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Rapid and sensitive qPCR-based quantification of Salmonella Gallinarum Bacteriophages cocktail (SAL3)

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Abstract

Bacteriophages have garnered significance as alternatives to antibiotics against multidrug-resistant pathogens. Their prophylactic and therapeutic use in poultry production is well documented against *Salmonella* spp. and *E. coli*. However, accurate and reliable quantification of each bacteriophage is crucial for cocktail development and quality assurance. However, conventional plaque assay method is laborious and requires at least 12 hours of incubation for phage quantification. Moreover, for a phage cocktail targeting a single host, plaque assay cannot differentiate between individual phages except when different phages produce morphologically different plaques. Hence, it can compromise the quality assurance during cocktail production. In this study, we developed a quantitative qPCR assay to identify and quantify three lytic bacteriophages (SGP9, SGP13, and Frank) within 'SAL3,' a bacteriophage cocktail formulated against *Salmonella* Gallinarum. Phage-specific primers and TaqMan probes were designed and optimized to achieve high sensitivity with a detection limit of 10^3–10^4 phage genomes per mL (equivalent to 2–20 genomes per reaction). Standard curves generated from plasmids containing phage genome fragments showed excellent linearity, with a correlation coefficient (R^2) of 0.99. Comparative analysis of freshly prepared phage stocks using direct qPCR and the double-layer agar (DLA) method revealed a consistent, phage-dependent ratio between the two techniques. Direct qPCR detected approximately one log higher phage count than DLA and DNase treated qPCR assay, reflecting total phage genomes in the preparation rather than only infectious phages. Phage titer obtained after treating the phage prep with DNase I was comparable with DLA count. qPCR-based quantifications reduced processing time to under 3 hours with high reproducibility (CV < 5%) compared to the conventional DLA method, which requires over 12 hours. Multiplex qPCR assay enabled a rapid identification and quantification of individual phages in SAL3 cocktail. Additionally, DNase pretreatment reduced the contamination of free DNA both in the cocktail and lysate.

Introduction

Bacteriophages have emerged as a promising alternative to antibiotics against multidrug resistant pathogens (Ranveer et al., 2024). Their ability to selectively target and eliminate bacterial pathogens has positioned them as impressive biotherapeutic in modern antimicrobial strategies. Apart from therapeutic uses, phages have also been exploited for various other applications including: phage display, cloning vectors, phage-based delivery methods, in genome editing and regulation (Wang & Zhao, 2022).

In poultry research, *Salmonella* enterica serovar Gallinarum is a serious concern in poultry due to its association with fowl typhoid, particularly in developing countries including countries of southern Asia and northern America (Farhat et al., 2023). Because of the rising antibiotic resistance in poultry pathogens, phage therapy against *Salmonella* has gained a lot of attention. Consequently, lytic phages are being isolated and studied for their therapeutic use against *Salmonella* Gallinarum (Rehman et al., 2025).

The double-layer agar (DLA) technique, introduced by Gratia and Adam (Ralston & Baer, 1960), is a traditional method for quantifying bacteriophages. It remains the gold standard for detecting and enumerating bacteriophages as plaque-forming units (PFU) (Jo et al., 2024; Stachurska et al., 2021). This assay involves mixing host bacteria with phage particles in soft agar, overlaying the mixture onto solid agar plates, and incubating to form plaques. Counting these plaques determines the phage titer as PFU per milliliter (PFU/mL).

Despite several advances in phage isolation and characterization, double layer agar method is still the gold standard for quantification of bacteriophages. Although this method is reproducible and reliable, it exclusively counts infective phage particles that are capable of producing distinctive plaques, ignoring the non-plaque forming or defective particles (Luo et al., 2022). Moreover, the factors such as physiological state of host, medium, and incubation conditions also affect the plaque forming ability of bacteriophages. This assay also requires an incubation time of more than 12 hours to form distinct plaques making it time intensive and tedious (Van Overfelt et al., 2024). These limitations became evident while quantifying a cocktail comprising more than two bacteriophages with same primary host bacteria, where phage morphology hinders discrimination and quantification using DLA.

Molecular techniques for accurate and rapid quantification of phage particles have been extensively used to complement traditional assay. Real-time PCR performs the detection and quantification of specific unique target region of bacteriophage genome, allowing quantification through comparison with the known standard DNA concentrations (Huang et al., 2022). This technique is independent of phage-host interaction, phage productivity and allows accurate detection and enumeration of bacteriophages (Van Overfelt et al., 2024). This method has been employed in various studies, aimed at developing therapeutic cocktails against *Escherichia coli, Acinetobacter, Pseudomonas aeruginosa* and several other pathogenic bacteria (Luo et al., 2023; Van Overfelt et al., 2024). Nevertheless, limited studies have been reported for bacteriophages against *Salmonella species*.

In the present study, we implemented real-time-based quantification strategy to enumerate three lytic bacteriophages isolated against *Salmonella* Gallinarum. Bacteriophages were quantified using standard curve to find out the exact viral particles count. Quantitative results obtained from real-time PCR were compared with DLA results for each phage. This study aims to compare culture-based technique and qPCR for the enumeration and detection of three bacteriophages in a phage cocktail (SAL3).

Material and Methods

Primer designing, cloning and standard curve plotting

Three double stranded DNA phages i.e. SGP9 (PV068287), SGP13(PV007926), and Frank (PV007924) were used in this study. To plot a standard curve, unique genomic sequences from phages SG9, SG13, and Frank were amplified using specific primers (Table 1). The PCR conditions were: initial denaturation at 95°C for 5 minutes, followed by 35 cycles of denaturation at 95°C for 35 seconds; annealing at 52°C for 35 seconds; extension at 72°C for 15 seconds; and a final extension at 72°C for 10 minutes.

Table 1
Primers for cloning and detection of bacteriophages SGP9, SGP13 and Frank.

Sr. NO.	Primer name	Sequence (5'-3')	Product size (bp)
1	SGP13-CLO-F	TCGCA GAATTC GGCTCCTACGAAAGAAGAGTTC	124
	SGP13-CLO-R	TAGTCCTCGAG AGCGGTTAGCAGATTAGTAACG	
2	SGP9-CLO-F	TCGCA GAATTC GTTAGCAGACCAGTCGAGAAG	122
	SGP9-CLO-R	TAGTCCTCGAG CTGGAGCCTCTTGTTGGATAA	
3	FRANK-CLO-F	TCGCA GAATTC GAGCAGCAGTTACCAACCTATAA	116
	FRANK-CLO-R	TAGTCCTCGAG CCTTACGCAAGAGCGGATAAA	
4	SGP9-RT-F	GTTAGCAGACCAGTCGAGAAG	100
	SGP9-RT-R	CTGGAGCCTCTTGTTGGATAA	
	Probe	FAM-AGGAAGTTGGTAAGGTTGTTGGAGGC-BHQ-1	
5	SGP13-RT-F	GGCTCCTACGAAAGAAGAGTTC	106
	SGP13-RT-R	AGCGGTTAGCAGATTAGTAACG	
	Probe	VIC- AGATGTGACTTCATTGCGCACTGC-BHQ-1	
6	FRANK-RT-F	GAGCAGCAGTTACCAACCTATAA	94
	FRANK-RT-R	CCTTACGCAAGAGCGGATAAA	
	Probe	ROX- TTGCGCATGAGTACGATAACCCGA-BHQ-1	

