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Gold Nanoparticle and PCR-Based Colorimetric Assay for the Identification of *Campylobacter* spp. in Chicken Carcass

Abstract

Campylobacteriosis is a common cause of gastrointestinal disease. In this study, we suggest a general strategy of applying gold nanoparticles in colorimetric biosensors to detect *Campylobacter* in chicken carcass. Polymerase chain reaction (PCR) was utilized for the amplification of the target genes, and the thiolated PCR products were collected. Following the blending of colloid gold nanoparticles (AuNPs) with PCR products, the thiol bound to the surface of AuNPs, forming gold nanoparticle-PCR (GNP-PCR) products. The PCR products had a sufficient negative charge, which enabled AuNPs to maintain a dispersed formation under electrostatic repulsion. This platform presented a color change as gold nanoparticles aggregate. It did not need additional time and optimization of pH for PCR amplicons to adhere to the gold nanoparticles. The specificity of gold nanoparticles of modified primer pairs for *mapA* from *C. jejuni* and *ceuE* from *C. coli* was activated perfectly (*C. jejuni*, *P*-value: 0.0085; *C. coli*, *P*-value: 0.0239) when compared to *Salmonella* Enteritidis and *Escherichia coli* as non-*Campylobacter* species. Likewise, *C. jejuni* was successfully detected from artificially contaminated chicken carcass samples. According to the sensitivity test, at least 15 ng/μL of *Campylobacter* PCR products or 1×10^3 CFU/ml of cells in the broth was needed for the detection using the optical method.

Keywords: *Campylobacter* spp., gold nanoparticle, polymerase chain reaction, chicken

Introduction

Campylobacteriosis is an infectious disease that can cause gastrointestinal symptoms including diarrhea, abdominal pain, and vomiting. Guillaine-Barre syndrome is one of the complications of campylobacteriosis that can damage nerve system of humans (Yang et al., 2013). The European Center for Disease Prevention and Control and the European Food Safety Authority ranked campylobacteriosis as the most common zoonosis in Europe (Euro surveillance Editorial Team, 2012). The most common species that can cause campylobacteriosis in humans is *C. jejuni*, followed by *C. coli* (Tam et al., 2003). Contact with live animals and consuming of raw poultry have been defined as the main sources of *Campylobacter* infection (Tam et al., 2003).

To confirm species origin for pathogens, analytical methods are mostly based on protein or DNA analysis (Ayaz et al., 2006). However, protein-based isolation method has a weakness. When the sample is exposed to high temperatures and pressure, the protein has a general tendency to degenerate (Murugaiah et al., 2009). Compared with protein-based methods, DNA based methods are more dependable because of their unique variability and high stability. Among DNA based methods, PCR methods, including conventional PCR, real-time PCR (Camm`a et al., 2012; Kesmen et al., 2012) and PCR-RFLP (Chen et al., 2010; Haider et al., 2012), have high specificity and accuracy. Recently, specific nanoparticles, including silver (AgNPs) and gold (AuNPs) nanoparticles, have been widely used as components of new technologies to detect pathogens (Du et al., 2013), hazardous materials (Li et al., 2009), DNA (Benedetto et al., 2011), small molecules (Lv et al., 2013), and aptamers (Ping et al., 2012). Aptamer-based detection has been carried out in our previous research (Kim et al., 2018). When various solutions change from dispersion to aggregation state, specific nanoparticles

exhibit significant color change because of their unique optical properties, that can be observed using a UV-visible wave-based spectrophotometer or the naked eye (Du et al., 2020; Shams et al., 2019). Thus, this research was designed to use AuNPs. A few types of AuNP-based colorimetric assays have been previously reported. In particular, the colorimetric assay based on thiol-labeled PCR primer and gold nanoparticles has been previously reported for the detection or diagnosis of disease (Htoo et al., 2019; Osmani Bojd et al., 2017,). The nanoparticle method is favored in the surveillance of pathogens over other PCR methods, due to its advantages over other PCR methods such as conveniency and time saving. To our knowledge, however, few studies have focused on the selective detection of fastidious foodborne pathogens such as *Campylobacter*. Since conventional ways to detect *Campylobacter* based on culture method require more than approximately 4 days (Masdor et al., 2016), various rapid detection methods have been developed for decades (Yang et al., 2013).

In this study, we aimed to develop a colorimetric assay integrating PCR and AuNP conjugation technology. The current study also outlined a colorimetric assay for the PCR-amplified *Campylobacter* genes, that could be directly identified by the naked eye.

Materials and Methods

Bacterial strains and cultivation

All oligonucleotides (Denis et al., 1999) were synthesized and purified by Bionics (Seoul, Korea) (Table 1). For the inclusivity test, some *C. jejuni* (A total of four *C. jejuni* strains: ATCC 33560 and 3 wild type strains from chicken carcasses) and *C. coli* strains (A total of four *C. coli* strains: ATCC 33559 and 3 3 wild type strains from

chicken carcasses) were used. *Campylobacter* strains were isolated from chicken carcasses from a local slaughterhouse and a poultry farm. Non-*Campylobacter* strains for the exclusivity test including *Salmonella* Enteritidis (*S.* Enteritidis) and *Escherichia coli* (*E. coli*), were also isolated from the collected chicken carcasses.

All *Campylobacter* strains were incubated in Bolton broth (Oxoid, Hampshire, UK) at 42℃ for 42 h under microaerobic condition (5% O_2, 10% CO_2, and 85% N_2), while non-*Campylobacter* strains were incubated in tryptic soy broth (Oxoid) at 37℃ for 24 h. Additionally, phosphate-buffered saline was applied to dilute samples in the sensitivity test of gold nanoparticle (GNP)-PCR and 4 log CFU of *Campylobacter* and non-*Campylobacter* strains were incubated in 30 g of chicken meat samples with 30 ml of Bolton broth (42℃ for 42 h) and tryptic soy broth (37℃ for 24 h). Thereafter, 10 µl of cultured bacteria was used for GNP-PCR direct detection test.

Genomic DNA isolation

Approximately 1 ml cultures were used for DNA extraction. DNA was extracted by using QIAamp minikit (Qiagen, Germany). The concentration of extracted DNA was measured by using Nanodrop 2000 (Thermo Scientific, Wilmington, DE, USA) and genomic DNA was stored at 4℃ before PCR amplification.

