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Colorimetric gold nanoparticles-based assay for direct detection of *Clostridium difficile* in clinical isolates from Qatar

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***Clostridium difficile* infection (CDI) is a significant health problem worldwide. Control and prevention strategies of *C. difficile* horizontal transmission require assays with fast detection with high specificity and sensitivity. Conventional diagnostic methods are time consuming and costly for clinical field settings. This study aims to develop gold nanoparticles (AuNPs)-based assay for direct qualitative detection of the nucleic acid of *C. difficile* and its toxins. A colloidal solution of AuNPs with a diameter of 13±1 nm was prepared and characterized. The qualitative colorimetric AuNPs assay was developed for restricted genomic *C. difficile* DNA detection, and results were confirmed by PCR. One hundred and five positive *C. difficile* isolates were collected from patients with diarrheal diseases and tested using AuNPs based-assay. Ninety-six samples (91.4%) were detected positive using AuNPs based assay, as indicated by the color change from red to blue within 1 min. All ninety-six positive samples were positive for toxin B. In conclusion, nano-gold assay prototype was developed for direct and inexpensive detection of *C. difficile*. The developed prototypes are simple, sensitive, rapid and can substitute PCR-based detection. The developed assay may show potential in the clinical diagnosis of *C. difficile*, especially in developing countries as it is less costly as compared to the commercially available assays.**

Key words: Gold nanoparticles, *Clostridium difficile,* colorimetric assay, polymerase chain reaction (PCR).

INTRODUCTION

Clostridium difficile is a Gram-positive, strictly anaerobic, spore-forming bacterium (Chankhamhaengdecha et al., 2013), first recognized in the late 1970s (Burnham and Carroll, 2013). It is documented as the causative agent of

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a broad spectrum of intestinal diseases ranging from mild self-limiting diarrhea to more serious and potentially life threatening manifestations such as *Pseudomembranous colitis* and is responsible for most cases of antibiotic-associated diarrhea (Carter et al., 2007; Goncalves and Decre, 2004). Pathogenicity of *C. difficile* is linked to two major toxins produced by the bacteria: toxins A (enterotoxin) and B (cytotoxin). Toxins A and B are encoded by the genes *tcdA* and *tcdB*, respectively, and are located in the 19.6-kb pathogenicity locus (PaLoc) of the *C. difficile* chromosome (Persson et al., 2011). Some strains of *C. difficile* also secrete binary toxin. CdtA and cdtB genes that are located outside the PaLoc (Goncalves and Decre, 2004) encode these binary toxins.

Rapid and reliable identification of toxigenic *C. difficile* is essential for appropriate patient management and implementation of timely infection control measures due to the rapidly increasing infection rate of *C. difficile* in health care facilities. Current laboratory diagnosis remains challenging with many limitations, as rapid test procedures relying on enzyme immunoassays (EIAs) show limited sensitivity. On the other hand, the gold standard toxigenic culture and cytotoxicity assays, which are considered as reference standard, are time-consuming (Dalpke et al., 2013). A two-step algorithms consisting of sensitive detection of glutamate dehydrogenase enzyme (GDH) followed by a confirmatory test using PCR have been proposed by Ticehurst et al. (2006) to increase sensitivity and specificity of the detection.

Gold has been used as a vital material in nanotechnology and incorporated in diagnostic procedures. Distinctive size-dependent optical properties of AuNPs, their inertness and strength make them one of the most robust materials utilized in nano diagnostics technology (Syed, 2014). They are spherical in shape with a typical diameter of approximately 2 to 100 nm that exhibit a unique phenomenon known as “localized surface plasmon resonance” or LSPR, which is responsible for their intense red color. Upon aggregation, AuNPs change color and that is easily detected visually without the aid of any instrumentation. Addition of salt (NaCl) during hybridization shields the surface charge on the AuNPs, which are typically negatively charged due to reduced citrate ions on their surfaces, leading to aggregation of AuNPs and thus change in color from red to blue (Hussain et al., 2013; Syed, 2014). This property is especially useful in colorimetric detection based assay, which is evaluated in this study (Azzazy et al., 2006; Jennings and Strouse, 2007; West and Halas, 2003). The principle of colorimetric AuNPs-based assay in microbial identification is illustrated in Figure 1.