Amplicons were purified using Gel extraction kit (NEB, cat# T10132573) and restricted using EcoRI and XhoI restriction enzymes for cloning in pET28(a) plasmid in separate reactions. Ligation was performed using T4 DNA ligase (Thermo Scientific™ cat# EL0011) and transformed into *E. coli* TOP10 cells. Plasmids with inserted cassettes were extracted using plasmid miniprep kit (Thermo Scientific™ cat# K0503). Purity of the DNA was checked by Colibri Nanodrop-spectrophotometer. Extracted plasmids were 10-fold diluted in triplicates for 10^8-10^1 copy numbers per µl and used as standards to plot standard curves. Copy number was calculated using following formula (Hodzic et al., 2023):

$6.02\times10^{23} \times (ng/\mu l\times10^{-9}) / bp\times660$

For the quantification of phages through real-time PCR, primers and hydrolysis probes were designed using Primer quest software (https://sg.idtdna.com/pages/tools/primerquest?returnurl=%2FPrimerquest%2FHome%2FIndex). Conserved and unique regions from each phage were selected to ensure specificity and to avoid cross reactivity. All three primers were designed with the following parameters: primer length ~ 20–24 bps, GC content 40–60%, with minimal self-complementarity and hair-pin formation. Primer dimmer formation, secondary loop formation, Tm was

cross checked using BLAST/nt. Subsequently, the specificity of each primer set was further investigated against other two phages and bacterial host to check the cross reactivity. TaqMan probes for SG9, SG13, Frank were labelled with FAM, VIC, and ROX fluorophores respectively. All the primers and probes were ordered from Macrogen, Korea. Primers and probes were reconstituted according to suppliers' instruction and stored at -20□C.

Production and quantification of bacteriophages

Phage lysates of SGP9, SGP13, and Frank were prepared as described elsewhere (Asif et al., 2021). Briefly, host bacteria and bacteriophages were mixed at 1 multiplicity of infection (MOI) in tryptic soy broth (Oxoid™ cat# CM0129B) and incubated overnight at 37°C in a shaking incubator (120 rpm). The following day, lysates were centrifuged at 9,000 × g for 10 min and filtered through 0.22-µm PVDF filters.

Phage titers in each preparation were determined using DLA (Paranos et al., 2024). Bacteriophage stocks were serially diluted 10-fold from 10^{-1} to 10^{-8} (Van Overfelt et al., 2024). For each dilution, 100 µL of host bacterium was mixed with 3 mL of 0.7% soft agar and poured over 1.5% solid agar plates. Plates were incubated overnight at 37°C. Plaques were enumerated, and titers were calculated as plaque-forming units per milliliter (PFU/mL). All experiments were performed in triplicate.

Correlation analysis of qPCR and DLA

Individual phage lysates were quantified using qPCR, DNase treated qPCR, and DLA. While the DNase treated and untreated cocktails of these phages were quantified by using multiplex qPCR. To evaluate the co-relation between the bacteriophage quantification from direct bacteriophage stock using qPCR without prior DNA extraction, three independent stocks of each phage were prepared. Aliquots from the same stocks were separately treated with DNase I prior to qPCR analysis. For DLA, three assays were performed using same bacterial suspension on the same day. Three separate titrations in saline were performed for each phage stock. For every titration, different phage dilutions were plated in triplicates. Plaques were counted and results were recorded in PFU/mL. For qPCR, a 100-fold dilution of each stock was made in PCR grade water in triplicates and ran with standard controls in the single thermal cycling run (Fig. 1). To remove the free DNA, 1U of DNase I was added to 500µL of phage prep with 10 minutes incubation at 37□C, followed by inactivation of enzyme at 95□C for 10 minutes. Serial dilutions were subsequently prepared and analysed by Taq Man based qPCR.

SAL3 cocktail (3mL) was prepared using equal volume ratio of (1:1:1) of three phages in triplicate. While each replicate was further quantified in triplicates. Cyclic quantification value (Cq) was used to calculate genomes/mL using calibration curve (Duyvejonck et al., 2019). For DNase treatment, 1mL of cocktail was treated with DNase I, using protocol discussed earlier. Expected cocktail values were calculated using the following formula:

Expected concentration of each phage in the cocktail = individual phage concentration/3

Optimization and validation of qPCR primers

Basic PCR was used to assess the specificity of primer sets, annealing temperature optimization, primer concentrations, and extension time for qPCR reaction. Annealing temperatures of primer sets were calculated using Thermo scientific Tm calculator for primers (https://www.thermofisher.com/pk/en/home/brands/thermo-scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/thermo-scientific-web-tools/tm-calculator.html). Each reaction of 12.5 µL contains 1.5µL of each primer (10pM), 6.25µl of 2X PCR Master Mix (Thermo Scientific ^{TM} cat # 3021473), 2µl of DNAse/RNAse free water and 1µl of DNA genome (86ng/1µl). Amplification was carried with 5min initial denaturation, followed by 35 cycles of denaturation at 95°C for 5 mins, annealing at 52°C for 35 sec, extension at 72°C for 15 sec, with 5 min initial denaturation at 95°C and final extension of 10 min at 72°C. PCR reaction (8µl) mix, mixed with 6x loading dye (5:1) were loaded onto 2.5% agarose gel with ethidium bromide prepared in 1x TAE buffer. After electrophoresis, gel was observed with gel documentation system (SYNGENE InGenius 3).

For standard curve formation, 1µl of each serial dilution from 10^1 – 10^8 of plasmid was added to 12.5µl reaction mixture containing 1.5µl (0.12pmol) of each primer, 4 µl of DNAse/RNAse free water, 0.2 µl of probe and 6.25µl of MasterMix (Van Overfelt et al., 2024). Dilutions of the phage lysates were performed in DNAse/RNAse free PCR grade water. Reactions were carried out in QuantStudio 3 (Applied Biosciences) using following program. Denaturation for 5 min at 95°C and amplification for 40 cycles with denaturation at 95°C for 30s, annealing at 52°C for 35 s and at extension at 72°C for 15 s using ramp rate of 1.6°C per s (Ye et al., 2025).

Data collection and statistical analysis

DLA was performed by manually counting plaques and calculating PFU/mL, taking into account the dilution factor and the volume plated (Wan et al., 2021). Cq values from the qPCR analysis were converted to concentration (genomes/mL) by calibrating against standard curve for each bacteriophage. Data was log transformed before the statistical analysis. Statistical and graphical analysis were done using Graph Pad Prism 8. Three independent samples from each freshly prepared stock were used for quantification, and each sample was considered as paired sample for DLA, qPCR, and DNase treated qPCR. Each phage was analyzed separately. Similarly, the quantity of all three phages in cocktail were determined through multiplex qPCR quantification. Both untreated and DNase treated phage cocktails were analyzed. The untreated cocktail determined the total qPCR detectable genome copies, including both viable and non-viable free genomes, while DNase treated cocktail showed only intact phage genomes quantification. Paired student's t-test (for normally distributed data) or Wilcoxon (for not normally distributed data) was used. P-value above 0.05 indicates that obtained differences are statistically significant. To assess the reproducibility of each method, coefficient of variation (CV %) was calculated. Values below 20% were reflective of the low variability (Duyvejonck et al., 2019). Observed and expected multiplex qPCR values were compared using paired analysis.

