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GOLD NANOPARTICLES-BASED ASSAYS FOR DIRECT AND COST EFFECTIVE DETECTION OF *CLOSTRIDIUM DIFFICILE* IN QATAR

A Thesis in

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By

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ABSTRACT

Background

Clostridium difficile Infection (CDI) is one of the common health problems worldwide. Prevention techniques require fast and precise detection with high sensitivity. Conventional diagnostic methods are time-consuming, costly and inappropriate for clinical field settings. Therefore, the aim of this study is to develop Gold Nanoparticles- based assay (AuNPs) for direct qualitative detection of the nucleic acid of *C.difficile* and its toxins. The proposed assay is expected to be highly sensitive, rapid and simple.

Methods

Total one hundred five *C.difficile* isolates were collected from Al-Khor hospital (a member of Hamad Medical Corporation). Results of Clostridium difficile isolates were confirmed by RT-PCR (GeneXpert, Cepheid, CA, USA). Different concentration of salts and different annealing temperature were all developed and optimized. Extracted DNA, hybridization buffer containing salt and a primer were mixed. The mixture was heated, annealed and then cooled to room temperature for 10 minutes followed by the addition of AuNPs. *C.difficile* toxins were also tested using the same AuNPs optimization.

Results

One hundred five positive *C.difficile* isolates were tested using the optimized AuNPs based-assay. In ninety-six samples out of one hundred five, the color of the solution changed from red to blue within 1 min, which is considered a positive result. On the other hand, there were no color change in nine samples out of 105 and were considered as negative. All Ninety-six positive samples were positive for Toxin B by RT-PCR (GeneXpert, Cepheid, CA, USA) and

AuNPs assay. Six samples were positive for binary toxins by RT-PCR (GeneXpert, Cepheid, CA, USA). However, binary toxins results using AuNPs assay were positive for all samples.

Conclusion

Our study showed a sensitivity of 91.4 % and a specificity of 100%. Furthermore, *C.difficile* toxins were tested using AuNPs, and it showed 100 % agreement with toxin B detection in comparison to RT-PCR (GeneXpert, Cepheid, CA, USA). However, the assay results were not compatible with RT-PCR (GeneXpert, Cepheid, CA, USA) results of Binary toxins. Further work is needed to improve the assay efficiency for detection of Binary Toxins and to validate the assay on clinical samples.

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List of Abbreviations

AAD: Antibiotic Associated Diarrhea

AuNPs: Gold Nanoparticles

CDC: Centre of Disease Control

CDF: *Clostridium difficile*

CDF-F: *Clostridium difficile* Forward primer

CDF-R: *Clostridium difficile* Reverse primer

cdtA: Binary toxin A

cdtB: Binary toxin B

CDI: *C.difficile* Infection

DMSO: Dimethyl Sulfide

DLS: Dynamic Light Scattering

ELISA: Enzyme Linked Immuno Sorbant Assay

EIA: ELISA Immuno Assay

FDA: Food and Drug Administration

FN: False Negative

FP: False Positive

GDH: Glutamate Dehydrogenase

HMC: Hamad Medical Corporation

MRSA: Methicillin Resistant Staphylococcus aureus

NAATs: Nucleic acid amplification techniques

NIBS**:** Non-Invasive Backscatter optics

NaCl: Sodium Chloride

NPV: Negative Predictive Value

PCR: Polymerase Chain Reaction

PBS: Phosphate Buffer Solution

PMC: Pseudomembranous colitis

PPV: Positive Predictive Value

SHEA/IDSA: Society for Healthcare Epidemiology of America and the Infectious Diseases Society of America

SPR: Surface Plasmon Resonance

TcdA: CDF toxin A

TcdB: CDF toxin B

TE: Tris-EDTA Buffer

TN: True Negative

TP: True Positive

+ctr: Positive Control

-ctr: Negative Control

WFI: Water For Injection

WHO: World Health Organizations

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List of Reagents and Kits

- QIAamp DNA mini kit, Qiagen (Cat# 51306)
- Ethanol (96-100%)
- Phosphate Buffer Saline, PBS (1x): 45ml distilled water and add 5 ml PBS
- Promega kit BamHI (REF # R6021)
- 3M Sodium acetate
- Isopropanol
- DNase/ RNase-Free Distilled Water
- Promega PCR Master Mix (REF # M7502)
- *Clostridium difficile* Forward primer (5- GTG CGG CTG GAT CAC CTC CT-3)
- *Clostridium difficile* Reverse primer (5'- CCC TGC ACC CTT AAT AAC TTG ACC-3')
- DMSO (5%): Dimethyl sulfoxide
- Gold nanoparticle solution (AuNPs): Trisodium citrate and $HAuCl_4$
- Different concentrations of Sodium chloride: 0.2M NaCl, 0.5M NaCl, 1M NaCl and 2M NaCl
- Tris (0.1 M): take 200 µl Trizma (Trizma hydrochloride buffer solution) and add 1.8 ml DW to have final volume of 2 ml
- 1:10 buffer (Tris borate) for gel electrophoresis
- 2 % Agarose gel
- *C.difficile* toxin B primer: (5- CAC GCC TGG AGA ATC TAT ATT TGT AGA AA-3)
- *C.difficile* Binary toxin primers:
    1. Binary toxins cdtA (5- ATG CAC AAG ACT TAC AAA GCT ATA GTG-3)

2. Binary toxins cdtB (5- CCA AAA TTT CCA CTT ACT TGT GTT G -3)

- GelPilot R 100 bp Plus Ladder 100 lanes, Qiagen (Cat # 239045)

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Chapter 1- Introduction

Communicable diseases represent one of the main causes of morbidity and mortality in the developing and developed countries with significant financial burden. The huge expansion of the world population and global travel has influenced their spread from one area to another in the world, making them one of the major worldwide health threats. Prevention techniques against those diseases mandate fast and precise detection and identification of the pathogenic organisms with highest sensitivity. Traditional diagnostic methods are time-consuming, costly and inappropriate for field conditions. Nano diagnostic assays have been promising for timely, sensitive, point-of-care and cost-effective detection of microbial agents (Hauck et al., 2010).

Clostridium difficile (CDF) is a significant health problem around the world. The prolonged incubation period of this agent before the development of clinical manifestations, makes the diagnosis and patient management challenging. C*lostridium difficile* is the most significant reason of nosocomial diarrhea (Dalpke et al., 2013). It is responsible for the majority of cases of infectious antibiotic-associated diarrhea (AAD) as well as pseudomembranous colitis that may result in death (Pancholi et al., 2012)

Gold has been an exciting material in nanotechnology and has been discovered to be a crucial diagnostic material. Gold nanoparticles (AuNPs) display a wide range of uses among NPs based assays for microbial detection and identification. Distinctive size-dependent optical properties of AuNPs, their inertness and strength make them one of the most robust materials utilized in Nano diagnostics (Syed, 2014).

Localized Surface Plasmon Resonance (LSPR) is one of the most significant criteria of gold nanoparticles (Au NPs). Because of these natural optical properties, colloidal solutions of Au

NPs have high extinction factors and diverse color in the visible area of the spectrum when they are well-spaced in comparison with when they are aggregated. Accordingly, a generally composed substance connection between the analyte and NPs surroundings prompts a change of color (red to blue from spread out to aggregated ones, respectively) permitting the visual recognition of the target analyte (Vilela et al, 2012). Moreover, this sort of colorimetric assays has established significant consideration in the analytical field on account of their effortlessness and low cost since they do not require any costly or sophisticated instrumentation. As a result of this, recognition of molecules with a high importance in the bio-medical and clinical fields including DNA, proteins and a wide range of organic and inorganic molecules have been impressively reported in the most relevant literature utilizing these assays (Vilela et al., 2012)

Aim & Objectives

The aim of this project is to develop Gold Nanoparticles- based assay for direct qualitative detection of the nucleic acid of CDF and its toxins from leftover preserved isolates. Moreover, to evaluate Sensitivity, Specificity for Gold Nanoparticles in CDF detection compared to RT-PCR (GeneXpert, Cepheid, CA, USA).

Hypothesis

Using Gold Nanoparticles for the detection of CDF will meet demands of the clinical laboratories to improve sensitivity and specificity compared to Real Time- Polymerase Chain Reaction (PCR). The proposed assay is expected to be highly sensitive, rapid, simple and minimize the need for expensive and sophisticated equipment.

Chapter 2- Literature review

2.1. *Clostridium difficile*

Clostridium difficile (CDF) is a Gram-positive, strictly anaerobic, spore-forming bacterium (Chankhamhaengdecha et al., 2013). It was first isolated in 1935 by Hall and O'Toole from newborn feces and meconium and was originally named *Bacillus difficile* because of its morphology and difficulty in cultivation (Burnham & Carroll, 2013). It was considered normal flora until late 1970s where it was recognized to be responsible for most cases of antibiotic-associated diarrhea (AAD) and was quickly expanding in prevalence (Goncalves et al., 2004). *Clostridium difficile* infection (CDI) is a major clinical and infection control issue in numerous health care facilities (Pancholi et al., 2012). This organism is carried asymptomatically in about 50% of neonates, 20% of hospitalized patients and only 2% of healthy adults. In fact, asymptomatic carriers usually exceed symptomatic patients. Therefore, the high level of healthy carriers among hospitalized patients coupled with the presence of patients under antibiotic treatment explains the high rate of nosocomial diarrhea associated with *CDF* (Belanger et al., 2003).

2.1.1. Prevalence and incidence

CDF is responsible for 10%-35% of AAD and nosocomial diarrhea that is associated with high morbidity and mortality and this subsequently leads to health care system burden. For example, the estimated annual cost in the USA is approximately 3.2$ billion where 80% of CDI is a hospital acquired infection (HAI) while 20% is a community-acquired infection (CAI) (Khan et al., 2014).

The prevalence of CDI in Qatar is 7.9%. This prevalence is comparable to data from other Middle-Eastern countries (prevalence range 4.6- 13.7%; average ~8.6%) (Al-Thani et al., 2014) The incidence of CDI in the USA, Canada, and Europe increased among long term and elderly (older than 65 years) hospitalized patients. It has been found that the overall incidence in these countries ranges from 2 to 6 cases per 10,000 patients days (Khan et al., 2014). On the other hand, it has been found that the overall incidence in Qatar was 1.6 cases/10,000 patient days.

2.1.2. Toxins and their contributed genes

CDF produces two major toxins that are toxin A (enterotoxin) and toxin B (cytotoxin). Toxins A and B are glucosyltransferases that are encoded by the genes TcdA and TcdB, respectively, and inactivate Rho- family within target cells. Release of toxins inactivates Rho- family and other GTPases, affecting their interactions with regulatory molecules and interrupting vital signaling pathways. Cells round up, shrink, and die, leading to significant loss of the intestinal epithelial barrier, and tight junctions are disrupted, permitting neutrophil migration. In addition, both toxins stimulate the release of pro-inflammatory cytokines such as interleukin-1 (IL-1), tumor necrosis factor-alpha (TNF), and IL-8 from activated macrophages. This subsequently leads to neutrophil recruitment, stimulating an inflammatory response; neutrophil aggregation is responsible for the pseudomembrane formation seen in severe colitis (PMC) (Burnham et al., 2013).

CDF toxin A and toxin B are located with three more genes (TcdC, TcdR, TcdD) on the *CDF* chromosome in a 19.6-kb pathogenicity locus (PaLoc) only present in pathogenic strains (Persson et al., 2011). TcdC and TcdD encode negative and positive regulators respectively that control the level of toxin production and TcdE facilitate toxin release from the bacterial cell wall (Luna et al., 2011). Most pathogenic strains are toxin A-positive, toxin B-positive (A+B+) strains

although toxin A-negative, toxin B-positive (A-B+) variant isolates have been emerged as pathogenic strain according to the literature (Sambol et al., 2000). Diverse genetic alterations in the TcdC gene have been observed. Most prominent are the in-frame deletion of 18, 39, or 54 bp and the mutation at position 117 (1-bp deletion) (Persson et al., 2011). Some strains of *CDF* also secrete an actin-specific ADP-ribosyltransferase called CDT or binary toxin. These binary toxins are encoded by two genes (*cdtA* and *cdtB*) and are located outside the PaLoc (Goncalves et al., 2004).

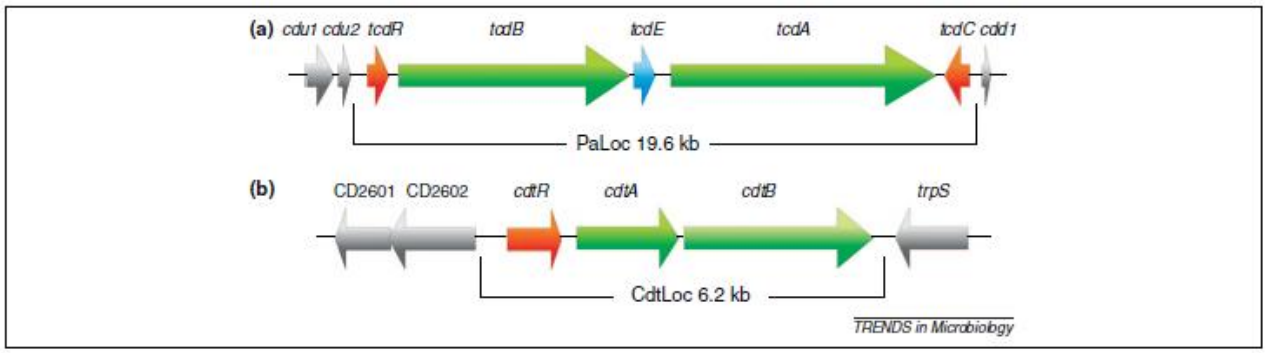

Figure 2.1.1: Genetic map of toxin loci in *Clostridium difficile*. (a) Structure of the pathogenicity locus (PaLoc). Toxin genes are shaded in green, regulatory genes are in red, tcdE is in blue. (b) Structure of CdtLoc (binary toxin locus). Toxin and regulatory genes are shaded in green and red, respectively.