To the best of the authors’ knowledge, this is the first study aimed to develop unmodified AuNPs - based assay for direct qualitative detection of the nucleic acid of *C. difficile* and its toxins. Additionally, it evaluated AuNPs assay sensitivity and specificity as compared to RT-PCR (GeneXpert, Cepheid, CA, USA).

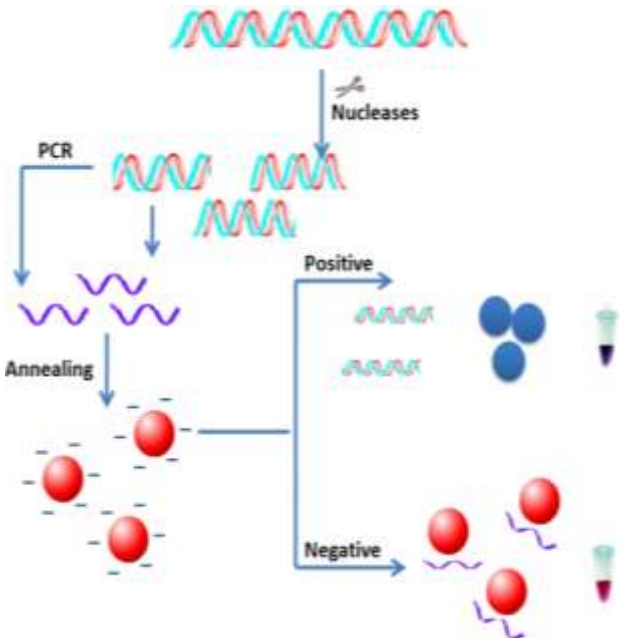

Figure 1. Schematic diagram illustrating the principle of colorimetric AuNPs-based assay in microbial identification. In positive samples, the primer is complementary to the DNA/RNA target, thus not available in the hybridization buffer, containing NaCl to protect and stabilize AuNp, leading to aggregation of AuNPs and blue color formation. On the other hand, in the absence of the target or non-specific DNA, the primer will be free in the reaction mixture and binds to AuNPs preventing aggregation and the color remains unchanged.

MATERIALS AND METHODS

Synthesis of AuNPs

Spherical AuNPs were prepared by citrate reduction of gold 111 chloride trihydrate ($HAuCl_4.3H_2O$). Synthesis was carried out as previously prescribed by Grabar et al. (1995). Briefly, the reflux system was cleaned by aqua regia and then rinsed with ultrapure water and left to dry. An aqueous solution of 1 Mm $HAuCl_4.3H_2O$ (Sigma Aldrich) was brought to boil under reflux conditions while stirring. When gold started to boil, 1% of trisodium citrate (Sigma Aldrich) was rapidly added. This resulted in a subsequent change in solution color from yellow - clear - black - purple - deep red. Afterwards, the solution was then refluxed for an additional 15 min and subsequently allowed to cool to room temperature. The colloidal solution was then stored into a clean dark storage glass bottle at 4°C until further use.

Characterization of AuNPs

The absorbance of the prepared AuNPs solution was measured by Agilent 8453 UV-visible spectrophotometer at wavelength 400 to 700 nm. The size and distribution was characterized using Zeta sizer (Malvern, Nano ZSP, UK) based on light scattering principle and scanning electron microscope, SEM (FE1 NOVA-NanoSEM, 450, USA). The recommended AuNPs size ranges from 12 to 15

nm with the absorbance of the visible range 400 to 700nm (Grabar et al., 1995).

