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Original Scientific Article

COMPARISON OF DNA EXTRACTION METHODS TO DETECT *SALMONELLA* SPP. IN TAP WATER

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Summary: Bacteria of the genus *Salmonella* cause a global health problem related to contaminated foodstuffs and faecally-polluted water supplies. The aim of our work was to determine the efficiency and rapidness of *Salmonella* detection in spiked tap water samples by comparison of different methods for DNA extraction. Water samples were spiked with different *Salmonella* loads. Four DNA extraction methods were employed: foodproof™ Sample Preparation Kit I (Biotecon Diagnostics) and QIAamp® DNA Stool Mini Kit (Qiagen) for DNA extraction after an overnight enrichment step, and Adiapure® Water DNA Extraction and Purification Kit (Adiagene) and SmartHelix® Complex Samples DNA Extraction Kit (developed recently in our laboratory) for direct DNA extraction. The extracted DNA was subjected to PCR amplification using *Salmonella* genus-specific primer pair. The SmartHelix extraction kit proved to be more efficient than the Adiapure Water kit for direct detection of *Salmonella* DNA in water. The overnight enrichment step improved the detection of *Salmonella* spp. in samples spiked with low bacterial load, however it extended the analysis time. Results suggest that the newly developed SmartHelix extraction kit should be regarded as a considerable choice when rapid detection of pathogens in water samples is aimed for to prevent disease outbreaks and support safe food assurance.

Keywords: DNA extraction; food safety; pathogen detection; PCR; *Salmonella*; water

Introduction

Salmonelloses are one of the most frequent foodborne zoonoses in industrialized countries caused by consumption of contaminated foodstuffs of animal origin (1). They are associated with abdominal cramps, diarrhea, nausea, vomiting and fever, but can occasionally evolve into severe localized infections or potentially fatal systemic sepsis (2). Since bacteria of the genus *Salmonella* are found in the digestive tract of humans and a variety of animals, faecal transmission to water supplies in regions with poor sanitary measures occurs continually. Contaminated drinking and food-processing water represents a significant source of infection for humans in the third world countries, mostly by the human-adapted *Salmonella enterica* subsp. *enterica* serovar Typhi and *Salmonella* serovar Paratyphi causing severe systemic disease (3). On account of easy dissemination by contamination of food and water supplies, bacteria of the genus *Salmonella* are also considered a potential bioterroristic agent belonging to the category B according to the established classification. There have been cases of intentional contamination reported in the past aiming for economical or political destabilization to create panic among civilian population and intimidate the authorities (4-6).

The main requirement for rapid detection of deliberate or non-deliberate contamination is a sensitive surveillance system that links local laboratories and clinicians with public health professionals to enable the recognition of unusual pattern of reported disease cases (5,7). Traditional cultivation-dependent methods based on ISO 6579:2002 (8), which is

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set as the golden standard for detection of *Salmonella* spp. in food and feedstuffs (9,10), require five working days to confirm positive results. Therefore, standardized procedures are not compatible with the demand for rapid assessment of *Salmonella* spp. contamination in water systems. In order to prevent the dissemination of bacteria, more rapid and sensitive molecular techniques, which are based on the detection of microbial nucleic acids, are needed.

The objective of our study was to investigate the efficiency and rapidness of direct DNA extraction method applying a commercially available kit that was recently developed in our laboratory (Institute of Physical Biology, Slovenia). This new method is especially adapted for DNA isolation from cells concentrated on filters. It was compared to other commercial extraction kits with or without the pre-enrichment of bacterial cells. Four methods for DNA extraction were selected according to their applicability for the type of samples and their ability to remove the inhibitors of the subsequent molecular detection that can be found in the field, clinical or alimentary samples. Detection of *Salmonella* spp. in tap water samples was based on microbial DNA extraction followed by the polymerase chain reaction (PCR).

Materials and methods

Bacterial strain for sample preparation

Reference strain of *Salmonella enterica* subsp. *enterica* serovar Enteritidis (CAPM 5439) was used in the present study. The overnight bacterial culture was grown at 37°C in buffered peptone water (BPW) prepared according to Anex B of ISO 6579:2002 (8), diluted in 2-fold series and used as an inoculum for the tap water samples.

