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Improving Detection of Shiga Toxin-Producing *Escherichia coli* by Molecular Methods by Reducing the Interference of Free Shiga Toxin-Encoding Bacteriophages

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Detection of Shiga toxin-producing *Escherichia coli* (STEC) by culture methods is advisable to identify the pathogen, but recovery of the strain responsible for the disease is not always possible. The use of DNA-based methods (PCR, quantitative PCR [qPCR], or genomics) targeting virulence genes offers fast and robust alternatives. However, detection of *stx* is not always indicative of STEC because *stx* can be located in the genome of temperate phages found in the samples as free particles; this could explain the numerous reports of positive *stx* detection without successful STEC isolation. An approach based on filtration through low-protein-binding membranes and additional washing steps was applied to reduce free Stx phages without reducing detection of STEC bacteria. River water, food, and stool samples were spiked with suspensions of phage 933W and, as a STEC surrogate, a lysogen harboring a recombinant Stx phage in which *stx* was replaced by *gfp*. Bacteria were tested either by culture or by qPCR for *gfp* while phages were tested using qPCR targeting *stx* in phage DNA. The procedure reduces phage particles by 3.3 $\log_{10}$ units without affecting the recovery of the STEC population (culturable or assessed by qPCR). The method is applicable regardless of phage and bacteria densities and is useful in different matrices (liquid or solid). This approach eliminates or considerably reduces the interference of Stx phages in the detection of STEC by molecular methods. The reduction of possible interference would increase the efficiency and reliability of genomics for STEC detection when the method is applied routinely in diagnosis and food analysis.

Shiga toxin-producing *Escherichia coli* (STEC) bacteria are a class of enteric pathogens capable of causing severe gastrointestinal disease (hemorrhagic colitis) that can develop undesirable complications, such as acute kidney failure (hemolytic-uremic syndrome [HUS]) that could require lifelong treatment (1, 2). STEC strains belonging to different serotypes, notably strains of serotype O157:H7, have been the causative agents of large outbreaks of food-borne disease. However, the emergence of non-O157 STEC strains with various combinations of virulence genes also represents a serious challenge for the protection of consumers from food-borne disease (3, 4).

The identification of STEC strains, which requires culture enrichment on selective medium, is advisable to confirm and characterize the pathogen. However, recovery of the strain responsible for the disease is not always possible because it could be present in low concentrations, the cells might not be in a culturable state, or there could be interference from commensal *E. coli* within the microbiota in the sample. The need for early identification of STEC demands the use of faster and more robust methods.

STEC strains present genomic plasticity that complicates discrimination of the pathogenic strains among other *E. coli* strains present in a sample. For this reason, DNA and protein detection methods have been developed to target the genes related to virulence (5–7) and genes that help to identify a specific serotype (8). Reliable methods focused on the detection of virulent serotypes (for instance, O26, O45, O111, O103, O121, and O145, accounting for the non-O157 serotypes, plus O157:H7) (9) are very useful for known pathotypes or for epidemiological purposes. However, the recent outbreak caused by *E. coli* O104:H4 in Germany (10) highlighted the limitations of methods based on the detection of only well-known serotypes when an unusual one is present. In order to better assess the presence of an unknown STEC serotype in a sample, it appears to be necessary to establish protocols that target specific virulence genes, alone or preferentially in combination, instead of serotypes.

Many DNA-based methods for STEC detection include PCR techniques (5–7, 11–13), including standardized methods approved by legislation of the European Union (14, 15). Moreover, next-generation sequencing (NGS) protocols are emerging, and their implementation is advised to improve detection (10, 16).

The main virulence determinant of STEC, responsible for the most undesirable complications of infection such as HUS, is the Shiga toxin (Stx). *stx* genes are encoded in the genome of temperate bacteriophages (Stx phages) inserted as prophages into the STEC chromosome (17) or in enteric bacteria other than STEC, such as *Citrobacter* (18). Moreover, Stx phages can be found as free phages in environments that include urban sewage and animal wastewater (19, 20), human feces (21, 22), and even food (23), and they are extremely persistent in the natural environment (24–26).