PCR amplification

PCR was conducted under the following conditions for the amplification of the *mapA* (*C. jejuni*) and *ceuE* (*C. coli*) genes. The reaction was carried out in a 50 µl mixture with 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl, 0.5 mM of deoxynucleoside triphosphate, 200 nM of forward/backward primer, 1 unit of Taq DNA polymerase (TaKaRa, Japan), and 2 µl (200 ng) of template DNA. The details of

primers used in this study is presented in Table 1. The amplification initiated with 10 min at 95℃, followed by 30 cycles of 30 s at 94℃, 90 s at 59℃, and 60 s at 72℃, and then 10 min at 72℃. PCR was conducted in a Veriti 96 well thermal cycler (Applied Biosystems, Waltham, Massachusetts, USA). Exactly 2 µl of PCR products were then used for a gel electrophoresis with 1.0% agarose gel. The samples were run at 100 V for 30 min, followed by some amount of PCR products being visualized with image analysis software (image lab software V3, Bio-Rad, CA, USA). The length of expected PCR product was 589 bp and 462 bp for *mapA* and *ceuE* gene, respectively.

AuNPs synthesis

Sodium citrate dehydrate and $HAuCl_4$ were from Sigma Aldrich (St. Louis, USA). GNPs (13 nm in diameter) were prepared via $HAuCl_4$ citrate reduction. All glassware was cleaned in KOH-IPA (Potassium hydroxide + isopropyl alcohol) solution and washed with pure H_2O. A 50 ml of a 38.8 mM trisodium citrate solution was rapidly mixed with $HAuCl_4$ solution (1 mM, 500 ml), resulting in a change of color from yellow to dark red. The solution was then refluxed for another 15 min to cool it down to 25℃. The characteristics of the GNPs were verified using a surface plasmon band centered at 520 nm (Grabar et al., 1995).

Colorimetric assay-based GNPs

The center of the surface plasmon band of GNPs was 520 nm. A 6 µl of PCR product was mixed with 40 µl of the gold colloid for the colorimetric assay. They were mixed for 1 min of mixing, and another 10 µl of 1 M NaCl was added. The color was identified by the naked eye, and they were quantified using Nanodrop 2000 (Thermo Scientific, Wilmington, DE, USA). Since the aggregations between GNPs at 520 and at

650 nm are different, the ratio of absorbance 520/650 (Abs520/Abs650) was chosen.

Transmission electron microscopy (TEM)

The colloidal gold solution was dropped onto 1000 mesh of copper grids that was coated by carbon, followed by being dried in ambient conditions. Images were acquired using TEM JEM-2100 (JEOL Ltd, Japan) from Seoul National University. At least ten locations on the TEM copper grid were examined.

Data analysis

Analysis of different Abs520/Abs650 data for *Campylobacter* spp. for specificity was compared to that for non-*Campylobacter* spp. and the data for various concentrations of PCR products in the sensitivity analysis were analyzed using one-way ANOVA, which was conducted using GraphPad Prism software (GraphPad Software, San Diego, CA).

Results and Discussion

The design of colorimetric assay for the detection of *Campylobacter*

Fig. 1 represents the main principle of the suggested method. The main idea behind the GNP colorimetric assay was the dispersion and aggregation of colloidal GNPs. It was effectively demonstrated that the interparticle movement and pulsive forces are the main reason for GNP stabilization or aggregation (Napper, 1983; Zhao et al., 2008). GNP electrostatic stabilization relies on the surface charge of the repulsive electric double layer. The layer can stabilize the colloids against Van der Waals forces (Evans and Wennerstrom, 1999). When the electric double layer of GNP is suppressed,

159 the electrostatic repulsion force dramatically decreases at high concentrations of salt.
160 That is why citrate-combined GNPs are stabilized in water but aggregated in salty
161 conditions (Zhao et al., 2008). Thus, the covalently combined complex of thiolated
162 double-strand DNA (dsDNA) and monodispersed GNP can resist the aggregation
163 regardless of salt addition.

164 AuNPs maintain a red color in stable state. When they aggregated, the state of
165 AuNPs becomes unstable, causing change of color from red to blue or purple. The main
166 focus of the current research was that AuNPs can be modified by thiolated PCR
167 products, that prevented AuNPs from salt-induced aggregation (Chan et al., 2014; Fu et
168 al., 2013; Jyoti et al., 2010). Because of this attribute, we modified unique
169 *Campylobacter* target primers with an unlabeled backward primer and a thiol-labeled
170 forward primer. Therefore, thiol-labeled target gene sequence was obtained. When
171 AuNPs were mixed with thiolated PCR products, they were surrounded by a thick layer
172 that consisted of dsDNA. Therefore, this structure tended to make a change of color.

173 For a successful result, GNPs need a thick obstacle with a lot of negative
174 charges to inhibit their aggregation. We modified the primer for *mapA* of *C. jejuni* (589
175 bp) and *ceuE* of *C. coli* (462 bp) for PCR amplification. *mapA*, which is usually called
176 membrane-associated protein A gene, does not cross-react with *C. coli* proteins
177 (Gonzalez et al., 1997; Stucki et al., 1995). *ceuE* is a lipoprotein-encoding gene which
178 has been used for *C. coli* identification (Gonzalez et al., 1997). To confirm PCR
179 efficiency, thiol-labeled PCR primers used for *C. jejuni* and *C. coli* were the same as
180 unlabeled primers used for the conventional PCR.

181 PCR and gel electrophoresis were conducted to analyze the PCR products. Fig. 2
182 shows that PCR products from thiol-labeled- and unlabeled primers are identical in size.
183 It shows that the result obtained using the thiolated primers did not show any size

184 difference to conventional PCR products.

185

186 **Thiolated PCR products**

187 For the detection of *Campylobacter* spp., thiol-labeled PCR products prevented
188 GNP aggregation based on salt-inducing. Both unlabeled and thiol-labeled PCR
189 products were added into the GNP solution and the color change was compared. After
190 adding NaCl solution, GNP solution with unlabeled PCR products turned from red to
191 blue and that with thiol-labeled products remained red. Spectroscopic analysis verified a
192 sharp peak of surface plasmon resonance (SPR) at approximately 520 nm. This narrow
193 SPR peak appeared due to the red colored solution containing thiol-labeled PCR
194 products after salt adjusting. A broad SPR band (530-650 nm) seemed in the blue
195 colored solution with unlabeled PCR products, indicating GNP aggregation after salt
196 addition (Fig. 3). To confirm the characteristic features of GNPs in the colorimetric
197 assay, different behaviors of the salt-induced aggregation of GNPs were observed using
198 TEM JEM-2100 (JEOL Ltd, Japan) (Fig. 4).