2.1.3. Pathophysiology

CDF is a major nosocomial pathogen causing CDI and life-threatening PMC (Eastwood et al., 2009). The pathogenic effects of *CDF* are mucosal damage to the colon that is caused by toxin A and toxin B (Sambol et al., 2000). Mature colonic bacterial flora in a healthy adult is resistant to *CDF* colonization. However, if the normal colonic flora is altered due to antibiotic as an example, resistance to colonization is lost (Eastwood et al., 2009). Following the colonization, an enterotoxin, TcdA, which is found in ~70% of *C.difficile* strains and a cytotoxin, TcdB, which is found in all *CDF* strains, can be produced, thereby disrupting tight junctions of the intestinal epithelial cells resulting in inflammation and increased permeability of the intestine. Once spores

are ingested via contact or environment, their acid-resistance allows them to pass through the stomach intact, grow and multiply into vegetative cells in the colon upon exposure to bile acids. This cause colonization in immunosuppressed patients and production of toxins (Carter et al., 2007). As a results of these effects, polymorph nuclear neutrophils (PMNs) are recruited to the site of toxin action and lead to PMC (Voth & Ballard, 2005). Approximately less than 10% of clinical *CDF* isolates possess binary toxins (cdtA/B), which have been associated with increased severity of the symptoms. The pathogenic role of *cdtA* (enzymatic component) and *cdtB* (Binding component) has been suggested to trigger microtubule protrusion, thereby increasing the adherence of *CDF* to the gut epithelium (Chankhamhaengdecha et al., 2013).

2.1.4. Clinical features

CDF causes a spectrum of clinical presentation ranges from mild, self-limiting diarrhea to serious Pseudomembranous Colitis (PMC) and toxic megacolon, leading to colonic perforation, peritonitis, and even death. Symptoms occur secondary to the production of two major toxins, toxin A and toxin B, which affect the integrity of the colonic mucosa (Tenover et al., 2010). The Society for Healthcare Epidemiology of America and the Infectious Diseases Society of America (SHEA/IDSA) guidelines define severe disease as colitis associated with a leukocyte count that is 15,000 cells/ L or higher. Other characteristics include markedly elevated temperature reaching 40°C, PMC, and hypoalbuminemia (serum albumin level of 2.5 mg/dl). Fulminant *C. difficile* occurs in 5% of patients and is characterized by severe abdominal pain, profuse diarrhea, or sometimes no diarrhea, as the patient rapidly progresses to development of an ileus or toxic megacolon (Burnham et al., 2013).

2.1.5. Diagnosis

The diagnosis if CDI is based on the clinical features, Laboratory confirmation for the presence of toxins in stool and sometimes endoscopy to verify PMC (Sambol et al., 2000). Since CDI rate is increasing rapidly in health care facilities and to implement timely infection control measures and appropriate patient management, a rapid and reliable identification of toxigenic CDF is necessary. However, laboratory diagnostics remain challenging, as rapid test procedures relying on enzyme immunoassays (EIAs) show limited sensitivity, whereas the more-sensitive (94%-100%) and more accurate (99%-100%) toxigenic culture and cytotoxicity assays which are considered as "gold standard" are time-consuming (long turnaround time), high cost and requires tissue culture facilities (Dalpke et al., 2013). EIA for testing TcdB alone or both TcdA/B offer a simple, rapid turnaround time (TAT) compared to conventional methods, tests for which the time to the final result can be 2 to 6 h. However, EIA is associated with reduced sensitivity (65%-85%) and specificity (95% - 100%), with performance largely dependent on which reference method is used for comparison, making its reliability questionable for an accurate diagnosis of CDI (Pancholi et al., 2012). Enzyme immunoassay (EIA) for CDF Glutamate dehydrogenase (GDH) antigen is used to detect the presence of the enzyme GDH, which is produced by all strains of CDF isolates (toxigenic and non-toxigenic). This method is highly sensitive (75%-90%) with high Negative Predictive Value (NPV) (95%-100%) but is not specific (≤ 50%) with low Positive Predictive Value (PPV) for toxigenic isolates; therefore, a 2-step method has been recommended by the Infectious Diseases Society of America/ Society for Healthcare of America guidelines (SHEA/IDSA) on diagnostic testing of CDF. This strategy uses GDH and then uses RT-PCR as the confirmatory test for GDH-positive stool samples. Recently, Nucleic acid amplification techniques (NAATs) for CDF testing have been developed to combine low

turnaround times with high sensitivity, nevertheless they are expensive, require skilled personnel and the platforms and ease of use vary considerably, fully automated PCR assays that combine nucleic acid extraction, amplification, and detection have been developed. Currently, there are a number of FDA-approved commercially available NAATs including (i) the Xpert *C.difficle*, (ii) the illumigene *C.difficile* assay (Dalpke et al., 2013). These methods have high sensitivity (88%-100%) and high specificity (96%- 100%). These assays detect conserved regions of toxin A (*tcdA*) or B (*tcdB*) genes located on the pathogenicity locus (PaLoc) of *C. difficile*. For epidemiological studies, positive *C. difficile* isolates are further analyzed by PFGE, PCR-ribotyping, and/or direct sequencing of the *tcdC* gene to detect the 18-bp or nt 117 deletions (Pancholi et al., 2012).

Prior to performing Laboratory tests, abdominal imaging studies, including CT scans, may reveal "thumbprinting" of colonic mucosa, which suggests the presence of mucosal edema, but these changes are not specific for PMC due to CDF. Direct visualization of colonic mucosa using either sigmoidoscopy or colonoscopy is required to determine the presence of PMC. However, *CDF* colitis or diarrhea may occur without pseudomembrane formation, and colitis may be missed if only proximal disease is present. In general, sigmoidoscopy and colonoscopy should be avoided in fulminant colitis because of the risk of toxic megacolon and perforation (Sambol et al., 2000)

Table 2.1.1: Laboratory diagnostic tests used for *C.difficile* detection. Different methods vary in sensitivity, specificity and turnaround time (Khan et al., 2014)

Laboratory test	Sensitivity (%)	Specificity (%)	Turnaround time (TAT)
Tissue culture cytotoxicity assay	94-100	99-100	1-3 days
Glutamate dehydrogenase enzyme assay	75-90	<50	15-45 minutes
Enzyme immunoassay for C.difficile toxin	65-85	95-100	2-6 hours
RT-PCR (Cepheid GeneXpert)	88-100	96-100	45 minutes- 1 hour
Anaerobic culture of stool	89-100	48-68	2-3 days

2.1.6. Treatment

CDI treatment often involves the first line treatment that is discontinuation of antibiotics if deemed to be medically appropriate and providing appropriate supportive care with hydration and electrolyte replacement. For mild disease, this is often sufficient for full recovery. For more severe disease, antimicrobial therapy directed against CDF is essential. Empirical treatment with oral metronidazole for two weeks is suggested. Vancomycin for two weeks as well is the recommended second-line treatment.

2.1.7. Preventive measures

Prevention of CDI is challenging health authorities. However, preventive measures are taken such as implementation of infection-control measures (careful attention to hand washing, Patient's isolation, barrier precautions (PPE), and cleaning of the physical environment with 10% hypochlorite solution throughout the duration of symptomatic disease (Okada, 2010). Because CDF spores may be, relatively resistant to alcohol, current and comprehensive guidelines recommend that health care workers wash their hands with soap and water. Strategies aimed at preventing the development of *CDF* diarrhea include antibiotic restriction, the use of probiotics

and passive and active immunization (Halsey, 2008). A multidisciplinary antibiotic management program to restrict the inappropriate use of antibiotics can lead to a significant decrease in HAI caused by CDF.

2.2. Gold Nanoparticles (AuNPs):

Nanotechnology has been promising for the development of rapid, accurate and cost-effective tool for the next generation of diagnostic assays with high sensitivity (Kaittanis et al., 2012). There are many types of Nanoparticles (e.g., Ag, ZnO, Co etc.) considered toxic to human and not used for *in vivo* applications. Nevertheless, Gold has low toxicity and is safe to be used in *in vivo* applications (Syed et al., 2011). The use of AuNPs to label with DNA was first discovered by Mirikin et al. in 1996. He added thiol group (-SH) to one end of the DNA probe since it has a strong affinity to gold. In 2009, Liandris et al. have designed AuNPs based assay for Mycobacterium DNA detection. In 2010, Uludag and coworkers had developed a biosensor for Herpes Simplex 1 virus (HSV-1) and DNA probe conjugated with AuNPs was used to hybridize with the target DNA.

Because of AuNPs unique optical and physiochemical properties, they have many applications in medicine, material sciences, imaging, therapeutics as well as diagnostics (Cai et al., 2008). Gold nanoparticles (AuNPs) exhibit a broad spectrum of applications among NP based assays for microbial detection and identification and among the most promising nanoparticles. They are gold spheres with a typical diameter of approximately 2- 100 nm. Gold nanoparticles (AuNPs) exhibit a unique phenomenon known as Localized Surface Plasmon Resonance (LSPR), which is responsible for their intense red color. This color changes to blue upon aggregation of AuNPs that are easily detected visually without the aid of any instrumentation. The addition of salt 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 red-to-blue color change (Syed, 2014). The above mentioned unique optical properties have allowed the use of AuNPs in simple and rapid colorimetric assays for clinical diagnosis that can offer simpler, faster, cheaper and reliable detection techniques for CDF. Development of such tests would support global, regional efforts to control CDF in developing countries with limited resources and high infection rates (Syed, 2014).

The principle of this assay is that citrate-coated AuNPs possess a surface negative charge which allows the adsorption of single-stranded DNA (ssDNA), which can uncoil and expose their nitrogenous bases, allowing electrostatic attraction to the AuNPs surface. Consequently, the negative charge on the AuNPs increases and so does the repulsion between the AuNPs, thus preventing their aggregation. Upon addition of AuNPs to a saline solution containing the target nucleic acid and its complementary target, and this double-stranded DNA structure cannot adsorb on AuNPs due to the repulsion between its negatively-charged phosphate backbone and the negatively-charged coating of citrate ions on the surface of the AuNPs. In this situation, primers are not free to stabilize the AuNPs and the solution color changes to blue, due to aggregation of AuNPs. On the other hand, in the absence of the target or the presence of a non-complementary target, the aggregation of the AuNPs is prevented due to the presence of free primers to stabilize them, and solution color remains red (Figure 2.2.1).

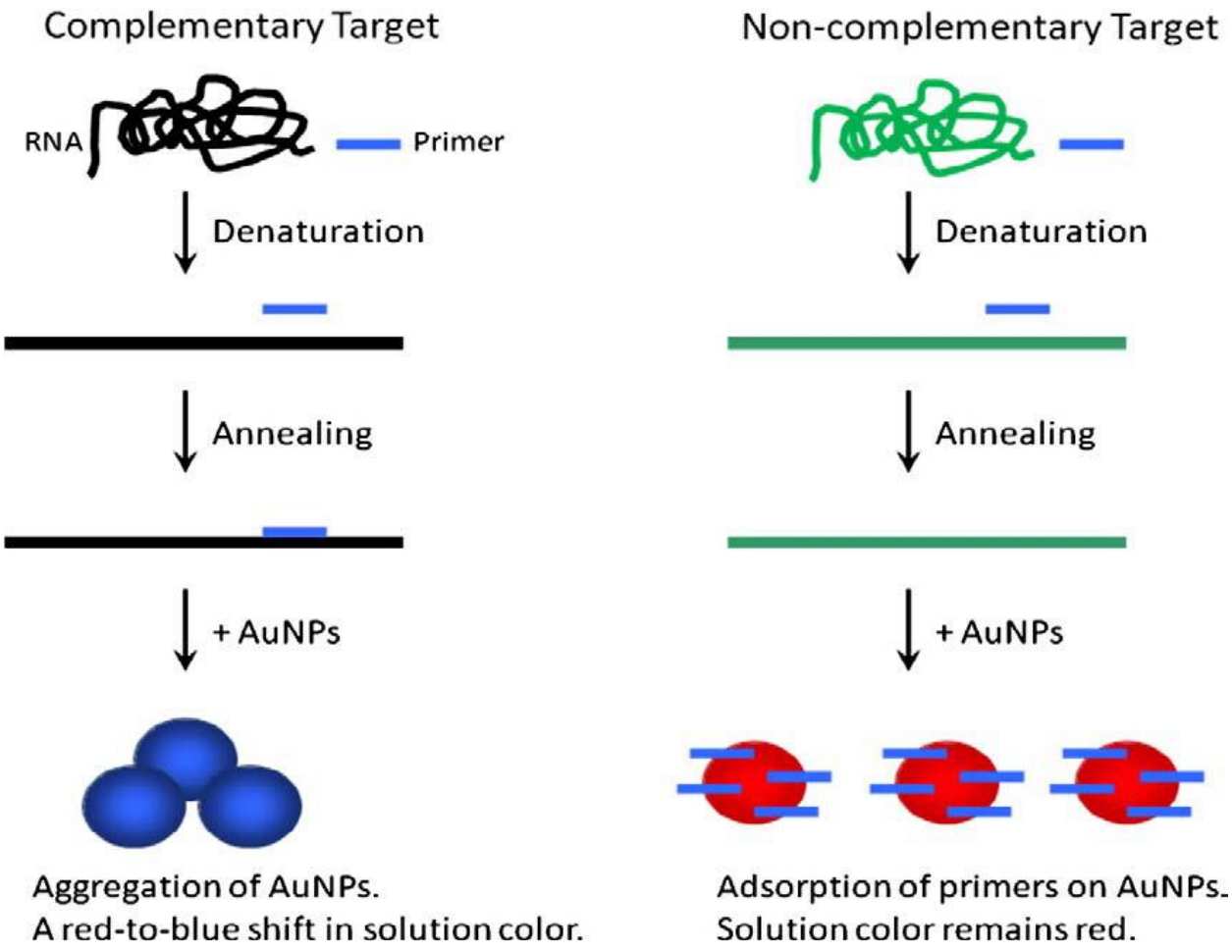

Figure 2.2.1: Principle of colorimetric AuNPs-based assay in microbial identification. If the primer is complementary to the DNA/RNA target, there will be no free primers in the mixture leading to aggregation of AuNPs and blue color formation. On the other hand, if primer is not complementary to the target, it will be free in the mixture and binds to AuNPs preventing aggregation and the color remains unchanged (Shawky et al., 2010)

Several studies had been published on the application of AuNPs in microbial detection. One study was conducted and published by Shawky et al., 2010 in the detection of Hepatitis C virus (HCV). Another study using AuNPs in the detection of *Mycobacterium Tuberculosis* Complex (MTBC) was published by Hussein et al., 2013. Recent studies in *Acinetobacter baumanni* identification also showed a high sensitive and accurate results compared to conventional biochemical methods and PCR (Khalil et al., 2014). Furthermore, a study was done on the identification of *E.coli* using AuNPs oligo-prob principle, and it was found that it is highly sensitive and specific (Padmavathy et al., 2012). Several studies on Intestinal organisms detection using Gold nanoparticles has been published. For example, In 2008, Wang and his team develop a quartz crystal microbalance (QCM) biosensor for real-time detection of *E. coli* O157:H7 DNA based on nanogold particles amplification and results showed that this developed biosensor enhance the detection of *E.coli* O157:H7 compared to conventional method (Wang et al., 2008). *Staphylococcus aureus* is also one of the most important human pathogens, causing more than 500,000 infections in the US each year. By using aptamers that specifically recognize *S. aureus*, Chang et al. (2013) developed an ultrasensitive aptamer-conjugated AuNPs for rapid bacterial detection. Their non-polymerase chain reaction (PCR)-based method measures the resonance light-scattering signal of aptamer-conjugated AuNPs to detect a single cell within 1.5 h. Accordingly to the authors this platform technology has the potential to develop a rapid and sensitive bacterial testing at point-of-care (Veigas et al., 2014). This new assay also had been currently developed and validated to provide faster and at a low cost diagnosis of resistant pathogens (MRSA, MDR-TB & XRD-TB) comparing to conventional culture and drug susceptibility tests (Veigas et al., 2014).