When PCR-based and NGS methods are applied to detect

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TABLE 1 Oligonucleotides used in this study

Name	Sequence (5′–3′)[a]	Amplimer size (bp)	Function or description	Reference or source
tRNAup	TCTGCATTATGCGTTGTT	224	Upstream stx_{2A}	This study
Stx2a-gfpTir5	TAAAGTTAATCAGAATTCAAAACCCAGTAACAGGCAC		Overlapping of *gfp* gene and stx_{2A}	This study
GfpTir up	GAATTCTGATTAACTTTA	753	Entire *gfp* gene from pGreen TIR vector	This study
GfpTir lp	TTATTTGTAGAGCTCATCC			
GfpTir lp-cm5	GAAGCAGCTCCAGCCTACACATTATTTGTAGAGCTCATCC		Overlapping of *gfp* gene and *cat*	
Gfp short-up	TCCATCTTCAATGTTGTGTCT	211	*gfp* fragment for qPCR standard	This study
Gfp short-lp	GAACTATAAGACACGTGCTGA			
Cm 5	TGTGTAGGCTGGAGCTGCTTC	1015	*cat* gene	31
Cm-3	CATATGAATATCCTCCTTAG			
Cm3-stx	CTAAGGAGGATATTCATATGAGGAGTTAAGTATGAAGAAG	281	Overlapping *cat-stx*	31
Stx2Blp	TCAGTCATTATTAAACTG		Final codon of stx_{2B}	This study
UP378	GCGTTTTGACCATCTTCGT	378	378-bp stx_{2A} fragment	32
LP378	ACAGGAGCAGTTTCAGACAG			
RR46 LP	GAGCTCTAAGGAGGTTAT	457	Red recombinase in pKD46	31
RR46-UP	GTGCAGTACTCATTCGTT			
GFPTir-F	GCTTCCATCTTCAATGTTGTGTCT	121	Real-time PCR for *gfp* gene	This study
GFPTir-R	CATTCTTGGACACAAATTGGAATACAACT			
GFPTir- probe	FAM-CATGGCAGACAAACAA-NFQ			
STX-Any f	ACGGACAGCAGTTATACCACTCT	65	Real-time PCR for stx_2 gene	29
STX-Any r	ACGTTCCGGAATGCAAATCAG			
STX-Any probe	FAM- CCAGCGCTGCGACACG-NFQ			

[a] Overlap region is underlined.

STEC in a sample, the presence of free Stx phages in the sample (or other phages that could harbor virulence genes) represents a challenge since the phages can generate amplimers (PCR) or reads (NGS) that may be interpreted as belonging to STEC while they in fact belong to the phages. Moreover, when the protocols include a previous step of selective enrichment of the target bacteria, the step may also maintain or even propagate bacteriophages. The present study attempts (i) to confirm this possibility and (ii) to develop a method to eliminate or reduce the presence of free Stx phages in different samples, with the goal of minimizing their interference in DNA-based STEC detection methods.

MATERIALS AND METHODS

Bacteria, bacteriophage plasmids, and media. Bacteriophage 933W was used in the experiments as a positive control. Phage 933W was induced from lysogenic *E. coli* strain C600 (933W) (27) and purified as described below. *E. coli* DH5α was used as a control of culturable bacteria. An *E. coli* C600 lysogen containing a modified 933W phage in which a fragment of the *stx* gene was replaced by the gene encoding the green fluorescent protein (*gfp*) and the gene encoding chloramphenicol resistance (*cat*), constructed as described below, was used to enumerate culturable bacteria and to distinguish bacteria from free Stx phages using quantitative PCR (qPCR). A pGEM-T-Easy cloning vector (Promega Co., Madison, WI) was used for the construction of standard curves. *E. coli* C600 (pGEM::stx_2) (19) or plasmid pGreen TIR (28) containing the *gfp* gene was used to prepare the standards for the qPCR assays. Luria-Bertani (LB) broth or LB agar was used for bacteria cultivation and phage assays. When necessary, medium was supplemented with chloramphenicol (Cm) (20 μg/ml) (Sigma-Aldrich, Steinheim, Germany).

