COMPARISON OF TWO *CLOSTRIDIUM DIFFICILE* TOXIN IMMUNOASSAYS AND A REAL-TIME PCR ASSAY FOR *C. DIFFICILE tcdC* TO TOXIGENIC CULTURE FOR DETECTION OF TOXIN-PRODUCING *C. DIFFICILE* IN CLINICAL SAMPLES

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DECLARATION

I, Trusha Nana declare that this research report is my own work. It is being submitted for the degree of Master of Medicine in the branch of Microbiology in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other university.

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ABSTRACT

Background: Accurate diagnostic methods for *Clostridium difficile* infection (CDI) are required for optimal patient management, appropriate implementation of infection control measures and surveillance. An assay that also provides rapid results, is easy to implement in a routine diagnostic lab and is cost-effective would be ideal. Laboratory testing for *Clostridium difficile* infection is rapidly evolving. Recently published literature has shown that immunoassays for toxin detection, whilst being cheap and easy to implement, lack sensitivity. Molecular diagnostics that are sensitive and provide rapid results are now available. However, the high cost of these assays is of concern. As reflected in the literature the optimal test or testing algorithm for *Clostridium difficile* infection diagnosis is not clear.

Objectives: This study aimed to compare the performance of a real-time PCR assay and two immunoassays, and to establish the optimal testing strategy for Charlotte Maxeke Johannesburg Academic Hospital (CMJAH).

Methods: Using toxigenic culture as the gold standard, the Roche PCR assay for the detection of the *tcdC* gene, the Immuno *Card* Toxins A & B immunoassay and the C. Diff Quik Chek Complete immunoassay were evaluated as stand alone assays and as part of testing algorithms.

Results: The sensitivity, specificity, positive predictive value and negative predictive value of the various assays and algorithms ranged from 38% to 81%, 98% to 100%, 92% to 100% and 85% to 95%, respectively. The charge per sample tested varied widely depending on the assay and algorithm used. The maximum turnaround time ranged between four and twenty four hours.

Conclusion: The algorithm combining glutamate dehydrogenase and toxin immunoassay testing of all samples followed by PCR testing of only a subset of samples, performed the best, providing accurate results rapidly and cost-effectively.

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Introduction

1.1 Background

1.1.1 Epidemiology

Clostridium difficile infection is defined as 'the presence of symptoms (usually diarrhoea) and either a stool test positive for *C. difficile* toxins or toxigenic *C. difficile*, or colonoscopic or histopathologic findings revealing pseudomembranous colitis' [Cohen, 2010]. In the last 10 years there has been widespread increase in CDI incidence. *C. difficile* has emerged as the most important cause of healthcare-associated diarrhoea. Published data from North America and Europe clearly show a rise in CDI in healthcare institutions [Barbut, 2011]. A prospective incidence survey carried out in European hospitals in late 2008 showed the mean incidence of CDI cases to be 5.5 cases per 10 000 patient days, higher than that recorded in 2005. The anticipated number of CDI cases in 2010 for the United States (US) were 450 000 to 750 000 [Freeman, 2010]. Due to the lack of local surveillance, data on CDI rates for Africa, and South Africa in particular, are deficient. There is also little data from the Middle East and Asian regions [Barbut. 2011].

The escalating rate of CDI is partly attributable to the emergence of the hyper-virulent 027/BI/NAP1 strain of *C. difficile* [Barbut, 2011]. Toxin A (TcdA) and toxin B (TcdB) are implicated in the pathogenesis of CDI. These toxins inactivate Rho family GTPases, resulting in cellular cytotoxicity. The 027//BI/NAP1 strain has an 18-base pair deletion in the gene encoding the TcdC protein, a putative negative regulator of toxin A and B production. This mutation results in a truncated protein,

which has been linked to increased toxin A and B production *in vitro*. In addition, the 027/BI/NAP1 strain has the binary toxin gene. The binary toxin is another postulated virulence factor of *C. difficile*. This toxin has been found to increase the adherence of clostridial cells to intestinal epithelial cells [Freeman, 2010]. Hyper-sporulation and toxin B binding domain polymorphisms are other mechanisms thought to contribute to the pathogenicity of the 027/BI/NAP1 strain [Barbut, 2011]. High rates of resistance to the fluoroquinolones in the current 027/BI/NAP1 strain, together with the widespread use of these antibiotics in recent years, may have facilitated the dissemination of the 027/BI/NAP1 strain [Cohen, 2010]. More recently other hypervirulent strains like the PCR078 have been described [Vecchio, 2012]. There is no locally published data on *C. difficile* strain distribution or on the 027/BI/NAP1 strain prevalence specifically.