Intestinal pathogens detection were developed using Gold nanoparticles to detect *salmonella* infection. It was conducted by Kalidasan et al., 2013 and showed that it is promising and sensitive method. One study related to *C.difficile* detection in 2013 in which Luo et al. had developed an aptamer biosensor for the detection of toxin A of *Clostridium difficile* using gold nanoparticles synthesized by *Bacillus stearothermophilus.* The results of the study showed good sensitivity in the detection of toxin A as well as good selectivity, stability ad recovery rate (Luo et al., 2014). Furthermore, many examples of different approaches of AuNPs-based assays for microbial detection and identification were discovered and evaluated. One of these methods is immunochromatographic strips. The principle of this assay is that gold is conjugated with antibodies impregnated in membrane chromatography. They are commercially available and have been developed to detect several viruses, bacteria, fungi and parasites (Syed et al., 2011). Moreover, AuNPs had been integrated in many assays to enhance sensitivity and specificity for example, Bio Barcode Based (BBB) Assays for Microbial DNA Detection (Syed et al., 2011).

Chapter 3: Material and Methods

Ethical consideration

This study was approved by Hamad Medical Corporations (HMC) Research Office, Doha, Qatar, Research Protocol #11136/11: "NPRP-4-1215-3-317 Gold Nanoparticles-based Assays for Direct and Cost Effective Detection of High Burden Diseases." See Appendix (1)

Collection of bacterial isolates:

Leftover CDF isolates cultured stool samples originally provided for regular clinical analysis were collected from Hamad Medical Corporation- Al Khor Hospital in Doha, Qatar, during the period of 2011 to 2012. One hundred forty-eight (148) Cryopreserved *Clostridium difficile* isolates were revived and sub-cultured onto Blood Agar enrichment medium and incubated anaerobically. Forty-three (43) CDF isolates were excluded from the study either due to duplication or failure of the strains to grow. One hundred five (105) samples were transported to QU (Health Science Department- Biomedical Research Center) in an icebox for extraction and testing with Gold Nanoparticles based assay. The isolated CDF were confirmed previously using RT-Polymerase Chain Reaction (PCR) (GeneXpert, Cepheid, CA, USA). Another method is the morphology of feathery spreading colonies on enrichment media, which is the characteristic feature of CDF. According to PCR results, all CDF isolates were positive for toxin B and 6 were positive for Binary toxin along with toxin B. 29 additional ATCC Bacterial Strains other than CDF and *Clostridium* species were used to assess specificity performance and cross-reaction.

DNA Extraction:

Genomic DNA from bacterial cultures (Sheep Blood Agar plate cultures) was extracted using QIAamp DNA Mini Kit (Qiagen; Cat. No.51306) according to manufacturer's instructions. Briefly, culture cells was suspended in 180 µl of Buffer ATL (Supplied in the QIAamp DNA Mini Kit) into 1.5 ml centrifuge tubes by vigorous stirring and then 20µl of Proteinase K was added and mixed by vortexing, then was incubated at 56^0C. The tube was centrifuged to remove drops from the inside of the lid. After that, four µl of RNase A (100 mg/ml) was added and mixed by pulse-vortexing for about 15 seconds, incubation was done for 2 minutes at room temperature and then centrifuged briefly. After that 200 µl Buffer AL was added to the sample, mixed again by pulse-vortexing for 15 seconds and incubated at 70^0C for 10 minutes. The tube was centrifuged briefly to remove drops from inside the lid. After that, 200 µl ethanol (96-100%) was added to the sample and mixed by pulse-vortexing for 15 seconds. After mixing, the tube was centrifuged briefly to remove drops from inside the lid. The lysate (including the precipitate) was transferred carefully onto QIAamp Spin Column without wetting the rim of this column that contain a filter to remove all the debris except the DNA of the bacteria. Centrifuge at 6000 x g (8000 rpm) for 1 minute was done, and the QIAamp Spin Column was transferred to a clean 2 ml collection tube, the collection tube containing the filtrate was discarded. Then 500 µl Buffer AW1 was added, centrifugation at 6000 x g (8000 rpm) for 1 min was done and the QIAamp Spin Column was transferred to a clean 2 ml collection tube, the collection tube containing filtrate was discarded. Later 500 µl of AW2 buffer was added, centrifuge at full speed 12000 x g (14000 rpm) for 3 min to dry the column. Next, the QIAmp Spin Column was transferred to a clean 1.5 ml microcentrifuge tube; the collection tube containing filtrate was discarded. Finally, centrifuge at 6000 x g (8000 rpm) for 1 min was done.

Measurement of DNA concentration using Spectrophotometer:

All the extracted DNA samples were measured for DNA concentration and purity by spectrophotometer technique using Infinite F200 PRO (TECAN) and 260/280 ratios were calculated automatically (data not shown).

DNA Restriction using Bam HI

Extracted DNA was restricted using Promega kit. In 1.5 ml microcentrifuge tube, a mixture was prepared by adding 2 µl Buffer (10x), 0.2 µl Acetylated BSA, 10 µl DNA Samples and 0.5 µl enzyme (Bam HI). To adjust the final volume to 20 µl 7.3 µl of free DNase water was added. Mixing gently by pipetting was done; the tube was centrifuged for a few seconds in a microcentrifuge, and then incubated at 37^0C for 1 hour and at 65^0C for 15 minutes.

Restricted DNA Precipitation

For DNA precipitation; 2 µl of (3M sodium acetate) was added to 20 µl of restricted DNA; and 22 µl of isopropanol. Then the mixture was incubated for 30 minutes in the freezer and centrifuged at 13000 rpm for 30minutes. After that, the supernatant was discarded carefully, and 20 µl of Nuclease-Free water was added. Finally, the DNA is either stored at -20^0C or at -80^0C for long term storage.

Amplification of *Clostridium difficile* by PCR:

To detect the CDF DNA in preserved isolates, we have carried out the conventional PCR assay as follows: 12.5 µl PCR Master Mix was combined with 1.25 µl of each forward CD-F (5'- GTG CGG CTG GAT CAC CTC CT- 3') and reverse CD-R (5'- CCC TGC ACC CTT AAT AAC TTG ACC- 3') primers. Then 2.5 µl of precipitated DNA template and 1.25 µl Dimethyl sulfoxide (DMSO, Promega) were added. Finally, the mixture was made up to 25 µl volume with Nuclease-Free water (6.25 µl). The PCR amplification was initiated at 95^0C for 2 minutes and

completed by 30 amplification cycles (denaturation at 95^0C for 30 seconds, annealing at 50^0C for 30 seconds and extension at 72^0C for 0.45 seconds and final elongation at 72^0C for 2 minutes). PCR amplification was carried out in ABI 9700 GeneAmp PCR System (Applied Biosystems)

Gel Electrophoresis (GE):

To confirm the quality of *C.difficile* DNA, Gel electrophoresis was run. First 1:10 buffer (Tris borate) was prepared by taking 100 ml of Tris/ borate buffer and dilute with distilled water to have a final volume of 1000 ml. After that 2 g of agarose gel was dissolved in 100 ml diluted buffer and kept in the oven until completely dissolved. Then was left to cool down at room temperature and 2µl ethidium bromide was added. CDF DNA Samples (10 µl after mixing with 2 µl dye) were loaded as well as a ladder (6 µl of 100 bp) at 100 voltage for 45 minutes. Then the result was read by gel imaging device using (BIO-RAD).

Synthesis of AuNPs:

Spherical AuNPs were prepared by citrate reduction of hydrogen tetrachloroaurate (III) ($HAuCl_4.3H_2O$). Briefly, the reflux system was cleaned by aqua regia (1 Nitric Acid: 3 HCL) and then rinsed with ultrapure water, and blown out with N_2. First working solution was prepared as following: 1 mM Gold Chloride (20 ml from 10mM stock to 200 ml de-ionized water) and 38.8 mM Sodium Citrate (9.7 ml from 200mM stock to 50 ml de-ionized water). An aqueous solution of 50 ml of 1 Mm $HAuCl_4.3H_2O$ was brought to reflux while stirring. Then cover the Gold Chloride containing beaker with aluminum foil and place in the sand bath so that it's fully immersed in sand. Later place 10 ml of 38.8mM sodium citrate in a 15 ml falcon tube and place it in a water-filled beaker, in the sand bath. When the Gold-Chloride starts to boil, 10 ml sodium citrate (1% trisodium citrate) was added quickly which resulted in a change in solution color from yellow to clear to black to purple to dark red. Afterward, the solution will be refluxed for an

additional 15 minutes and then allowed to cool to room temperature. The colloidal solution will then transferred to a clean storage bottle. The concentration of the trisodium acetate in the reaction will determine the final AuNPs size i.e. as the concentration of sodium acetate decreases, the AuNPs size increases. See Appendix (II)

Au weight (?) = $\frac{\text{MW x 0.001 x 50ml}}{1000} = \frac{393.83\text{g x 0.001 x 50ml}}{1000}$ = 0.0197g = 19.7mg in 50ml

Characterization of AuNPs:

The absorbance of the prepared AuNPs solution was measured by spectrophotometry. The recommended AuNPs size ranges from 12-15 nm with the absorbance of the visible range 400-700nm (Shawky et al., 2010) (Hussain et al., 2013). The AuNPs average size was measured by Zetasizer Nano ZSP (Malvern, UK). It is used for the characterization of proteins and nanoparticles. The system incorporates a two angle particle and molecular size analyzer for the enhanced detection of aggregates and measurement of small or dilute samples, and samples at very low or high concentration using dynamic light scattering with 'NIBS' optics. Dynamic Light Scattering (DLS) is used to measure particle and molecule size. DLS measures the diffusion of particles moving under Brownian motion, and converts this to size and a size distribution using the Stokes-Einstein relationship. Non-Invasive Back Scatter technology (NIBS) is incorporated to give the highest sensitivity simultaneously with the highest size and concentration range. Brownian motion principle suggests that the movement of particles is due to the random collision with the molecules of the liquid that surrounds the particle. The ZSP also incorporates a zeta potential analyzer that uses electrophoretic light scattering for particles, molecules, and surfaces.

Colorimetric AuNPs assay for detection of CDF DNA: development and optimization

Optimization of Colorimetric Qualitative AuNPs Assays for *C. difficile* was done through optimization 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 (Appendix IV). Hybridization buffer was prepared using 0.50 M NaCl and 10 μM primer. Different volumes of the AuNPs were tested (data not shown), and 25 μL of the prepared AuNPs (12-15 nm) was selected for use in the final assay. As for the primer used in the assay, reverse CD-R (5'- CCC TGC ACC CTT AAT AAC TTG ACC- 3') was used due to its high specificity to all CDF. The assay was performed as follows, 22 μL of the extracted DNA were placed in a sterile PCR tube and 13 μL of the hybridization buffer (4.8 ul NaCl + 5.4 ul primer + 2.8 ul Nuclease-Free Water) were added and mixed well (final concentration of the primer and NaCl after addition of AuNPs was 0.9 μM and 0.04 M, respectively) to have a final concentration of 35 ul 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). Positive, negative control and *E.coli* were run with every run. Positive control tube contains (4.8 ul NaCl + 30.2 ul Nuclease-Free Water) while negative control tube contains (4.8 ul NaCl + 5.4 ul primer + 24.8 ul Nuclease-Free Water).

While cell culture cytotoxicity assays have been considered the "gold standard" historically, there is no currently universally agreed upon gold standard for toxigenic *C. difficile* detection. In this study, we consider RT-PCR (GeneXpert, Cepheid, CA, USA) as our "reference method"

since it is the method used in the clinical laboratory in Qatar as confirmatory assay for CDF detection.

