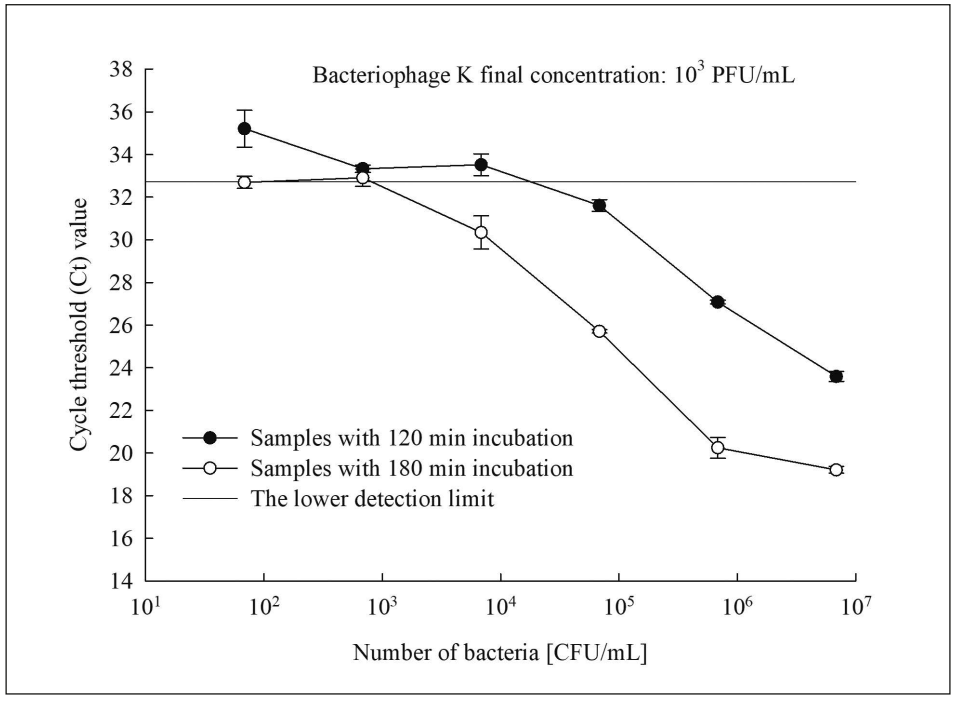

Figure 1 - *Comparison of 120 min and 180 min incubation period of serially diluted S. aureus ATCC 25923 culture with bacteriophage K at a final concentration of 10^3 PFU/mL, showing the lower limit of positive detection with qPCR. Results are expressed as mean values ± SD. The limit of positivity is determined as 3 times the sample's SD below the mean of the reference control sample's Ct.*