Collection of bacterial isolates

A total of 105 *C. difficile* clinical isolates used in this study were, originally provided for routine laboratory diagnosis at Hamad Medical Corporation (HMC) - Al Khor Hospital, Qatar during the period of 2011 to 2012. Cryopreserved *C. difficile* isolates were revived and sub-cultured onto blood agar enrichment medium, and incubated anaerobically. Samples were transported to Qatar University (Biomedical Research Center) for DNA extraction and testing with gold nanoparticles-based assay. Isolates were confirmed to be *C. difficile* using RT-polymerase chain reaction, RT-PCR, (GeneXpert, Cepheid, CA, USA). According to RT- PCR results, all *C. difficile* isolates (105) were positive for toxin B. Twenty nine additional ATCC bacterial isolates other than *C. difficile* were used to assess specificity of the assay. The Research Ethical Committee of Hamad Medical Corporation, Qatar, approved the study.

DNA extraction

Genomic DNA was extracted from bacterial cultures following manufacturer's instructions of QIAamp DNA Mini kit (Qiagen; Cat. No. 51306).

Restriction of genomic *C. diff* DNA

The entire extracted genomic *C. diff* DNA was digested using *Bam* HI, according to the conditions recommended by the supplier of the enzyme kit (Promega). Briefly, 17.3 µl of the extracted genomic DNA were restricted by addition of 0.5 µl of the enzyme, 2 µl of the buffer, 0.2 µl of the acetylated bovine serum albumin. Then incubated at 37°C for 1 h after that inactivated at 65°C for 20 min.

Colorimetric AuNPs assay for detection of *C. difficile* DNA: Development and optimization

The colorimetric qualitative AuNPs assay for *C. difficile* was optimized through adjustment of the assay parameters such as annealing temperature, salt concentration and targeting oligonucleotide sequences. Different concentrations of NaCl and primer concentrations were tested to determine the optimum concentrations for performing the assay (data not shown). Hybridization buffer was prepared using 0.50 M NaCl and 10 µM primer. Different volumes of the AuNPs were tested, and 25 µL of the prepared AuNPs (12 to 15 nm) was selected for use in the final assay. Forward CD-R 5'- CCC TGC ACC CTT AAT AAC TTG ACC-3' (Integrated DNA Technologies, Inc. Belgium) primer was used in the assay due to its high specificity to all *C. difficile*. The assay was performed as follows: 22 µL of the extracted DNA (1.7ng/µl), were placed in a sterile PCR tube. Then, 13 µL of the hybridization buffer were added and mixed well (final concentration of the primer and NaCl after addition of AuNPs were 0.9 µM and 0.04 M, respectively) to obtain a final volume of 35 µl per PCR tube. The mixture was then heated at 95°C for 30 s, and annealed at 50°C for 30 s and then cooled to room temperature for 10 min. 25 µl of colloidal AuNPs were then added to the mixture, and the color was observed within 1 min (Shawky et al., 2010).

Colorimetric AuNPs assay for detection of *C. difficile* toxin B

All *C. difficile* positive samples were further tested for toxin B following similar methods for detection of *C. diff* DNA by AuNPs but using specific toxin B (Ted B) primer (5-CAC GCC TGG AGA ATC TAT ATT TGT AGA AA-3).

RESULTS

Characterization of AuNPs

The SEM image (Figure 2) showed that the synthesized AuNPs prepared in our laboratory were well dispersed and spherical in shape. The extinction spectrum of the prepared AuNPs demonstrated a single peak in the visible region (400 to 700nm) with λ_{max} at 519 to 521 nm (Figure 3). The diameter of AuNPs was found to be 13±1 nm (Figure 4), as characterized by both SEM and Zeta sizer.

C. difficile gold nanoparticles assay prototype

Optimization of the AuNPs-based assay is affected by four main factors namely, concentrations of NaCl, AuNPs size, primer and the assay annealing temperature. In the positive samples, blue color indicates the presence of primers complementary to the *C. difficile* DNA sequence and this leads to the aggregation of AuNPs. On the other hand, red color indicates that the primer is free in the mixture that leads to stabilization of AuNPs and prevents their aggregation. Any minimal change in color from red to blue or purple is considered as a positive result (Figure 5).

