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ABSTRACT

The high incidence of foodborne diseases caused by pathogenic bacteria raises concerns worldwide and imposes considerable public healthcare challenges. This is especially observed with dormant spores of *Bacilli,* which can often survive treatments used by the food industry to kill growing bacteria. The early and rapid detection of bacterial spores is essential to ensure food safety. Commercial availability of such a test will present a high potential for food sector. We present a point-of-need colorimetric assay for detection of *Bacillus cytotoxicus* spores in food. The detection principle is based on spore-enhanced peroxidase-like catalytic activity of gold nanoparticles. The sensing platform consists of a microtube containing gold nanoparticles (AuNPs), and magnetic particles (MPs), both conjugated with specific aptamer BAS6R that recognize *B. cytotoxicus* spores. Upon the addition of the sample, spores were determined as present by the enhanced color change of the solution, due to the oxidation of tetramethylbenidine (TMB) with H_2O_2. The assay was evaluated by the naked eye (on/off) and quantitatively with use of a spectrophotometer. BAS6R@AuNPs aptasensor coupled to BAS6R@MPs proved to be highly sensitive, achieving the naked-eye limit of detection as low as 10^2 cfu/mL in water and milk, and 10^4 cfu/mL in mashed potatoes. Moreover, discrimination between spores of *B. cytotoxicus* and *B. subtilis* as well as bacterial vegetative cells was achieved in contaminated food samples, providing a good selectivity. This work provides a promising proof of concept for the development of instrument-free, low-cost and rapid assay for *Bacillus cytotoxicus* spore detection, which is able to compete in sensitivity with conventional costly and time-consuming laboratory analyses.

1. Introduction

Bacillus cytotoxicus is a recently described bacterial pathogen that causes severe foodborne illness [1]. It belongs to *Bacillus cereus* group also known as a *B. cereus sensu lato*, which is comprised of closely related species: *B. cereus sensu stricto*, *B. anthracis*, *B. thuringiensis*, *B. cytotoxicus*, *B. pseudomycoides*, *B. mycoides*, *B. weihenstephanensis*, *B. toyonensis*, *B. wiedmannii*, and at least nine new strains isolated from marine sediments [2]. *B. cytotoxicus* is the only member of the *B. cereus* group with the ability to grow at high

Abbreviations: MPs, Magnetic particles; AuNPs, Gold nanoparicles; TMB, Tetramethylbenidine; cfu, Colony forming unit; SDS, Sodium dodecyl sulfate; BHI, Brain Heart Infusion broth.

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temperatures (up to 52 °C) and to produce a diarrhoeic enterotoxin, named cytotoxin K-1 [3–5].

The diarrhoeal illness caused by *B. cytotoxicus* is thought to be a toxicoinfection due to vegetative cells, which are ingested as spores or viable cells that then produce cytotoxins in the gastrointestinal tract [6,7]. The incubation time is usually around of 8–16 h and the duration of illness is usually 12–24 h. Typical syndromes include abdominal pain, watery diarrhoea and occasionally nausea and emesis, although more serious and even lethal cases have occurred. *B. cytotoxicus* was first isolated in association with fatal cases of diarrheal disease in France in 1998 [3]. This outbreak reported 44 cases of illness, including six cases of bloody diarrhoea and three deaths from necrotic enteritis. The strain involved, called NVH 391-98, was isolated from a vegetable puree sample and has been used in this study. *B. cytotoxicus* was linked to other outbreaks, all involving plant-based food matrices such as dehydrated vegetables, cereals and spices [8]. Recently, the UK Food Standards Agency detected *B. cytotoxicus* in products containing insect flour [9], which raises concerns regarding the increasing popularity of insect related food. Due to the nature and extent of cytotoxicity in *B. cytotoxicus,* further monitoring is required in a wider range of foodstuffs as well as in the environments it may arise.

Traditional microbiological methods used to detect bacterial vegetative cells or spores are time consuming and show limited efficiency and sensitivity [10–12]. Official methods to enumerate and detect presumptive B. cereus *sensu lato* cells are ISO 7932 (for direct plate counting) and ISO 21871:2006 (for detection and counting of low numbers through the most probable number). The Codex Alimentarius Commission of the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) Codex standard the maximum acceptable number of *B. cereus* is 10^2 CFU/g in infant formulas [13]. The EU legislation imposes systematic checking for *B. cereus* for powdered formulas intended for infants under 6 months [14]. Nevertheless, a reliable methodology to detect low levels of *B. cereus* cells/spores ($<10^2$ CFU/g) in a timely manner is still missing. The majority of foodborne outbreaks due to *B. cereus* cells/spores in adults have been caused by more than 10^5 CFU/g of food product [13]. Detection of germinating spores usually requires an additional step to germinate spores, as diagnostic protocols are mostly adapted for vegetative cells [2]. In addition to classical methods, molecular techniques are used to discriminate the phylogenetic group of *B. cereus* strains (Group VII for *B. cytotoxicus*) or to search for the *cytK*-1 gene [15,16]. However, molecular methods are not adapted for screening food contamination by spores, as it is difficult to extract DNA from spores that are highly resistant against chemical or enzymatic lysis.