199

200 **The specificity of the assay**

201 A color change was observed in the GNP solution containing non-labeled PCR
202 products, whereas the GNP solution with thiol-labeled PCR products are still red (Fig.
203 5). This can be identified with the naked eye.

204 Abs520/Abs650 of *C. jejuni* and *C. coli* isolates were significantly different (*C.*
205 *jejuni*: 4.86±0.27; *C. coli*: 4.81±0.28) compared to that of *S.* Enteritidis and *E. coli* (*C.*
206 *jejuni P*-value: 0.0085; *C. coli P*-value: 0.0239) in five replicates. Thus, 200 ng/µl of
207 *mapA* and *ceuE* were detected in the sample with thiol-labeled products after salt
208 adjusting.

The sensitivity of the assay

For the determination of the sensitivity, different concentrations of *mapA* from *C. jejuni* were tested. Abs520/Abs650 of the detected samples are shown in Fig. 6. Fig. 6 (A) shows that at least 15 ng/μl of *C. jejuni mapA* PCR product was needed to detect *C. jejuni* using the optical method. At this concentration, Abs520/Abs650 was 3.38±0.17 (mean±standard deviation (S.D.)). Fig. 6 (B) shows that at least 1×10^3 CFU/ml of *C. jejuni* was needed to use the colorimetric method. At this concentration, Abs520/Abs650 was 3.26±0.35 (mean±S.D.). These results represented that the Abs520/Abs650 increased with the increasing number of PCR products and cells. To verify the precision of the assay, one-way ANOVA was used to determine the *P*-value after measuring the absorption at different concentrations in five replicates.

More PCR products can be obtained by applying more amplification cycles. However, too many amplification cycles lead to low efficiency because of the formation of non-specific bands due to DNA degeneration (Cha and Thilly, 1993). Therefore, 30 cycles of PCR protocol was selected in this study. The colorimetric system was controled by NaCl. The concentration of NaCl affects the dynamics of the method as well as the sensitivity of the detection. The limit of detection (LOD) of various types of detection methods are compared in Table 2. qPCR is slightly more sensitive than GNP-PCR (Table 2). However, the detection limit of the GNP-PCR was similar or superior to other detection methods such as ELISA, GNP-adjusted aptasensor, and Lateral flow (Table 2).

As we stated above, the thiol-labeled PCR primer and gold nanoparticles have been applied in many research fields for the detection or diagnosis of disease. Htoo et al. (2019) used thiol-labeled PCR primer and unmodified gold nanoparticles for the detection of target RNA for the diagnosis of prostate cancer and found that the assay

was specific and sensitive for the RNA detection in prostate cancer cell lines with a visual detection limit of 31.25 ng/reaction. Osmani Bojd et al. (2017) reported application of thiolated AuNP probes and multiplex PCR for the detection of *Staphylococcus epidermidis*. They found that the minimum quantity of target DNA for multiplex PCR was 1 ng/mL and for color and absorption altercation of solution in colorimetric assay was 20 ng/mL. Since *Campylobacter* is fastidious bacteria that requires long incubation time and labors, development of this types of rapid and convenient isolation method is highly useful for the screening of the pathogen. In the present study, the newly developed method showed slightly lower sensitivity compared to qPCR according to Table 2. However, this method could be favored over qPCR especially in field tests as the assay could be conducted without further steps that require cost, labor, and detection device. With the assay, the positives could be easily distinguished from the negatives with the naked eye. Thus, the developed assay could be finished only with thermal cycler and optical detection, while conventional or qPCR still need further steps after PCR cycles. Even though qPCR is superior to this assay in terms of sensitivity, the developed method could be widely used in many research fields considering its convenience for the detection of pathogens.

Direct detection of *Campylobacter* in foods

To estimate the detection ability of GNP-PCR in food pathogenic bacteria, the method was applied to a food sample. Three groups of chicken meat samples were purchased from local retails. A group was artificially spiked with an overnight culture of *C. jejuni*, while the others were contaminated with *S.* Enteritidis and *E. coli*. Artificially contaminated samples were cultured at 42°C overnight and 10 µl of each sample was directly adjusted to the GNP-PCR assay. The results of these assays are shown in Fig. 7.

When thiol-labeled primers were used in the rinsate of fresh chicken meat, the color of the GNP solution changed from red to blue after salt adjusting. Abs520/Abs650 was 1.06±0.22 (mean±S.D.). However, if the *C. jejuni* template was present, the color of GNP solution is still red after salt adjusting. This result suggested that the GNPs remained dispersed, while the Abs520/Abs650 of *mapA* was 4.30±0.3. With the spectroscopic results, the sample groups can be clearly distinguished.

The detection ability of the GNP-PCR assay for *C. jejuni* and *C. coli* was well defined according to sensitivity assays results. The length of PCR amplicons and amplification efficiency were important factors for the detection. Previous research found that GNPs highly tend to aggregate when the PCR amplicons were shorter than 400 bp. When the amplicons were longer than 400 bp, it became a limiting factor for efficient detection (Fu et al., 2013). As we stated above, GNPs require a thick barrier with a lot of negative charges to inhibit the aggregation. We selected the 589 bp *mapA* for *C. jejuni* detection and the 462 bp *ceuE* for *C. coli* detection.

Conclusion

The suggested assay enables the generation of a PCR product with high amplification efficiency and the convenience of a colorimetric assay. Moreover, leading to a limit of optical detection of density around 15 ng/µl of *Campylobacter* verified high specificity. The suggested assay has a couple of advantages. Above all, the PCR colorimetric method has high sensitivity, specificity, and amplification efficiency. Furthermore, this method avoids bacterial culture steps, reduces the time needed, and requires no complicated apparatus. Moreover, the proposed method allows the detection of pathogens even by the naked eye. Therefore, it appears that this assay has high potential for a various bio-related detections. Lastly, as we stated above, this method

does not require complex devices, while detecting conveniently with the naked eye.

The current research reported a novel and unique colorimetric assay for the detection of *Campylobacter* spp. in raw poultry. Considering high specificity of the assay, the accurate identification of *Campylobacter* spp. from other environmental samples is also available with this method.

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References

Ayaz Y, Ayaz ND, Erol I. 2006. Detection of species in meat and meat products using enzyme-linked immunosorbent assay, J Muscle Foods 17:214–220.

Benedetto A, Abete MC, Squadrone S. 2011. Towards a quantitative application of real-time PCR technique for fish DNA detection in feedstuffs, Food Chemistry 126:1436–1442.

Cammà C, Domenico MD, Monaco F. 2012. Development and validation of fast Real-Time PCR assays for species identification in raw and cooked meat mixtures, Food Control 23: 400–404.