Results

Primer optimization and specificity assessment

The primary evaluation using conventional PCR and gel electrophoresis resulted in single, sharp band with the desired amplicon size, validating the primers specificity and efficacy. The optimum annealing temperature for all the primer sets were 52□C. Primers concentration for each primer per 12.5µL of reaction mixture was 0.1µM. qPCR results indicate that each phage was correctly quantified, without the cross reactivity of each primer pair with any of other two phages. Limit of detection per reaction was 10^3-10^4 phage genomes per mL i.e., 2–20 genomes of phages per 12.5µL PCR mixture. Similarly, the DLA limit was around 10–20 PFU/mL.

Calibration curve plotting

Calibration curve of all three standards showed strong linearity with correlation coefficients (R^2) exceeding the value of 0.99 indicating high reproducibility and precision across the dilutions. (Fig. 2). Standard curve provides absolute quantification with Cq values converted into genome copy/mL by interpolating from the standard curves.

Comparative analysis of direct qPCR, DNase treated phage preps and double layer agar method for phage quantification

Direct qPCR from phage prep always yielded a higher number of phages as compared to DLA and DNase treated qPCR. For each freshly prepared stock, a constant ratio between the number of phages through the qPCR and the DLA was obtained. This ratio, in each case was phage dependent (Table 2). Bacteriophage titer obtained after DNase I treatment was comparable to that determined by DLA and lower than titers determined by using direct lysate (Fig. 3, Fig. 4, and Fig. 5). All the three assays showed low variation in reproducibility, with qPCR being the most reproducible method (CV% < 5%).

SAL3 cocktail quantification

Multiplex qPCR detected and quantified all phages of the cocktail through probe specific detection. The amount of each phage detected shows the number of phage genome per microliter of the final concentration of the cocktail. The observed multiplex qPCR for each phage was compared with the expected phage concentration. Paired t-test of untreated cocktail (P value of SGP9 = 0.4473, SGP13 = 0.0670 and Frank = 0.2870) and DNase treated phage cocktails (P value of 0.381, 0.1561, 0.1877) shows no significant difference between expected and observed values.

Table 3

Estimated number of bacteriophages detected through qPCR, DLA, DNase treated qPCR prep, and their mean ratios. The number of phages in each stock was calculated by taking mean of three replicates of the three stocks (1,2,3) each.

Bacteriophages	Stocks	Mean genomes/mL			qPCR/DLA ratio	qPCR of DNase treated prep /DLA ratio
		qPCR	DLA	qPCR of DNase treated prep		
SGP9	1	1.38E9	2.0E8	1.43E8	6.74	0.72
	2	3.2E9	1.88E8	1.34E8		
	3	1.88E9	5.7E8	4.1E8		
SG13	1	3.2E10	8.10E + 09	6.32E9	7.90	0.68
	2	4.9E10	7.20E + 09	3.76E9		
	3	5.1E10	1.40E + 09	1.34E9		
Frank	1	2.80E + 10	7.50E + 09	4.59E + 09	6.36	0.62
	2	4.40E + 10	9.80E + 09	5.99E + 09		
	3	7.50E + 10	5.80E + 09	3.72E + 09		

Table 4
Comparison of expected and observed genome copies in untreated phage cocktails quantified by multiplex qPCR. The expected qPCR concentration was calculated by dividing original lysate by 3.

Cocktails	Phage	Expected qPCR (genomes/mL)	Observed qPCR (genomes/mL)
Stock 1	SGP9	4.60E + 08	4.49E + 08
	SGP13	1.07E + 10	1.23E + 10
	Frank	9.33E + 09	9.98E + 09
Stock 2	SGP9	1.07E + 09	1.97E + 09
	SGP13	1.63E + 10	1.98E + 10
	Frank	1.47E + 10	2.47E + 10
Stock 3	SGP9	6.27E + 08	6.00E + 08
	SGP13	3.70E + 10	3.89E + 10
	Frank	3.50E + 10	4.55E + 10

Table 5
Expected and observed genome copies/mL in DNase treated cocktail. expected concentrations were in close agreement with the expected genome copies/mL.

Cocktails	Phage	Expected qPCR (genomes/mL)	Observed qPCR (genomes/mL)
Stock 1	SGP9	4.77E + 07	4.37E + 07
	SGP13	2.11E + 09	2.01E + 09
	Frank	1.53E + 09	1.52E + 09
Stock 2	SGP9	4.47E + 07	4.02E + 07
	SGP13	1.25E + 09	1.15E + 09
	Frank	2.00E + 09	1.88E + 09
Stock 3	SGP9	1.37E + 08	1.34E + 08
	SGP13	4.47E + 08	4.40E + 08
	Frank	1.24E + 09	1.18E + 09

Discussion

In this study, we developed and optimized a qPCR assay for the detection and quantification of bacteriophages in the cocktail (SAL3) and compared its performance with the double layer agar method. The designing and optimization of specific and efficient primers and probes is the first step in establishing a robust qPCR assay (Rodríguez et al., 2015). We designed and optimized the primer sets and TaqMan probes for quantification of bacteriophages SGP9, SG13, and Frank, with high specificity and efficacy. First the amplicon lengths were determined by conventional PCR in a standardized amplification mixture, with an optimum concentration of primers, and buffer, followed by gel electrophoresis and later confirmed the absence of non-specific amplification (Zhao et al., 2021). Moreover, all the primer pairs presented no cross reactivity and high specificity against other phages from the cocktail and the bacterial host which indicates that correct quantification of individual phages would be possible even in a multiplex reaction mixture. Bacteriophages were quantified directly with qPCR without prior DNA extraction. Dilutions of freshly prepared stocks were prepared in PCR grade water, to avoid any PCR inhibition by the media content (Francioli et al., 2021).

Standard curves obtained for SG9, SG13, and Frank using standard plasmids showed excellent linearity with correlation coefficients of above 0.99. The high reproducibility and precision within a broad range of dilutions from 10^1 to 10^8 copies per µL confirms the reliability of qPCR assay for absolute quantification of phage based on its genomic DNA. Based on this established parameter, the minimum limit of detection in our qPCR assay was determined as 10^3 -10^4 phage genomes per mL, equivalent to 2–20 genomes per reaction allowing sensitive detection. In comparison, the DLA has a limit of ~ 10–20 PFU detection per ml.

Since, freshly prepared stocks of phages were used which primarily consist of intact phage particles, there are less chances that qPCR overestimate the phages in the stocks (Parcey et al., 2020). However, there is still possibility of phages' impairment in freshly prepared stocks due to centrifugation or filtration steps. This may lead to the overestimation of phage DNA by qPCR in comparison with the DLA method. This agrees with the theoretical consideration that DLA counts only the infective phage particles that can form plaques, not considering non-plaque forming or defective virions, as well as phage DNA from inactive or lysed phages. In contrast, direct qPCR quantifies all existing phage DNA independently of the infectivity or structural integrity of the particle (Ács et al., 2020). Our comparative analysis showed a constant higher estimation by qPCR in comparison with DLA method (Fig. 3,Fig. 4, and Fig. 5). Furthermore, we observed a phage-dependent qPCR/DLA ratio which seems to be rather constant for each phage. This ratio, determined based on three, (DLA) and (qPCR), assays of three biological replicates (different stocks), was 6.90(± 0.21) for SGP9, 7.90(± 0.81) for SGP13, 6.36(± 0.34) for Frank. The consistency in the quantitative results of three different phages obtained by using phage DNA indicate that TaqMan based qPCR is reliable, rapid, and precise method, aligning with findings from other studies (Gregorova et al., 2022). Phage quantification by qPCR can be accomplished in less than 3 hours, in contrast with traditional DLA method requiring around 48 hours. (Ács et al., 2020).