In response to the need for an analytical tool that allows for convenient and easy-to-perform spore detection, in this work, we propose a reliable colorimetric assay exploring the peroxidase-like activity of gold nanoparticles (AuNPs) [17]. To provide a recognition of spores, a specific aptamer BAS-6R was grafted to AuNPs. When attached to spores, BAS6R@AuNPs complexes exhibited higher nanozyme color reaction of 3,3′,5,5′-tetramethylbenzidine (TMB) then BAS6R@AuNPs alone. This phenomenon was used for development of aptamer-based biosensors for detection of *B. cytotoxicus* spores. Furthermore, to improve the sensitivity of detection, magnetic particles modified with aptamer (BAS6R@MPs) were used to concentrate spores. Finally, the feasibility of B. cytotoxicus spores detection in food samples was shown in spiked milk and mashed potato samples. This simple sensing platform provides a new and affordable method to detect spores of *B. cytotoxicus* and may have value in food microbiological surveillance.

2. Materials and methods

2.1. Reagents

Gold nanoparticles (10 nm), H_2O_2, inosine, Tris-HCl, EDTA, NaCl were purchased from Sigma Aldrich (Saint Quentin Fallavier, France). Sodium dodecyl sulfate 4X sol 20% (SDS) was purchased from Euromedex (Souffelweyersheim, France). Gold nanoparticles (20 nm), Dynabeads M − 280 Streptavidin magnetic particles (MPs) and 1-Step Turbo TMB-ELISA were purchased from Fisher Scientific (Illkrich, France). Phosphate buffer saline (PBS) was purchased from VWR Chemicals (Rosny-sur-Bois, France). Binding and washing buffer (B&W 2X) was 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 2 M NaCl. Aptamers were obtained from Eurofins Genomics France. Milk powder and powdered mashed potatoes were purchased in the local supermarket.

2.2. Bacterial strains and sporulation

The strains used in this study are listed in Table 1. Bacterial vegetative cells were grown in the Brain Heart Infusion broth (BHI: 7.5 g/L calf brain infusion, 10 g/L beef heart infusion, 10 g/L peptone mixture, 2 g/L glucose, 5 g/L NaCl, 2.5 g/L Na_2HPO_4) and Brain Heart Infusion Agar (BHIA) at 37 °C while sporulation was performed in the sporulation-specific medium HCT (136 g/L KH_2PO_4, 12.3 g/L $MgSO_4$, 0.169 g/L, 1.44 g/L Zn_2SO_4, 11 g/L ferric ammonium citrate, 14.7 g/L $CaCl_2$, 5 g/L tryptone, and 2 g/L bacto casamino

Table 1
Bacterial strains used in this study.

Microorganisms	Collection code
B. cereus ssp. *cytotoxicus*	NVH 391-98[A]
*Escherichia coli*K12	ATCC 14948[B]
Bacillus cereus	ATCC 14579[B]
Bacillus subtilis	NDmed[C]
Staphylococcus aureus	RN7044[D]

Source/Reference: [A], [3]. [B], American Type Culture Collection (Manassas, VA, USA) [C], [20]; [D], [21].

acid) at 30 °C with shaking (200 rpm). For sporulation, a single bacterial colony was transferred into flasks containing 20 mL of BHI broth and incubated at 37 °C overnight under shaking (200 rpm). Sporulation was obtained by diluting this preculture in the HCT medium as previously described [18]. The final solutions containing spores were washed to eliminate the remaining medium and debris of bacterial cells using a protocol described by Ref. [19] with some modifications. Briefly, the spores were collected by centrifugation (5 min at 5000 rpm) and washed one time with sterile water, and a further three times with 50% ethanol aqueous solution, followed by an overnight incubation in 4 °C water. Finally, spores were washed once more, to eliminate cell lysis residues. The solutions were stored at 4 °C. A plate count was made to evaluate the spore concentration in solutions. Spore germination was performed by adding a high inosine concentration of 5 mM to the solution. The germination was measured by following the optical density at 600 nm (OD_{600}) of the cultures.

2.3. Aptasensor construction

The aptamer-gold nanoparticle complex (BAS6R@AuNPs) was prepared by formation of Au–S bonds between thiolated BAS-R6 and AuNPs. Briefly, 200 µl of citrate stabilized AuNPs (0.1 g/L) were mixed with 32 µL of thiol-BAS-6R aptamer (10 µM). The mixture was incubated at room temperature overnight under stirring. The obtained aptasensor was stored at 4 °C and used within a week. The sequences of BAS-6R and control aptamers are given in Table 2.

2.4. Colorimetric bioassay

Following a previously published protocol with some modifications [22], 200 µl of a sample were incubated with 58 µl of BAS6R@AuNPs for 20 min at room temperature with shaking. The sample was then centrifuged at 13,000 rpm for 3 min and 120 µl of the supernatant was removed to get rid of the unbound BAS6R@AuNPs. 250 µl of TMB was then added to the samples, followed by a 20 min incubation at room temperature with shaking in the dark, before the addition of 13 µl of H_2O_2 (30%) and 5 µl of SDS (20%). After 15 min of incubation, 200 µl of each sample was moved into a 96 well cell culture plate (Greiner bio-one Gmbh, Kremsmünster, Austria). Absorption at 672 nm was recorded using the Infinite 200 PRO microplate reader (TECAN, Salzburg, Austria).

2.5. Magnetic beads functionalization and spores concentration

Magnetic particles (MPs) carrying streptavidin were functionalized with biotinylated BAS-6R aptamer. For this, 250 µl of the magnetic bead solution (~6-7 x 10^8 beads/mL) was firstly washed 3 times with B&W buffer 1X using an external magnet to discharge the supernatant. The washed beads were then resuspended in 250 µl of B&W buffer 2X and mixed with 250 µl of biotin-BAS-6R (0.1 µM). The aptamer-magnetic beads complex BAS6R@MP was obtained upon 15 min incubation at room temperature under stirring. The aptamer coated beads were then 3 times washed with B&W buffer 1X and finally resuspended in 250 µL of B&W buffer.