Ninety six (96) out of 105 (91.4%) *C. diff* positive samples showed blue and 9 out of 105 (8.6%) showed a red color. The nine negative samples were retested and yielded same results (Figure 6). Reference strain *E. coli* ATCC 25922, *Bacteroides fragilis*, *Peptostreptococcus anaerobious* and *Campylobacter jejuni* tested negvative with our assay as expected (Figure 6B). Additionally, all twenty-nine ATCC strains of *Clostridium* species other than *C. diff* were also tested negative with AuNPs-based assay (red color) (Figures 6A, B and C) indicating high specificity.Control

Assay performance assessment

No cross-reactivity was observed between *C. difficile* and other bacteria tested (Figure 6). The AuNPs-based assay performance was then compared with commercially available RT-PCR (GeneXpert, Cepheid, USA), and yielded 91.4% positivity, all samples were positive with RT-PCR.

Gold nanoparticles assay to detect *C. diff* toxin B

The assay was then optimized to detect toxin B gene(*tcdB*) of *C. difficile*. All ninety-six samples positive

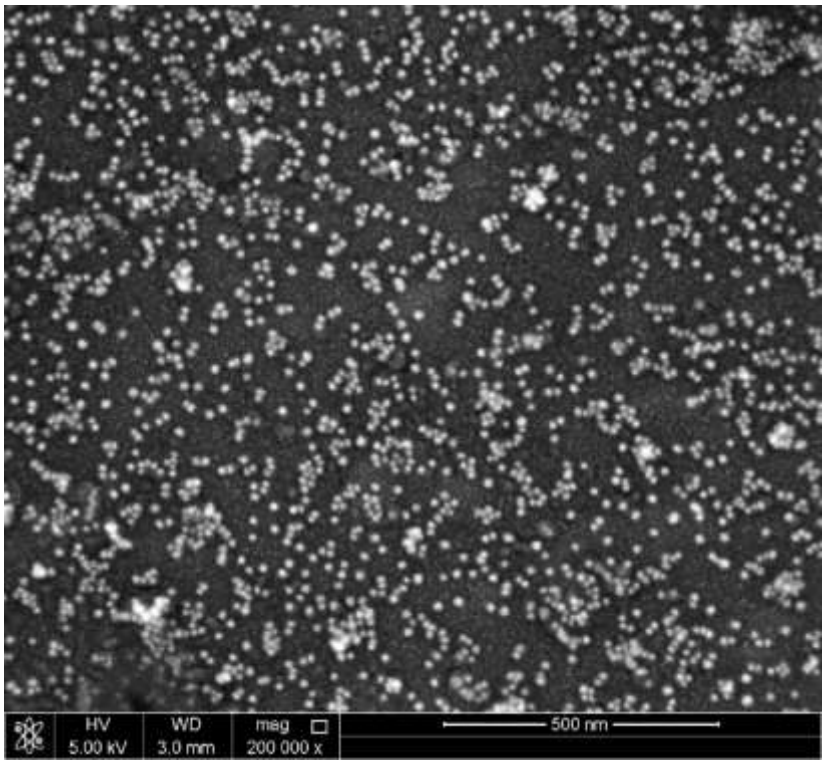

Figure 2. SEM image showing spherical AuNPs, with a size of 13±1 nm, prepared using citrate reduction method. The image was analyzed using the professional image analysis software (Clemex vision P.E 4).