Assay performance assessment

The following performance parameters of the developed AuNPs assays will be determined then compared to (RT-PCR) (GeneXpert, Cepheid, CA, USA) to evaluate the developed assays:

1. **Specificity**

   Using the following formula

   Specificity= True negative (TN)/ True negative (TN) + False positive (FP) X 100

$$\frac{TN}{TN + FP} \times 100$$

To measure specificity, a total of 29 enteric bacterial organisms, consisting of ATCC organisms (*Bacteroides fragilis* ATCC 25285; *Campylobacter jejuni* ATCC 33291; *Clostridium perfringens* ATCC 13124; *Escherichia coli* ATCC 35218, ATCC 25922, and O157: H7 ATCC 35150; *Enterococcus faecalis* ATCC 29212; *Enterobacter aerogenes* ATCC 13048; *Klebsiella pneumoniae* ATCC 700603; *Peptostreptococcus anaerobius* ATCC 27337; *Proteus mirabilis* ATCC 12453; *Proteus vulgaris* ATCC 13315; *Pseudomonas aeruginosa* ATCC 27853; and *Salmonella typhi* ATCC 14028, *Shigella flexeneri* ATCC 12022, *Brevibacillus agri* ATCC 51663, *Enteropathogenic E.coli* type (2, 3 & 4), *Hafnia* species, *Yersinia enterocolitica* ATCC 27729, *Stenotrophomonas maltophilia* ATCC 51331, *Vibrio cholera*, *Candida albicans* ATCC 90028 , *Staph.aureus* ATCC 29213, *Staph. epidermidis* ATCC 12228, *Staphylococcus saprophyticus* ATCC 15305, *Streptococcus pyogenes* ATCC 19615 and *Streptococcus agalactiae* ATCC 12386 were individually analyzed by TaqMan PCR. In brief, each organism was cultured into Blood agar medium, and Genomic DNA was extracted from isolated colonies, restricted, precipitated, PCR and GE. DNA purity and quantity were measured by absorbance

spectrophotometry. In specificity formula, True negative samples are those which are negative with RT-PCR (GeneXpert, Cepheid, CA, USA and AuNPs-based assay. While False positive samples are those which are negative by RT-PCR (GeneXpert, Cepheid, CA, USA) and positive by AuNPs-based assay

2. **Sensitivity**

   Using the following formulas:

   Sensitivity= True positive (TP)/ True positive (TP) + False negative (FN) X 100

$$\frac{TP}{TP + FN} \times 100$$

To measure Sensitivity, the results of AuNPs based assay were compared with RT-PCR (GeneXpert, Cepheid, CA, USA) which is considered in this study as "Gold standard" since it is the method used in Clinical Microbiology Laboratory for C.difficile detection with high sensitivity and specificity. In sensitivity formula, True positive samples are those which are positive with RT-PCR (GeneXpert, Cepheid, CA, USA) and AuNPs-based assay. While False negative samples are those which are positive by RT-PCR(GeneXpert, Cepheid, CA, USA) and negative by AuNPs-based assay

Gold Nanoparticles assay to detect CDF toxins:

CDF isolates were used to detect tcdB, as well as cdtA and cdtB genes. Same optimization was used but with Toxin B (Tcd B) primer (5- CAC GCC TGG AGA ATC TAT ATT TGT AGA AA-3) and binary toxins (cdtA & cdtB) primers cdtA (5- ATG CAC AAG ACT TAC AAA GCT ATA GTG-3) & cdtB (5- CCA AAA TTT CCA CTT ACT TGT GTT G -3) to detect different CDF toxins.

Statistical analysis

Excel program was used for simple calculations (Frequencies, percentage, mean and standard deviation of the age). Sample characteristics including age, gender, clinical findings and nationality were summarized using frequency distributions to generate the numbers and percentages (Table 4.1)

Chapter 4: Results

4.1. Demographics and clinical diagnosis characteristics of the study population

In this study, a total of 105 cryopreserved (leftover) CDF isolates were collected Hamad Medical Corporation- Al-Khor Hospital, Doha, Qatar.

Table 4.1 shows demographic data in 105 cases including nationality, gender, age and clinical data using frequency distributions to generate the numbers and percentages. The majority of patients were elderly (≥ 50 years) and most of them suffer from diarrhea (49.5%)

The age range was 1-95 years with a mean age of 46 years and Standard deviation of 27.2, 61.9% were males and 38.1% were females. The distribution of patients based on their age groups < 10, 10-30, 31-50, and ≥50 was 17 (16.2%), 18 (17.1%), 16 (15.2%) and 54 (51.4%) respectively. Total 38.1% of the patients in the sample population were Qataris and 61.9 % were non-Qataris. Twenty-three patients 23 (21.9 %) were addmitted to the hospital with fever, 52 (49.5 %) with diarrhea, 1 (0.95 %) with gastrointestinal bleeding and 37 (35.2 %) have no clinical data record.

Table 4.1: Demographic data of the study population. Demographic data in 105 cases including nationality, gender, age and clinical data

Variable	Category	No. Of subjects n (%)
Total number of subjects	Qatari residents	40 (38.1 %)
	Non-Qatari	65 (61.9 %)
Gender	Males	65 (61.9 %)
	Females	40 (38.1 %)
Age	<10 yrs	17 (16.2%)
	10-30 yrs	18 (17.1 %)
	31-50 yrs	16 (15.2 %)
	≥ 50 yrs	54 (51.4 %)
Clinical data	Diarrhea	52 (49.5 %)
	Fever	23 (21.9 %)
	GI Bleeding	1 (0.95 %)
	No data	37 (35.2 %)

4.2. Characterization of AuNPs:

the absorbance of the synthesized Gold Nanoparticle solution was measured by spectrophotometry using a visible light wavelength of 400-700 nm. The graph below (Figure 4.1) shows the absorbance vs. wavelength of the synthesized colloidal gold nanoparticles solution prepared in our laboratory. The peak represents the AuNPs λ_{max} which was within 518-521 nm that is considered suitable for testing (Shawky et al., 2010).

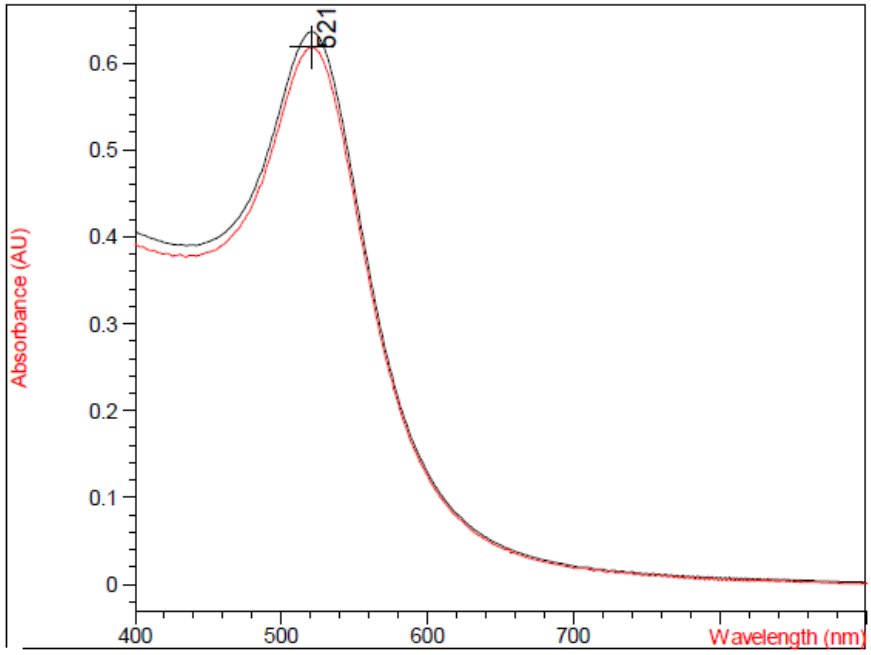

Figure 4.1: Extinction spectra of the prepared AuNPs solution. It displays the peak in the visible light region with λ_{max} 518-521 nm

After that, the average size of AuNPs was measured using Zetasizer Nano ZSP (Malvern, UK) via DLS. The peak represents the size distribution by number (percent) and it was within range 12-15 nm (Hussain et al., 2013) (Figure 4.2).

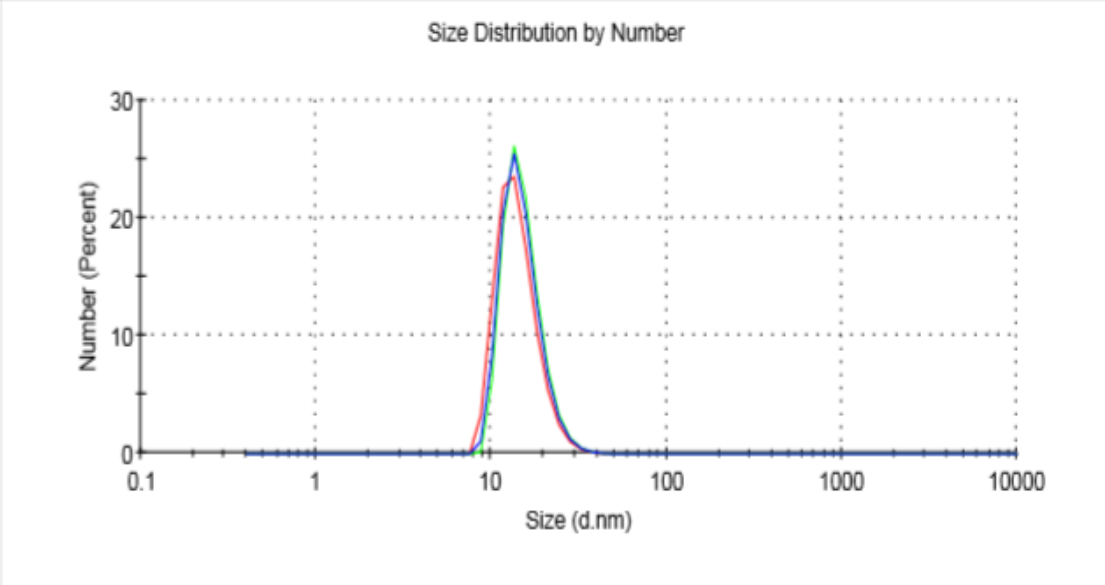

Figure 4.2: AuNPs size measurement using Zetasizer Nano ZSP (Malvern, UK). The peak represents the number (percent) of AuNPs that have size within 12-15 nm

4.3. CDF Gold Nanoparticles assay prototype:

The AuNPs-based assay is affected by four main factors that should be optimized for best results. These factors are concentrations of NaCl, AuNPs and primer used, and the assay annealing temperature. As indicated in chapter 3, the optimized AuNPs-based assay had been initially developed for the detection of CDF isolates. The assay was performed as follows, 22 µL of the extracted DNA were placed in a sterile PCR tube and 13 µL of the hybridization buffer (4.8 µL NaCl + 5.4 µL primer + 2.8 µL Nuclease-Free Water) were added to have a final concentration of 35 ul per PCR tube. The mixture was then heated at 95 °C for 30 s and annealed at 50 °C for 30 s. after cooling the mixture at room temperature for 10 minutes, 25 µL of 12-15nm AuNPs were then added. The photograph was taken within 1 minute from the addition of the AuNPs. Note the change in color from red to blue in the positive samples (Figure. 4.3 A and B). Blue color indicates the presence of primers complementary to the CDF DNA sequence and this leads to the aggregation of AuNPs together. On the other hand, red color indicates that the primer is free in the mixture that will bind to AuNPs and prevent their aggregation. Any minimal change in color from red to blue or purple is considered positive results.

96 out of 105 CDF positive samples gave a blue color and 9 out of 105 gave a red color (Table 4.2). Negative samples were retested and yielded the same results (Figure 4.3 C). *E.coli* ATCC 25922 was used as a negative control.

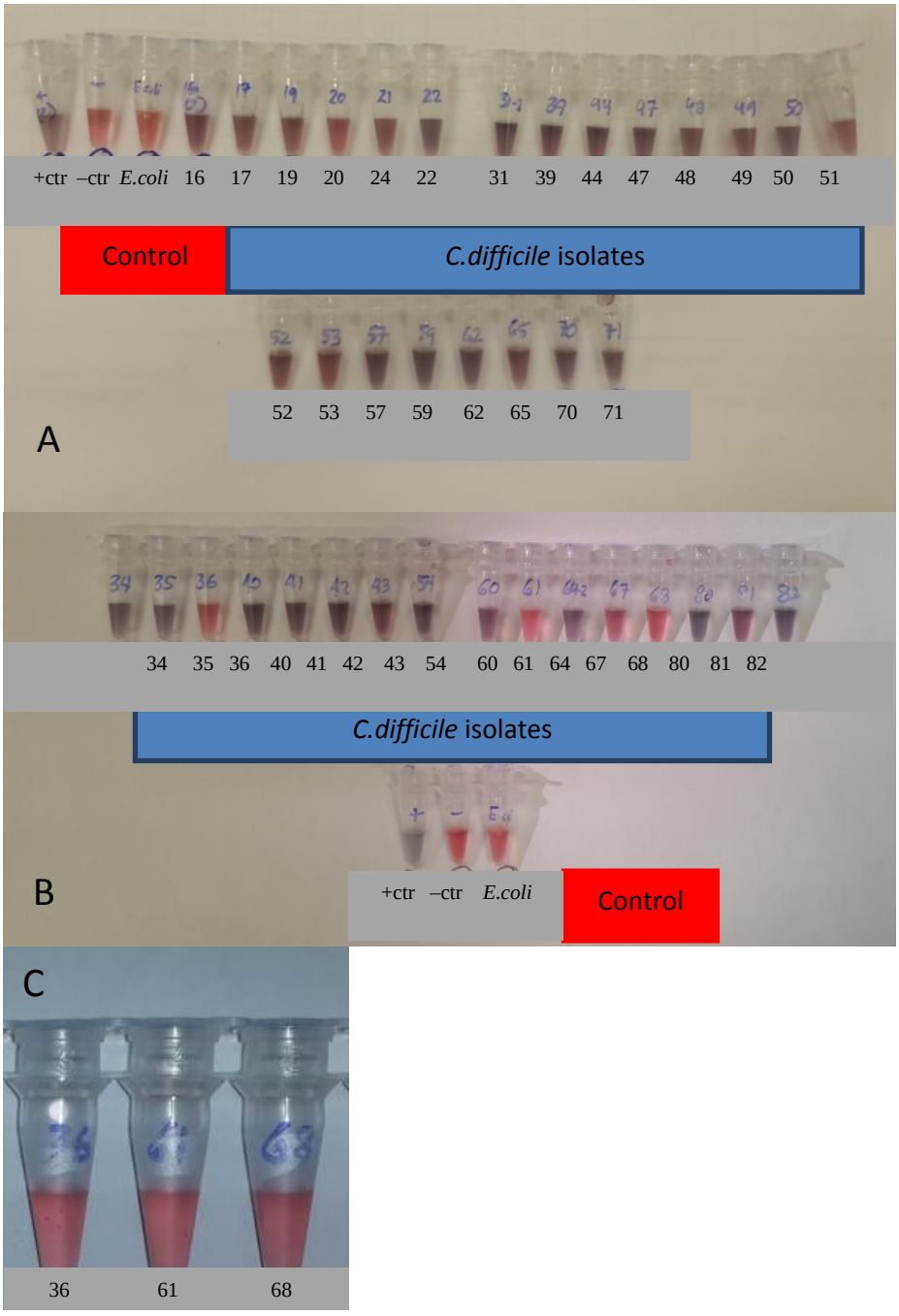

Figure 4.3: AuNPs-based assay for *C.difficile* detection (Qualitative). Positive control (+ctr) = blue, Negative control (-ctr) = red. *E.coli* ATCC 25922 as negative control= red. A) All CDF samples were positive (change in color from red to blue). B) All CDF samples were positive (blue color) except 36, 61 & 68. Negative samples were retested and yielded the same results as seen in Figure C.

29 ATCC Strains of *Clostridium* species and other than *Clostridium difficile* were also tested for AuNPs and all samples were negative (red color) to measure specificity and cross reaction (Figure 4.4 A, B and C).