To concentrate spores, BAS6R@MPs (from 5 to 100 µL) was added to 1 mL of a spore suspension. The mixture was incubated at room temperature under stirring for 30 min to allow spore capturing. The formed BAS6R@MP-spore complexes were collected using the magnet and resuspended in 200 µL of water for the colorimetric test. The spore capturing efficiency was calculated using the following formula:

$$\text{Capturing efficiency } (\%) = (\text{Nt-Ne})/\text{Nt} \times 100\%$$

where N_t and N_e are the number of spores in a sample and the number of uncaptured spores in the sample, respectively. The spore number of each sample was enumerated using the standard colony counting method after plating 100 µL of the sample on BHIA plate.

2.6. Colorimetric bioassay with magnetic concentration

The colorimetric bioassay coupled with the magnetic concentration was performed by mixing 1 mL of the spore suspension with 20 µl of BAS6R@MP and 58 µl of BAS6R@AuNPs and incubated for 30 min at room temperature with shaking. A magnet was then used to collect the magnetic beads and 900 µl of supernatant was removed from the mixture. Subsequently TMB, H_2O_2 and SDS were added to initiate reaction as described in 2.4.

Table 2
DNA aptamers used in this study.

DNA sequences	
BAS-6R	5′ - ATCCGTCACACCTGCTCTGCACGGGCTCAGTTTGGCTTTGTATCCTAAGAGGATGGTGTTGGCTCCCGTAT - 3′
S14	5′ - AGCAGCACAGAGGTCAGATGATATGTTTACGCCAGTGGTATTATTGGGGTTGATATGTCACCTATGCGTGCTACCGTGAAAAA - 3′
Campy	5′- GGGAGAGGCAGATGGAATTGGTGGTGTAGGGGTAAAATCCGTAGA - 3′

2.7. Microscopy

Optical microscopy was performed to visualize the spores binding to BAS6R@MPs. Observations were performed using an AxioObserver Z1 Zeiss inverted microscope (Zeiss, Oberkohen, Germany) with a 100x oil immersion objective lens. Images were processed using the ZEN software package.

Transmission electronic microscopy (TEM) analysis was performed to visualize spores binding to BAS6R@AuNPs. Five μL of *B. cereus* spore solution (10^6 cfu/mL) was added to 50 μL of BAS6R@AuNPs, and incubated at room temperature for 30 min. Two μl of the solution was collected onto 200-mesh copper grids, and visualized using a Hitachi HT7700 electron microscope operated at 80 kV (Milexia, France). Digital images were acquired using a charge-coupled device camera system (AMT).

2.8. Spectrophotometry

The specificity of aptamer binding to spores was evaluated using the UV–Vis spectrophotometer Biochrom Libra S22 (Biochrom Ltd., Cambridge, UK). Spores (10^4 cfu/mL) were mixed with 5 μM of aptamer in 10 mM Tris-HCl, pH 8 and incubated at room temperature for 4 h with shaking. Following this, the spores were centrifuged at 3000 rpm for 5 min. The supernatant was then removed and the pellet washed 3 times before being resuspended in 10 mM Tris-HCl, pH 8, for analysis. In control experiments, BAS-6R was replaced by the S14 DNA oligomer, and *B. cytotoxicus* spores were replaced with spores of *B. subtilis*.

2.9. Food matrices preparation

Milk 1% was prepared dissolving 1 g of milk powder in 100 mL of water. Mashed potatoes were prepared by mixing 1 g of the powdered mashed potatoes in 100 mL of water and stirring the solution for 30 min. Food matrices were prepared daily and spiked with different concentration of spores without any addition food processing to facilitate detection.

3. Results and discussion

3.1. Aptamer binding to B. cytotoxicus spores

The BAS-6R aptamer used in this study was originally selected as a *B. anthracis* spore-binding DNA aptamer beacon [23] but was later shown to also bind spores of other *B. cereus* strains [24]. To verify whether BAS-6R specifically recognizes *B. cytotoxicus* spores, the spores were incubated with the aptamer and characterized by UV–Vis spectroscopy. Nucleic acids show a strong UV absorbance around 260 nm arising from π–π* transitions in the aromatic purine (A, G) and pyrimidines (U, C, T) nucleotide basis (Fig. 1). In