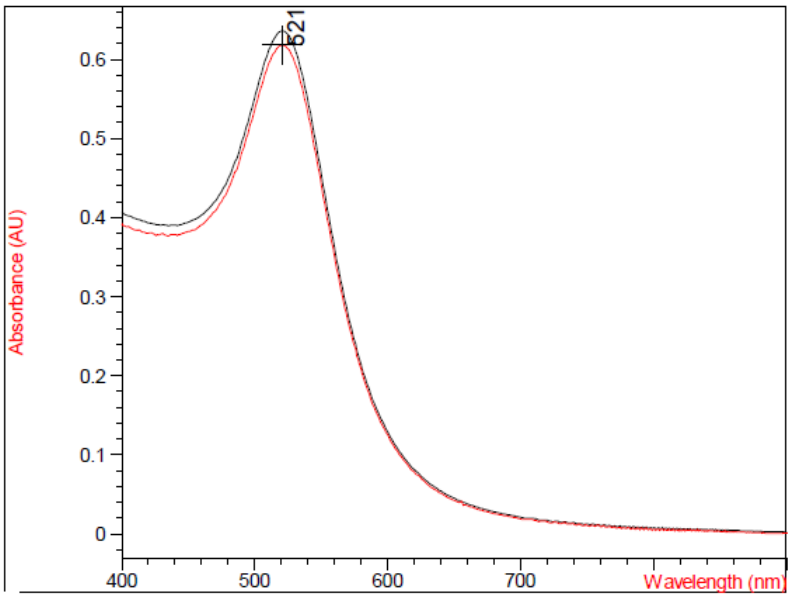

Figure 3. Extinction spectra of the prepared AuNPs solution. The peak is displayed in the visible light region with λ_{max} 518-521 nm.

with *C. difficile* prototype detection assay were also positive for toxin B gene (Figure 7). Equivalent result was obtained when using RT-PCR method. *C. perfringens* ATCC 13124 was used as a negative control in toxin B

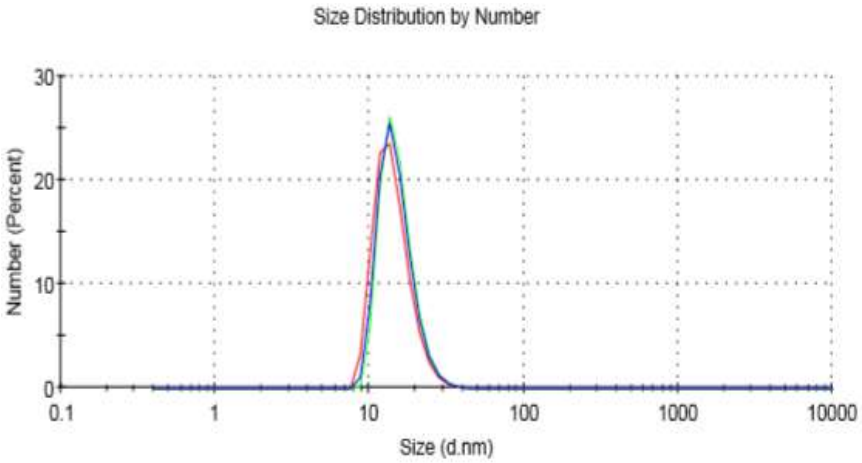

Figure 4. Dynamic light scattering analysis of size distribution of the prepared AuNPs using Zetasizer Nano ZSP (Malvern, UK). The peak represents the number (percent) of AuNPs that have size between 12 and 15 nm.