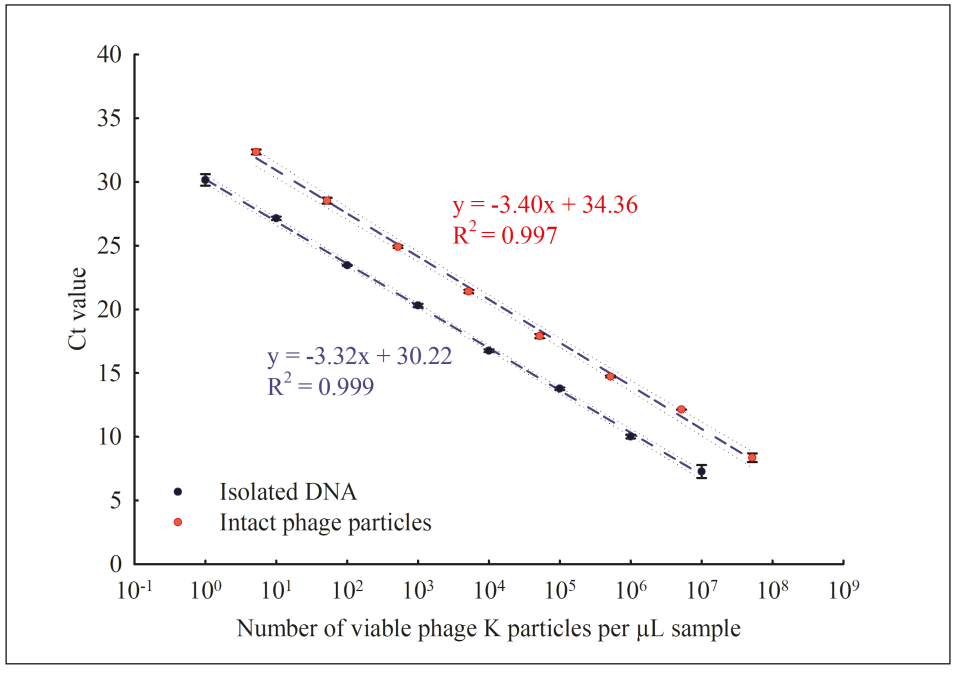

Figure 2 - *Performance testing of qPCR with isolated DNA from bacteriophage K and with intact phage particles. Results are analyzed with the linear regression method with 95% confidence interval.*

ples after 180 min incubation with bacteriophage K at a final concentration of 10^4 PFU/mL improved bacterial detection by lowering the limit of detection from 7.8×10^5 CFU/mL to 7.8×10^2 CFU/mL (*Figure 4*). The addition of chloroform after incubation with bacteriophage K was therefore included in the assay as an important step to improve bacterial detection.

To determine the suitability of the method for the detection of staphylococci in sonicate fluid obtained from the sonication of removed prosthetic joints, the method was further tested on SF samples obtained from 25 patients undergoing revision surgery due to suspected infection. Results were compared to results obtained from standard microbiological diagnostic procedures. Following the definition of PJI, 11 patients were diagnosed with PJI and 14 patients with aseptic loosening of prosthesis. Out of those, 4 PJI were caused solely by *S. aureus*, 1 by *S. capitis*, 1 by *S. epidermidis*, 2 were mixed infections caused by *S. epidermidis* along with bacteria from other species (*Cuticubacterium acnes* and in the other case with *E. coli*), 1 PJI was caused by *Enterococcus faecalis,* 1 with *Serratia marcescens* and 1 with *C. acnes*. With the method of qPCR detection of bacteriophage K DNA, all 8 SF where staphylococci were present were confirmed positive, whereas 17