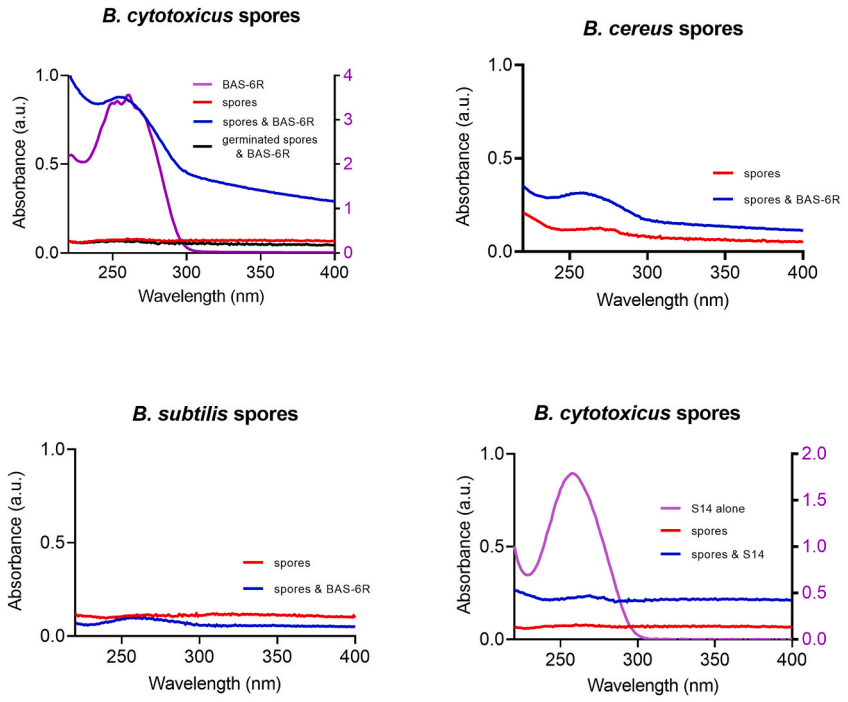

Fig. 1. Spectrophotometric detection of BAS-6R aptamer binding to (a) *B. cytotoxicus* spores, (b) *B. cereus* spores and (c) *B. subtilis* spores. (d) Non-specific aptamer S14 failed to bind to *B. cytotoxicus* spores.

contrast, spores have no absorbance in this wavelength region. When *B. cytotoxicus* spores (10^4 cfu/mL) were incubated with BAS-6R and then thoroughly washed, a peak at 260 nm was observed indicating the binding was successful (Fig. 1a). A similar peak around 260 nm was also observed when BAS-R6 bound to spores of *B. cereus* ATCC 14579 (Fig. 1b). This was expected because spores of different bacterial strains within *B. cereus* group show high similarities [2,25]. In contrast, BAS-6R showed no affinity to bind germinated *B. cytotoxicus* spores (Fig. 1a), nor *Bacillus subtilis* spores (Fig. 1c). Taking into account that the outmost layer of *B. cereus* spores is the exosporium, a layer that is absent in *B. subtilis* [26], our results suggest that BAS-6R binds to an epitope located at the exosporium.

In addition, *B. cytotoxicus* spores incubated with a non-related DNA aptamer (S14) showed no significant peak intensity increase at 260 nm (Fig. 1d). Thus, BAS-6R aptamer seems to specifically recognize spores of *B. cytotoxicus*. Moreover, BAS-6R does not recognize germinated spores that have lost the extreme resistance of dormant spores and are relatively easy to kill by classical food treatments.

3.2. Feasibility of the spore detection by the aptasensor

The working principle of the label free colorimetric detection of *B. cytotoxicus* spores is illustrated in Fig. 2. BAS-6R aptamer was 5′-end chemically modified with thiol group and covalently grafted to gold nanoparticles. The aptamer and AuNPs bioconjugation was verified by the UV–Vis absorption spectra (Fig. 3a). The peak at 260 nm confirmed the binding. In the presence of *B. cytotoxicus* spores, BAS6R@AuNPs bound to their surface and concentrated on them. Then, the formed spores/BAS6R@AuNPs complexes were able to catalyze the H_2O_2-mediated oxidation of TMB and to induce a dramatic color change (Fig. 3b and c). In this assay, an inherent peroxidase-like activity of BAS6R@AuNPs to oxidase TMB in the presence of H_2O_2, generated a blue color with lower intensity, while the reaction between TMB/H_2O_2 and spores remained colorless (Fig. 3c). This result demonstrated that spores themselves had no peroxidase-like activity but promoted the formation of the blue products (namely, diamine/diimine charge transfer complex) in the catalytic oxidation of TMB. The phenomenon may be attributed to the enrichment of BAS6R@AuNPs on the spores' surface that increased the concentration of the enzyme-mimetic nanoparticles. TEM measurements confirmed the binding of BAS6R@AuNPs on spores (Fig. 4).

3.3. Detection performances of the aptasensor

To allow an effective and reliable naked-eye detection of *B. cytotoxicus* spores, the optimization of the experimental conditions to achieve reproducible formation of the blue TMB oxidation intermediate is crucial. To this regard, we first observed that anionic surfactant, SDS, stabilized the blue chromogen. Such a finding was previously described for chromogenic catalysis of TMB with horseradish peroxidase enzyme [27]. SDS improved the stability of the diamine/diimine charge transfer complex resulting in a more obvious blue color with the appearance of a strong absorption peak at 672 nm. Hence, the absorbance at 672 nm obtained following the addition of SDS was used as a signal value. We secondly optimized the time required to obtain a relative stable signal. After the addition of TMB, H_2O_2 and SDS, the absorbance at 672 nm increased rapidly and reached a maximum level at 15 min, after which it slowly decreased due to the partial sedimentation of the complex. The absorbance in the range of 15–20 min did not show a significant difference. Therefore, 15 min incubation was used for subsequent experiments. Third, the effect of temperature was assayed. There was no significant difference in signal intensity when the reaction was allowed at room temperature or at 37 °C, although the higher temperatures were expected to favor the interaction between spores and BAS6R@AuNPs. To simplify the procedure, room temperature was chosen for subsequent experiments.

