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FULL PAPER

Magnetic nanoparticle-enhanced PCR for the detection and identification of *Staphylococcus aureus* and *Salmonella enteritidis*

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SUMMARY

This paper evaluated magnetic nanoparticle-enhanced PCR for the detection and identification of *Staphylococcus aureus* and *Salmonella enteritidis*.
Two different types of magnetic nanoparticles designated MPIO (iron concentration 2.5 mg/ml, size 1 µm) and NP (iron concentration 8.7 mg/ml, size 60 nm), both conjugated with *S. aureus* or *S. enteritidis* antibodies were evaluated as an enrichment procedure for PCR-detection of the pathogens in Trypticase Soy Broth, milk, blood and meat broth. Bacterial suspensions ($1.5x10^8$ cfu/ml) were prepared and serial diluted 10^{-1}. The MPIO and NP nanoparticles were added, followed by incubation for 1 hour at room temperature, magnetic separation of the pellet, DNA extraction and PCR, targeting the *fem*A and *inv*A sequences. The nanoparticle-free and the NP-supplemented dilutions were positive down to the $1.5x10^2$ cfu/ml concentration for both bacteria. The MPIO-supplemented dilutions were positive down to approx. $2x10^0$ cfu/ml concentration, respectively. Bacteria-free TSB was negative by PCR.
MPIO nanoparticles (size 1 µm) enhanced the detection of *S. aureus* and *S. enteritidis* by PCR, whilst NP nanoparticles (size 60 nm) did not, thus indicating that the size of the magnetic nanoparticles play a significant role in the enrichment procedure.

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INTRODUCTION

Food-borne illness poses serious public health problems, responsible for up to 76 million cases every year, of which 325,000 cases may require hospital admission, as well as 5,000 deaths (http://www.cdc.gov/foodsafety/outbreaks/, last accessed 16 October 2016). According to the European Food Safety Authority (EFSA), bacteria such as *Salmonella enteritidis*, *Escherichia coli* serotype O157:H7 and *Listeria monocytogenes* are considered the most important pathogens, due to either the number of recorded incidents, or the infection severity (Anonymous, 2006), while viral agents are less common.
Rapid and accurate detection, and in certain circumstances enumeration of the bacterial load, are crucial, both in the clinical setting and for food safety and public health (Rossmanith and Wagner, 2011). Many culture-based tests currently in use require a high concentration of bacterial cells, typically from 10^6-10^7 cfu/ml of specimen, and rely on enrichment procedures to achieve this concentration (Chiu, 2014). This, however, may result in a turnaround time of several days. In the meantime the patient may deteriorate, or food products may become obsolete. Immunologic methods, such as those based on the enzyme-linked immunosorbent assay (ELISA), may offer faster turnaround times, but those too rely on time-consuming enrichment steps, as their detection limit is 10^5 to 10^7 cfu/ml. Molecular techniques offer fast alternatives, but they do not always ensure true viability of the microorganism, since they detect DNA and not living bacterial cells.
Therefore, the development of simplified, cost-effective and accurate procedures for pathogen detection, especially regarding the preliminary crucial enrichment step, is important in order to increase food safety and improve society's quality of life. In particular, a simple rapid and sensitive detection of trace amounts of bacterial pathogens is critical for minimizing or eliminating possible infections.

Key words:
Magnetic nanoparticles, Foodborne pathogens, PCR, Staphylococcus aureus, Salmonella enteritidis.

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Magnetic nanoparticles have recently been used in the separation of pathogenic bacteria from food, clinical or environmental samples (Kloepfer *et al.*, 2003; Olsvik *et al.*, 1994; Chu *et al.*, 2013; Duan *et al.*, 2012; Laube *et al.*, 2014), in order to detect pathogens such as *Salmonella* or *Staphylococcus aureus*. They can be prepared in the form of single domain or superparamagnetic iron oxides, e.g. magnetite (Fe_3O_4) or maghemite (γ-Fe_2O_3). When dispersed in a suitable solvent, they form a magnetic fluid. Separation of the particles from this magnetic fluid requires an external magnetic field to be used. The magnetic particles together with the surrounding solvent molecules are drawn towards the magnetic poles, as if the whole liquid were magnetic, hence its name. Due to relaxation processes, these particles lose their magnetic moment when the external magnetic field is removed. The ability to couple covalently with proteins, enzymes, antibodies, and other ligands makes magnetic nanoparticles suitable for direct use in bioassays or as affinity ligands for the capture of target molecules and cells (Varshney *et al.*, 2003). They can be coated with antibodies targeted to surface proteins or antigens on the microorganism, leading to the formation of magnetically labelled cells which could be easily separated from the specimen matrix. In comparison with other techniques, the magnetic separation offers several advantages (Safarík *et al.*, 1999) as it allows rapid simple separation and enrichment of target cells from crude samples (blood, culture media or food) (Safarik *et al.*, 2012).