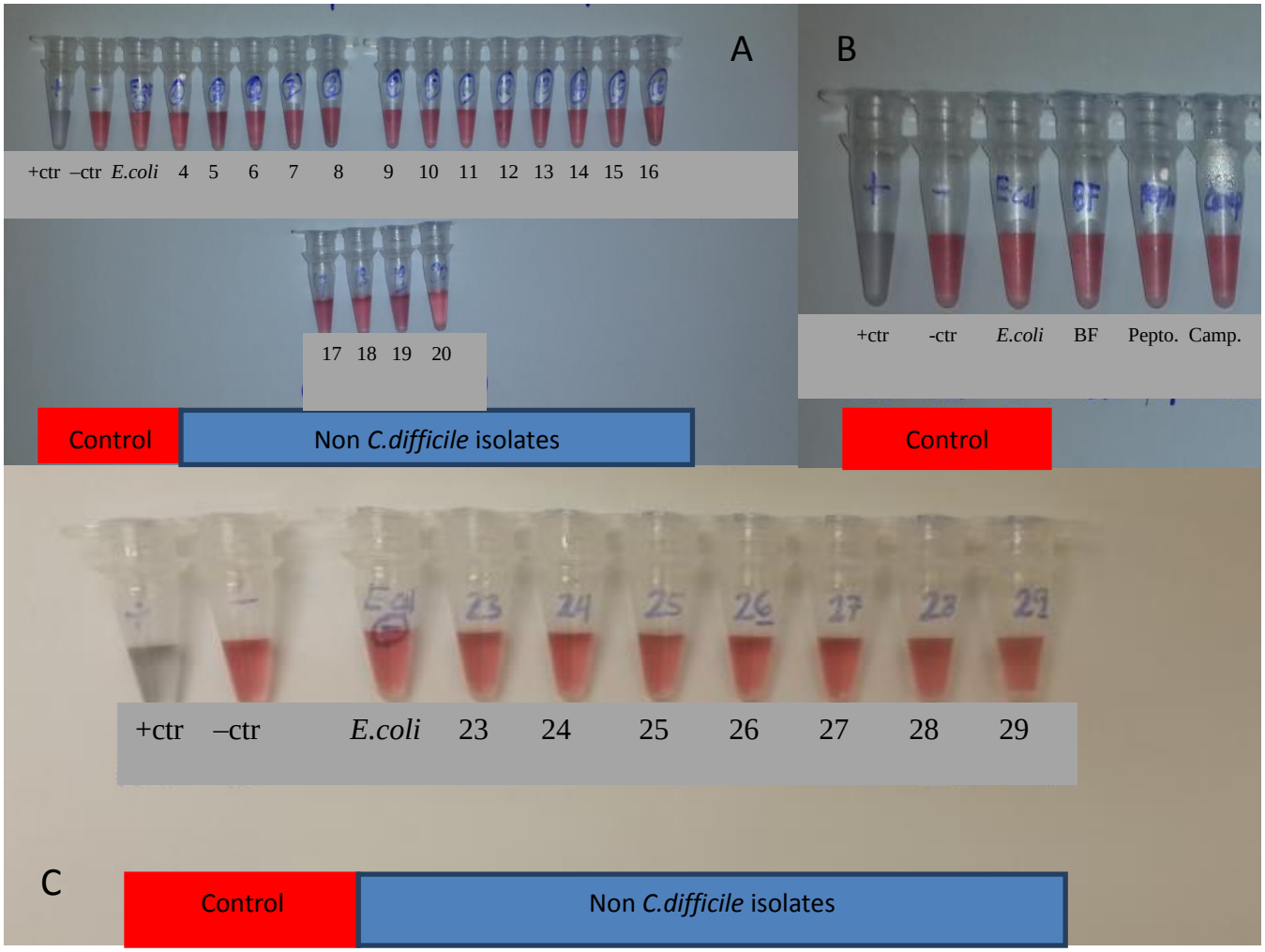

Figure 4.4: AuNPs based assay for other than *Clostridium difficile* and other *Clostridium* species (Qualitative)(A-C). Positive control (+ctr) = blue, Negative control (-ctr) = red. All samples were negative (red color). *E.coli* ATCC 25922 as negative control= red. BF: *Bacteroides fragilis*, Pepto.: *Peptostreptococcus anaerobious* and Camp.: *Campylobacter jejuni.*

4.4. Assay performance assessment:

Based on the above results, the AuNPs-based assay performance has been assessed in comparison to the results of RT-PCR (GeneXpert, Cepheid, USA) using simple statistical formula of sensitivity and specificity.

Sensitivity= True positive (TP)/ True positive (TP) + False negative (FN) X 100

$\frac{TP}{TP+FN} \times 100$= 96/ (96+9) X 100= 91.4%

Specificity= True negative (TN)/ True negative (TN) + False positive (FP) X 100

$\frac{TN}{TN+FP} \times 100$= 29/ (29+0) X 100= 100%

These initial results showed that the assay has a sensitivity of 91.4 % and a specificity of 100% in comparison to RT-PCR (GeneXpert, Cepheid, USA) (Table 4.2). Initially, no cross-reactivity was observed between *C.difficle* and other bacteria tested (Figure 4.4).

Table 4.2: AuNPs-based assay performance results of *C.difficile* isolates. 96 out of 105 positive *C.difficile* samples were positive using AuNPs-based assay. As a result, the new developed assay has a sensitivity of 91.4% compared to RT-PCR. 29 ATCC strains non *C.difficile* were negative by AuNPs-based assay compared to RT-PCR and the new developed assay has a specificity of 100 %

Samples	Real time PCR	AuNPs Assay
C.difficile positive	**105**	**96 (91.4%)**
C.difficile negative	**29**	**29 (100%)**
Total	**134**	**125**

4.5. Gold Nanoparticles assay to detect CDF toxins

All CDF positive samples with AuNPs were tested for toxins by AuNPs. Same optimization for the hybridization buffer (4.8ul NaCl + 5.4 ul primer + 22 ul CDF extracted DNA + 2.8 Nuclease-Free Water) was used in the CDF toxins detection but using specific Toxin B (Tcd B) primer (5-CAC GCC TGG AGA ATC TAT ATT TGT AGA AA-3) & binary toxin cdtA primer (5- ATG CAC AAG ACT TAC AAA GCT ATA GTG-3) & cdtB primer (5- CCA AAA TTT CCA CTT ACT TGT GTT G -3). Ninety-six samples were positive for toxin B using the optimized AuNPs assay and same result when using RT-PCR (GeneXpert, Cepheid, USA) (Figure 4.5). For the binary toxin, only 6 samples were positive out of 96 samples using the RT-PCR. However, all AuNPs assay tested samples (96) were positive for the binary toxin (Figure 4.6). *Clostridium perfringens* ATCC 13124 was used as a negative control in toxins detection with AuNPs.

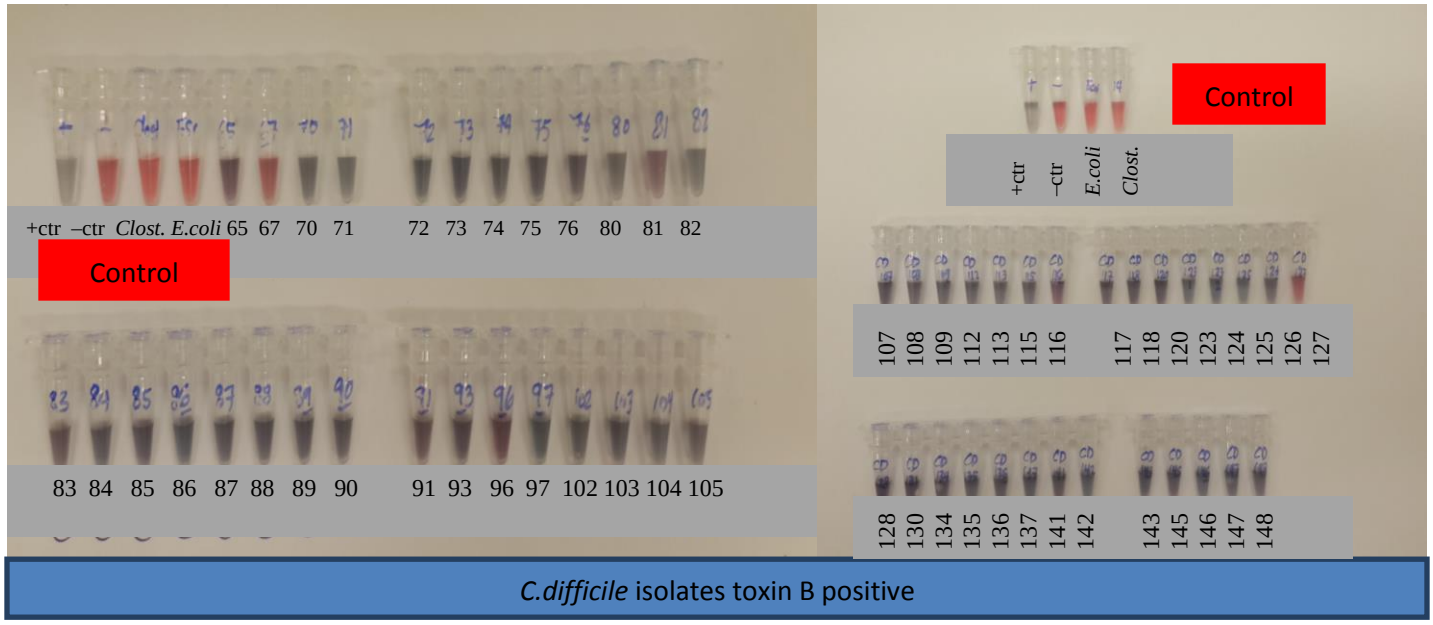

Figure 4.5: AuNPs based assay for C.difficile toxin B detection (Qualitative). Positive control (+ctr) = blue, Negative control (-ctr) = red, Clost.: *C.perfringens* ATCC 13124 and *E.coli* ATCC 25922 as negative control= red. All samples were positive (blue color).

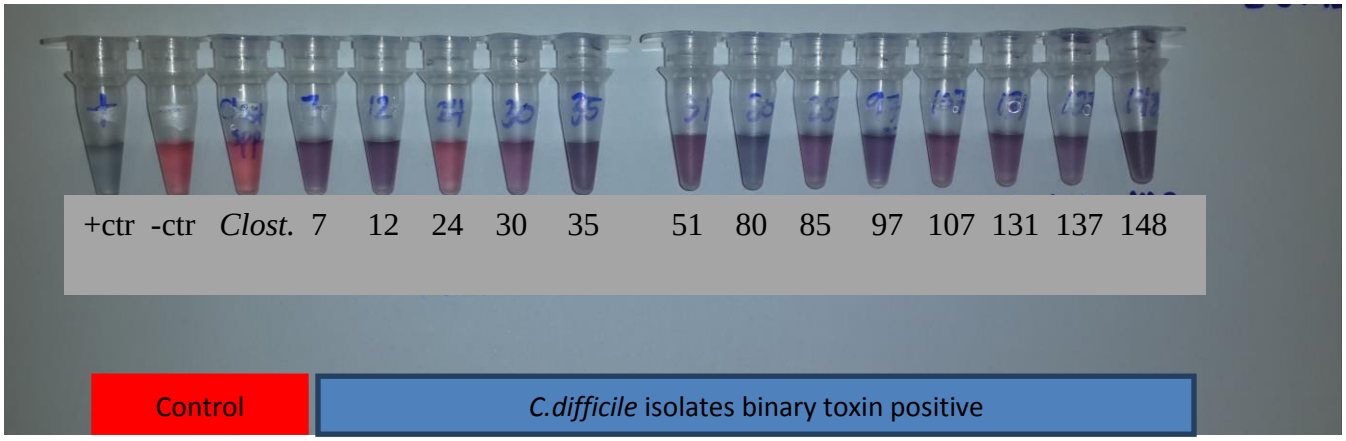

Figure 4.6: AuNPs based assay for *C.difficile* Binary toxins (cdtA & cdtB) detection (Qualitative). Positive control (+ctr) = blue, Negative control (-ctr) = red, Clost.: *C.perfringens* ATCC 13124 as negative control= red. All samples were positive (blue color). Samples (7, 24, 35, 85, 131 & 137) were positive for binary toxins by RT-PCR (GeneXpert, Cepheid, USA). Other samples were negative for Binary toxins but positive for Toxin B by RT-PCR.

Chapter 5: Discussions

Clostridium difficile (CDF) is a significant health problem in hospital and community acquired infection. It is responsible for the majority of cases of infectious antibiotic-associated diarrhea (AAD) as well as pseudomembranous colitis (PMC) which may result in death (Dalpke et al., 2013). The use of the appropriate antibiotic therapy is crucial to prevent the progression of *C.difficile* pathogenesis. Thus, the rapid diagnosis of this pathogen is crucial in patient's management and Infection control surveillance (Okada, 2010). The incidence of *C. difficile* infection (CDI) is increasing throughout the world with the universal use of antibiotics (Okada, 2010). Several diagnostic tools for the detection of this pathogen in clinical microbiology laboratories are available. Although widely used and very rapid, conventional diagnostic methods are time-consuming, costly and inappropriate for clinical field settings. Although RT-PCR has many advantages to overcome problems with the conventional methods and to be used as standalone method, it had some disadvantages that affect its use worldwide for example, it is expensive, require sophisticated infrastructure and skilled staff (Khalil et al., 2014). In contrast, nanodiagnostics assays have been promising for timely, sensitive, point-of-care and cost-effective detection of microbial agents (Hauck et al., 2010). Therefore, the aim of this study was to develop Gold Nanoparticles- based assay (AuNPs) for direct qualitative detection of the nucleic acid of CDF and its toxins.

Currently in Clinical laboratories in Hamad Medical Corporation, *C.difficile* is detected using 2-step algorithm as suggested by CDC SHEA/IDSA. This strategy uses Glutamate Dehydrogenase (GDH) as screening test because of its high sensitivity and then uses the RT-PCR (GeneXpert, Cepheid, USA) as confirmatory test for GDH-positive stool samples only.

In this study, isolated CDF strains were originally confirmed by RT-PCR (GeneXpert, Cepheid, USA) in clinical laboratory (Al-Khor Hospital). Negative and positive controls were first run to optimize the assay conditions before testing CDF isolates. Optimized AuNPs-based assay was developed for detection and identification of CDF, which includes the extracted DNA from colonies followed by detection of CDF and their toxins using specific primers and colloidal AuNPs solution.