The assay was performed in a polypropylene microtube, which was used as a reservoir and detection platform. For evaluation of analytical parameters of the aptasensor, water, milk and mashed potatoes were inoculated with different concentrations of

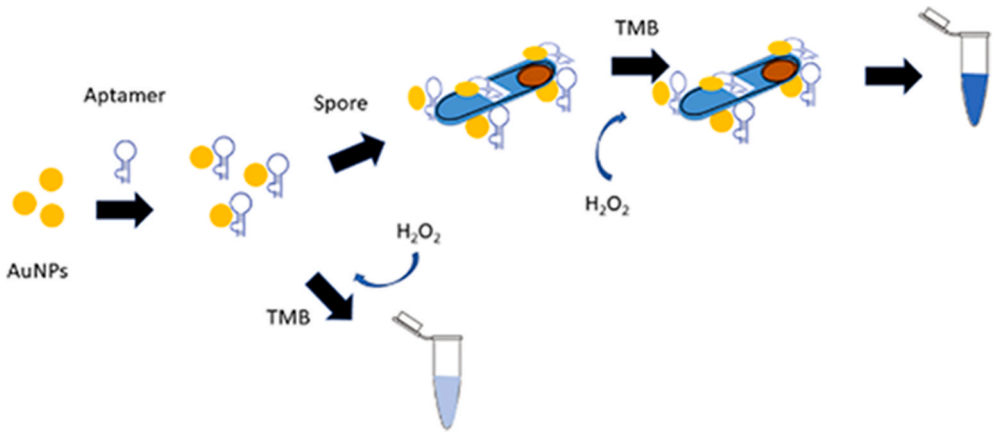

Fig. 2. Scheme of the colorimetric assay for detection of *B. cytotoxicus* spores based on spore-enhanced peroxidase-like catalytic activity of gold nanoparticles. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

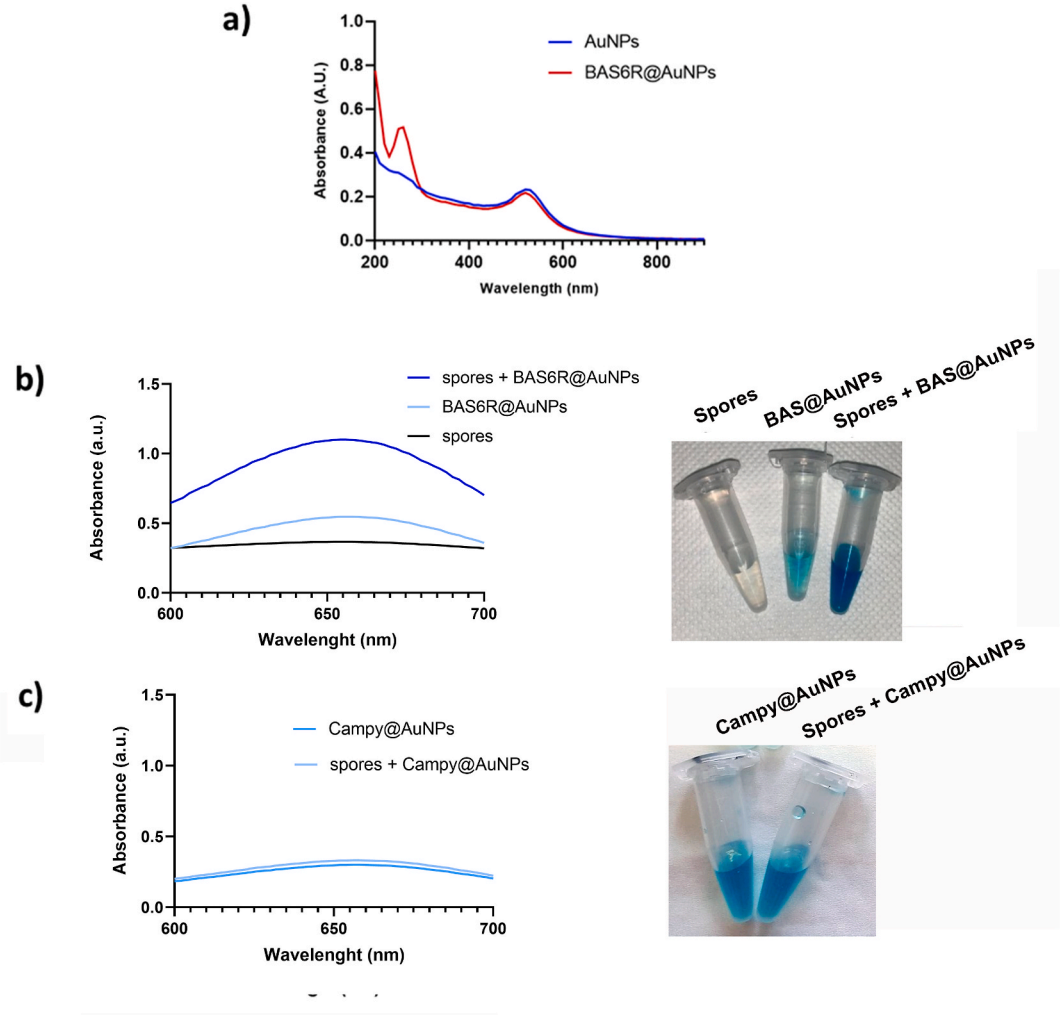

Fig. 3. (a) UV–Vis absorption spectra of bare and aptamer functionalized AuNPs, BAS6R@AuNPs. (b) UV–Vis absorption spectra of TMB solutions containing 10^8 cfu/mL of *B. cytotoxicus* spores, BAS6R@AuNPs (4×10^{10} nanoparticles) or their mixture and photographs to illustrate the color changes. (c) UV–Vis absorption spectra of TMB solutions containing Campy@AuNPs (4×10^{10} nanoparticles) and Campy@AuNPs (4×10^{10} nanoparticles) with *B. cytotoxicus* spores (10^8 cfu/mL). Photographs illustrate no color changes. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

B. cytotoxicus spores ranging from 10^3 cfu/mL to 10^8 cfu/mL. Fig. 5 displays absorbance at 627 nm taken from at least three independent tests per concentration. Absorbance was increased compared to corresponding controls for water and milk samples containing 10^7 and 10^8 cfu/mL of *B. cytotoxicus* spores and only for mashed potatoes samples inoculated with 10^8 cfu/mL of *B. cytotoxicus* spores. Such high concentrations are out of the clinically relevant range. We thus attempted to increase the assay sensitivity by coupling it to a magnetic concentration of spore.