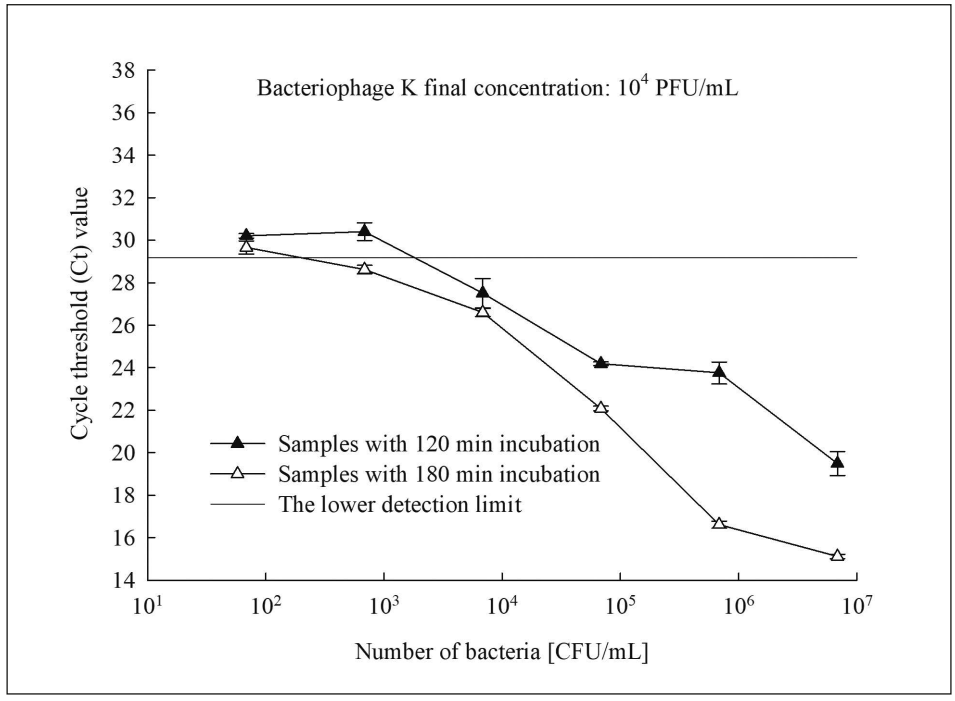

Figure 3 - *Comparison of 120 min and 180 min incubation period of serially diluted S. aureus ATCC 25923 culture with bacteriophage K at a final concentration of 10^4 PFU/mL, showing the lower limit of positive detection with qPCR. Results are expressed as mean values ±SD. The limit of positivity is determined as 3 times the sample's SD below the mean of the reference control sample's Ct.*

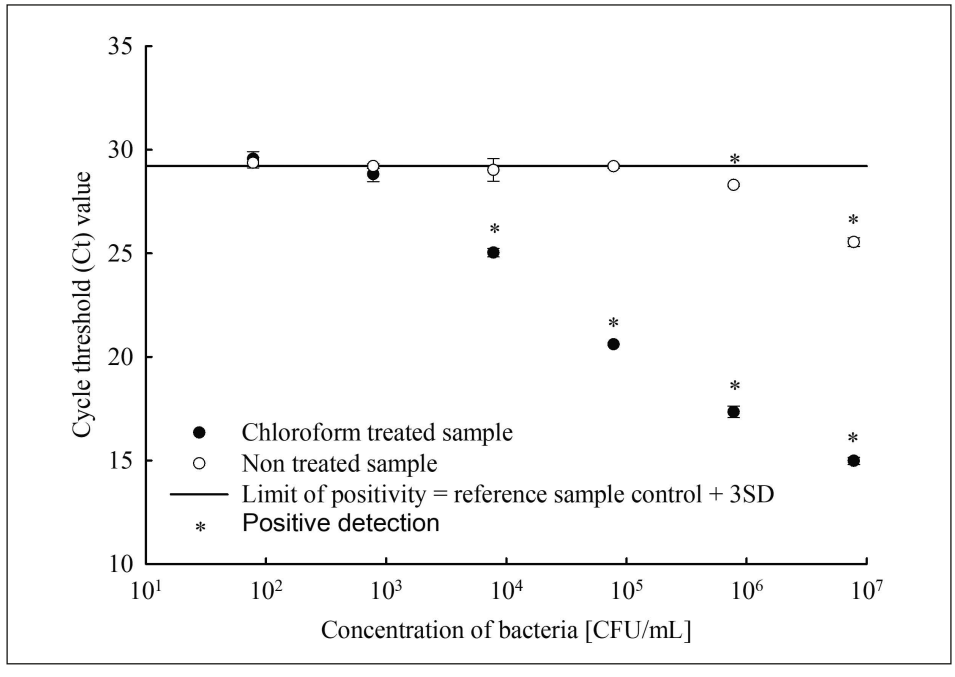

Figure 4 - *Comparison of qPCR results on S. aureus ATCC 25923 serial dilutions with and without the addition of chloroform after incubation with bacteriophage K. Results are expressed as mean values ± SD. The limit of positivity is determined as 3 times the sample's SD below the mean of the reference control sample's Ct. The detection is considered positive in samples with a Ct value below the limit of positivity of a sample.*

Table 1 - *Bacterial detection results of 25 clinical SF samples obtained with standard microbiological methods and results from the method of indirect detection with bacteriophage K and qPCR. The detection is considered positive in samples with a Ct value below the limit of positivity of a sample.*