The AuNPs-based assay is affected by four main factors: annealing temperature, size of AuNPs, primers and salt concentrations. Of these, the constant parameter in this study was the AuNPs size. Control of the particles size was achieved by using the suitable concentration of the sodium citrate, which acts as reducing agent. This citrate reduction causes the AuNPs to be negatively charged. The remaining factors were optimized for detection of CDF. High annealing temperatures can result in AuNPs aggregation. However, in this assay, AuNPs is added after the annealing step, which enabled the use of optimal temperature for annealing without interference with AuNPs solution stability. Different primer concentrations were tested to stabilize the AuNPs colloidal in the presence of appropriate NaCl concentration (Hussain et al., 2013). In this study, the optimal concentration for the primer was found to be 0.9-1 µM in the total assay volume. Moreover, the optimal final concentration of NaCl used was 0.04 M that is sufficient for aggregation of AuNPs and visual detection of the color change.

The principle of the AuNPs assay is based on the ability of ssDNA primers to stabilize the colloidal AuNPs preventing their salt-induced aggregation. In the positive sample (target present), primers will bind with their complementary DNA sequence in the target; therefore, addition of AuNPs will lead to its aggregation by the salt available in hybridization buffer, resulting in blue color development. On the other hand, if target is absent, ssDNA primers will

remain free in the solution, stabilizing colloidal AuNPs, preventing their salt-induced aggregation and maintaining the red color of the solution (Khalil et al., 2014).

The initial AuNPs-based assay prototype was developed and optimized to detect CDF positive isolates and their toxins. The results showed a high sensitivity and specificity (91.4 % and 100% respectively) compared to RT-PCR. These initial results suggest that the new assay has a comparable performance to RT-PCR. False negative results can be due to very high concentration of the primer that will prevent aggregation of AuNPs. In contrast, very low concentration of the primer may cause false positive results. The AuNPs assay detected toxin B in all 96 isolates identified as tcdB-positive by RT-PCR. These results declared complete concordance with RT-PCR results. On the other hand, Binary toxins were positive in 6 CDF isolates by RT-PCR assay, approximately 6 to 12.5% of strains of *C. difficile* produce binary toxin, which confirms the low prevalence of binary toxin. However all the samples (96) show positive results when was tested for Binary Toxin using AuNPs assay. This is may be related to the fact that the Binary Toxin is encoded by the Cdt locus (CdtLoc) (Burnham et al., 2013). It was found that there is a correlation between the presence of the PaLoc and the CdtLoc. More than 98% of CdtLoc-positive strains also have the PaLoc (Carter et al., 2007). Using other Binary primer sequences with more specificity for binary toxin Cdt locus could contribute to overcome the result interference. In our study, the overall agreement (accuracy) between the AuNPs-based assay and RT-PCR was 93.3%.

The turnaround time for the developed assay was found to be 15-30 minutes, which is shorter than RT-PCR (45minutes- 1 hour). Moreover, the use of AuNPs eliminates the need for expensive detection instrumentation (Shawky et al., 2010).

Because of the high sensitivity and specificity of AuNPs-based assays for detection of nucleic acid targets, 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. In comparison to several studies uses AuNPs in microbial detection and identification, results showed acceptable sensitivity and specificity in detecting HCV using AuNPs-based assay (93.3% and 88.9% respectively) (Shawky et al., 2010). Another study showed 96.6% sensitivity and 98.9% specificity for the detection of *Mycobacterium tuberculosis* Complex (MTBC) and 94.7% sensitivity and 99.6% specificity for the detection of MTB (Hussain et al., 2013). Recent studies in *Acinetobacter baumanni* identification also showed a high sensitive and accurate results compared to conventional biochemical methods and PCR (Khalil et al., 2014). Furthermore, a study was done on the identification of *E.coli* using AuNPs oligo-prob principle, and it was found that it is highly sensitive and specific (Padmavathy et al., 2012). Several studies on Intestinal organisms detection using Gold nanoparticles has been published. For example, In 2008, Wang and his team develop a quartz crystal microbalance (QCM) biosensor for real-time detection of *E. coli* O157:H7 DNA based on nanogold particles amplification and results showed that this developed biosensor enhance the detection of *E.coli* O157:H7 compared to conventional method (Wang et al., 2008). Another study using Gold nanoparticles to detect *salmonella* infection was conducted by Kalidasan et al., 2013 and his collegue. It showed promising results and sensitive method (Kalidasan et al., 2013). One study related to *C.difficile* detection in 2013 in which Luo et al. had developed an aptamer biosensor for the detection of toxin A of *Clostridium difficile* using gold nanoparticles synthesized by *Bacillus stearothermophilus.* The results of the study showed good sensitivity in the detection of toxin A as well as good

selectivity, stability ad recovery rate (Luo et al., 2014). The use of gold nanoparticles for screening molecular signatures of drug resistance (MRSA, MDR-TB & XDR-TB) that has been reported thus far, and provides a critical evaluation of current and future developments of these technologies assisting pathogen identification and characterization (Veigas et al., 2014). MRSA detection using AuNPs with clinical samples demonstrated very good agreement with the "gold standard" (94.44%). In addition the sensitivity and specificity were 97.14% and 91.89% respectively compared to conventional standard method(Veigas et al., 2014).

These results were consistent with the initial results that were obtained in this study except the detection of HCV using AuNPs showed low specificity (88.9%) compared to our study (100%). This is because clinical serum samples were used in this study.

Current trends in *Clostridium difficile* detection involve using microarray. Hicke et al., 2012 combine the advantages of molecular testing (sensitivity) and immunoassays (low cost) and developed an assay for toxigenic *C. difficile* that couples isothermal DNA amplification to array-based hybridization using clinical samples. This idea can be implemented in AuNPs-based assay development since there is integration of specific primers (molecular) and colorimetric assay (immunoassay) to have a sensitive and cost effective method in the near future and could be used as Point of Care Testing (POCT).

In this study, the AuNPs-based assay requires simple preparation and nucleic acid extraction. It does not require target amplification prior to detection, which reduces the cost, time, the need for highly trained staff and expensive and complex instrumentation along with providing high sensitivity and specificity. However, most of the work being carried out is in the

initial stage of development, and further simplification and commercialization of the assays are likely to be achieved soon.

Although AuNPs showed acceptable sensitivity and specificity compared to RT-PCR, the limitations of this study is that *C.difficile* isolates tested is leftover isolates that may be prone to contamination or inability to grow. Another limitation is missing other diagnostic laboratory data that could be useful in the study. Further limitation of the study is that the assay is not yet validated on clinical stool samples. This AuNPs assay would have a significant impact especially in low-resource settings if it was tested with stool samples directly to evaluate Positive predictive value, Negative predictive value and other performance criteria. It is also recommended to have a quantitative measurement along with qualitative analysis. A future plan in the phase II of this study is to test clinical stool samples to determine the following performance parameters of the developed AuNPs assays: PPV, NPV, Linearity, Limit of Quantification and interference assessment.

In conclusion, *Clostridium difficile* (CDF) is a significant health problem around the world. The need for simple, rapid and precise method for CDF detection is a critical step for appropriate therapy and infection control measures implementation. A colorimetric assay has been developed for rapid detection of CDF and their toxins using AuNPs-based assay. Direct detection of genomic DNA by colloidal AuNPs-based assay was performed on 105 clinical CDF isolates in addition to twenty-nine reference strains other than *Clostridium difficile*. The initial results for the developed AuNPs-based colorimetric assay were positive in Ninety-six and negative in nine samples. The assay maybe considered cost effective, sensitive, reliable and rapid that can compete with commercial immunoassays and RT-PCR methods as routine tests for the management of CDF patients. This colorimetric assay is the first unmodified AuNPs-based assay

for the detection of CDF. Our future prospectives include the development of an AuNPs quantitative prototype using Froster Resonance Energy Transfer (FRET). Quantitative analysis is very important for patient's management and therapy. It is used in early diagnosis to find out the concentration of the pathogen and to determine if it is early or late infection. Moreover, quantitative assay is useful for monitoring therapy. If concentration of the organism is still high after treatment, this may indicate resistance pattern. However if the concentration decreases, it gives indication that patient is responding to therapy. In addition, a wide range of clinical features is considered for early diagnosis. Further to evaluate AuNPs-based assay detection limit on samples with serial dilution and finally to vlidate this developed assay on clinical samples to determine the following performance parameters of the developed AuNPs assays: PPV, NPV and Linearity and interference assessment.

Chapter 6: Conclusions

Clostridium difficile Infection (CDI) is a worldwide health problem with the increase in antibiotic consumption and absence of antibiotic restriction and stewardship. Diagnosis of *C.difficile* (CDF) is considered crucial in patient management and infection control measures and surveillance. Several diagnostic methods are available but with some disadvantages for example, Real-time PCR method that is used for *C.difficile* detection has a high sensitivity and specificity but it is costly, requires skilled staff and complex infrastructure. Therefore the aim of this study is to develop Gold Nanoparticles- based assay for direct qualitative detection of nucleic acid of CDF and its toxins. The proposed assay is expected to be highly sensitive, specific, rapid, simple, and minimize the need for expensive and complex equipment. Based on the collected data and results, 96 out of 105 of C.difficile samples showed positive results while 9 samples showed negative results compared to RT-PCR. The sensitivity of AuNPs was 91.4% compared to RT-PCR 88-100% while specificity was 100% compared to RT-PCR 96-100%. These results showed acceptable performance as an initial study for the AuNPs developed assay.

A colorimetric assay has been developed using AuNPs for the direct qualitative detection of CDF in leftover (cryopreserved) isolates. The developed assay has several advantages including acceptable sensitivity, specificity and short turnaround time. This developed, optimized AuNPs-based assay may improve the management of *C. difficile* infection and may lead to a more rational use of antibiotics, as the clinicians will rapidly obtain the clinical microbiology results. However, a large-scale clinical trial is needed to further validate this assay. Eventually this assay may have a great impact on clinical diagnosis in low-resources countries for patient's management and infection control measures.

Appendix I: Ethical approval

مؤسسة حمد الطبية
Hamad Medical Corporation
HEALTH · EDUCATION · RESEARCH صحة · تعليم · بحوث

مركز البحوث الطبية
Medical Research Center

Ref. No: RC/11680/2011
Date: 3rd July 2011

Dr. Asma A. J. Al-Thani
Associate Professor
Medical Micro Biologist
BioMedical Program
Health Departmenrt
PO Box: 2713
Doha, Qatar

Dear Dr.Asma,

Research Proposal 11136/11: NPRP 4-1215-3-317 "Gold Nanoparticles-based Assays for Direct and Cost Effective Detection of High Burden Diseases"

The above Research Protocol submitted to the Medical Research Center has been reviewed.

On behalf of the Research Committee we inform you that the above Research Proposal meets with the ethical requirements of the Hamad Medical Corporation and approval is granted for one year from 3rd July 2011.

A study progress report should be submitted semi-annually and a final report at the study's completion.

We wish you all success and await the results in due course.

Yours sincerely,

Dr.Anjum Susan John
Coordinator, Research Committee

Cc:
1) Dr. Ahmad Muhammed Ahmadna, Director, Research Department, College of Arts & Sciences
2) Dr.Tahra El Obeid, Head of the Depart.of Health Sciences,College of Arts & Sciences, QU
3) Dr. Hassan M. Azzazy,Professor in Chemistri Department & Associate Dean , ACU
4) Dr. Neama Al Molawi,Clinical Scientist, Virology Laboratory,HMC
5) Chaiman, Department of Laboratory Medicine & Pathology
6) Chief of Staff, Hamad General Hospital