Preparation of bacteriophage 933W suspension. Lysogen *E. coli* C600 (933W) was incubated under agitation (180 rpm) at 37°C to an optical density at 600 nm (OD_{600}) of 0.3, as measured with a spectrophotometer (Spectronic 501; Milton Roy, Belgium). To induce 933W bacteriophages, mitomycin C (0.5 μg/ml) was added to the culture and incubated overnight at 37°C in the dark in a shaker. Phage lysates were prepared by filtration of the bacterial cultures through low-protein-binding 0.22-μm-pore-size membrane filters (Millex-GP; Millipore, Bedford, MA, USA). The supernatant was treated with DNase (100 units/ml of the phage lysate) to eliminate free DNA outside the phage particles. Phage DNA was extracted using different methods, as described below.

Standard and commercial DNA extraction methods. A phage DNA purification method was used to extract DNA from the 933W suspension as described previously (29). The concentration and purity of the phage DNA extracted were determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies/Thermo Scientific Instruments, Wilmington, DE, USA).

In addition, phage 933W DNA was extracted using three commercial DNA extraction methods applied for bacterial or total DNA extraction, according to the manufacturers' instructions, as follows: QIAamp DNA Blood minikit (Qiagen GmbH, Hilden, Germany), QIAamp DNA Stool minikit (Qiagen), and NucliSENS miniMAG (bioMérieux España, Madrid, Spain). The phage count results from the commercial methods were compared with the specific protocol applied for bacteriophage DNA extraction.

Construction of the *E. coli* C600 (933W Δ*stx*::*gfp*::*cat*) recombinant lysogen. *E. coli* C600 lysogenic for phage 933W was used to prepare a recombinant strain in which *stx* was replaced by the *gfp* gene amplified from plasmid pGreen TIR (28) and a *cat* (chloramphenicol resistance) gene. A 224-bp fragment including the 5′ region of the stx_{2A} subunit was amplified with tRNAup/Stx2a-gfpTir5 primers (Table 1), where the second primer overlaps *gfp*. The 753-bp *gfp* gene was amplified from plasmid pGreen TIR with primers GfpTir up/GfpTir lp-cm5, with the second primer overlapping *cat*. The 1,015-bp *cat* gene was amplified with primers cm5/cm3, and, finally, a 281-bp fragment of the stx_{2B} subunit was amplified with primers Cm3-stx/Stx2Blp, with the first primer overlapping *cat*. A single stx_{2A}-*gfp*-*cat*-stx_{2B} amplimer was generated by overlap extension PCR and then purified and electroporated into the laboratory lysogen *E. coli* C600 (933W) containing pkD46 (30) with the Red recombinase system. The recombinant phage 933W Δstx_2::*gfp*::*cat* (hereafter, 933Wgfp) was constructed using previously described methods (31) by recombination of the fragment into the late gene region of the Stx phage, replacing the sequence of the stx_2 target gene (Fig. 1). The *cat* gene was introduced in the construct to prevent spontaneous induction of phage 933Wgfp

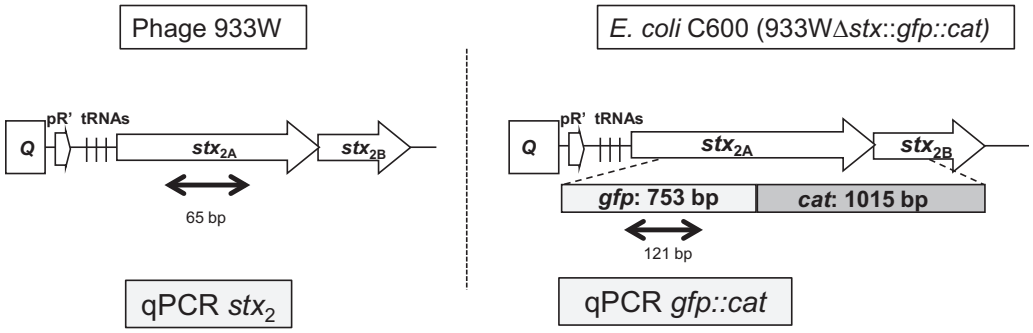

FIG 1 Schematic representation of the genetic organization of the *stx* operon in phage 933W in the STEC strain and the replacement of the stx_2 gene by the *gfp-cat* fragment in *E. coli* C600 (933Wgfp). The schemes indicate where the fragments amplified by the qPCR assays for *stx* and *gfp* are located. *Q*, antiterminator gene; pR, late phage promoter.

from the *E. coli* C600 (933Wgfp) lysogen by growing the strain in the presence of Cm.