Inoculation of water samples

Thirty liters of tap water were collected and further subdivided into 500-mL samples. Four different bacterial dilutions were spiked in five parallels into water samples to obtain 20 samples with *Salmonella* loads of 18, 36, 72 and 144 colony forming units (CFU) per liter, respectively. The same spiking procedure was repeated three times to obtain sample parallels for different DNA extraction procedures, namely two series of 20 samples for direct extraction and one series of 20 samples for the overnight enrichment prior to extraction. CFU load of the bacterial culture was determined using the standard plate count technique (11) on Rambach agar plates (12).

Extraction of microbial DNA from spiked water samples

Bacteria in spiked water samples were concentrated by filtration through 0.45 μm membrane filters (Sartorius, Germany). After filtration, one series of 20 samples was used for the overnight pre-enrichment in 10 mL of BPW at 37°C. One mL of the obtained enrichment culture was used for DNA extraction using foodproof™ Sample Preparation kit I (Biotecon Diagnostics, Germany; abbreviated as FP-e) and one mL for the extraction using QIAamp® DNA Stool Mini kit (Qiagen, Germany; abbreviated as S-e) according to the manufacturers' instructions. Two other series of 20 samples were used for direct extraction from the filter-concentrated bacteria using Adiapure® Water DNA extraction and purification kit (Adiagene, France; abbreviated as AW-d) and by the method developed in our laboratory, namely SmartHelix® Complex Samples DNA extraction kit (Institute of Physical Biology, Slovenia; abbreviated as SH-d), according to the manufacturers' instructions. Direct extraction was performed immediately after sample filtration. The extraction methodology is summarized in Table 1. More information on the SmartHelix technology is available at http://smart-helix.com/.

Molecular detection of Salmonella spp. in water samples

The isolated microbial DNA was subjected to PCR amplification using *Salmonella*-specific primers ST11 and ST15 (13,14). PCR was performed according to the modified protocol as described before (15). Each individual sample in five parallels was PCR amplified in four replicates to obtain 20 results of detection per each of the four extraction methods. Amplified PCR products were separated on agarose gels by electrophoresis, stained with ethidium bromide (10 μg/mL; Invitrogen, USA) and documented using the GeneGenius bio-imaging system (Syngene, UK).

Data analysis

To statistically evaluate the obtained data, the most probable number (MPN) method was coupled

Table 1: Commercially available kits for DNA extraction from water samples employed in the present study

DNA extraction kit	Manufacturer	Abbreviations	Type of analysis	Type of extraction method	Hands-on time (h)
Foodproof™ Sample Preparation kit I	Biotecon Diagnostics	FP-e	Overnight pre-enrichment in BPW prior to extraction	Chemical/thermal cell disruption and DNA purification with glass fiber spin columns	≥12 + 0.5
QIAamp® DNA Stool Mini kit	Qiagen	S-e		Thermal/proteinase K cell disruption and DNA purification with silica spin columns	≥12 + 1
Adiapure® Water DNA extraction and purification kit	Adiagene	AW-d	Direct extraction	Chemical/thermal cell disruption and DNA purification with ultrafiltration columns	1.5
SmartHelix® Complex Samples DNA extraction kit	Institute of Physical Biology	SH-d		Mechanical cell disruption and DNA purification with detergent/phenol	3

Note: At hands-on time, the overnight pre-enrichment in BPW prior to extraction is depicted as ≥12h.
Legend: e, pre-enrichment; d, direct extraction; BPW, buffered peptone water

with PCR and calculated using the Most Probable Number Calculator program version 4.04 (http://www.epa.gov/microbes/mpn.exe) with four dilution and 20 tubes per dilution parameter settings.

Results

Differences in *Salmonella* detection limit could be observed between the two groups of DNA extraction kits based on the employment of sample pre-enrichment step. The overnight pre-enrichment prior to DNA extraction markedly improved the detection, since bacteria of the genus *Salmonella* were detected in all spiked water samples with some trivial discrepancies observed for higher bacterial loads with the method S-e (Table 2). Detection limit was at least 18 CFU/L, which was the lowest contamination level tested.