CDI has a spectrum of severity, with most cases presenting as uncomplicated diarrhoea with or without colitis. However fulminant disease, with megacolon, prolonged ileus, bowel perforation, hypotension and renal dysfunction can occur. Increased severity of CDI in recent years has been reported in Canada and the US. This has in part been attributed to an increased proportion of CDI being caused by the 027/BI/NAP 1 strain. Prospective surveillance in 88 Canadian hospitals in 2005 showed that compared to other strains, the 027/BI/NAP1 strain was associated with increased CDI incidence and increased disease severity [Freeman, 2010]. Similarly, across Europe the 027/BI/NAP1 strain has been associated with increased CDI-related morbidity. Increased occurrence of toxic megacolon, bowel perforation, septic

shock and poor clinical response to therapy with metronidazole, has been associated with this strain [Barbut, 2011].

Thirty-day attributable mortality during outbreaks of CDI in Montreal in the early to mid 2000's was 6.9%, with CDI having indirectly contributed to an additional 7.5% of deaths [Cohen, 2010]. Overall CDI-related mortality in the US is estimated at 15 000-20 000 patients per annum [Barbut, 2011]. At the Charlotte Maxeke Johannesburg Academic Hospital (CMJAH), a tertiary 1200 bed hospital, cases of severe CDI seem to be uncommon [anecdotal evidence-personal experience and discussion with clinicians].

Prolonged hospitalization for management of CDI results in substantial excess hospital costs. In Massachusetts over the period of 1999-2003, 55 380 days of hospitalization and $55.2 million in healthcare costs resulted from CDI management. In the US 10 years ago, the estimated excess cost related to CDI was $3.2 billion per annum [Cohen, 2010]. In South Africa and in the developing world in particular, extended hospital stays arising from any cause, including nosocomial infections, and the associated costs place an enormous strain on scarce healthcare resources.

1.1.2 Risk factors for CDI

The most commonly cited risk factors for CDI include antimicrobial use, advanced age, hospitalization, exposure to other patients with CDI and therapy with anti-neoplastics [Cohen, 2010]. Broad spectrum antibiotics by inhibiting normal bowel

flora provide a selection pressure for *C. difficile*. Cephalosporins, fluoroquinolones and clindamycin are antibiotics that have been associated with a particularly high risk for CDI [Freeman, 2010]. Prolonged and recurrent hospital admissions as risk factors may reflect transmission pressure. The cummulative exposure to *C. difficile* spores from healthcare workers' hands and contaminated fomites in the hospital environment may increase the chances of colonization and subsequent infection with *C. difficile* [Cohen, 2010; Freeman, 2010]. Some studies have found that the use of proton pump inhibitors and histamine 2 receptor antagonists may predispose to CDI [Freeman, 2010]. CDI in persons without these traditional risk factors are also increasing [Freeman, 2010].

Community-acquired CDI (CA-CDI), paediatric infections and infections in young females in the peri-partum period have been recently reported. Varying intervals since discharge from hospital have been used to define CA-CDI. This may impact on the proportion of CA-CDI cases reported in various publications, which varies from 11-41% of all CDI cases [Freeman, 2010; Khanna, 2012]. The SHEA/IDSA guidelines define CA-CDI as 'disease in persons with no overnight stay in an inpatient healthcare facility (HCF) in at least the 12 weeks prior to symptom onset' [Cohen, 2010]. The risk factors and source(s) of exposure in CA-CDI are not clear [Freeman, 2010]. Inflammatory bowel disease, irritable bowel syndrome, hospitalization two years prior to CDI presentation, renal failure and proton pump inhibitor use, has been associated with CDI. However, cases with no exposure to antibiotics or hospitalization have been documented. Increased pressure from the

high rates of CDI in hospitals, as well as farm animals and meat as a source of *C. difficile*, is being investigated as likely sources for CA-CDI [Freeman, 2010].