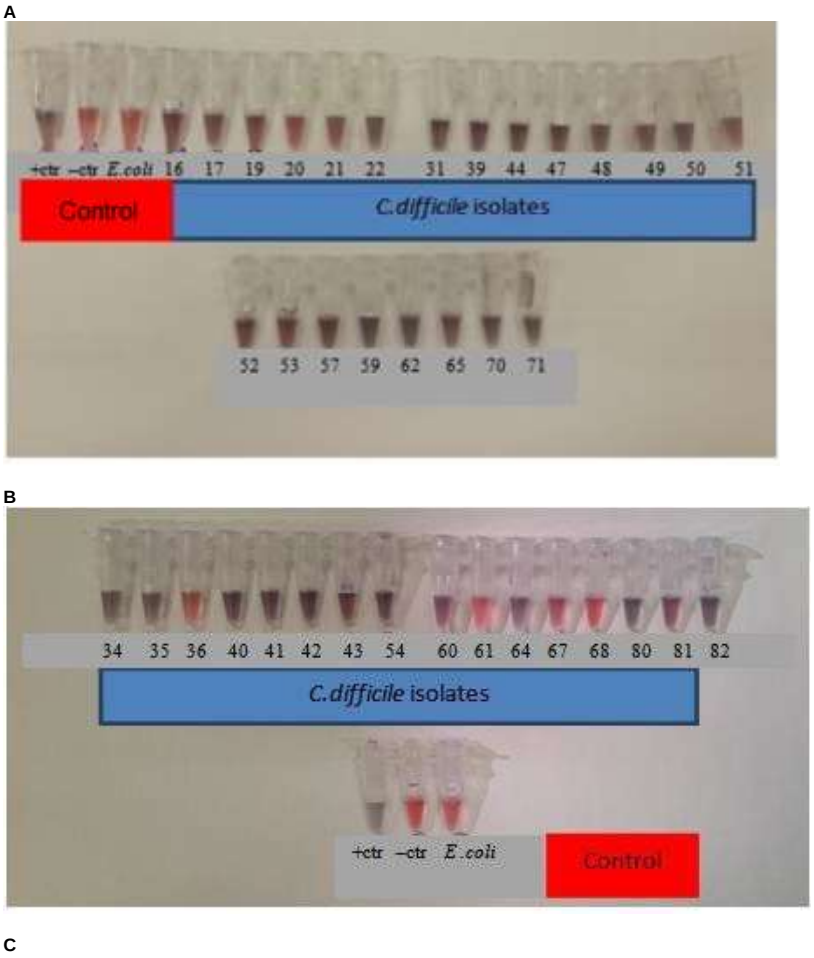

Figure 5. Qualitative AuNPs-based assay for *C. difficile* detection. Positive control (+ctr) = blue, Negative control (-ctr) = red. *E.coli* ATCC 25922 as negative control = red. A) All *C. difficile* samples were positive (change in color from red to blue). B) All *C. difficile* samples were positive (blue color) except 36, 61 and 68. C) Negative samples were retested and yielded the same results.