Cha RS, Thilly WG. 1993. Specificity, efficiency, and fidelity of PCR. PCR Methods Appl 3:18-29.

309 Chan WS, Tang BSF, Boost MV, Chow C, Leung PHM. 2014. Detection of methicillin-
310 resistant *Staphylococcus aureus* using a gold nanoparticle-based colourimetric
311 polymerase chain reaction assay. Biosens Bioelectron 53:105–111.
312 Che Y, Li Y, Slavik M. 2001. Detection of *Campylobacter jejuni* in poultry samples
313 using an enzyme-linked immunoassay coupled with an enzyme electrode.
314 Biosens Bioelectron 16: 791–797.
315 Chen SY, Liu YP, Yao YG. 2010. Species authentication of commercial beef jerky
316 based on PCR-RFLP analysis of the mitochondrial 12S rRNA gene. J Genet
317 Genomics 37:763–769.
318 Denis M, Soumet C, Rivoal K, Ermel G, Blivet D, Salvat G, Colin P. 1999.
319 Development of a m-PCR assay for simultaneous identification of
320 *Campylobacter jejuni* and *C. coli*. Lett Appl Microbiol 29: 406–410.
321 Du J, Wu S, Niu L, Li J, Zhao D, Bai Y. 2020. A gold nanoparticles-assisted multiplex
322 PCR assay for simultaneous detection of *Salmonella* Typhimurium, *Listeria*
323 *monocytogenes* and *Escherichia coli* O157:H7. Anal Methods 12:212-217.
324 Du J, Yin S, Jiang L, Ma B, Chen X. 2013. A colorimetric logic gate based on free gold
325 nanoparticles and the coordination strategy between melamine and mercury
326 ions. Chem Commun 49:4196–4198.
327 Euro surveillance Editorial Team. 2012. The European Union summary report on trends
328 and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2010.
329 Euro Surveill 17:20113
330 Evans DF, Wennerström H. 1999. The colloidal Domain: Where Physics, Chemistry,
331 and Biology Meet, Wiley-VCH New York.
332 Fu Z, Zhou X, Xing D. 2013. Rapid colorimetric gene-sensing of food pathogenic
333 bacteria using biomodification-free gold nanoparticle. Sens Actuators B Chem

182:633–641.

Gonzalez I, Grant KA, Richardson PT, Park SF, Collins MD. 1997. Specific Identification of the Enteropathogens *Campylobacter jejuni* and *Campylobacter coli* by Using a PCR Test Based on the *ceuE* Gene Encoding a Putative Virulence Determinant. J Clin Microbiol 35:759–763.

Grabar KC, Freeman RG, Hommer MB, Natan MJ. 1995. Preparation and characterization of Au colloid monolayers. Anal Chem 67:735–743.

Haider N, Nabulsi I, Al-Safadi B. 2012. Identification of meat species by PCR-RFLP of the mitochondrial COI gene. Meat Sci 90:490–493.

He K, Zhang X, Chen L, Zhao R, Wang L. 2018. Sensitive detection of *Campylobacter jejuni* using one-step strategy based on functional nanospheres of immunomagnetic capture and quantum dots. Ecotoxicol Environ Saf 163:274–278.

Htoo KPP, Yamkamon V, Yainoy S, Suksrichavalit T, Viseshsindh W, Eiamphungporn W. 2019. Colorimetric detection of PCA3 in urine for prostate cancer diagnosis using thiol-labeled PCR primer and unmodified gold nanoparticles. Clin Chim Acta 488:40-49.

Jyoti A, Pandey P, Singh SP, Jain SK, Shanker R. 2010. Colorimetric detection of nucleic acid signature of shiga toxin producing *Escherichia coli* using gold nanoparticles. J Nanosci Nanotechnol 10:4154–4158.

Kesmen Z, Yetiman AE, Sahin F, Yetim H. 2012. Detection of chicken and turkey meat in meat mixtures by using real-time PCR assays, J Food Sci 77:167–173.

Kim YJ, Kim HS, Chon JW, Kim DH, Hyeon JY, Seo KH. 2018. New colorimetric aptasensor for rapid on-site detection of *Campylobacter jejuni* and *Campylobacter coli* in chicken carcass samples. Anal Chim Acta 1029:78-85.

Li L, Li B, Qi Y, Jin Y. 2009. Label-free aptamer-based colorimetric detection of mercury ions in aqueous media using unmodified gold nanoparticles as colorimetric probe. Anal Bioanal Chem 393:2051–2057.

Lv Z, Liu J, Zhou Y, Guan Z, Yang S, Li C, Chen A. 2013. Highly sensitive fluorescent detection of small molecules, ions, and proteins using a universal label-free aptasensor. Chem Commun 49:5465–5467.

Masdor NA, Altintas Z, Tothill IE. 2016. Sensitive detection of *Campylobacter jejuni* using nanoparticles enhanced QCM sensor. Biosens Bioelectron 78:328-336.

Murugaiah, C., Noor, Z. M., Mastakim, M., Bilung, L. M., Selamat, J., Radu, S. 2009. Meat species identification and Halal authentication analysis using mitochondrial DNA. Meat Sci 83:57–61.

Napper DH. 1983. Polymeric Stabilization of Colloidal Dispersion. Academic Press, London.

Ping H, Zhang M, Li H, Li S, Chen Q, Sun C, Zhang T. 2012. Visual detection of melamine in raw milk by label-free silver nanoparticles, Food Control 23:191–197.

Osmani Bojd M, Kamaladini H, Haddadi F, Vaseghi A. 2017. Thiolated AuNP probes and multiplex PCR for molecular detection of *Staphylococcus epidermidis*. Mol Cell Probes 34:30-36.

Shams S, Bakhshi B, Moghadam TT, Behmanesh M. 2019. A sensitive gold-nanorods-based nanobiosensor for specific detection of *Campylobacter jejuni* and *Campylobacter coli*. J Nanobiotechnology 17:43.

Stucki U, Frey J, Nicolet J, Burnens AP. 1995. Identification of *Campylobacter* jejuni on the basis of a species-specific gene that encodes a membrane protein. J Clin Microbiol 33:855–859.