Table 2: Efficiencies of DNA extraction kits used in the present study for water samples inoculated with *Salmonella* Enteritidis (CFU/L)

		Number (%) of Salmonella-positive PCR reactions			
		pre-enrichment		direct extraction	
CFU/L	Number of tested PCR reactions	FP-e	S-e	AW-d	SH-d
18	20	20 (100)	20 (100)	0 (0)	0 (0)
36	20	20 (100)	20 (100)	2 (10)	4 (20)
72	20	20 (100)	19 (95)	4 (20)	11 (55)
144	20	20 (100)	19 (95)	12 (60)	20 (100)

Note: Samples were prepared in five parallels and for each parallel four PCR replicates were performed (i.e. 20 tested PCR reactions). See Table 1 for details on DNA extraction kit abbreviations

When comparing methods for DNA extraction not preceded with the additional manipulation after sample collection, differences in *Salmonella* detection limit were observed as well. Both methods for direct DNA extraction from water samples generated positive results at 36 CFU/L and/or at higher bacterial loads. However, all parallels and replicates tested positive only with SH-d for water samples spiked with 144 CFU/L (Table 2). In comparison to AW-d, SH-d supported a markedly higher number of positive results in all spiked samples. Likewise, when PCR results were quantified using the MPN method (Figure 1), the estimated level was significantly higher for the method SH-d in comparison to AW-d for the highest tested contamination level (144 CFU/L). Other contamination levels with the exception of the lowest one (18 CFU/L), where detection failed for both methods, showed no statistical difference ($P<0.05$). For the method SH-d, the estimated levels of contamination (MPN/L) were similar to the inoculated *Salmonella* loads (CFU/L).

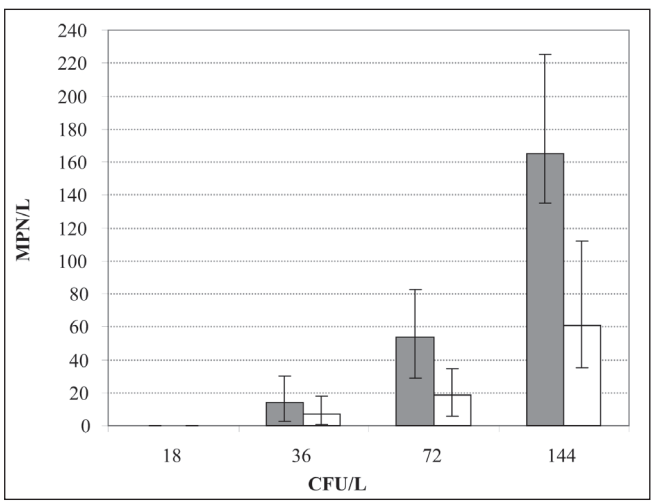

Figure 1: The inoculated *Salmonella* load (CFU/L) plotted against the estimated load (MPN/L) for water samples processed by the two direct DNA extraction methods: SmartHelix® Complex Samples DNA extraction kit (gray bars) and Adiapure® Water DNA extraction and purification kit (white bars); error bars represent 95% confidence levels

Discussion

Detection limit for *Salmonella* spp. in tap water samples employing the PCR amplification depended on the method for microbial DNA extraction. Bacterial enrichment in a selective or non-selective growth medium prior to molecular detection enables multiplication of targets to the detectable concentration, and it also dilutes the PCR inhibitory substances that can be present in the investigated samples. As expected, the overnight pre-enrichment step employed with the methods FP-e and S-e prior to molecular detection proved to be significant for lowering the detection limit of *Salmonella* spp. in comparison to direct methods. Increasing the number of samples in the future research will enable determination of the exact detection limit of direct extraction methods like SH-d, and the detection probability as reported before (16) will enable more objective calculation stating the expected relative frequency of positive PCR results at various contamination levels over a certain period of time.

Many PCR assays employed for spiked water, food, soil, and faecal samples included the pre-enrichment step that enabled detection of only few *Salmonella* cells per sample (17-19). However, when an instant response of the public health-assuring authorities is needed for the prevention of *Salmonella*-associated epidemic outbreaks, a protocol requiring the pre-enrichment step represents a major disadvantage. The methods for direct DNA extraction from environmental samples enable at least four to five time faster response, obtaining DNA within three hours after sampling, although not distinguishing between viable and dead bacterial cells. Samples can be processed immediately after collection and results obtained at the same day, rendering possible the prevention of dissemination of pathogens in water supplies.