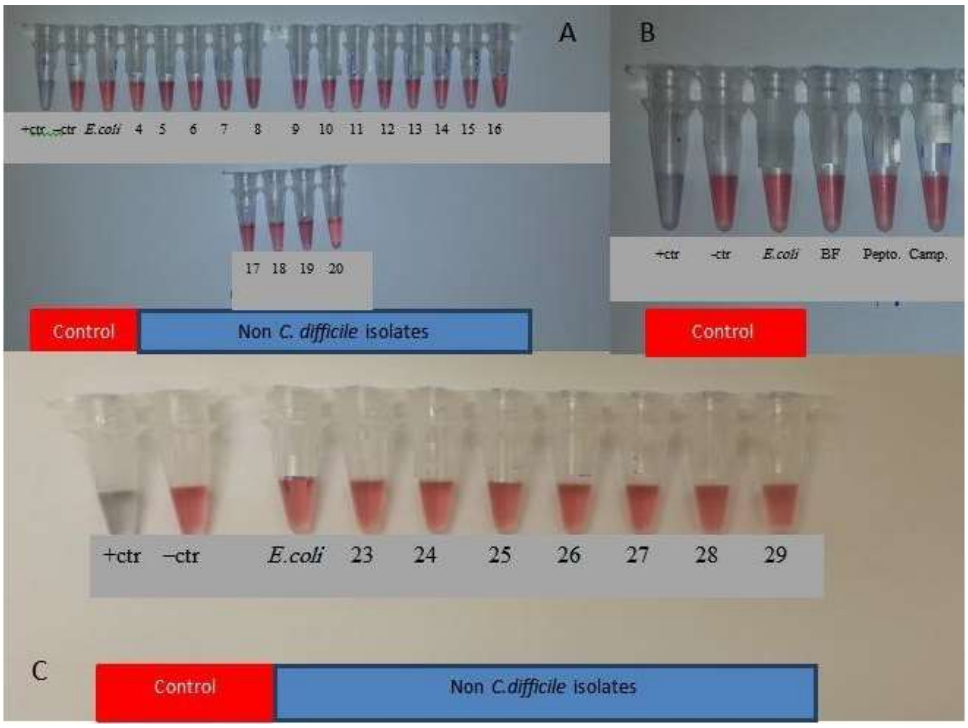

Figure 6. Qualitative AuNPs based assay for isolates other than *C. difficile* and other *Clostridium* species. Positive control (+ctr) = blue, Negative control (-ctr) = red. All samples were negative (red color). *E.coli* ATCC 25922 as negative control = red. BF: *Bacteroides fragilis*, Pepto: *Peptostreptococcus anaerobious* and Camp: *Campylobacter jejuni.*

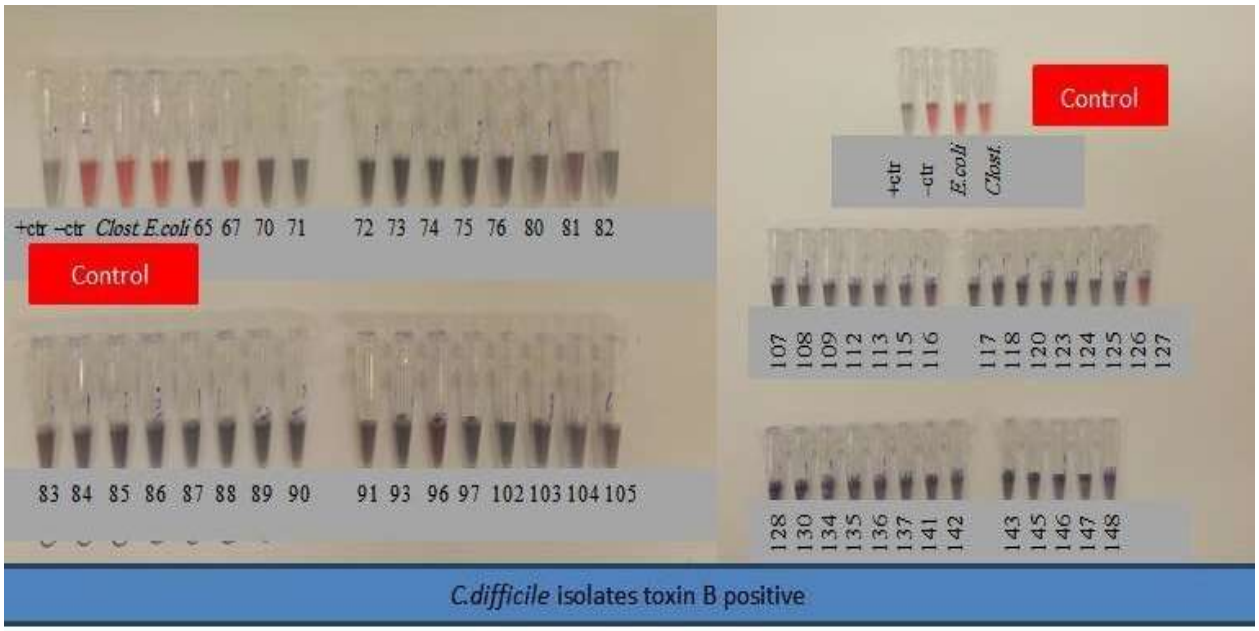

Figure 7. Qualitative AuNPs based assay for *C. difficile* toxin B detection positive control (+ctr) = blue, Negative control (-ctr) = red, Clost.: *C. perfringens* ATCC 13124 and *E. coli* ATCC 25922 as negative control= red. All samples were positive (blue color). AuNP: Gold nanoparticles; +ctr: Positive control; -ctr: Negative control; BF: *Bacteroides fragilis*; Pepto.: *Peptostreptococcus anaerobious*; Camp: *Campylobacter jejuni*; cdtA & cdtB: Binary toxins; Clost.: *Clostridum. Perfringens*; *C. diff*: *Clostridium difficile*

detection with AuNPs.