Phage reduction procedure. Phosphate-buffered saline (PBS) was spiked with various volumes of the 933W phage suspension. The spiked samples were evaluated in parallel with and without a previous filtration step. Volumes of 10 ml of each sample were filtered through 0.45-μm-pore-size mixed cellulose ester filters, commonly used for filtration in microbiological analyses (EZ-Pak membrane filter; Millipore, Bedford, MA, USA), or through 0.45-μm-pore-size low-protein-binding polyvinylidene fluoride (PVDF) membranes (Durapore membrane filter; Millipore, Bedford, MA, USA), which allowed bacteriophages to pass through but retained bacteria. To further remove phages retained in the filters, one or three washing steps were additionally applied. For each wash, 10 ml of PBS was added to the surface of the filter, gently agitated, and removed by filtration.

In addition, the phage reduction procedure with PVDF membranes was tested in PBS spiked with various volumes of *E. coli* DH5α or *E. coli* C600 (933Wgfp) bacterial cultures, and bacteria were recovered after three washing steps. Results of culturable bacteria were compared with and without a previous filtration step.

Phages and/or bacteria retained in the filter after the filtration procedure and the washing steps were recovered by suspending the membrane in the same volume of PBS as the original sample, shaking the membrane for 10 min by gentle rotation (horizontal shaker, Magna-AS15; Magna-Equipaments, Barcelona, Spain) at room temperature, and vortexing for 1 min. The filtrate was recovered in sterile 50-ml bottles and monitored for the presence of phages or bacteria as described below. Phages were monitored by qPCR as described below. Culturable *E. coli* was enumerated by serial decimal dilutions in PBS, and 100 μl was spread on LB agar plates or LB medium supplemented with Cm using a surface spread plate technique. The plates were incubated at 37°C for 18 h. To confirm that the reductions observed were due to the filtration procedure, in some experiments the filtrate was also analyzed for the presence of phages and bacteria.

Samples. The procedure was tested in different matrices in addition to PBS. A mixture of stools obtained from five healthy adult volunteers (aged 25 to 30 years) was used in this study. Stool samples were collected, coded, and manipulated anonymously and were used only for this study. As food matrices, five salad and five minced-beef samples were purchased at different dates from local retailers. The samples of beef were freshly minced on request at local supermarkets, and salad samples were a commercially prepared mixture. Five river water samples were collected at different dates in the lower course of the River Llobregat, located in the Barcelona (Spain) area.

Samples were spiked with phages and bacteria as described above. Liquid samples were homogenized by magnetic stirring for 2 min and directly processed. Solid samples were mixed 1:5 (wt/vol) in phosphate-buffered saline (PBS), placed in stomacher bags with filters (Afora, Barcelona, Spain), and homogenized using a Masticator (IUL Instruments GmbH, Königswinter, Germany) for 2 min. The samples were then allowed to settle to precipitate the larger particles, and the homogenate was processed. To avoid filter clogging in solid samples, an additional soft centrifugation step (300 × *g* for 10 min) was added. The phage reduction procedure (with PVDF filters and three washing steps) was then applied to the samples, and phages and/or bacteria were recovered from the filters as described above. Phages and bacteria were monitored by qPCR as described below, and the results obtained were compared with the values of the controls processed without filtration.

Standard and qPCR procedures. Endpoint PCR amplifications were performed with a GeneAmp PCR 2400 system (Perkin-Elmer, PE Applied Biosystems, Barcelona, Spain) with the primers described in Table 1.

Phage 933W quantification. A stx_2 qPCR assay was used for phage 933W quantification, as previously described (19). Since Stx phages are known to carry only one *stx* copy, the *stx* gene copy (GC) values can be extrapolated to the number of Stx phages in each sample.