384 Suh SH, Dwivedi HP, Jaykus LA. 2014. Development and evaluation of aptamer
385 magnetic capture assay in conjunction with real-time PCR for detection of
386 *Campylobacter jejuni*, LWT–Food Sci Technol 56:256–260.
387 Tam CC, O'Brien SJ, Adak GK, Meakins SM, Frost JA. 2003. *Campylobacter coli*-an
388 important foodborne pathogen. J Infect 47:28-32.
389 Wadl M, Polzler T, Flekna G, Thompson L, Slaghuis J, Kofer J, Hein I, Wagner M. 2009.
390 Easy-to-use rapid test for direct detection of *Campylobacter* spp. in chicken
391 feces. J Food Prot 72:2483–2488.
392 Yang X, Kirsch J, Simonian A. 2013. *Campylobacter* spp. detection in the 21st century:
393 a review of the recent achievements in biosensor development, J Microbiol
394 Meth. 95:48-56.
395 Zhao W, Brook MA, Li Y. 2008. Design of gold nanoparticle-based colorimetric
396 biosensing assays. ChemBioChem 9:2363–2371.
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398

399 **Table 1. Primer pairs for *Campylobacter* spp.**

Target gene	Sequence (5′- 3′)	Amplicon size	Reference
mapA (C. jejuni) forward	SH-C12-CTA TTT TAT TTT TGA GTG CTT GTG	589 bp	(Denis et al., 1999)
mapA (C. jejuni) reverse	GCT TTA TTT GCC ATT TGT TTT ATT A		
ceuE (C. coli) forward	SH-C12-AAT TGA AAA TTG CTC CAA CTA TG	462 bp	(Denis et al., 1999)
ceuE (C. coli) reverse	TGA TTT TAT TAT TTG TAG CAG CG		

400 *Thiol (-SH) formations were applied to 5′ end of the primer sequence.

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413 **Table 2. Comparison of the analytical performance of the developed GNP-PCR assay with several**

414 **reported assays.**

Methods	Target bacteria	LOD (cfu/mL)	Reference
ELISA	*C. jejuni*	4.5×10^4 cfu/mL	(Che, Li, & Slavik, 2001)
GNP-adjusted aptasensor	*C. jejuni*	7.2×10^5 cfu/mL	(Kim et al., 2018)
QD adjusted Fluorescent biosensor	*C. jejuni*	1×10^3 cfu/mL	(He et al., 2018)
Lateral flow	*C. jejuni*	1×10^7 cfu/mL	(Wadl et al., 2009)
qPCR	*C. jejuni*	1.25×10^2 cfu/ml	(Suh, Dwivedi & Jaykus 2014)
GNP-PCR assay	*C. jejuni*	1×10^3 cfu/mL	The present study

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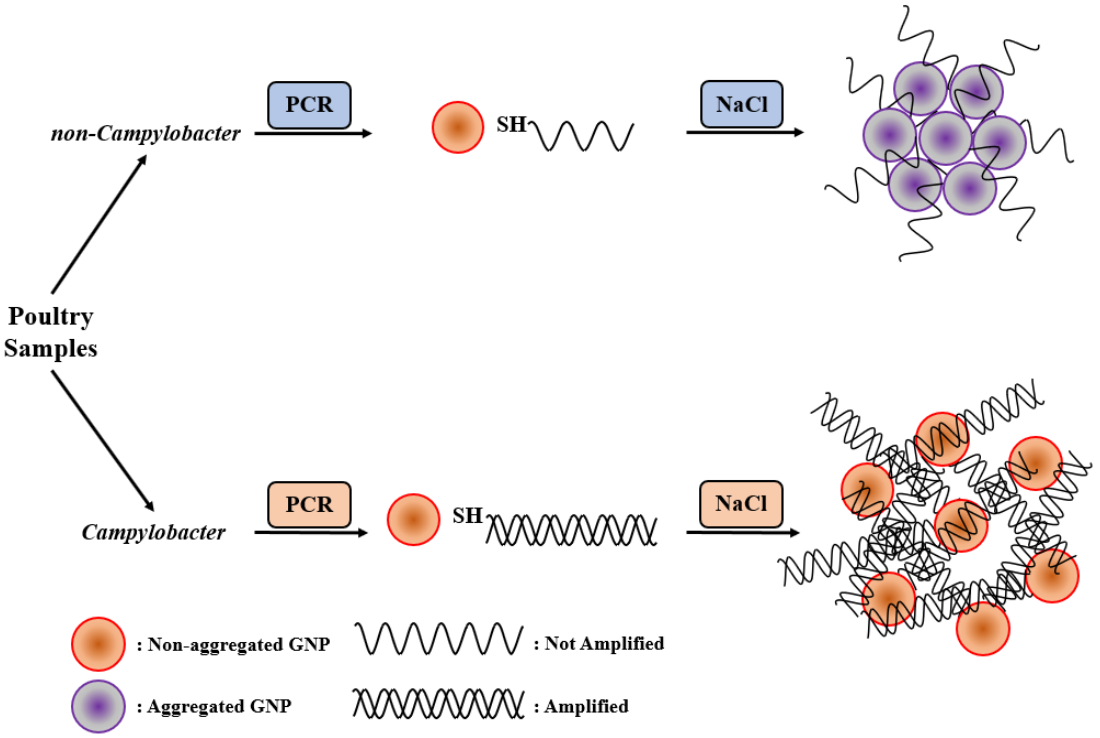

Fig. 1. The schematic diagram for the PCR-based gold nanoparticle colorimetric assay for detection of *Campylobacter*. *mapA* and *ceuE* were used for *C. jejuni* and *C. coli*, respectively.