To develop qPCR, the primers should be designed against the targeted region, which require prior sequencing. For the clinical application of phages, it is desirable to determine the phage genome

sequence first to ascertain that bacteriophages are free of any virulence or antibiotic-resistant genes. Additionally, qPCR may overestimate the number of infective phage particles in clinical samples due to amplification of free phage DNA from degraded phage particles. Pre-treatment of the phage lysate with the DNase can reduce the bias by degrading the free DNA. (Bertolini et al., 2023). These DNA degrading enzymes precisely degrade the non-encapsidated DNA in the sample, however, not penetrate into intact phage capsids, thus preserving DNA inside the viable phages (Luo et al., 2023). DNase treatment reduces extracellular free phage DNA and improved infective phage count.

The pool of amplifiable non-encapsidated DNA can be reduced effectively by enzymatic digestion of free-floating DNA, thereby enhancing the specificity of qPCR towards intact phage particles (Peng et al., 2018). However, the success of DNase I pretreatment depends on fully optimized enzymatic conditions such as incubation time, enzyme concentration, and sample matrix characteristics to ensure complete digestion of free DNA without compromising phage integrity (Bertolini et al., 2023).

As for reproducibility, all three assays proved to have low variability in case of phage prep quantification. However, qPCR was more reproducible, as indicated by its lower coefficient of variation. Improved reproducibility in qPCR can be explained by the fact that most conditions affecting plaque formation in DLA, like the physiological state of the host bacteria, culture medium composition, and incubation conditions, did not affect qPCR. Moreover, qPCR offers a much faster turnaround than DLA assay which require around 48 hours of incubation (Bagdonaitė et al., 2025). It is thus considered more suitable for high-throughput quantifications (Van Overfelt et al., 2024).

For DLA, phages and their respective bacterial host are required, whereas qPCR enables accurate quantification of phage genomes independently of a bacterial host (Willis et al., 2025). Furthermore, in cases where phages exhibit indistinguishable plaque morphologies, it is not possible to resolve their individual abundances from a cocktail. Although direct qPCR quantifies nucleic acid genomes rather than necessarily viable phage particles and may therefore overestimate phage counts, however, pretreatment of phage prep with DNase enzyme, provides a valuable approximation of infectious phage titers in preps (Paranos et al., 2024).

For practical application of bacteriophages cocktail, accurate enumeration of component bacteriophages is necessary. In cocktail applications, equal volume mixing of phages does not always produce equal titer cocktails. As evident in the present study, SGP9 has a lower initial titer than rest of the phages. After equal volume mixing, SGP9 detected at proportionally lower concentration than SGP13 and Frank.

As 1mL of each phage was mixed to make a cocktail volume of 3mL, therefore, the expected concentration of each phage was calculated as one third of corresponding phage lysate titer. The theoretical expected value was then compared with real time values through multiplex qPCR. The expected values were then compared with the observed values obtained by multiplex qPCR. The close agreement between the observed and expected values of multiplex qPCR confirms that the assay produces reliable quantification of the cocktail.

In this study, the multiplex qPCR strategy developed successfully quantitate SGP9, SGP13 and Frank in the cocktail. The higher detection of genomes/µL in the non-treated compared to DNase treated cocktail suggests the detection of free genome copies along with encapsidated phage genomes.

In summary, this study successfully applied real-time qPCR-based quantification approaches for lytic bacteriophages prep of *Salmonella* Gallinarum and directly compared their performance with DLA method. The results also demonstrate that the optimized multiplex qPCR assay is efficient and reliable in quantification of individual phage in the cocktail. DLA remains valuable for enumerating infective phages, however if all the phages infect the same host, it would be difficult to differentiate phages on plaque morphology. qPCR is faster, more sensitive, and more reproducible for the estimation of total phage particles. Such a molecular approach may provide a deeper understanding of phage populations, which is important for developing precise phage cocktails and optimizing dosing in phage therapy, where the total number of phage particles might influence therapeutic outcomes or ecological interactions. Overall, qPCR can provide a rapid and phage specific estimation of genome copies number in complex biological samples, thereby strengthening clinical interpretations and therapeutic monitoring.

Declarations

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Author Contribution

• Conceptualization: Shafiq Ur Rehman. • Data Collection: Zulquernain Haider, Hafsa Naseem and Asad Aslam Khan. • Data Analysis: Zulquernain Haider and Hafsa Naseem. • Manuscript Preparation: Hafsa Naseem. • Review and Editing: Zulquernain Haider, Hafsa Naseem, Muhammad Ahsan and Asad Aslam Khan • Supervision: Shafiq Ur Rehman

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Figures

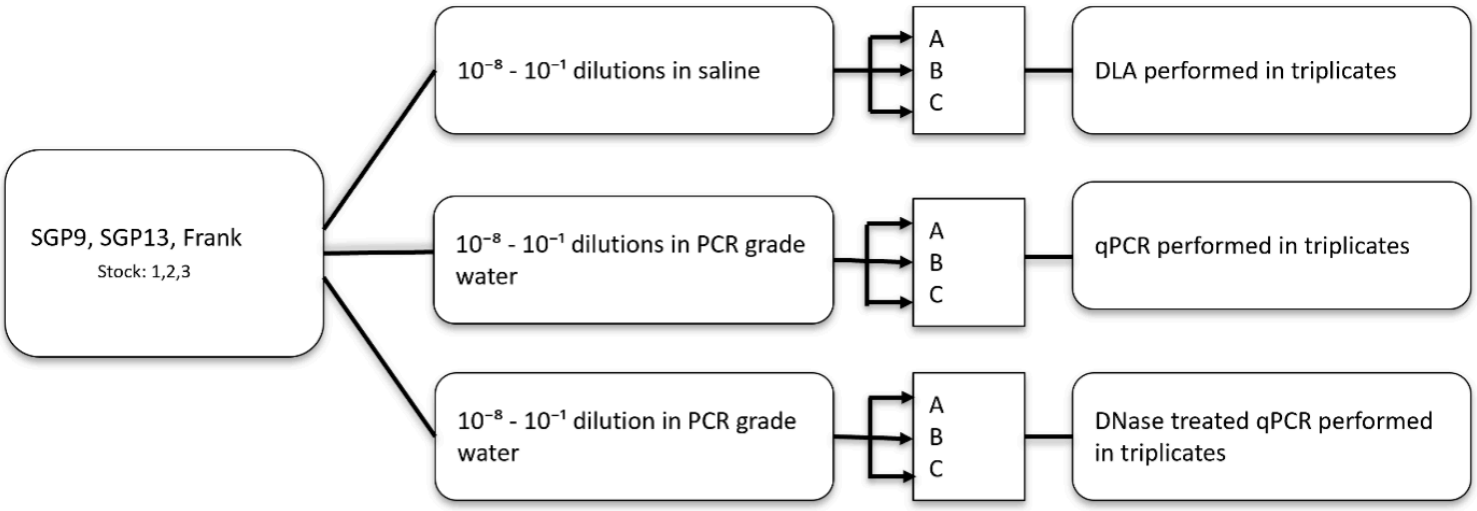


Figure 1

Work flow of quantitative and qualitative comparison of DLA, qPCR, and DNase treated qPCR. Three freshly prepared stocks of SGP9, SGP13 and Frank were diluted in saline and titrated with bacterial host. For TaqMan based qPCR, dilutions were made in PCR grade water from 10^{-8}-10^{-1} concentrations.