SF sample	*Standard microbiological methods*	*qPCR detection with phage K*	*SF sample Ct ± SD*	*Reference sample control Ct*
1	MRSA	D	16.51 ± 0.07	29.55 ± 0.27
2	*S. aureus*	D	17,13 ± 0,44	29.55 ± 0.27
3	*S. aureus*	D	19,78 ± 0,53	30.27 ± 0.21
4	*S. aureus*	D	16,88 ± 0,11	30.17 ± 0.12
5	*S. epidermidis*	D	25,04 ± 0,14	30.49 ± 0.44
6	*S. epidermidis + C. acnes*	D	18,22 ± 0,19	29.55 ± 0.27
7	*S. epidermidis + E. coli*	D	23,84 ± 0,98	30.27 ± 0.21
8	*S. capitis*	D	16,35 ± 0,17	30.17 ± 0.12
9	*E. faecalis*	ND	29,18 ± 0,19	29.55 ± 0.27
10	*S. marcescens*	ND	30,18 ± 0,18	29,89 ± 0.14
11	*C. acnes*	ND	30,12 ± 0,27	30.49 ± 0.18
12	ND	ND	31,02 ± 0,56	29.55 ± 0.27
13	ND	ND	31,73 ± 0,43	30.27 ± 0.21
14	ND	ND	30,9 ± 0,09	30.27 ± 0.21
15	ND	ND	30,62 ± 0,15	29,89 ± 0.14
16	ND	ND	30,91 ± 0,22	29,89 ± 0.14
17	ND	ND	30,69 ± 0,30	30.49 ± 0.44
18	ND	ND	30,67 ± 0,01	30.49 ± 0.44
19	ND	ND	30,41 ± 0,15	30.49 ± 0.44
20	ND	ND	30,63 ± 0,18	30.70 ± 0.22
21	ND	ND	31,13 ± 0,20	30.70 ± 0.22
22	ND	ND	30,81 ± 0,10	30.70 ± 0.22
23	ND	ND	31,40 ± 0,12	30.70 ± 0.22
24	ND	ND	30,23 ± 0,15	30.17 ± 0.12
25	ND	ND	30,85 ± 0,11	30.17 ± 0.12

D, detection; ND, no detection.

SF without the presence of staphylococci were found negative with the method (*Table 1*). The method was found to be suitable for the detection of staphylococci in SF of removed prosthetic joints and had comparable results with routinely used microbiological culturing procedures.

DISCUSSION

The purpose of this study was to optimize and assess an alternative molecular approach with the use of bacteriophage K for a more rapid and specific detection of clinically relevant bacteria causing PJI. For the detection of staphylococci, SF represented a good sample and no inhibitory effect on bacteria-bacteriophage interaction has been shown. Obtained results matched those obtained from routine microbiological culturing methods for the identification of the pathogen. Importantly, the qPCR method with the use of bacteriophage K provided results much quicker than standard microbiological methods, where at least 24 to 72 h of culturing is needed for infection confirmation and identification of the causative microorganism (Mancini *et al.*, 2010); in the case of slow-growing or anaerobic bacterial species even 10 to 14 days are needed (Kocjančič *et al.*, 2014).

The optimized qPCR method gave positive results in all SF samples with a microbiologically confirmed staphylococcal PJI, regardless of whether staphylococci formed part of a mixed bacterial culture or not, confirming the specificity of bacteriophage K. SF from a non-infected prosthetic joint and SF from a non- staphylococcal PJI were found negative with the method. Similar findings were observed in our previous study (Šuster *et al.*, 2017), where SF were assessed with phage K in an ATP based assay. However, the method of qPCR appears to be less labor intensive and has a 10× lower detection limit. In the literature, bacteriophage K has already been described as a broad-spectrum staphylococcal bacteriophage, capable of infecting a wide range of clinically relevant staphylococci (O'Flaherty *et al.*, 2005; Cerca *et al.*, 2007; Alves *et al.*, 2014; Šuster *et al.*, 2017). The occurrence of bacterial resistance to phage infectivity is possible, but can be resolved by multiple passaging of the phage on bacterial strains showing resistance. Obtained spontaneously mutated phages can be pooled together into a cocktail of phages extending the host range even more (O'Flaherty *et al.*, 2005; Kelly *et al.*, 2011; Botka *et al.*, 2019).