Recombinant *E. coli* C600 (933Wgfp) quantification. In the samples spiked with bacteria and phages, and to discriminate bacteria from free Stx phages, recombinant *E. coli* C600 (933Wgfp) was used; to quantify these recombinant bacteria, a TaqMan qPCR assay for the detection of *gfp* was designed using the primers GFPTir-F/GFPTir-R and a minor groove binding probe, GFPTir, with a 5′ 6-carboxyfluorescein (FAM) reporter and a nonfluorescent quencher (NFQ). The qPCR assays were performed under standard conditions in a StepOne Real Time PCR System (Applied Biosystems) in a 20-μl reaction mixture with TaqMan Environmental Real-Time PCR master mix 2.0 (Applied Biosystems). All the samples and standards were run in triplicate, and the GC number was the average of the triplicate data obtained. For the generation of a *gfp* qPCR standard, plasmid pGreen TIR (28) was used and prepared as described previously (19). The assay showed an efficiency of 99.39%.

Statistical analyses. Data and statistical tests were performed using the Statistical Package for Social Science software (SPSS). One-way analysis of variance (ANOVA) with a *P* value of 0.05 was used to evaluate the differences between the DNA extraction methods and between filtered and nonfiltered samples.

RESULTS

Comparison of DNA extraction methods. As expected, phage DNA was extracted from the 933W suspension with similar efficiencies by all the methods used, and quantification of *stx* in the phage DNA did not show any significant ($P > 0.05$) differences between the method designed specifically for phage DNA extraction and the commercial methods intended for total (bacterial and phage) DNA extraction (Fig. 2). These results confirm that common laboratory protocols can indeed include phage DNA in the results when food, environmental, and clinical samples are pro-

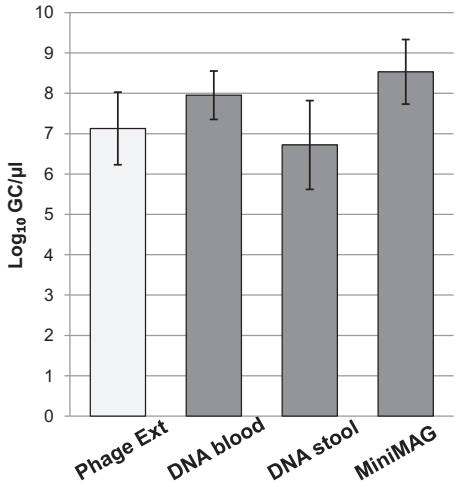

FIG 2 Concentrations of phage 933W evaluated by measurement of numbers of stx_2 GC/μl from phage DNA extracted using conventional phage DNA extraction (Phage Ext) methods and commercial DNA extraction kits (QIAamp DNA Blood minikit, QIAamp DNA Stool minikit, and NucliSENS miniMAG).

cessed. The experiments we detail below were performed with a QIAamp DNA Blood minikit, and a single DNA extraction was used to quantify either bacterial or phage DNA.

Phage reduction procedure. To determine whether the filtration step reduced the number of phage in a given sample, phage 933W was spiked in PBS. Samples were filtered through two different membrane filters: a 0.45-μm-pore-size mixed cellulose ester membrane and a 0.45-μm-pore-size PVDF membrane, described as a low-protein-binding membrane. To reduce phage content further, the filtration was followed by one washing step with PBS. After filtration, 933W was recovered from the filter and quantified by qPCR; results were then compared with those from the nonfiltered sample. All the filtration procedures significantly ($P < 0.05$) reduced the number of phage particles in the samples, but the use of low-protein-binding membranes resulted in a better reduction (Fig. 3A) (2.4 $\log_{10}$ GCs/μl) than filtration through mixed cellulose esters (0.8 $\log_{10}$ GCs/μl). Additional washing steps (three) were applied to the PVDF membrane to achieve a phage reduction of 3.3 $\log_{10}$ GCs/μl after filtration, compared with the initial sample (Fig. 3A). The experiments we describe below were therefore performed with filtration through PVDF membranes and three consecutive washing steps.

Analysis of the filtrate recovered after three washing steps allowed detection of 3.6 (0.13 standard deviation) $\log_{10}$ GCs/μl phage 933W, which is equivalent to the reduction observed by the filtration procedure.