DISCUSSION

AuNPs-based methods have been established for detection of several pathogenic organisms such as *Mycobacterium tuberculosis* (MTB), Hepatitis C virus (HCV), methicillin resistant *Staphylococcus aureus* (MRSA) and others, owing to the high sensitivity and specificity of AuNPs- based assays for detection of nucleic acid targets (Shawky et al., 2010; Hussain et al., 2013). AuNPs study carried out by Shawky et al. (2010), showed sensitivity and specificity of 93.3 and 88.9%, respectively, in detecting HCV. Another study by Hussain et al. (2013). indicated 96.6% sensitivity and 98.9% specificity for the detection of *M. tuberculosis* complex (MTBC) and 94.7% sensitivity and 99.6% specificity for the detection of MTB. These results are consistent with our findings in this study which, demonstrated 91.4% sensitivity and 100% specificity as compared to RT-PCR. PCR assays include amplification steps and has always been considered more sensitive than enzymetic/cloromertic assays. Additionaly, the lower sensitivity of AuNPs- assay (91.4%) could be clarified by the following reasons, 1) possible loss of the targeted genes due to long cryoperservation period, 2) PCR does not cover the same region used to design the primers for PCR.

Different gold nanoparticles approaches have been explored to detect *C. difficile* and its toxins, such as using an aptamer biosensor with gold nanoparticles synthesized by *Bacillus stearothermophilus* (Luo et al., 2013). Additionally, Zhu et al. (2015) considered the use of single domain antibody coated gold nanoparticles as enhancer for *C. difficile* toxin detection by electro chemical impedance immunosensors. This confirms that using AuNPs with different methodologies resulted in excellent performance with high sensitivity and is in concordance with our findings.

This study revealed that AuNPs-based assay could be used to detect *C. difficile* and *C. difficile* toxin B from unamplified genomic DNA with detection limit of 35.5 ng DNA. The assay was highly sensitive, specific, rapid, simple and minimized the need for expensive and complex equipment. Cost effectiveness is one of the advantages of synthesis of AuNPs that was explored in this study. The approximate cost of 1 g of gold chloride is 220 US Dollars which is enough to conduct 40,000.00 assays, as compared to ~ 55 USD per test for RT-PCR, (GeneXpert, Cepheid, CA, USA). Additionally, the prepared gold nanoparticles have a long shelf life and can be stored at 4°C for one year or more. Moreover, this method has short turnaround time, only 15 to 20 min, after DNA extraction; consequently, the method can be utilized by many laboratories especially in low-income countries with low resources. This developed assay may improve the management of *C. difficile* infection by early isolation of infected patient to prevent horizontal transmission in health care facilities, and may lead to a more rational use of antibiotics, as the clinicians will rapidly obtain the clinical microbiology results.

Conclusions

In conclusion, this assay will have a crucial and great impact on clinical diagnosis in low-resources countries in terms of patient's management and infection control measures. Moreover, the assay can compete with RT-PCR since it has comparable performance results.

ABBREVIATIONS

CDI, *Clostridium difficile* Infection; **AuNPs,** gold nanoparticles; **EIAs,** enzyme immunoassays; **GDH,** glutamate dehydrogenase enzyme; **LSPR,** localized surface plasmon resonance; **SEM,** scanning electron microscope; **RT-PCR,** reverse transcription-polymerase chain reaction.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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