In the current study, a PCR-based detection method, with the addition of an enrichment procedure based on magnetic particle-antibody conjugates was evaluated for the detection of *Salmonella enteritidis* (*Salmonella enterica* serotype *enteritidis*) and *Staphylococcus aureus* from different artificially spiked food sources and clinical specimens.

MATERIALS AND METHODS

The following nanoparticles were prepared:

a) iron concentration 8.7 mg/ ml and size 60 nm, designated NP;
b) iron concentration 2.5 mg/ml, $4x10^{10}$ particles per ml, size 1 μm, designated MPIO.

Both particles were made of iron oxide cores coated with dextran, or polystyrene, respectively. Both NP and MPIO were conjugated with species specific antibodies to *Staphylococcus aureus* (*S. aureus* Antibody, ThermoFisher Scientific, Paisley PA4 9RF, UK) and *Salmonella enteritidis* (BacTrace Anti-Salmonella, CSA-1 Antibody, KPL, Maryland, USA). Nanoparticles were suspended in 1x PBS, pH 7.3 prior to addition in the clinical specimen. Only a single master solution was prepared for each bead type/antibody type combination (NP/Staphylococcus, NP/Salmonella, MPIO/Staphylococcus, MPIO/Salmonella). These four master solutions were used throughout the study for all experiments.

The strains *S. aureus* ATCC 29213 and *Salmonella enteritidis* 35135 (clinical isolate from a positive blood culture) were used throughout the study. Bacterial suspensions were prepared in Tryptone Soy Broth (TSB) to a 0.5 McFarland standard turbidity equivalent (approx. $1.5x10^8$ cfu/ml), and serial tenfold dilutions were prepared in 5 ml final volume, to a final concentration of 2 cfu/mL in the following matrices:

a) in TSB (for all bacteria);
b) in whole blood from healthy volunteers collected in EDTA supplemented tubes (for *S. aureus*);
c) in milk (for *S. aureus*);
d) in meat broth (for *S. enteritidis*).

In that respect, five series of dilutions were prepared, two in TSB (one containing *S. aureus* and one containing *S. enteritidis*), one in blood (containing *S. aureus*), one in milk (containing *S. aureus*) and one in meat broth (containing *S. enteritidis*).

Each series of serial dilution was separated into three parts. The first part contained solely the bacterial suspension, whilst 30 μl of MPIO and 30 μl of NP coupled with their respective antibodies (MPIO-Staph, MPIO-Sal, NP-Staph and NP-Sal) were added in the second and the third parts. In that respect, the second and the third parts of the TSB-*S. aureus* suspension were mixed with the MPIO-Staph and the NP-Staph, respectively. The same was applied to the TSB-Salmonella suspensions (mixed with the MPIO-Sal and NP-Sal), the blood-*S. aureus* and milk-*S. aureus* suspensions (mixed with the MPIO-Staph and NP-Staph) and the broth-Salmonella suspensions (mixed with the MPIO-Sal and NP-Sal).

From all suspensions (with and without the nanoparticles) 10 μl were used for quantitative cultures on 5% sheep blood agar plates (Bioprepare, 16547, Gerakas, Greece), incubated for 24h in an 37°C, to ensure correct bacterial suspensions.

The first part of the suspension was centrifuged at 12.000 x g and the pellet was used for DNA extraction. The second and third parts of all dilution series (those with the MPIO and NP magnetic particles) were incubated for 1 hour at room temperature (allowing coupling of the antibody-conjugated nanoparticles with the bacteria). Magnetic separation of the magnetic pellet (nanoparticles+antibodies+bacteria) was performed using a DynaMag-15 magnet (Life Technologies, ThermoFisher Scientific, Paisley PA4 9RF, UK). The pellet was re-suspended in 200 μl of distilled H_2O, washed twice, and used for DNA extraction.