Influence of the procedure on culturable bacteria. The phage reduction procedure did not negatively affect recovery of culturable bacteria, as observed in experiments with PBS samples that were spiked with *E. coli* DH5α or with *E. coli* C600 laboratory lysogen containing phage 933Wgfp (Fig. 3B). This phage-containing lysogen was used to avoid manipulation of a pathogenic STEC strain and also as an STEC surrogate to evaluate an *E. coli* lysogen carrying an Stx phage under conditions as similar to those of an STEC strain as possible. No spontaneous release of the 933Wgfp phage during the cultivation of the strain before spiking the samples was expected due to the antibiotic selection, which allows growth of only the cells containing the phage. The numbers of culturable cells evaluated in LB agar for *E. coli* DH5α or in LB plus Cm for *E. coli* C600 (933Wgfp) at 37°C were not significantly different ($P > 0.05$) before and after the procedure (Fig. 3). Moreover, culturable *E. coli* strains DH5α and lysogen C600 (933Wgfp) (CFU/ml) were absent in the filtrate.

Evaluation of the procedure at different bacterial and phage concentrations. To assess the influence of various densities of Stx phages or culturable bacterial cells in PBS, we evaluated the phage removal protocol at decreasing concentrations (from 10^5 to 10^2 CFU/ml) of culturable *E. coli* C600 (933Wgfp) in combination with decreasing concentrations (from 10^4 to 10^1 GCs/μl) of phage 933W as evaluated by qPCR. DNA extraction was performed for each mixture with and without application of the phage removal procedure (Fig. 4).

As before, differences in numbers of culturable bacteria between the filtered and unfiltered samples were not significant ($P > 0.05$), indicating that the procedure did not interfere with bacterial determination. In contrast, the concentration of 933W fell below the limit of detection in the filtered samples. At the highest

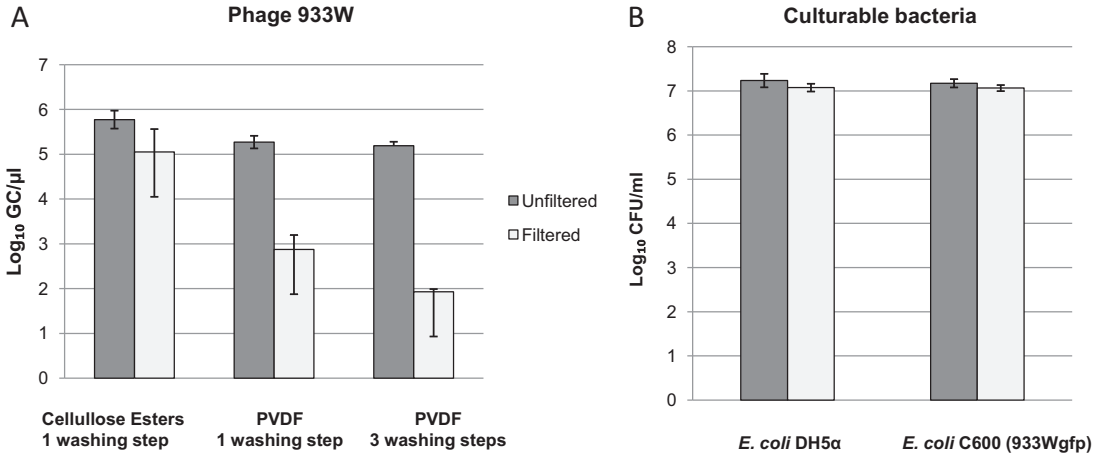

FIG 3 Concentrations of phage 933W (GC/μl) (A) and culturable *E. coli* strains DH5α and lysogen C600 (933Wgfp) (CFU/ml) (B) with or without application of the filtration procedure used to remove bacteriophage.

		E. coli C600 (933Wgfp) (log_{10} CFU/ml)			
FILTERED		10^5	10^4	10^3	10^2
933W (log_{10} GC/μl)	10^4	5.73 / 1.25*	4.89 / 1.31*	3.25 / 1.58*	2.30 / 1.66*
	10^3	5.93 / ND	4.97 / ND	3.28 / ND	2.60 / ND
	10^2	5.15 / ND	4.95 / ND	3.04 / ND	2.50 / 0.72*
	10^1	5.05 / ND	4.83 / ND	3.30 / ND	2.30 / ND
UNFILTERED					
933W (log_{10} GC/μl)	10^4	5.61 / 4.37	4.81 / 4.37	3.10 / 4.15	2.11 / 4.30
	10^3	5.45 / 3.34	4.61 / 3.18	3.28 / 3.15	2.00 / 3.15
	10^2	5.46 / 2.35	4.72 / 2.25	3.25 / 2.02	2.29 / 2.25
	10^1	5.30 / 1.76	4.72 / 1.69	3.20 / 1.74	2.63 / 1.60*