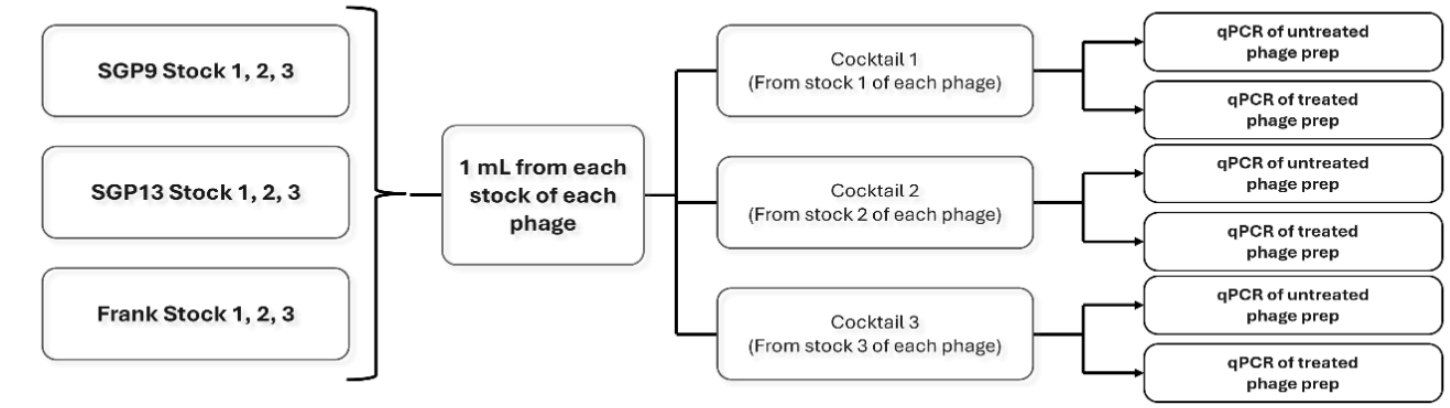


Figure 2

Sequential workflow of cocktail preparation. Three independent cocktails from each stock of SGP9, SGP13 and Frank were prepared separately. An aliquot of 1mL of each cocktail was treated with the DNase enzyme and proceeded for qPCR along with untreated cocktail qPCR.

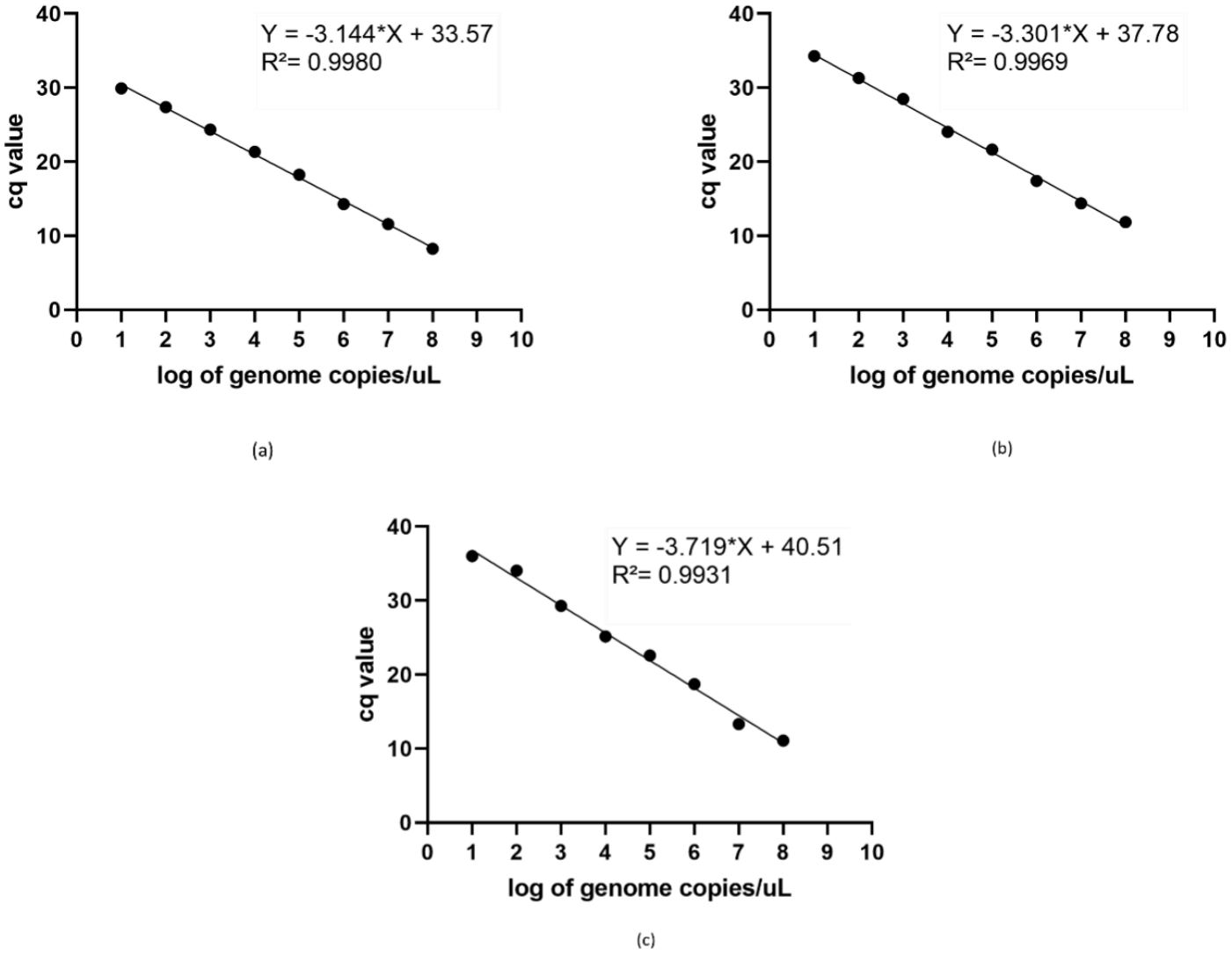


Figure 3

Standard curves of (a)SGP9, (b) SG13 and (c)Frank from TaqMan qPCR. Linear regression was plotted for genome copies vs Cq values to standardize the assay. At each concentration, every sample has a cq value. Linear regression was plotted using cq versus log (genome copies/mL) concentration.