DNA extraction from all TSB, milk and meat broth suspensions was performed using the NucleoSpin Tissue kit (Macherey-Nagel, 52355, Düren, Germany) according to the manufacturer's instructions, with an additional modification step of an overnight incubation with the Lysis Buffer and the Proteinase K at 65° C. DNA extraction from the EDTA-blood suspensions was performed using the QIAamp Blood DNA MiniKit (Qiagen, 40724, Hilden, Germany) according to the manufacturer's instructions.

PCR, targeting a 686 bp fragment of the *fem*A gene specific for *S. aureus* and a 560 bp fragment of the *inv*A gene, specific for *S. enteritidis*, was performed as described previously (Vannuffel *et al.*, 1995; Suo *et al.*, 2010). All experiments were performed in triplicate. PCR products were separated in a 2% agarose gel, stained with ethidium bromide (0.5 μg/ml), and documented under UV illumination.

Specificity of the enrichment procedure was tested using a mixture of the two previously described pathogens (*S. aureus* and *S. enteritidis*), together with *Escherichia coli* ATCC 25923 and a *Listeria monocytogenes* clinical isolate from a positive blood culture. Four different suspensions of 0.5 McFarland turbidity concentrations were prepared, each containing one of the four bacteria, and then a master suspension was prepared by adding an equal amount

of each of the four single suspensions. Serial dilutions of the master suspension were prepared and divided into two parts; 30 μl of MPIO-staph were added in the first part, whilst 30 μl of MPIO-Sal were added in the second part. The magnetic enrichment procedure and the PCR were performed as described previously. Both *S. aureus* and *Salmonella* PCR procedures were performed on all parts and dilutions.

Specificity of the PCR was tested using DNA extracted from all four of the previously described pathogens.

Multiplexing of the procedure was performed on TSB and meat broth spiked with the mixed bacterial suspensions used during the specificity testing. Both MPIO-Staph and MPIO-Sal particles were used at the same time, followed by DNA extraction and PCR detection of *S. aureus* and *S. enteritidis*.

RESULTS

Tables 1 and *2* show a comparative summary of the results of *S. aureus* and *S. enteritidis* PCR, respectively, on the unsupplemented media, as compared to the NP and MPIO-supplemented ones. More specifically, *S. aureus* PCR on unsupplemented TSB, blood and milk was positive in all dilutions between $1.5x10^8$ down to the $1.5x10^2$ cfu/ml, no media-specific differences were detected and all results were the same in all three media. The same was evident using the NP-supplemented TSB, blood and milk. In contrast, the MPIO-supplemented media performed better and the PCR amplified the target sequences two dilutions lower (down to the $2x10^0$ cfu/mL dilution).

Regarding *S. enteritidis*, enhancement of the PCR results was also observed on MPIO-supplemented media (TSB and meat broth) as compared to the unsupplemented and the NP-supplemented media.

The magnetic enrichment procedure specificity revealed that the procedure was highly specific, as the *S. aureus*-specific PCR was positive only after the MPIO-Staph enrichment and not the MPIO-Sal enrichment. The same was evident with the Salmonella-specific PCR and the MPIO-Sal enrichment procedure. In addition, no loss of the PCR analytical sensitivity was detected, as the PCR detection of both pathogens was achieved down to the $2x10^0$ cfu/mL dilution.

Multiplexing of the procedure using the four-pathogen mixture resulted in similar detection limits (data not shown). PCR on MPIO-supplemented media resulted in one log lower results than PCR on NP-supplemented media and no interference was recorded between the species. The whole procedure was tested in triplicate and the results were identical, indicating good reproducibility.

DISCUSSION

The present study was focused on the evaluation of an enrichment procedure based on immune-magnetic sepa-

Table 1 - *Results of PCR of S. aureus in unsupplemented, MPIO-supplemented and NP-supplemented matrices (TSB, milk and blood).*

Serial dilution no.	*Bacterial concentration (cfu/ml)*	*Unsupplemented matrices*	*MPIO-supplemented matrices*	*NP-supplemented matrices*
1	1.5×10^8	Positive	Positive	Positive
2	1.5×10^7	Positive	Positive	Positive
3	1.5 x 106	Positive	Positive	Positive
4	1.5×10^5	Positive	Positive	Positive
5	1.5×10^4	Positive	Positive	Positive
6	1.5×10^3	Positive	Positive	Positive
7	1.5×10^2	Positive	Positive	Positive
8	1.5×10^1	Negative	Positive	Negative
9	2×10^0	Negative	Positive	Negative