FIG 4 Concentrations of phage 933W (GC/μl) (dark gray) and culturable *E. coli* lysogenic strain C600 (933Wgfp) (CFU/ml) (light gray) in mixtures containing different phage and bacteria densities with or without the application of the filtration procedure used to remove bacteriophage. *, C_T below the detection limit; ND, not detected.

phage densities (10^5 GCs/μl), the phage was still detectable in the filtered samples although the values obtained were below the limit of detection required for positive samples in our qPCR assay, as determined by a threshold cycle (C_T) of <32.0 (19). These results correspond to samples marked with an asterisk in Fig. 4. For the other dilutions, Stx phages were undetectable by the qPCR assay (C_T of >33.5 or undetermined) (Fig. 4).

Application of the procedure for reduction of Stx phages in food and stool samples. The phage reduction method was applied in different matrices: PBS (control), river water, salad, minced beef, and human feces. The samples were spiked with *E. coli* C600 (933Wgfp) and phage 933W at known densities (approximately 10^5 GCs/μl of bacteria or phage). To reproduce the real procedure, bacteria and phages were evaluated from a single DNA extraction and by qPCR (*stx* assay for phages and *gfp* assay for bacteria) (Fig. 1), and values were compared between filtered and unfiltered samples.

Significant ($P < 0.05$) reductions in the number of Stx phages were obtained by the procedure in all the matrices analyzed without any significant ($P > 0.05$) reduction in the densities of *E. coli* C600 (933Wgfp) (Fig. 5). The reduction efficiency varied between the different matrices. Liquid samples showed the best reductions; in solid samples, the procedure required sedimentation of the sample to avoid collapse of the filter membranes, which would reduce the efficacy of the procedure. Sedimentation was conducted by including soft centrifugation (300 × *g* for 10 min) that was confirmed not to reduce bacterial counts (Fig. 5).

Salad, meat, and stool samples were also used to evaluate the presence of Stx phages by endpoint PCR using primers UP378/LP378 (32). The samples were directly analyzed or spiked with 10^4 GCs/μl of phage 933W suspension, with or without filtration. Nonspiked samples were negatives for stx_2. In the spiked samples, only unfiltered samples showed positive stx_2 detection, while the filtered ones were negative by endpoint PCR.

DISCUSSION

An important volume of work reports positive detection of *stx* and therefore STEC by PCR or qPCR techniques but then fails to isolate an STEC strain (33, 34). This could be attributed to several causes, such as previous antibiotic treatment of the patient, a dis-