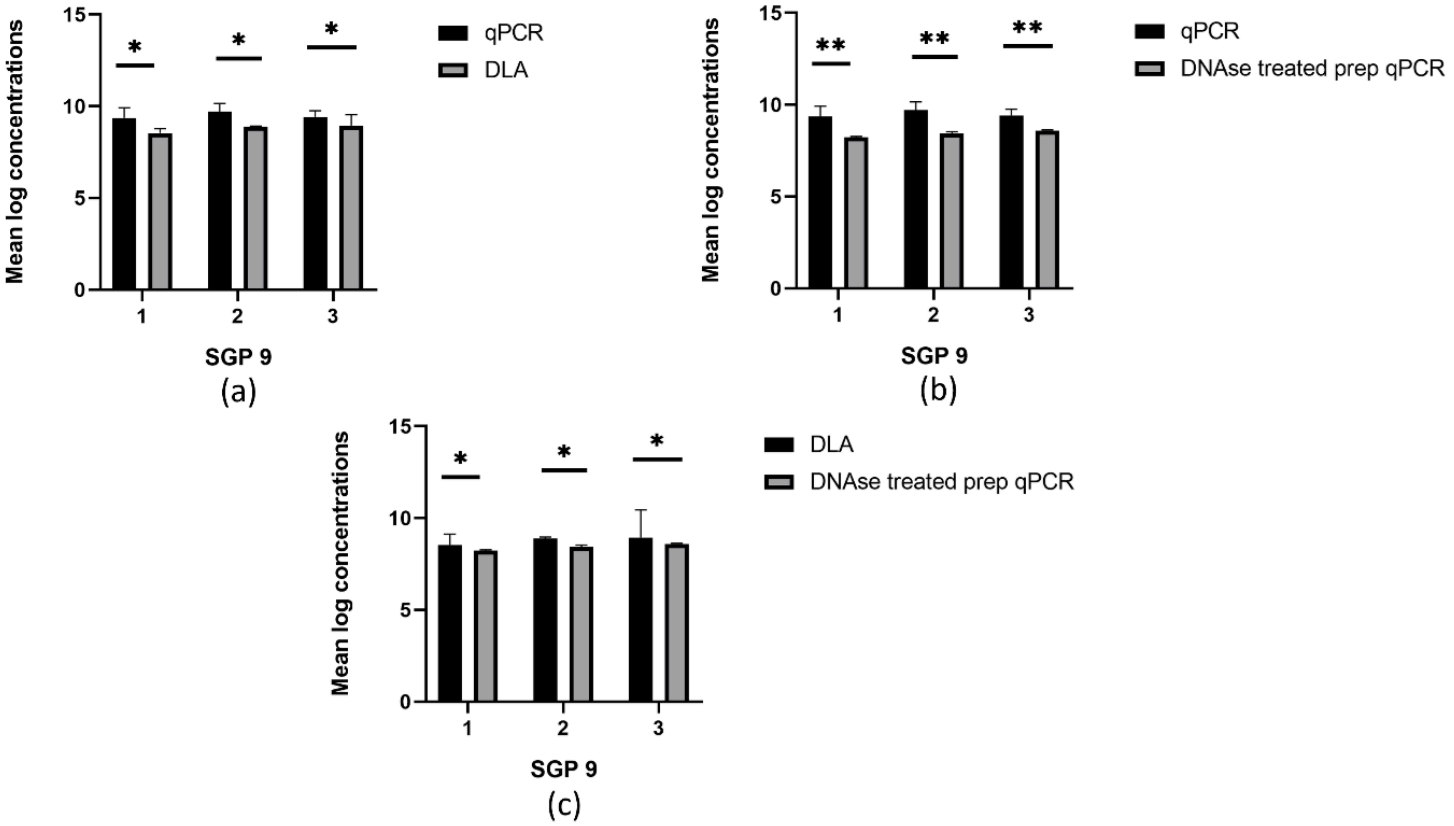


Figure 4

Quantification of bacteriophages by qPCR, DLA and DNase treated phage prep, with error bars showing standard deviation among the replicates. Normality and paired t-test analysis was done using Graph pad prism 8. Data was presented using mean log concentrations of triplicates of SGP9, with 95%CI, (a) qPCR and DLA, (b) qPCR and DNase treated prep qPCR, (c) DLA and DNase treated prep qPCR. qPCR in every phage prep estimates highest phage copy number than double layer agar assay (DLA), indicating the count of both infective and non-infective particles. DNase treatment detected PFU/ml comparable to DLA. * Indicates $P<0.05$, and ** Indicates $P<0.01$ between the qPCR and DLA.

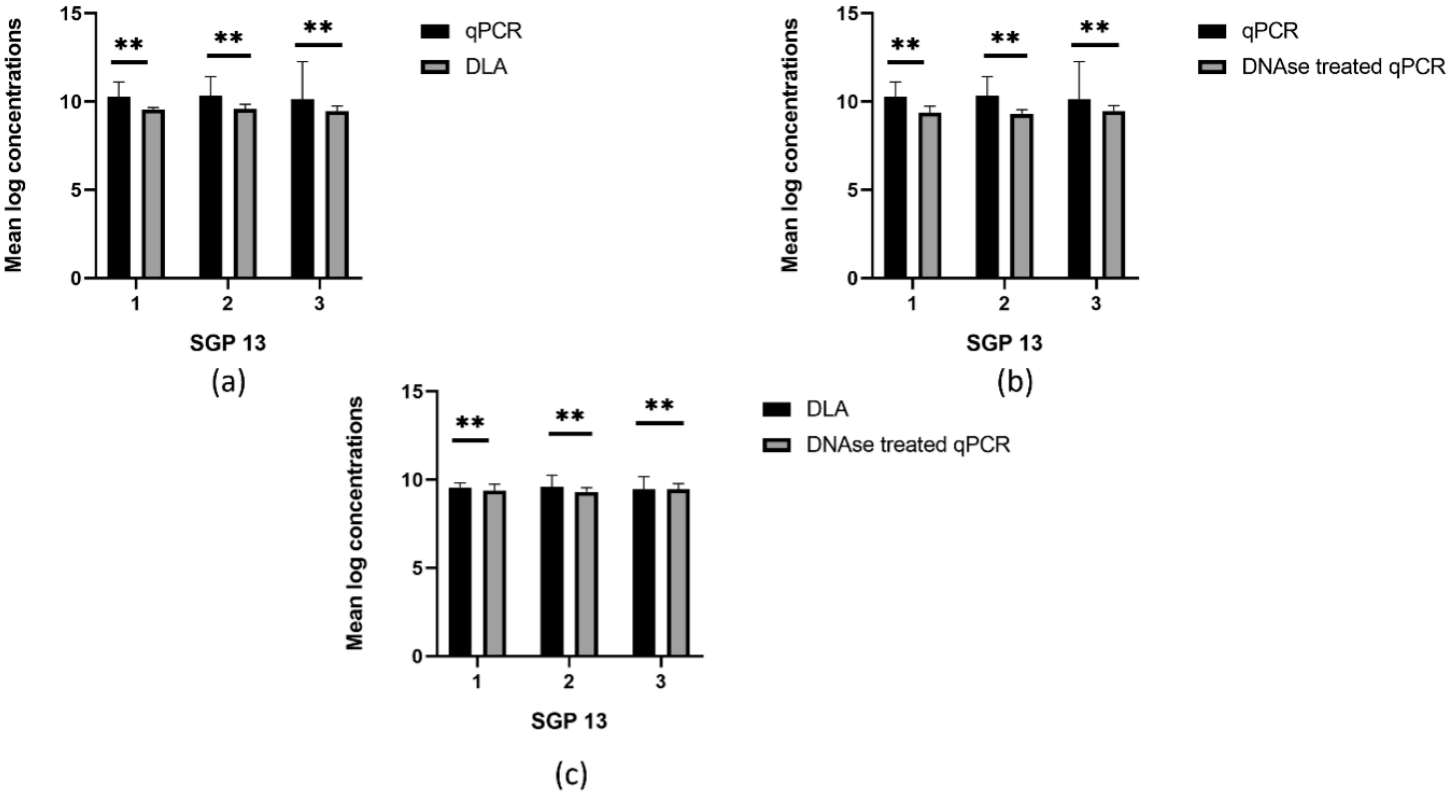


Figure 5

Comparative analysis of BacteriophageSGP13 (PFU/ml) quantified by (a) qPCR and DLA, (b) qPCR and DNase treated prep qPCR, (c) DLA and DNase treated prep qPCR with error bars showing standard deviation among the replicates. Data was presented using mean log concentrations of triplicates, with 95%CI.