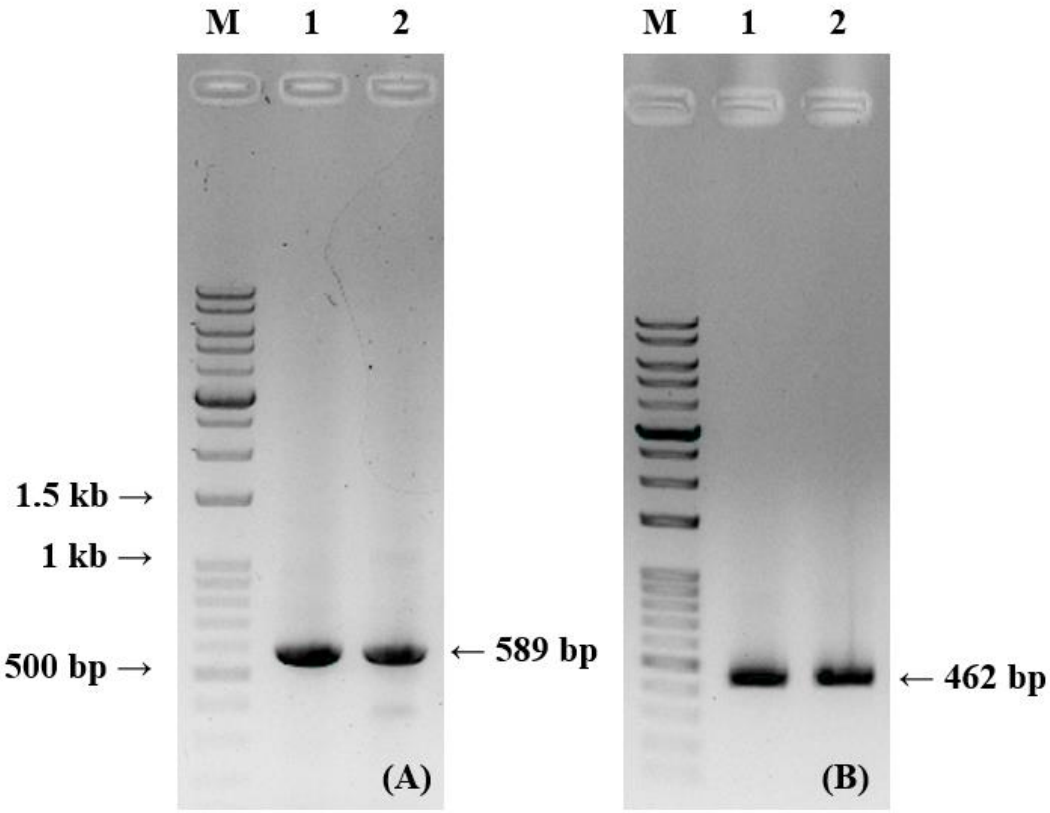

Fig. 2. Verification of PCR. PCR products were electrophoresed in a 1% agarose gel. The DNA ladder is indicated in lane M. Lane 1 indicates PCR products with no thiol-labeled primers and lane 2 represents PCR products with thiol-labeled primers. Thiol-labeled and non-labeled PCR products had similar sizes. Thiolated primer did not cause size differentiation. (A) Agarose gel electrophoresis of *mapA* from *C. jejuni* ATCC 33560 (589 bp). (B) Agarose gel electrophoresis of *ceuE* gene from *C. coli* ATCC 33559 (462 bp).

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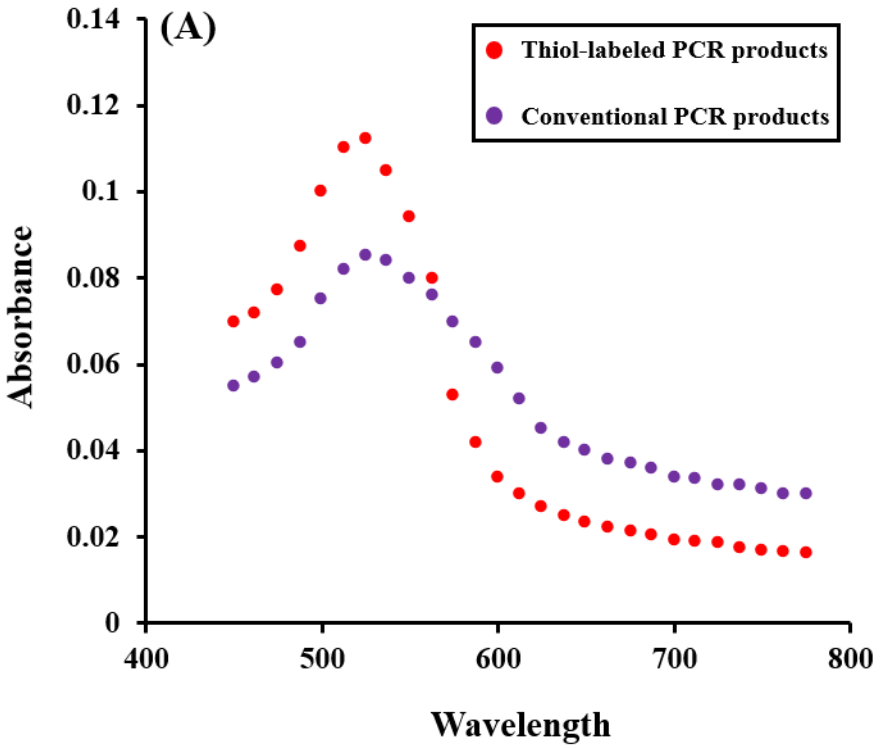

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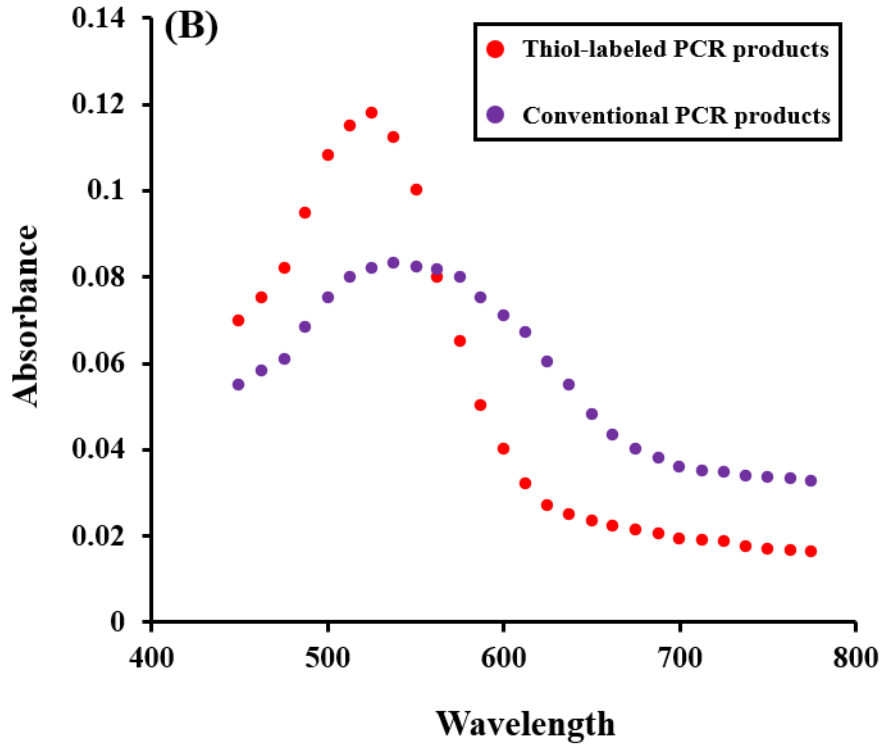