3.4. Detection performances of the aptasensor combined with concentration

Since spores are micro-sized particles and multiple epitopes are present at their surface, many BAS-6R molecules may bind to the same spore. Consequently, spores can be captured in a sandwich between BAS6R@AuNPs and magnetic particles functionalized with BAS-6R aptamer (BAS6R@MPs), as illustrated in Fig. 6a. The sandwich detection format was expected not only to increase the sensitivity of detection but also to enable the development of a point-of-need assay, because the centrifugation step can be replaced by a bead collection step, by using an external magnetic field.

BAS-6R was 5′-end chemically modified with biotin group and attached to commercial streptavidin coated magnetic particles using the manufacturer protocol. The aptamer immobilization was confirmed by the UV–Vis absorption spectra that showed a DNA absorption peak at 260 nm (Fig. 6b). Then, BAS6R@MPs were admixed to a water solution containing 10^5 cfu/mL of *B. cytotoxicus* spores, washed and observed under an optical microscope. Bright monodisperse metallic beads of a spherical shape were observed before

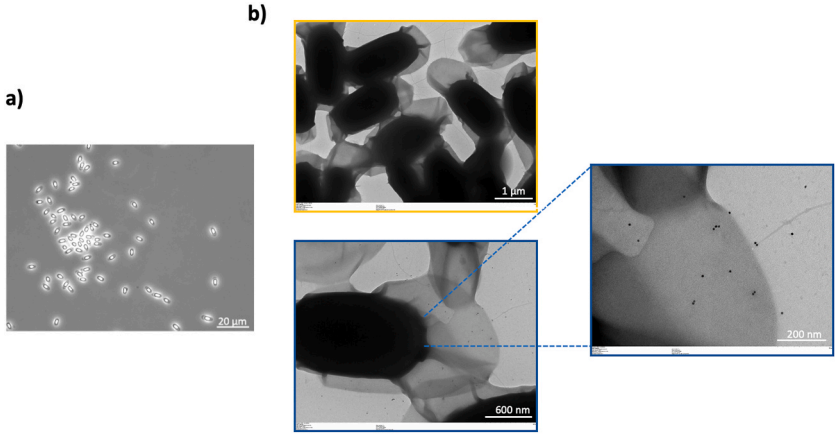

Fig. 4. (a) Optical microscopy image of *B. cereus* spores. (b) Transmission electronic microscopy (TEM) images reveal binding of BAS6R@AuNPs to spores.

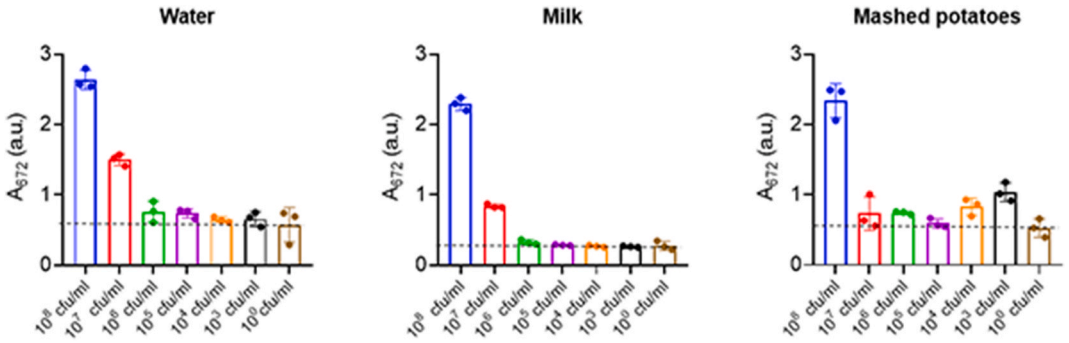

Fig. 5. Detection results for *B. cytotoxicus* spores in three different matrices using BAS6R@AuNPs aptasensor. Quantitative evaluation was performed by measuring absorption at 672 nm. Individual data points (n = 3 independent experiments) are shown; error bars represent means ± SD.

spore capturing, while beads were covered with spores after the incubation and washing steps. Fig. 6c shows that several spores can bind to a single MN and that bound spores are exposed to the solution and, thus, may capture BAS6R@AuNPs.

Next, we optimized the amount of BAS6R@MPs to obtain a sensitive spore detection. The tests were performed in water solutions containing 10^5 cfu/mL of *B. cytotoxicus* spores in the presence of the fixed concentration of BAS6R@AuNPs (~3.5×10^{10} particles) and different amount of functionalized magnetic beads ranging from 5 µg/mL to 100 µg/mL final concentration. Before the TMB oxidation, solutions in polypropylene microtubes were of yellow to brown color due to the colored beads (insert in Fig. 6d at T_0). After addition of TMB/H_2O_2/SDS, only the microtubes containing ≤20 µg/mL of BAS6R@MPs turned to an easy-to-observe blue color (insert in Fig. 6d at T_{15}). To facilitate the interpretation of the results, 20 µg/mL of BAS6R@MPs were used in subsequent tests. The capturing efficiency was estimated to 93 and 85% for 10^3 cfu/mL and 10^4 cfu/mL of *B. cytotoxicus* spores, respectively.