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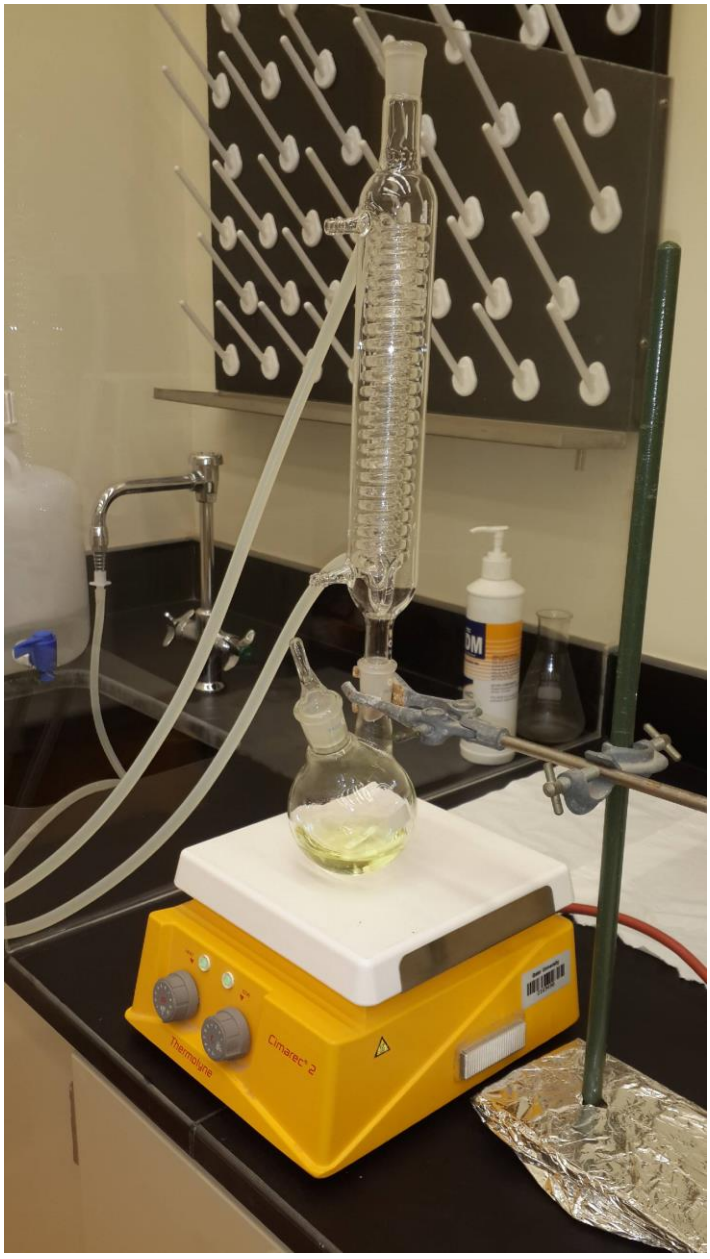
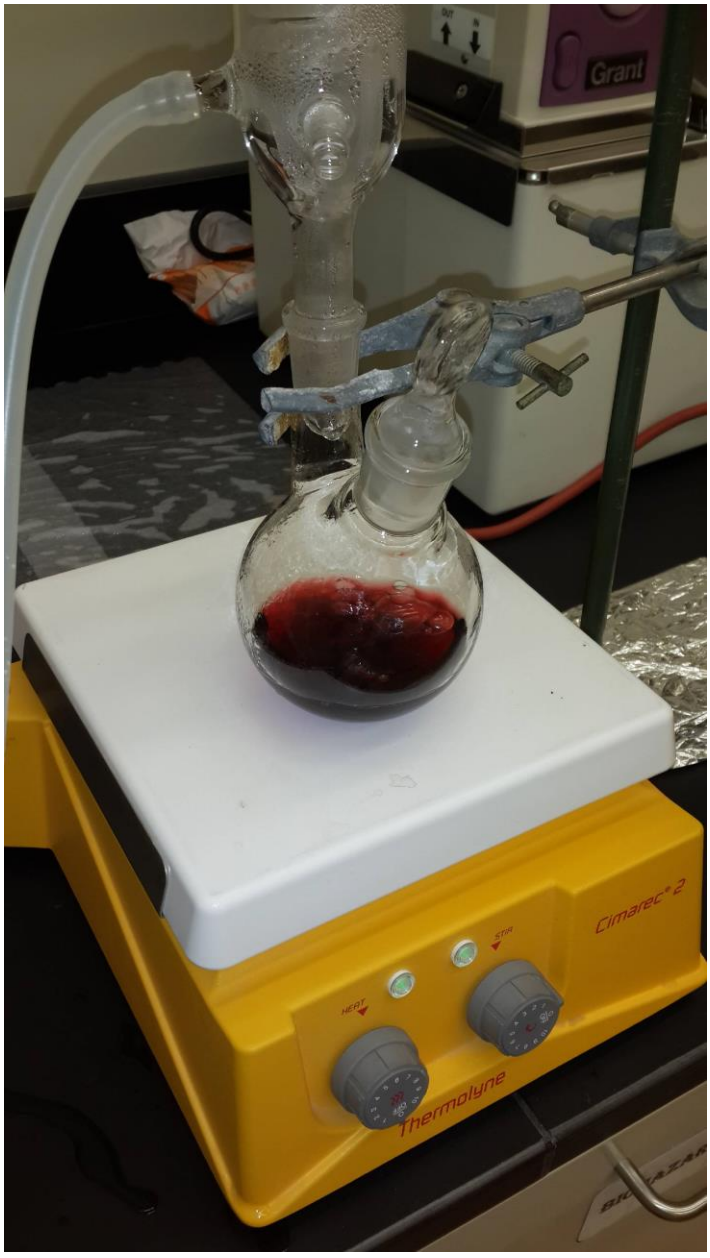
Appendix II: AuNPs Synthesis in QU Research center

Change in color from colorless or light yellow to brick red



0.020 g of AuCl4 dissolved in 50 ml Distilled Water



Addition of 1% Tri-sodium citrate

Appendix III: ATCC Strains other than *C.difficile* used in AuNPs-based assay

Organism name	Assigned sample ID	Organism name	Assigned sample ID
E.coli ATCC 25922	*E.coli* ATCC 25922	*E.coli* ATCC 35218	15
Bacteroides fragilis ATCC 25285	1	*Stenotrophomonas maltophilia* ATCC 51331	16
Peptostrptococcus anaerobious ATCC 27337	2	*Proteus vulgaris* ATCC 13315	17
Campylobacter jejuni ATCC 33291	3	*Proteus mirabilis* ATCC 12453	18
Salmonella typhi ATCC 14028	4	*Vibrio cholera* (patient's isolates)	19
Brevibacillus agri ATCC 51663	5	*Enterobacter aerogenes* ATCC 13048	20
Enteropathogenic E.coli (EPEC) 2 (patient's isolates)	6	*Clostridium perfringens* ATCC 13124	21
Enteropathogenic E.coli (EPEC) 3 (patient's isolates)	7	*Staph.aureus* ATCC 29213	23
Enteropathogenic E.coli (EPEC) 4 (patient's isolates)	8	*Staphylococcus saprophyticus* ATCC 15305	24
Hafnia species (patient's isolates)	9	*Staph. epidermidis* ATCC 12228	25
Shigella flexeneri ATCC 12022	10	*Streptococcus agalactiae* ATCC 12386	26
Klebsiella pneumonia ATCC 700603	11	*Candida albicans* ATCC 90028	27
E.coli O157 ATCC 35150	12	*Enterococcus faecalis* ATCC 29212	28
Pseudomonas aeruginosa ATCC 27853	13	*Streptococcus pyogenes* ATCC 19615	29
Yersinia enterocolitica ATCC 27729	14		

Appendix IV: Different concentrations of salt and primer results in the development and optimization step

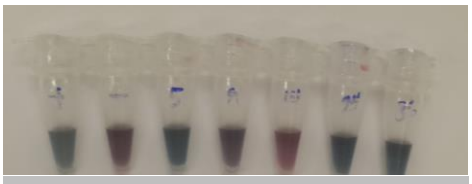
NaCl:

NaCl final concentration range from 0.04-0.08 M. Primer concentration and volume was fixed (1uM, 3 ul)

2 ul NaCl (0.2 M)

\+ + QC fail

Primer volume change from 3 to 2.9 ul (NaCl volume 2 ul (0.2 M)

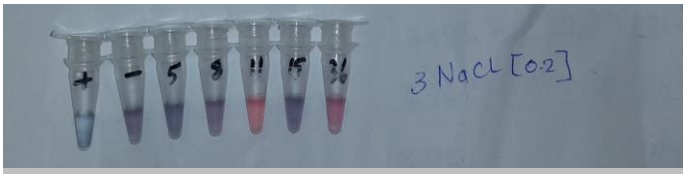


\+ + QC fail

2.5 ul NaCl (0.2 M)

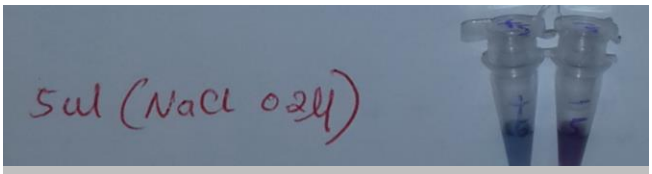
\+ + QC fail

3 ul NaCl (0.2 M)



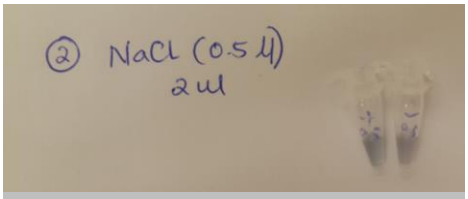
\+ + QC fail

5 ul NaCl (0.2 M)



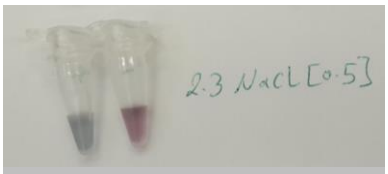
QC fail + +

2 ul NaCl (0.5 M)



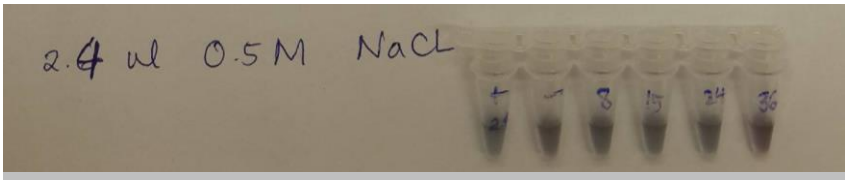
QC fail + +

2.3 ul NaCl (0.5 M)



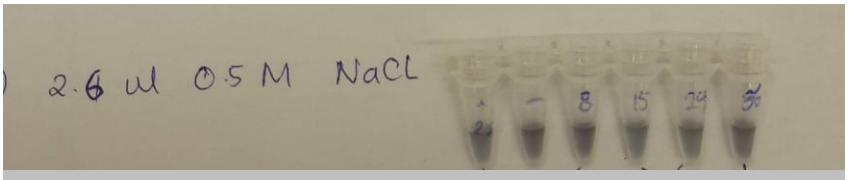
+ + QC fail

2.4 ul NaCl (0.5 M)



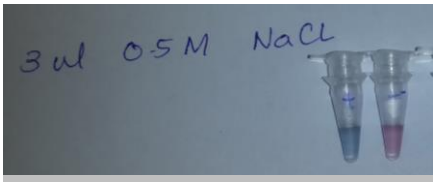
+ + QC fail

2.6 ul NaCl (0.5 M)



+ + QC fail

3 ul NaCl (0.5 M)



QC fail + +

4.8 ul NaCl (0.5 M) + 5.4 ul primer

\+ - *E.coli*

QC pass

Appendix V: Results of *C.difficile* sample in RT-PCR (GeneXpert, Cepheid, CA, USA) and Gold nanoparticles-based assay

No. of specimens	RT-PCR (GeneXpert, Cepheid, CA, USA)	AuNPs-based assay	No. of specimens	RT-PCR (GeneXpert, Cepheid, CA, USA)	AuNPs-based assay
1	Positive	Positive	32	Positive	Positive
2	Positive	Positive	33	Positive	Positive
3	Positive	Positive	34	Positive	Positive
4	Positive	Positive	35	Positive	Positive
6	Positive	Positive	36	Positive	Negative
7	Positive	Positive	39	Positive	Positive
9	Positive	Positive	40	Positive	Positive
10	Positive	Positive	41	Positive	Positive
12	Positive	Positive	42	Positive	Positive
15	Positive	Positive	43	Positive	Positive
16	Positive	Positive	44	Positive	Positive
17	Positive	Positive	47	Positive	Positive
19	Positive	Positive	48	Positive	Positive
21	Positive	Positive	49	Positive	Positive
24	Positive	Positive	50	Positive	Positive
25	Positive	Negative	51	Positive	Positive
26	Positive	Positive	53	Positive	Positive
27	Positive	Weak Positive	54	Positive	Positive
30	Positive	Positive	57	Positive	Positive
31	Positive	Positive	59	Positive	Positive

67	Positive	Weak Positive	103	Positive	Positive
68	Positive	Negative	104	Positive	Positive
70	Positive	Positive	105	Positive	Positive
71	Positive	Positive	106	Positive	Negative
72	Positive	Positive	107	Positive	Positive
73	Positive	Positive	108	Positive	Positive
74	Positive	Positive	109	Positive	Positive
75	Positive	Positive	112	Positive	Positive
76	Positive	Positive	113	Positive	Positive
80	Positive	Positive	115	Positive	Positive
81	Positive	Positive	116	Positive	Positive
82	Positive	Positive	117	Positive	Positive
83	Positive	Positive	118	Positive	Positive
84	Positive	Positive	120	Positive	Positive
85	Positive	Positive	123	Positive	Positive
86	Positive	Positive	125	Positive	Positive
87	Positive	Positive	126	Positive	Positive
88	Positive	Positive	127	Positive	Weak Positive
89	Positive	Positive	128	Positive	Positive
90	Positive	Positive	129	Positive	Negative
91	Positive	Positive	131	Positive	Positive
93	Positive	Positive	132	Positive	Negative
96	Positive	Positive	133	Positive	Negative
97	Positive	Positive	134	Positive	Positive
102	Positive	Positive	135	Positive	Positive

136	Positive	Positive			
137	Positive	Positive			
140	Positive	Negative			
141	Positive	Positive			
142	Positive	Positive			
143	Positive	Positive			
145	Positive	Positive			
146	Positive	Positive			
147	Positive	Positive			
148	Positive	Positive			

Appendix VI: Results of *C.difficile* toxin B in RT-PCR

(GeneXpert, Cepheid, CA, USA) and Gold nanoparticles-based assay

Sample CDF ID #	AuNPs-based assay	RT-PCR (GeneXpert, Cepheid, CA, USA)	Sample CDF ID #	AuNPs-based assay	RT-PCR (GeneXpert, Cepheid, CA, USA)
	Toxin B	Toxin B		Toxin B	Toxin B
1	Positive	Positive	34	Positive	Positive
2	Positive	Positive	35	Positive	Positive
3	Positive	Positive	39	Positive	Positive
4	Positive	Positive	40	Positive	Positive
6	Positive	Positive	41	Positive	Positive
7	Positive	Positive	42	Positive	Positive
9	Positive	Positive	43	Positive	Positive
10	Positive	Positive	44	Positive	Positive
12	Positive	Positive	47	Positive	Positive
15	Positive	Positive	48	Positive	Positive
16	Positive	Positive	49	Positive	Positive
17	Positive	Positive	50	Positive	Positive
19	Positive	Positive	51	Positive	Positive
21	Positive	Positive	53	Positive	Positive
24	Positive	Positive	54	Positive	Positive
26	Positive	Positive	57	Positive	Positive
27	Positive	Positive	59	Positive	Positive
30	Positive	Positive	60	Positive	Positive
31	Positive	Positive	62	Positive	Positive
32	Positive	Positive	64	Positive	Positive
33	Positive	Positive	65	Positive	Positive