The selected direct extraction methods differed to some extent in the required hands-on time, but more importantly in their efficiency. Results indicated that the method SH-d was more suitable than the other selected method for rapid detection of *Salmonella* spp. in tap water samples. It is suitable for DNA extraction from water samples for pathogen detection in the case of both high and low contamination levels. To optimize DNA extraction after sample filtration to concentrate microbial cells, the method SH-d was adapted to completely disrupt the filters and bacterial cell membranes using the mechanical force (*i.e.* beads were added to filtered samples prior to bead-beating disruption) in combination with detergent/phenol treatment to obtain high DNA extraction yields. As used in the method AW-d, chemical/thermal cell disruption without filter disintegration in combination with DNA purification columns proved to be less efficient for obtaining high DNA extraction yields possibly due to insufficient detachment of cells from

filters, therefore excluding a certain proportion of microbial cells from DNA extraction procedure. Our preliminary studies indicated that the mechanical disintegration increased the extraction yields from bacterial cells for at least five times, which was based on the measurement of DNA concentration (unpublished data). As strong mechanical forces can cause DNA fragmentation, subsequent PCR amplification can be less efficient. However, the duration of mechanical disruption was optimized to achieve complete disintegration of filters and cells but to obtain microbial DNA of satisfactory quality for PCR amplification.

The present study enabled the first insight into the applicability of the newly developed DNA extraction kit (SH-d). In addition to the present study, a preliminary research was conducted on spiked water samples collected from a pond, representing the naturally contaminated water source. For pond water, it could be observed that the sensitivity of detection was at least six times lower than for tap water when applying direct extraction methods but similar when including the pre-enrichment step (unpublished data). More research will be performed on raw water samples to study the effect of the background microbiota and the inhibitory chemical compounds. The collected data from our and other laboratories will enable us to introduce the potential method improvements. The practical operating range of PCR detection (16) preceded by the SH-d extraction will be determined and an internal amplification control (20) will be included if needed to give the newly developed kit a promising entry into the routine diagnostics. The analysis could be performed even faster without the DNA extraction step (21). However, the low contamination levels require a detection procedure of greater sensitivity and reproducibility. The extraction step employing a method not preceded with the pre-enrichment meets the demand for fast response.

We can conclude that direct methods for DNA extraction from bacterial cells concentrated on filters can be successfully used in place of procedures employing cultivation for the detection of *Salmonella* spp. in tap water samples. The new direct method SH-d, namely SmartHelix® Complex Samples DNA extraction kit developed in our laboratory, showed an efficient performance, therefore represents a good choice when rapid pathogen detection is needed for safe food and water assurance.

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PRIMERJAVA METOD OSAMITVE DNK ZA ODKRIVANJE BAKTERIJ IZ RODU *SALMONELLA* V PITNI VODI

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Povzetek: Bakterije iz rodu *Salmonella* predstavljajo splošen zdravstveni problem, povezan z okuženimi živili in fekalno onesnaženo vodo. Namen našega dela je bil s primerjavo različnih metod osamitve DNK ugotoviti učinkovitost in hitrost odkrivanja salmonel v vzorcih pitne vode, ki smo jim poprej dodali omenjene bakterije v različnih koncentracijah. Uporabili smo štiri različne metode osamitve DNK: komercialna kompleta foodproof™ Sample Preparation Kit I (Biotecon Diagnostics) in QIAamp® DNA Stool Mini Kit (Qiagen) za osamitev DNK po čeznočni obogatitvi ter kompleta Adiapure® Water DNA Extraction and Purification Kit (Adiagene) in SmartHelix® Complex Samples DNA Extraction Kit, ki smo ga nedavno razvili v našem laboratoriju, za direktno osamitev DNK. DNK smo uporabili za pomnoževanje v reakciji PCR s parom začetnih oligonukleotidov, ki je specifičen za rod *Salmonella*. Komplet SmartHelix se je za direktno odkrivanje DNK salmonel v vodi izkazal kot bolj učinkovit v primerjavi s kompletom Adiapure Water. Čeznočna obogatitev je izboljšala odkrivanje salmonel v vzorcih, ki smo jim dodali majhno koncentracijo bakterij, vendar pa je podaljšala čas analize. Rezultati kažejo na to, da bi morali na novo izdelani komplet SmartHelix upoštevati kot eno izmed možnih izbir, ko želimo v vzorcih vode patogene bakterije odkriti hitr,o z namenom preprečevanja izbruhov bolezni in zagotavljanja varne hrane.

Ključne besede: osamitev DNK; varna hrana; odkrivanje patogenov; PCR; *Salmonella*; voda