Detection of bacteria with the qPCR method improved with longer incubation with bacteriophage K. As reported in a previous study, where adenosine-5'-triphosphate release by phage K mediated lysis was followed, results showed that phage K starts to lyse staphylococci 60 min post infection and that most of bacteria already lyse at 120-180 min of incubation with phage K (Šuster *et al.*, 2017). When incubating samples with phage K for 180 min before assessing with the qPCR method, we were able to detect 10× lower bacterial concentrations compared to a 120 min incubation. A similar correlation between longer incubation time and improved bacterial detection was found when incubating samples with a higher bacteriophage K concentration. According to our previous

work, the use of higher phage K concentrations results in faster bacterial lysis (Šuster *et al.*, 2017); however, a high initial phage K concentration could have a high impact on qPCR results. Kutin *et al.* (2009) reported that the ability to detect small amounts of *Ralstonia solanacearum* with phage and qPCR was reduced when a high (5×10^5 PFU/mL) initial phage concentration was used, but obtained remarkable results with a low phage concentration of 5×10^2 PFU/mL. On the other hand (Sergueev *et al.*, 2010) successfully used phage at a final concentration of 10^5 PFU/ml for the indirect detection of *Yersinia pestis* with qPCR. To minimize the impact of the initial amount of phage K on qPCR results, two concentrations (10^3 PFU/mL and 10^4 PFU/mL) were tested with qPCR. The best results were obtained after incubating samples with phage K at a final concentration of 10^4 PFU/mL. The addition of chloroform to samples after incubation with bacteriophage K further improved the detection of bacteria by shifting the detection limit from $\approx10^5$ CFU/mL to $\approx10^2$ CFU/mL. This was due to the increase in the number of available bacteriophage DNA in the sample because of chloroform-induced premature lysis of infected bacteria. Bacteriophage-infected bacteria present in a sample coexist at different stages of the bacteriophage lytic cycle, when formation of new bacteriophages or their components is taking place. As a result, bacteria that have not yet lysed are present in the sample containing a number of new non-formed bacteriophages. During their lytic cycles, bacteriophages regulate the timing of bacterial lysis and the release of new bacteriophages, with holins. Holins are necessary for the activation of cytoplasm-accumulated endolysins and for the formation of pores in the host's cell membrane, thus allowing lysins to reach and degrade the peptidoglycan. Without holin, endolysin is not capable of passing the cell wall and accumulates in the cytoplasm (Rice and Bayles, 2008). Chloroform disrupts the cell membrane and provides access to the peptidoglycan, thus triggering a premature lysis of bacteria and the release of already accumulated progeny bacteriophages and/or their components and DNA (Carlson, 2005). Consequently, the best results with the method of qPCR were obtained after incubating samples with bacteriophage K at a final concentration of 10^4 PFU/mL for 180 min and chloroform addition. The limit of detection was determined at 7×10^2 CFU/mL approx., meaning that the method allowed the detection of less than one bacteria per µL sample tested in a single qPCR reaction. (Reiman *et al.*, 2007) obtained a similar detection limit (2.07×10^2 CFU/mL) for the detection of the bacterium *Bacillus anthracis* within a 5 h processing time. Similarly, the research of (Sergueev *et al.*, 2010) focused on possibilities for the use of the qPCR method for the indirect detection of *Yersinia pestis* by using two different bacteriophages. With a less specific bacteriophage that could also detect *Y. pseudotuberculosis*, the method reached a limit of detection of approx. 10^3 CFU/mL; on the other hand, with the use of a more specific bacteriophage the limit of detection dropped to about 10^5 CFU/mL. For that reason, they suggested the parallel use of both bacteriophages, which of course makes interpretation of the result more complex and the whole diagnostic process more labor intensive.