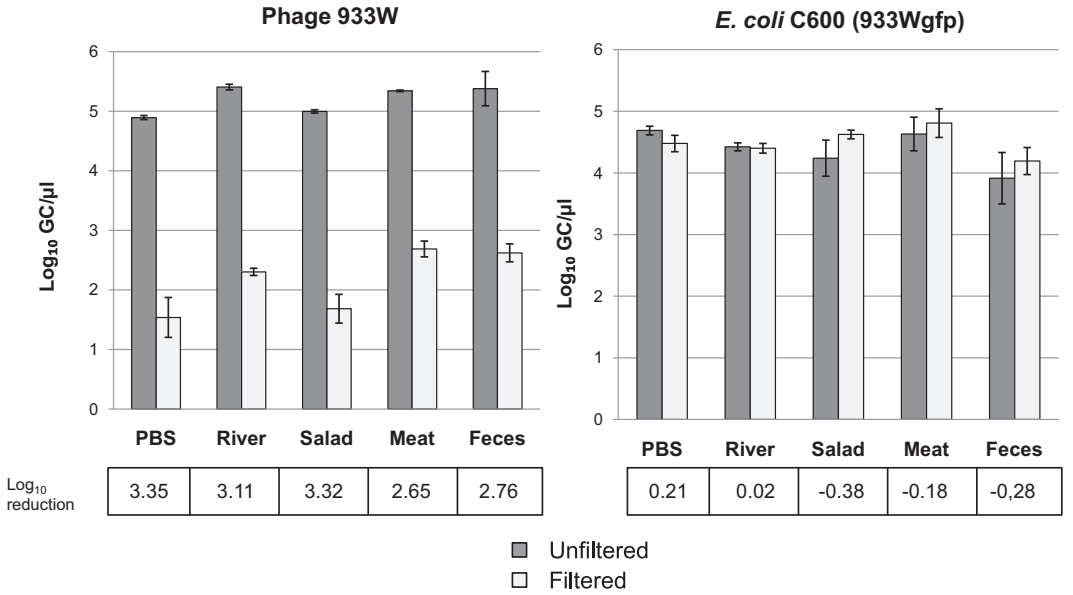

FIG 5 Simultaneous evaluation by qPCR of the densities of 933W and *E. coli* C600 (933Wgfp) in different matrices with and without the application of the filtration procedure.

ease diagnosed late in its course, the presence of commensal *E. coli* and other commensal flora in the sample, even to the presence of viable but nonculturable microorganisms, or just because the target bacteria is present in very low numbers. However, here we present a new consideration: the presence of Stx phages in the samples and their interference in positive PCR detection or their inclusion in sequences generated by NGS. This possibility is very real since it has previously been shown that Stx phages are abundant in many environments, including the human gut (20, 21, 23, 29).

Our study confirms that the methods used for bacterial DNA extraction in these complex matrices (14, 35, 36) also extract phage DNA. While this result was expected, the efficiency of recovery of phage DNA had not been previously evaluated. Therefore, any virulence genes present in phage DNA, particularly *stx* but also other virulence genes described in prophages (e.g., *nle* and *cdt*), could be detected in the sample. When NGS is used, there is no easy way to establish whether the target detected is from bacteria or from a prophage if the contig is not a sequence long enough to contain bacterial DNA. Although it could be argued that detection of *stx* could be considered indicative of the presence of STEC, if it is from a phage, the threat for virulence in humans is much less important, and this should be considered. In terms of diagnosis or food safety analyses, false-positive results could interfere with determination of the real causative agent. Many PCR assays include multiple target genes that could help rule out false STEC detections (5, 7, 36), but, again, there is no confirmation that all genes detected are located in the same bacterial chromosome.

Moreover, from a more practical point of view, enrichment cultures used to selectively isolate bacteria can cause the propagation of any sort of phages present in a sample. Many natural samples carry virulent phages that will propagate during the culture by causing the lysis of the bacteria that is the intended target of the detection; this would cause a bias in the population of enriched bacteria (37). Phage reduction procedures offer the possibility of reducing the numbers of any sort of phages in food or stool samples, enhancing detection and plausibly isolation of the target pathogen.

Complete elimination of all phages present in the samples cannot be accomplished by the procedure presented here, and the fact that phages were still present in the samples after the procedure, even at low concentrations, could represent a problem because enrichment cultures will likely be performed with these samples, allowing phages to multiply. Nevertheless, a compromise can be found between an easy and feasible approach that could be applied in routine laboratories and that does not affect bacterial determination while causing a reasonable reduction on phage populations. In addition, the efficiency of the filtration would always be higher when aqueous samples or clear homogenates of solid samples are treated than when complex solid samples that require additional sedimentation steps are treated. In general, the additional time required to apply the procedure is around 15 min while the cost is negligible (limited only to the membranes), and the procedure requires basic laboratory equipment; the benefits should therefore be evaluated. Although the method has been checked on a laboratory basis using spiked samples, more studies using real STEC-positive samples, analyzed on a routine basis in parallel with or without phage reduction, should be conducted in future studies to validate and optimize this method.

New perspectives for pathogen detection are offered by NGS techniques (22, 38, 39). Regarding the implementation of these techniques for routine diagnosis of pathogens, the interference that phages could cause in the assembly of a genome sequence should be considered when DNA is obtained directly from the samples and not from isolates, particularly from the less abundant members of a microbial community (40). For example, virulence genes (*stx* or others) or antibiotic resistance genes, both reported in phages or phage-like elements (41–43), will certainly be detected, but the strain or species to which they belong, if any, will be unknown. The reduction of possible interferences would improve the efficiency and reliability of genomics when routinely applied.

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