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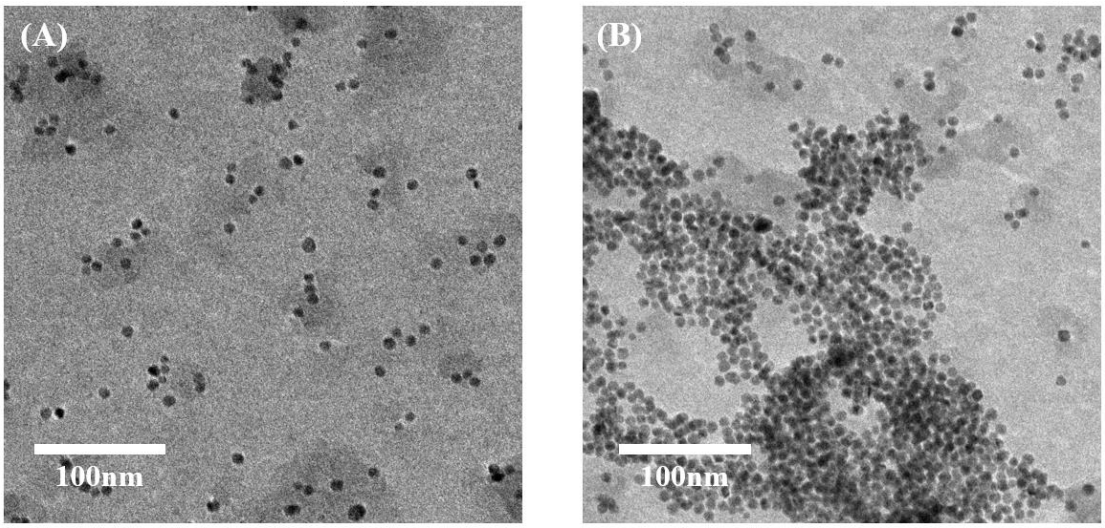
439 **Fig. 3. Colorimetric assays**. The effect of thiol-labeled PCR products is verified based

440 on *mapA* of *C. jejuni* (A) and *ceuE* of *C. coli* (B) isolated from chicken carcass samples.



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442 **Fig. 4.** TEM images of AuNPs after the addition of thiol-labeled PCR product with salt

443 (A) and unlabeled PCR product with salt (B). TEM, transmission electron microscopy.

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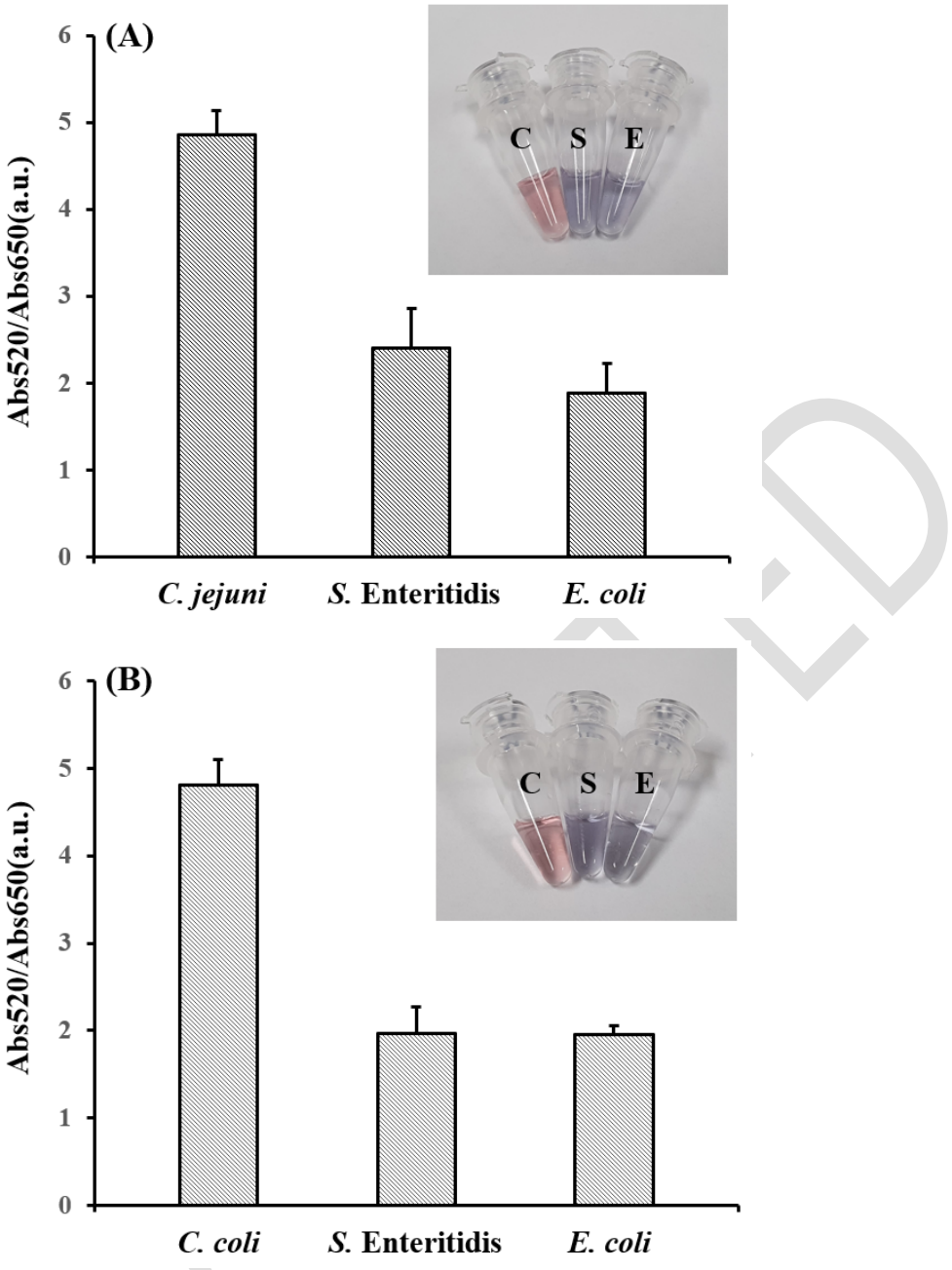

Fig. 5. Specificity analysis. Specificity evaluation of absorption spectra analysis of GNPs mixed with the PCR products of *C. jejuni* and *C. coli*. (A) Specificity evaluation of absorption spectra analysis of GNPs mixed with the PCR products of *C. jejuni*. Tube C shows the result of the colorimetric assay for *C. jejuni*, tube S shows that for *S.* Enteritidis, and tube E shows that for *E. coli*. (B) Specificity evaluation of absorption spectra analysis of GNPs mixed with the PCR products of *C. coli*. Tube C shows the