The method of detecting bacteriophage DNA in qPCR for the indirect detection of bacteria offers several advantages compared to the direct detection of bacteria with qPCR. It avoids problems of bacterial DNA contamination that lead to false-positive results; besides, bacteriophages are species specific and only multiply in living bacterial cells. Sensitivity also increases due to phage amplification and subsequent increase in the target DNA (Kutin *et al.*, 2009; Reiman *et al.*, 2007). The method is less expensive, less time consuming, and requires less labor compared to conventional qPCR assays targeting bacterial genes (Espy *et al.*, 2006), since there is no need for DNA isolation, purification, sample concentration and subsequent sequencing. For instance, a 16S rRNA PCR assay needs 14 hours of manipulation to obtain results (Loïez and Wallet, 2018). In addition, bacteriophages are easy to maintain and their production is quick and inexpensive without the need for expensive laboratory equipment (Brovko *et al.*, 2012). Results of qPCR with the phage lysate were in accordance with the data obtained with phage DNA, for which the step of DNA isolation prior to qPCR analysis proved unnecessary. Comparable results were obtained by (Sergueev *et al.*, 2010) using phages ϕA1122 and L-413C. (Jäckel *et al.*, 2017) further improved the detection of *Campylobacter* phages DNA in qPCR by introducing an initial heating step of 20 min at 95°C in the qPCR cycling program protocol.

One key drawback in the use of bacteriophage K for diagnosing PJI is its specificity, as the method has been optimized only for the detection of bacteria from the genus *Staphylococcus* spp. So far, no false negative results have been obtained with the method in SF, although some staphylococcal strains resistant to phage K have been reported in the literature. O'Flaherty *et al.* (2005) found that 14 out of 53 tested staphylococci were weakly sensitive to phage K. Cerca *et al.* (2007) found that 1 out of 11 tested *S. epidermidis* strains, capable of biofilm formation, showed resistance, while on the other hand Kelly *et al.* (2011) tested 180 *S. aureus* strains and only 29 strains showed resistance. Recently, Botka *et al.* (2019) tested 186 MRSA strains, out of which 57 strains were reported non-susceptible to phage K; however, 12 of the resistant strains originated from livestock or the environment. Despite the fact that resistance is possible, O'Flaherty *et al.* (2005) showed that initially unsusceptible strains become susceptible after one passage of the phage on strains showing resistance. This was also demonstrated by Kelly *et al.* (2011) and Botka *et al.* (2019). In the future, this could be an elegant way of overcoming resistance to phage K in the case of false negative results occurring with the method on SF. To use the method for the detection of a wider spectrum of causative bacteria, however, future research needs to focus on the integration of additional bacteriophages, specific for other bacterial species, and on further method optimization. However, the optimized method as-is can detect about half of PJI cases and could represent a stepping-stone to a more rapid and informative method for diagnosing PJI.

In conclusion, the optimized method with bacteriophage K and qPCR for indirect detection of staphylococci in SF of removed prosthetic joints offers several advantages compared to other diagnostic techniques that are currently in use. Importantly, the method allows for rapid and specific detection of less than one bacteria per µL sample tested in a single qPCR reaction. Additionally, the detection of only live bacteria represents an important breakthrough in comparison to molecular detection methods that focus on bacterial DNA detection and do not differentiate between live and dead bacteria. The results presented

in this study indicate the existence of potential for the use of bacteriophages for a rapid, specific and sensitive diagnosis of PJI.

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Symbols and Abbreviations
PJI, prosthetic joint infection; SF, sonicate fluid; PCR, polymerase chain reaction; BR-PCR, broad-range PCR; MRSA, methicillin resistant *Staphylococcus aureus*; MALDI, matrix-assisted laser desorption/ionization; Phage K, *Staphylococcus aureus* subsp. *aureus* bacteriophage K ATCC 19685-B1™; BHI, brain heart infusion broth; PFU/mL, plaque forming units per mL; qPCR, real-time PCR; Ct, threshold cycle; CFU/mL, colony forming units per mL; SD, standard deviation; LOD, limit of detection; NTC, non-template control; BLAST, Basic Local Alignment Search Tool; NCBI, National Center for Biotechnology Information; MOI, multiplicity of infection; SE, standard error.

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