To demonstrate the applicability of the sandwich assay as a point-of-care diagnostic platform, we employed it for the detection of *B. cytotoxicus* spores in spiked water, milk and mashed potatoes. The spore concentration varied from 10^1 to 10^4 cfu/mL. BAS6R@AuNPs and BAS6R@MPs were added together to polypropylene microtubes to have no additional operating step compared to the assay without concentration. Blank solutions contained no spores. Fig. 7 shows that the sensing signal increased linearly with the increasing spore concentration. The naked eye detection was possible starting from 10^2 cfu/mL of *B. cytotoxicus* spores in water and milk and 10^4 cfu/mL in mashed potatoes. These experimental limits of detection (LOD) were measured based on a signal–noise ratio of 3 independent experiments (according to formula 3 s/d, where s is the standard deviation of a blank and d is a slope of the linear calibration graph), and had values lower than the allowable level of *B. cereus* cells set by the authorities in France. In addition, each test costed around €0.5 due to the small amount of reagents used. This cost is nearly ten order of magnitude less expensive then commercially available assay for foodborne pathogen detection.

3.5. Specificity of the aptasensor

To evaluate the specificity of the colorimetric aptasensor, we further cross-tested several bacterial strains including *B. subtilis* (cells and spores), *B. cytotoxicus* cells, *Staphylococcus aureus* and *Escherichia coli*. As shown in Fig. 8, no significant blue color was observed in the presence of control strains indicating a good compatibility of the assay with other bacteria that can be found in milk or vegetables.

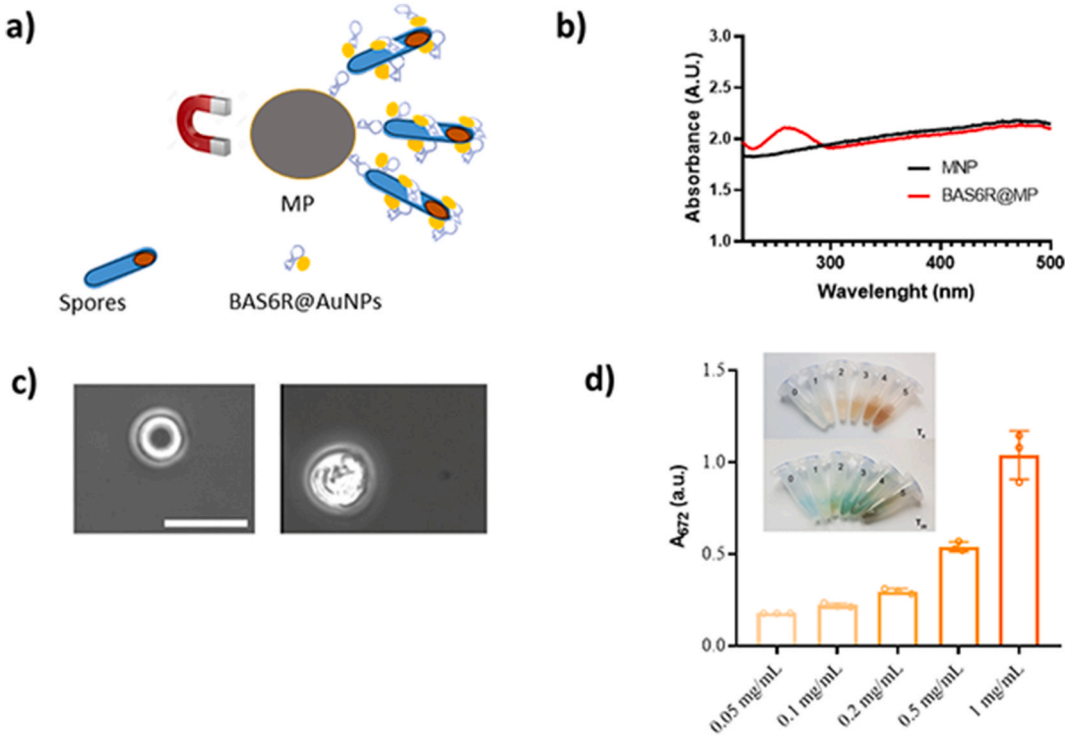

Fig. 6. (a) Schematic illustration of *B. cytotoxicus* spores forming into a 'sandwich' between magnetic particles functionalized with BAS-6R (BAS6R@MN) and BAS6R@AuNPs. (b) Absorption spectra of bare magnetic particles and BAS-6R aptamer functionalized. (c) Phase contrast transmission micrographs of BAS6R@MNs before (left panel) and after *B. cytotoxicus* spores capturing (right panel). Scale bar, 10 μm stains for both images. (d) Representative images from analytical performances of aptasensor coupled with various amounts of BAS6R@MNs to detect 10^5 cfu/mL of *B. cytotoxicus* spores in a 200 μL volume. Insets show experimental microtubes before (T_0) and after substrates addition (T_{20}). 0, no magnetic particles; 1, 0.05 mg/mL; 2, 0.1 mg/mL; 3, 0.2 mg/mL; 4, 0.5 mg/mL and 5, 1 mg/mL of BAS6R@MNs.