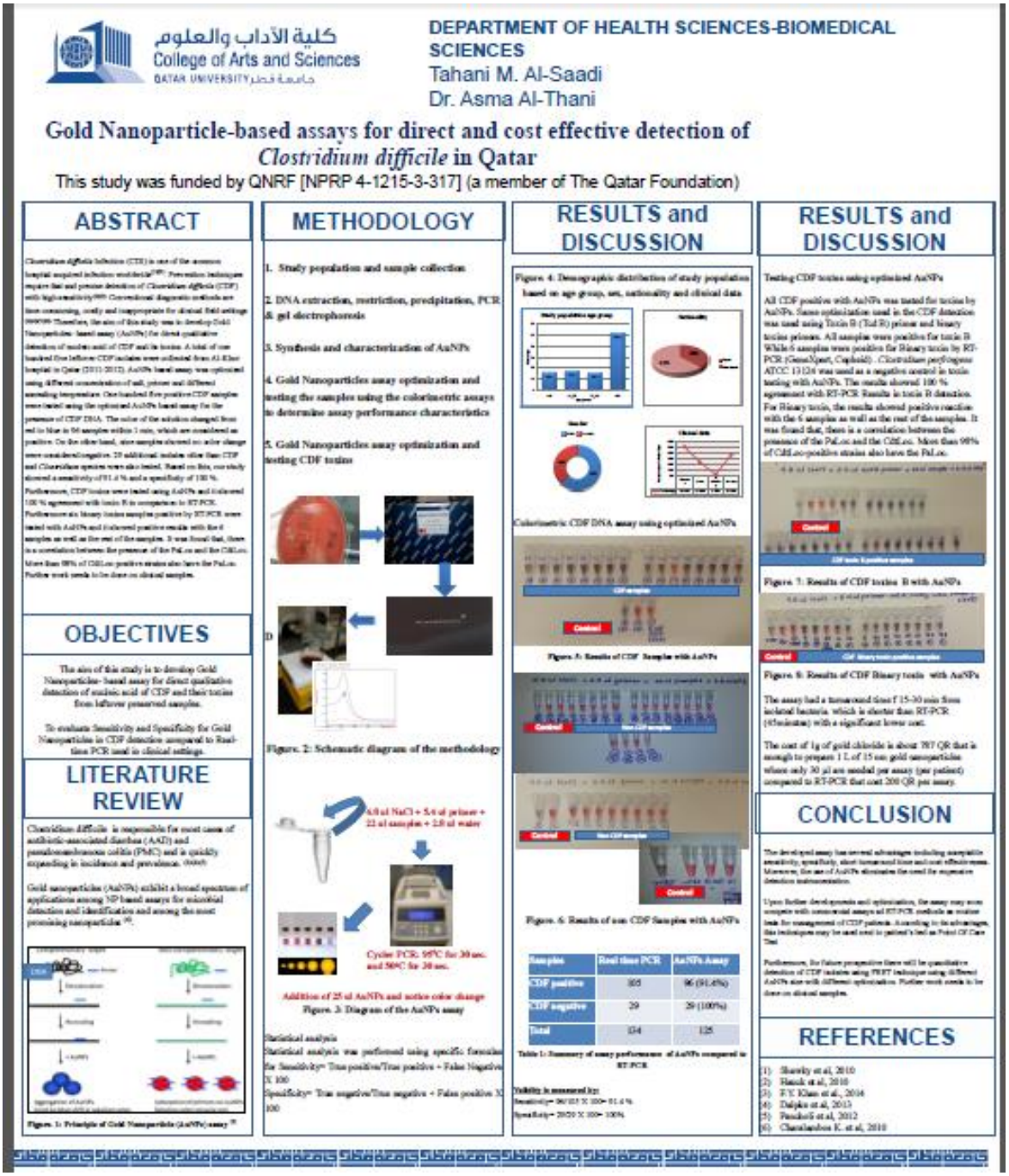
67	Positive	Positive	107	Positive	Positive
70	Positive	Positive	108	Positive	Positive
71	Positive	Positive	109	Positive	Positive
72	Positive	Positive	112	Positive	Positive
73	Positive	Positive	113	Positive	Positive
74	Positive	Positive	115	Positive	Positive
75	Positive	Positive	116	Positive	Positive
76	Positive	Positive	117	Positive	Positive
80	Positive	Positive	118	Positive	Positive
81	Positive	Positive	120	Positive	Positive
82	Positive	Positive	123	Positive	Positive
83	Positive	Positive	125	Positive	Positive
84	Positive	Positive	126	Positive	Positive
85	Positive	Positive	127	Positive	Positive
86	Positive	Positive	128	Positive	Positive
87	Positive	Positive	131	Positive	Positive
88	Positive	Positive	134	Positive	Positive
89	Positive	Positive	135	Positive	Positive
90	Positive	Positive	136	Positive	Positive
91	Positive	Positive	137	Positive	Positive
93	Positive	Positive	141	Positive	Positive
96	Positive	Positive	142	Positive	Positive
97	Positive	Positive	143	Positive	Positive
102	Positive	Positive	145	Positive	Positive
103	Positive	Positive	146	Positive	Positive
104	Positive	Positive	147	Positive	Positive
105	Positive	Positive	148	Positive	Positive

Appendix VII: Results of *C.difficile* Binary toxins in RT-PCR (GeneXpert, Cepheid, CA, USA) and Gold nanoparticles-based assay

Sample CDF ID #	AuNPs-based assay	RT-PCR (GeneXpert, Cepheid, CA, USA)	Sample CDF ID #	AuNPs-based assay	RT-PCR (GeneXpert, Cepheid, CA, USA)
	Binary toxins (cdtA & cdtB)	Binary toxins		Binary toxins (cdtA & cdtB)	Binary toxins
1	Positive	Negative	34	Positive	Negative
2	Positive	Negative	35	Positive	Positive
3	Positive	Negative	39	Positive	Negative
4	Positive	Negative	40	Positive	Negative
6	Positive	Negative	41	Positive	Negative
7	Positive	Positive	42	Positive	Negative
9	Positive	Negative	43	Positive	Negative
10	Positive	Negative	44	Positive	Negative
12	Positive	Negative	47	Positive	Negative
15	Positive	Negative	48	Positive	Negative
16	Positive	Negative	49	Positive	Negative
17	Positive	Negative	50	Positive	Negative
19	Positive	Negative	51	Positive	Negative
21	Positive	Negative	53	Positive	Negative
24	Positive	Positive	54	Positive	Negative
26	Positive	Negative	57	Positive	Negative
27	Positive	Negative	59	Positive	Negative
30	Positive	Negative	60	Positive	Negative
31	Positive	Negative	62	Positive	Negative
32	Positive	Negative	64	Positive	Negative
33	Positive	Negative	65	Positive	Negative

67	Positive	Negative	107	Positive	Negative
70	Positive	Negative	108	Positive	Negative
71	Positive	Negative	109	Positive	Negative
72	Positive	Negative	112	Positive	Negative
73	Positive	Negative	113	Positive	Negative
74	Positive	Negative	115	Positive	Negative
75	Positive	Negative	116	Positive	Negative
76	Positive	Negative	117	Positive	Negative
80	Positive	Negative	118	Positive	Negative
81	Positive	Negative	120	Positive	Negative
82	Positive	Negative	123	Positive	Negative
83	Positive	Negative	125	Positive	Negative
84	Positive	Negative	126	Positive	Negative
85	Positive	Positive	127	Positive	Negative
86	Positive	Negative	128	Positive	Negative
87	Positive	Negative	131	Positive	Positive
88	Positive	Negative	134	Positive	Negative
89	Positive	Negative	135	Positive	Negative
90	Positive	Negative	136	Positive	Negative
91	Positive	Negative	137	Positive	Positive
93	Positive	Negative	141	Positive	Negative
96	Positive	Negative	142	Positive	Negative
97	Positive	Negative	143	Positive	Negative
102	Positive	Negative	145	Positive	Negative
103	Positive	Negative	146	Positive	Negative
104	Positive	Negative	147	Positive	Negative
105	Positive	Negative	148	Positive	Negative

Appendix VIII: Poster



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Tahani M. Al-Saadi
Dr. Asma Al-Thani

Gold Nanoparticle-based assays for direct and cost effective detection of *Clostridium difficile* in Qatar

This study was funded by QNRF [NPRP 4-1215-3-317] (a member of The Qatar Foundation)

ABSTRACT

METHODOLOGY

1. Study population and sample collection
2. DNA extraction, restriction, precipitation, PCR & gel electrophoresis
3. Synthesis and characterization of AuNPs
4. Gold Nanoparticles assay optimization and testing the samples using the colorimetric assays to determine assay performance characteristics
5. Gold Nanoparticles assay optimization and testing CDF toxins

Figure. 2: Schematic diagram of the methodology

4.0 ul NaCl + 5.6 ul primer + 22 ul samples + 2.8 ul water

Cycler PCR: 95°C for 30 sec. and 50°C for 30 sec.

Addition of 25 ul AuNPs and notice color change

Figure. 3: Diagram of the AuNPs assay

Statistical analysis

Statistical analysis was performed using specific formulas for Sensitivity= True positive/True positive + False Negative X 100

Specificity= True negative/True negative + False positive X 100

RESULTS and DISCUSSION

Figure. 4: Demographic distribution of study population based on age group, sex, nationality and clinical data

Colorimetric CDF DNA assay using optimized AuNPs

Figure. 5: Results of CDF Samples with AuNPs

Figure. 6: Results of non CDF Samples with AuNPs

Samples	Real time PCR	AuNPs Assay
CDF positive	105	96 (91.4%)
CDF negative	29	29 (100%)
Total	134	125

Table 1: Summary of assay performance of AuNPs compared to RT-PCR

Validity is measured by:

Sensitivity= 96/105 X 100= 91.4 %

Specificity= 29/29 X 100= 100%

RESULTS and DISCUSSION

Testing CDF toxins using optimized AuNPs

All CDF positive with AuNPs was tested for toxins by AuNPs. Same optimization used in the CDF detection was used using Toxin B (TcdB) primer and binary toxins primers. All samples were positive for toxin B While 6 samples were positive for Binary toxin by RT-PCR (GeneXpert, Cepheid) . *Clostridium perfringens* ATCC 13124 was used as a negative control in toxin testing with AuNPs. The results showed 100 % agreement with RT-PCR Results in toxin B detection. For Binary toxin, the results showed positive reaction with the 6 samples as well as the rest of the samples. It was found that, there is a correlation between the presence of the PaLoc and the CdtLoc. More than 98% of CdtLoc-positive strains also have the PaLoc.

Figure. 7: Results of CDF toxins B with AuNPs

Figure. 8: Results of CDF Binary toxin with AuNPs

The assay had a turnaround time f 15-30 min from isolated bacteria which is shorter than RT-PCR (45minutes) with a significant lower cost.

The cost of 1g of gold chloride is about 787 QR that is enough to prepare 1 L of 15 nm gold nanoparticles where only 30 µl are needed per assay (per patient) compared to RT-PCR that cost 200 QR per assay.

CONCLUSION

OBJECTIVES

The aim of this study is to develop Gold Nanoparticles- based assay for direct qualitative detection of nucleic acid of CDF and their toxins from leftover preserved samples.

To evaluate Sensitivity and Specificity for Gold Nanoparticles in CDF detection compared to Real-time PCR used in clinical settings.

LITERATURE REVIEW

Clostridium difficile is responsible for most cases of antibiotic-associated diarrhea (AAD) and pseudomembranous colitis (PMC) and is quickly expanding in incidence and prevalence.

Gold nanoparticles (AuNPs) exhibit a broad spectrum of applications among NP based assays for microbial detection and identification and among the most promising nanoparticles

Figure. 1: Principle of Gold Nanoparticle (AuNPs) assay

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ملخص

خلفية

تعتبر العدوى البكتيرية *Clostridium difficile* من المشاكل الصحية المنتشرة عالمياً. يشترط الكشف السريع والدقيق للكائنات المسببة للمرض جودة عالية . بالإضافة إلى ذلك، يتطلب التشخيص الدقيق استخدام تقنيات مكلفة والتي تتطلب معدَات متخصصة ذات تكلفة مرتفعة وتستغرق وقتا طويلا كما انها غير مناسبة لإعدادات الحقل السريرية. ولذلك، فإن الهدف من هذه الدراسة هو تطوير اختبار يتميز بتوفير الوقت وإمكانية الإعتماد عليه في العينات السريرية باستخدام قياس اللون والتألق لجزيئات النانو جولد وذلك للكشف النوعي عن الأحماض النووية لبكتيريا *Clostridium difficile* والسموم التي تفرزها. ومن المتوقع أن يكون الإختبار الجديد ذات جودة عالية وبسيط ولا يستغرق وقتاً طويلاً.

الطرق

تم جمع مائة وخمس عينات من مستشفى الخور (عضو في مؤسسة حمد الطبية) وتم تأكيد ايجابية العينات باستخدام RT-PCR (GeneXpert, Cepheid, CA, USA). وقد تم إعداد جزيئات النانو جولد المثلى من خلال استعمال تراكيز مختلفة من الملح والمعالجة الحرارية (annealing temperature). بعد ذلك تم خلط الحمض النووي المستخرج من بكتيريا *Clostridium difficile* مع المحلول الأمثل الذي يحتوي على الملح وprimer المناسب. ثم تم تسخين المحلول وتبريده الى درجة حرارة الغرفة لمدة عشر دقائق تليها إضافة جزيئات النانو جولد. بالإضافة الى ذلك تم استعمال نفس جزيئات النانو جولد المثلى في الكشف عن سموم *Clostridium difficile*.

النتائج

تم اختبار مائة وخمسة من عينات *Clostridium difficile* الإيجابية باستخدام فحص جزيئات النانو جولد المثلى وأظهرت العينات نتائج ايجابية لستة وتسعين عينة من بين مائة وخمس عينات حيث تغير لون المحلول من اللون الأحمر إلى اللون الأزرق في دقيقة واحدة. ومن ناحية أخرى لوحظ نتائج سلبية وعدم تغير لون المحلول في تسع عينات. أما بالنسبة للسموم فقد كانت جميع العينات الإيجابية الستة والتسعون إيجابية ل للسم B باستخدام فحص RT- PCR (GeneXpert, Cepheid, CA, USA) و فحص جزيئات النانو جولد. بينما ست عينات إيجابية للسموم الثنائية من بين ستة وتسعين عينة ايجابية باستخدام RT- PCR (GeneXpert, Cepheid, CA, USA). ومع ذلك، كانت النتائج للسموم الثنائية باستخدام فحص جزيئات النانو جولد إيجابية لجميع العينات.

الخاتمة

النتائج أظهرت أن تخصصية الإختبار كان 91.4 ٪ وخصوصية الفحص كان 100 ٪. وعلاوة على ذلك ، تم اختبار السموم C.difficile باستخدام جزيئات النانو جولد، حيث أظهرت اتفاق 100 ٪ مع الكشف عن السم B بالمقارنة مع RT- PCR (GeneXpert, Cepheid, CA, USA) . ومع ذلك، فإن نتائج الفحص بالنسبة للسموم الثنائة كانت غير متوافقة مع RT-PCR (GeneXpert, Cepheid, CA, USA). وبالتالي فإن هناك حاجة إلى مزيد من العمل ل تحسين كفاءة الفحص للكشف عن ثنائي السموم كما أن الأختبار الجديد يتطلب تجربته على عينات سريرية مباشرة للمرضى.

جامعة قطر

قسم الدراسات العليا

كلية الآداب والعلوم

استخدام جزيئات النانو جولد في الكشف السريع والغير مكلف عن عدوى *Clostridium difficile* في قطر

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