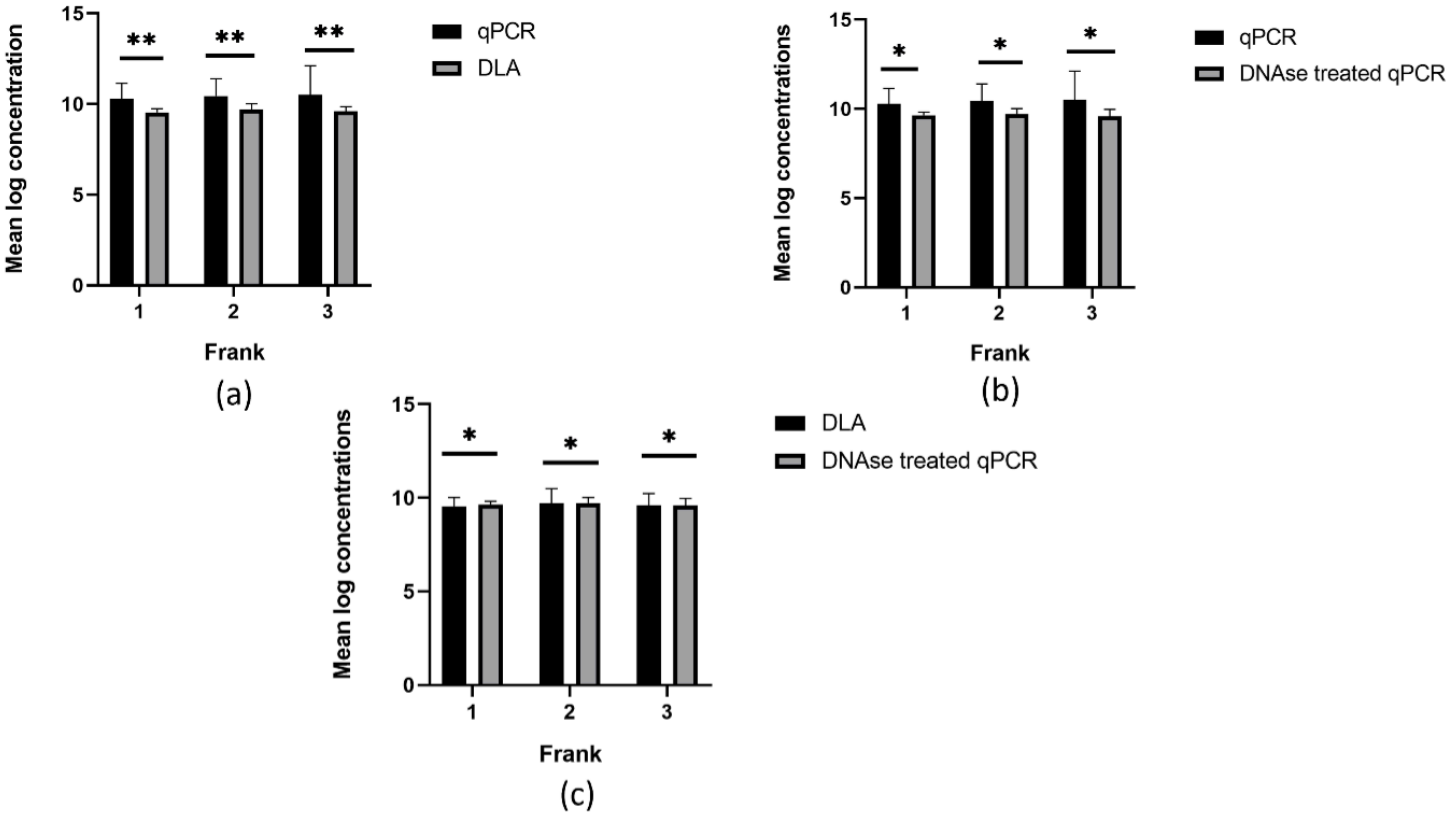


Figure 6

Comparative analysis of Bacteriophage Frank (PFU/ml) quantified by (a) qPCR and DLA, (b) qPCR and DNase treated prep qPCR, (c) DLA and DNase treated prep qPCR with error bars showing standard deviation among the replicates. Data was presented using mean log concentrations of triplicates, with 95%CI.

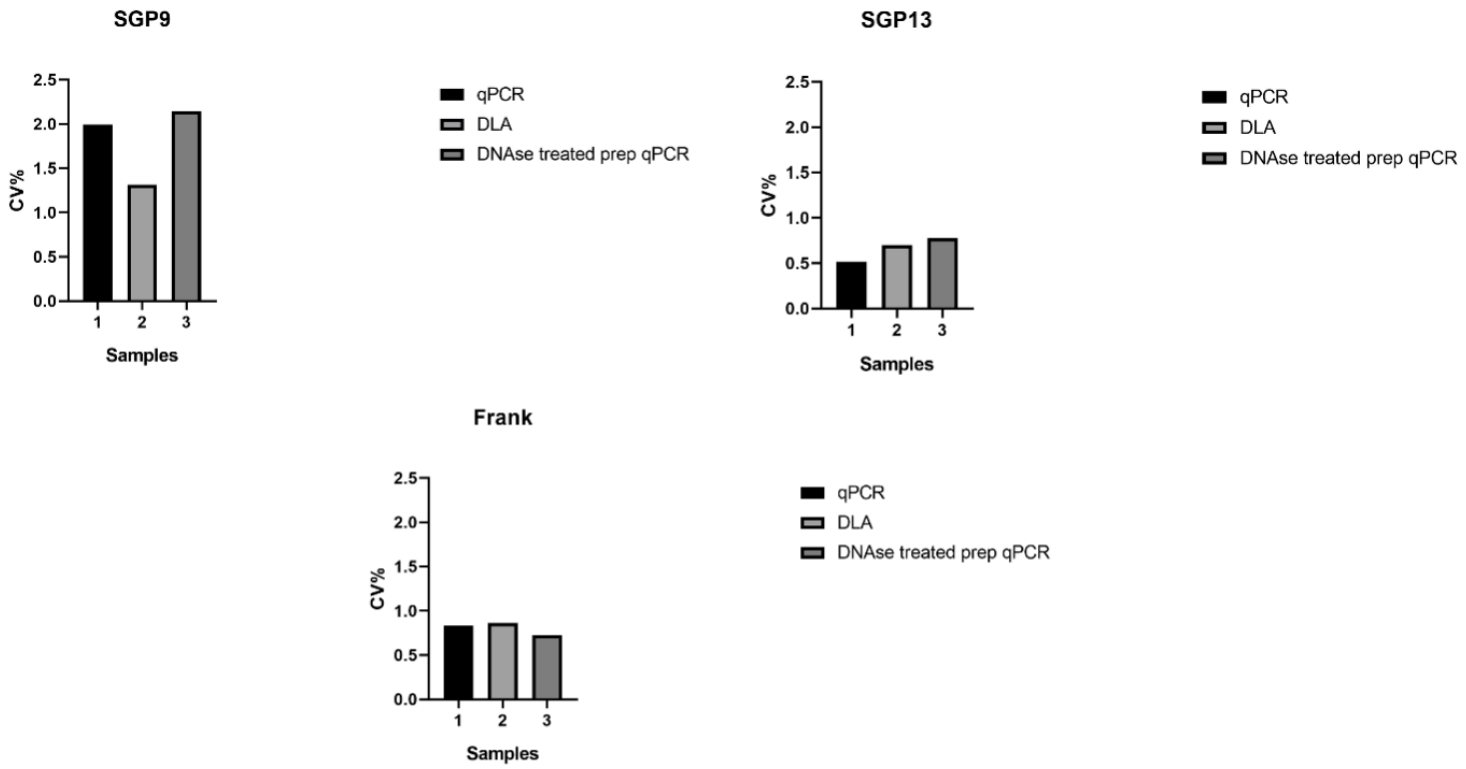


Figure 7

Coefficient of Variation (CV%): Comparison of reproducibility of quantification by means of qPCR, and DNase treated qPCR prep DLA. (a) SGP9, (b) SGP13, (c) Frank. The mean values from each stock of all three bacteriophages were considered.