455 result of the colorimetric assay for *C. coli*, tube S shows that for *S.* Enteritidis, and tube
456 E shows that for *E. coli*. GNP, gold nanoparticle.
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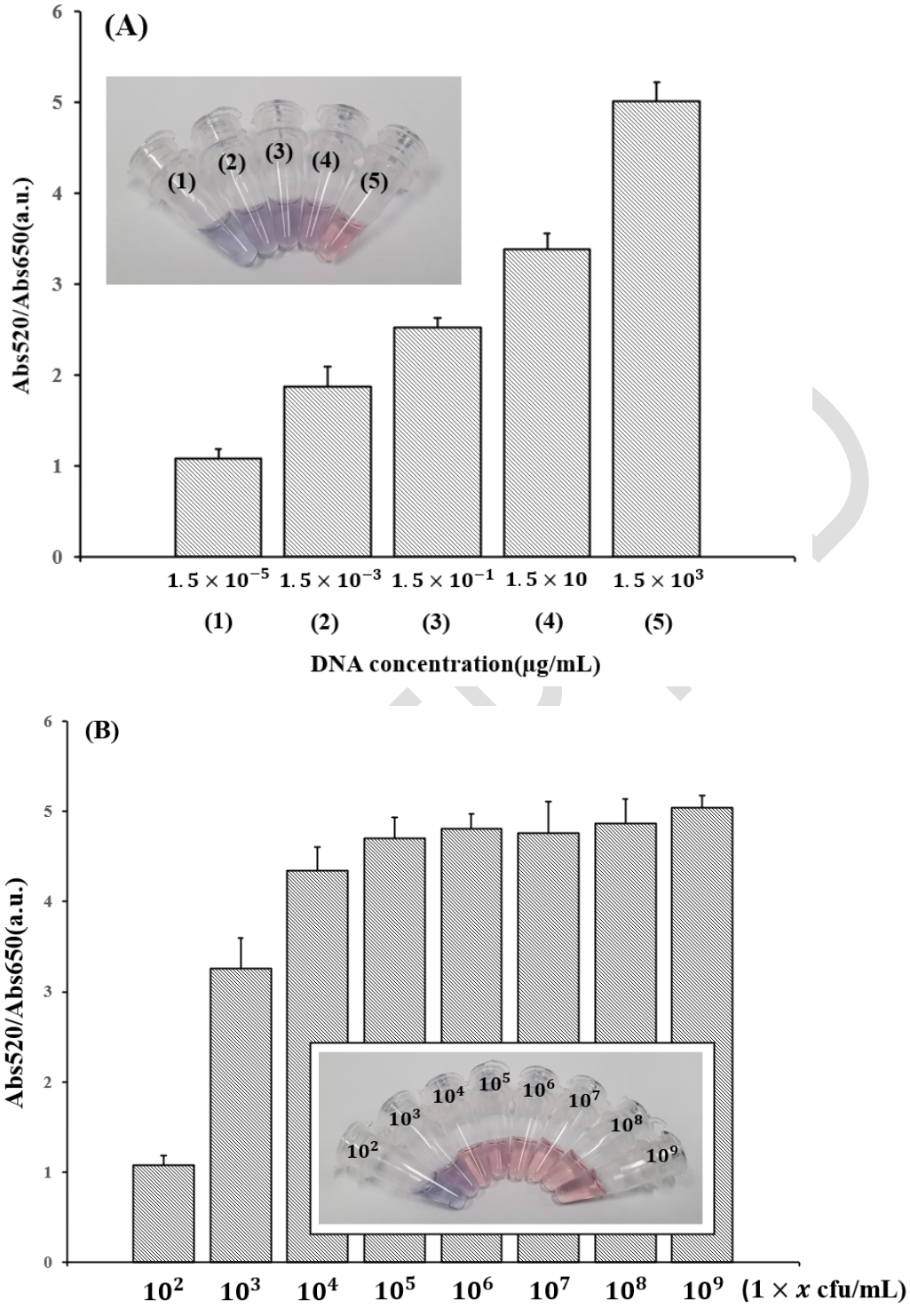

Fig. 6. Sensitivity analysis. The sensitivity of different concentrations of GNP-PCR colorimetric assay for DNA (A) and cells (B) based on *mapA* of *C. jejuni*.

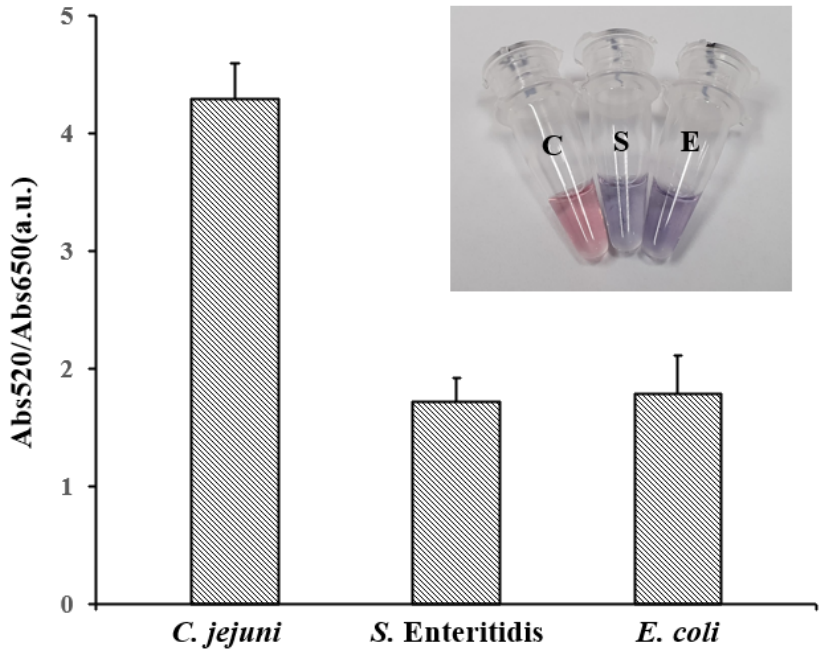

Fig. 7. *C. jejuni* detection in artificially contaminated chicken meat. Tube C shows the result of the colorimetric assay for *C. jejuni*, tube S shows that for *S.* Enteritidis, and tube E shows that for *E. coli*. All three samples were obtained from artificially contaminated chicken meat samples.