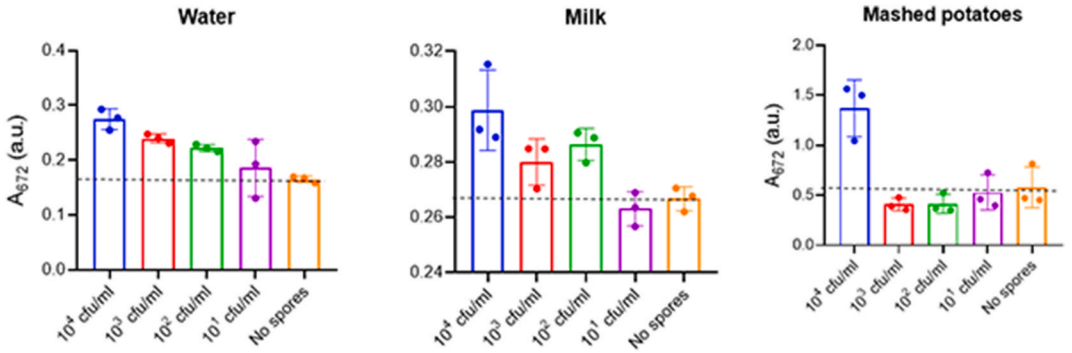

Fig. 7. Detection results for *B. cytotoxicus* spores in three different matrices using BAS6R@AuNPs aptasensor coupled to magnetic concentration. Quantitative evaluation was performed by measuring absorption at 672 nm. Individual data points (n = 3 independent experiments) are shown; error bars represent means ± SD.

Controls were performed in water using 10^7 cfu/mL of bacterial cells/spores and 10^4 cfu/mL for the preconcentration assay and for the sandwich assay, respectively. The results suggest that the aptasensor has a good specificity and selectivity to target *B. cytotoxicus* spores, which can be attributed to the specific recognition properties of the BAS-6R aptamer.

4. Conclusions

We constructed an affordable strategy for the simple and rapid detection of *B. cytotoxicus* spores in food matrices based on the use of enhanced peroxidase-like activity of AuNPs. Through this rapid colorimetric test, spores of *B. cytotoxicus* were quantified in low-cost polypropylene microtubes, to a concentration of 10^2 cfu/mL. The detection strategy was demonstrated by absorbance response and color change of the substrate which was catalyzed by AuNPs concentrated on the spores via the specific aptamer. This approach takes

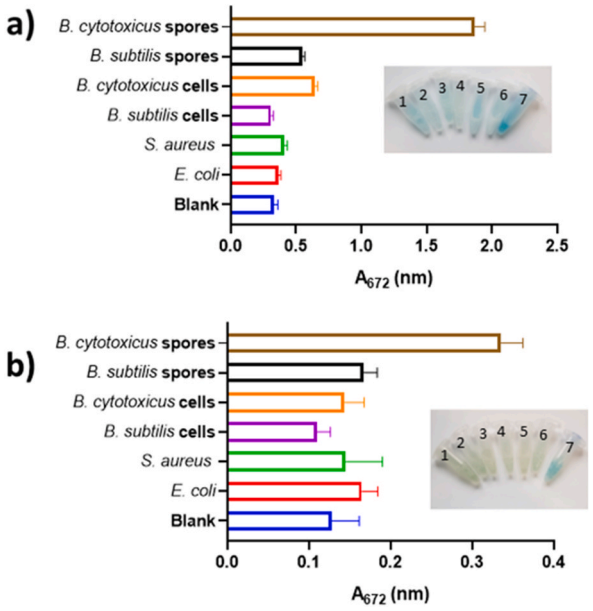

Fig. 8. Specificity study against bacterial vegetative cells and spores in assays performed without (a) and with BAS6R@MNs (b). Concentration of *B. cytotoxicus* spores was 10^7 cfu/mL whereas the other concentrations were 10^8 cfu/mL in tests without spore concentration (a) while concentrations of all strains were 10^4 cfu/mL in tests with concentration (b). Individual data points (n = 3 independent experiments) are shown; error bars represent means ± SD. Insets are the corresponding images of TMB oxidation: 1, blank; 2, *E. coli*; 3, *S. aureus*; 4, *B. subtilis* cells; 5, *B. cytotoxicus* cells; 6, *B. subtilis* spores; and 7, *B. cytotoxicus* spores.

advantage of the aptamer binding to an unknown but specific epitope on spores' surface. Targeting surface epitopes instead of intracellular biomarkers enabled detection without complex and time-consuming procedures for extraction, purification and amplification of biomarkers. Although thermally preheated ready-to-use foods are rarely positive to bacterial spores, a high prevalence *B. cytotoxicus* spores have been found in dehydrated potatoes products [28]. Thus, powder for mashed potatoes could pose a microbiological risk for consumers. The colorimetric biosensor in this study provides efficient screening method for spores due to lower detection limit, wide linear range and minimal sample processing. It may be used for preliminary monitoring of food products that represent high risk, while confirmation tests and strain identification can be performed only on positive food items. This work provides a promising proof of concept for the development of label-free, instrument-free, low-cost and rapid assay for spore detection which is able to compete in sensitivity with conventional costly and time-consuming laboratory analyses.

Author contribution statement

Francesco Rizzotto: Performed the experiments; Analyzed and interpreted the data. Marco Marin: Conceived and designed the experiments. Christine Péchoux: Performed the experiments. Sandrine Auger: Contributed reagents, materials, analysis tools or data; Wrote the paper. Jasmina Vidic: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Data availability statement

Data will be made available on request.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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