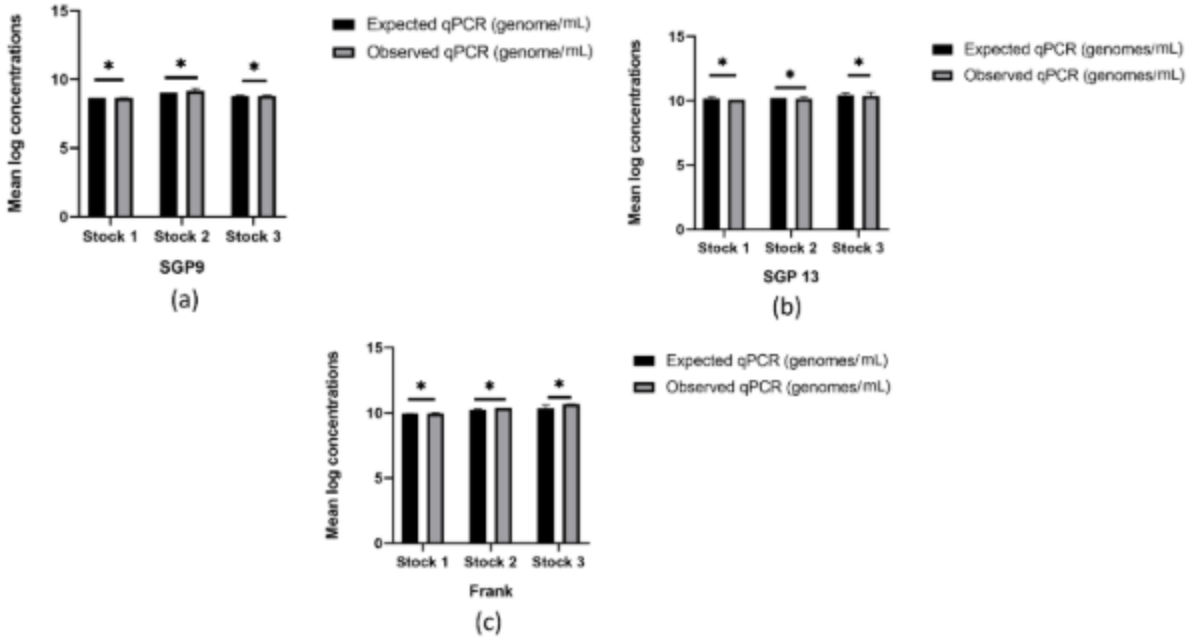


Figure 8

Multiplex qPCR quantification of untreated phage cocktail. For each phage (a) SGP9, (b) Sgp13, (c) Frank, the expected and observed values are comparable across all three stocks with no significant variation

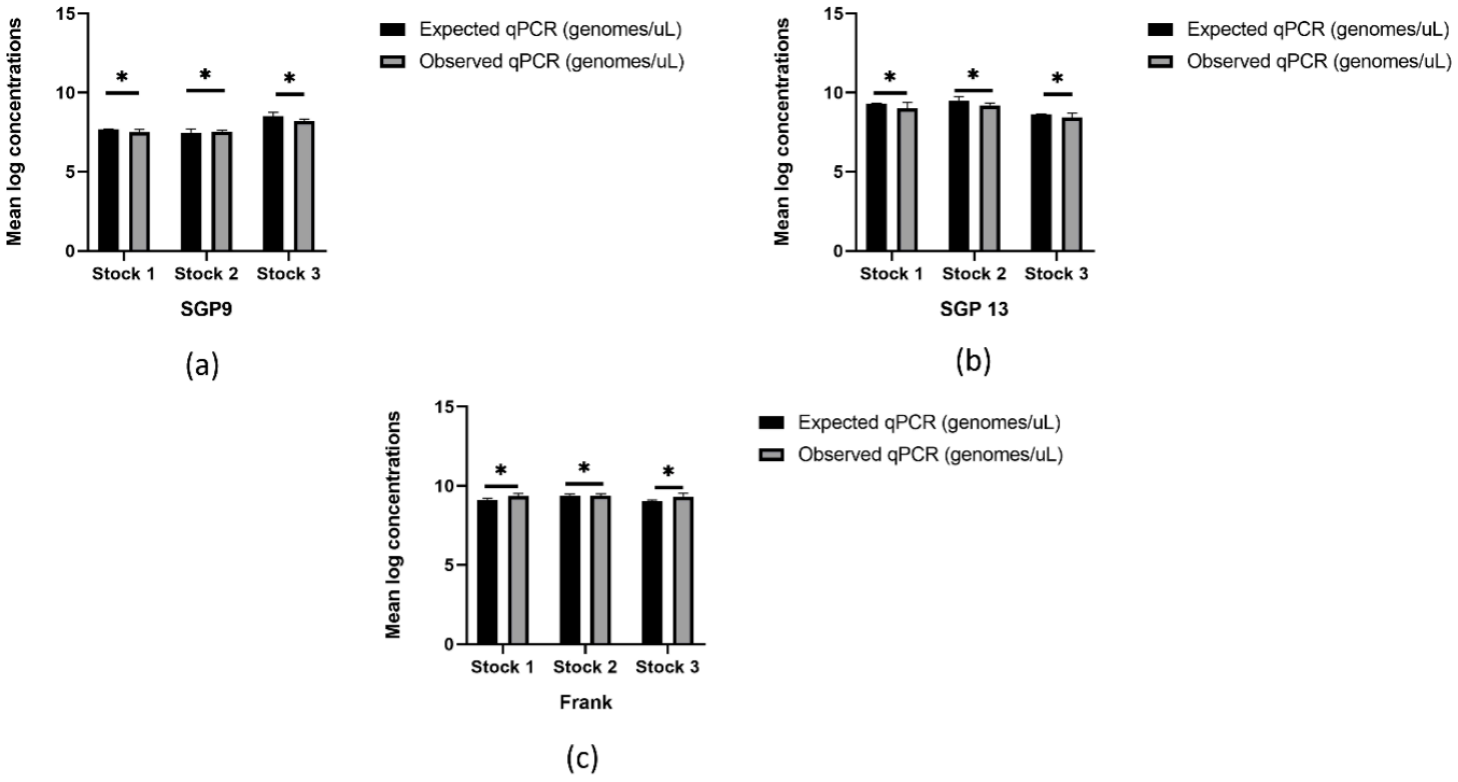


Figure 9

Multiplex qPCR quantification of DNase treated phage cocktail. For each phage (a) SGP9, (b) sgp13, (c) Frank, the expected and observed values are comparable across all three stocks with no significant variation. Lower copy number in indicates the removal of free DNA from the cocktail following DNase treatment.