Table 2 - *Results of PCR of Salmonella enteritidis in in unsupplemented, MPIO-supplemented and NP-supplemented matrices (TSB and meat broth).*

Serial dilution no.	*Bacterial concentration (cfu/ml)*	*Unsupplemented matrices*	*MPIO-supplemented matrices*	*NP-supplemented matrices*
1	1.5×10^8	Positive	Positive	Positive
2	1.5×10^7	Positive	Positive	Positive
3	1.5 x 106	Positive	Positive	Positive
4	1.5×10^5	Positive	Positive	Positive
5	1.5×10^4	Positive	Positive	Positive
6	1.5×10^3	Positive	Positive	Positive
7	1.5×10^2	Positive	Positive	Positive
8	1.5×10^1	Negative	Positive	Negative
9	2×10^0	Negative	Positive	Negative

ration of magnetic nanoparticles for molecular detection of *S. aureus* and *S. enteritidis* in clinical and food specimens. The ability of the magnetic nanoparticles to couple covalently with various ligands (Gao *et al*., 2009) makes them suitable for direct use in bioassays or as affinity ligands for the capture of target molecules and cells. In the present study, the magnetic nanoparticles were coated with antibodies targeted to surface-specific antigens of *S. aureus* or *S. enteritidis*, leading to the formation of magnetically labelled cells which could easily be separated from the system using an appropriate magnetic separator.

Among the two nanoparticle forms under evaluation in the present study, MPIO resulted in enhancement of the final result in both PCR assays, as compared to the standard centrifugation process, whilst NP did not. The larger size of the MPIO nanoparticles (1 μm, as compared to the 60 nm of the NP particles) seemed to allow better coupling of the target molecule and faster magnetic separation, thus allowing enhancement of the procedure. In addition, although we did not test for binding differences of the antibodies between the two types of magnetic beads, given the differences in the size (60 nm and 1 μm), the better results of the larger MPIO beads should have been due to the larger amount of antibody coupling in each bead.

The optimized MPIO-enrichment-PCR-detection method showed high specificity and sensitivity, with a detection limit at least one log lower than the PCR procedure with the standard centrifugation method. In addition, it proved a simple technique for the detection and identification of foodborne pathogens in a modestly equipped laboratory, requiring only a magnetic separator and a conventional PCR cycler.

In recent years, much effort has been directed to the study and development of rapid detection methods for foodborne pathogens, as an alternative methodology to standard culture oriented procedures. Immunological assays, immune-magnetic separation, and biosensors technology are available for the identification of bacteria (Yu *et al*., 2000) as standalone novel techniques, replacing conventional or molecular procedures, but requiring major infrastructure and/or protocol changes. By contrast, the present technique is a simple enrichment procedure that improves previously described PCR assays and can easily be implemented in laboratories without major changes in their protocols or infrastructure.

Similar techniques are scarce in the literature so that direct comparison of the results of the present study was limited. A magnetic immune-sensor with electrochemical readout was reported for the detection of *Salmonella* spp. in milk (Liebana *et al*., 2009). The bacteria were captured and pre-concentrated from milk samples with magnetic particles through an immunological reaction. The enrichment procedure resulted in a detection limit of 1 cfu in 25 g of milk, but it also had a turnaround time of six hours to complete. By contrast, the procedure described here was completed after only one hour. In another study (Maa *et al.*, 2014), the combination of magnetic particles with a PCR-based methodology resulted in rapid and simultaneous detection of *Salmonella*, *Shigella* and *S. aureus* in fresh pork at a detection limit of 10 cfu/g, within 6 h. The detection limits of these two studies are comparable to the values reported here. In addition, we showed that the same detection limit was recorded for the two species, thus indicating that the parameter of the species details (Gram stain, cell differences between bacteria and cocci) was not an important factor.

The major advantage of the present study, however, is the turnaround time, which is faster, thus allowing rapid detection of the pathogen.

A limitation of the study is that we used only a single isolate of each of the two species under investigation to establish the proof of principle of our procedure. In order for the method to find its way to industry (possibly as a ready-to-use kit) more experiments are needed, using more species and isolates. Nevertheless this was not the aim of the present study.

In conclusion, the present study showed that the optimized MPIO-enrichment-PCR-detection method resulted in high specificity and sensitivity, with a detection level one log more sensitive than centrifugation-based PCR, thus allowing rapid detection of pathogens without major infrastructure changes in modestly equipped laboratories.

Conflicts of interest

The authors declare no conflicts of interest

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