C. difficile is mainly acquired in HCFs [Cohen, 2010]. The risk of colonization with *C. difficile* increases with the duration of admission. This risk can reach up to 40 % after a four week admission period. *C. difficile* spores are shed in stool and transmission is through the faecal-oral route. Horizontal transmission in HCFs occurs through healthcare workers' hands, contaminated environmental surfaces and direct contact with *C. difficile*-infected patients. Studies have demonstrated that between 14 and 59% of healthcare workers' hands are contaminated following care of patients with CDI [Barbut, 2011]. Patients with CDI-related diarrhoea heavily contaminate their surroundings [Barbut, 2011]. *C. difficile* spores are hardy and able to persist in the environment. These spores are also resistant to alcohol-based hand hygiene products. Appropriate infection control measures include hand hygiene (washing hands with soap and water), contact precautions (gloves and gowns), isolation or cohorting of infected patients and environmental decontamination. Implementation of such measures has been shown to reduce CDI rates [Cohen, 2010].

1.1.3 CDI Surveillance

Active surveillance of CDI is important to establish baseline rates of infections. Ongoing surveillance allows for detection of outbreaks and facilitates appropriate targeting of infection control efforts. The recommended denominator for HCF CDI rates is the number of patient days [Cohen, 2010]. When an increase in the CDI

rate is detected, unit-specific data must be analysed to identify possible hotspots of CDI transmission. Surveillance is also required to monitor the impact of infection control measures and antibiotic stewardship programs on CDI rates. Clinical practice guidelines recommend that 'at a minimum (conduct) surveillance for HCF-onset, HCF-associated CDI in all inpatient HCFs to detect outbreaks and monitor patient safety' [Cohen, 2010]. Despite improvements in surveillance efforts in North America and Europe, the implementation of uniform diagnostic methods and standardized reporting protocols even in these regions, is variable [Freeman, 2010].

In addition to the use of standardized case definitions, surveillance also requires accurate laboratory diagnostics. Numerous test methods are available for CDI laboratory diagnosis. The accuracy of these different test methods is highly variable. This variation in the performance characteristics of the different test methods, impacts on the patient management and on reported rates of infection. Much research effort has been focused on evaluating diagnostic assays.

1.1.4 CDI diagnosis

Asymptomatic colonization with both toxin- and non-toxin producing *C. difficile* strains can occur. Between one and three percent of healthy adults have intestinal colonization with *C. difficile* [Barbut, 2011]. In the paediatric population this rate of asymptomatic colonization is believed to be higher [Freeman, 2010]. Hence, testing for *C. difficile* must only be performed on unformed stools (samples from patients with diarrhoea). The one exception to this rule is for samples from patients with an ileus related to severe CDI. Only toxin producing strains are pathogenic.

At present there are a number of testing methods with different diagnostic targets available. Laboratory diagnosis may be based on the detection of glutamate dehydrogenase (GDH), toxigenic strains, toxin encoding genes (*tcdA* or *tcdB*), the *tcdC* gene or toxin (by IAs or cytotoxicity assays) [Sloan, 2008; Carroll, 2011]. GDH is an enzyme produced by all strains of *C. difficile*, both toxigenic and non-toxigenic [Shetty, 2010]. Hence, the detection of this enzyme in a stool sample using monoclonal antibodies to *C. difficile*-specific GDH, indicates the presence of *C. difficile*.

Cell culture cytotoxicity neutralization assays (CCCNAs) were previously regarded as the "gold standard" for CDI diagnosis [Carroll, 2011]. More recently when compared to nucleic acid
amplification tests (NAATs) and toxigenic culture, CCCNAs (both in-house and commercial assays) have shown sensitivities of less than 90%. Toxigenic culture (anaerobic culture of stool samples, followed by detection of toxin from cultured isolates by CCCNA or molecular detection of toxin genes or toxin-regulating genes), is more sensitive. Toxigenic culture is now regarded as the 'standard against which other clinical tests results should be compared' [Cohen, 2010]. This method of testing can however be laborious and time-consuming [Sloan, 2008]. The minimum turnaround time is two to three days, but this may extend up to nine days [Cohen, 2010]. A brief discussion of the various available testing methods follows.

Toxigenic Culture:

Toxigenic culture requires anaerobic culture using selective and differential culture media. Due to the abundance of normal flora in stool, the ability of the inhibitory additives in the culture medium to suppress bacteria other than *C. difficile* is of paramount importance. Various media, including chromogenic agars, are available commercially. The sensitivity and selectivity of these agars vary. A recent publication reported on the performance of a new chromogenic agar for *C. difficile* isolation [Perry, 2010]. Ninety-four percent of all *C. difficile* isolates were recovered as characteristic grey-black colonies following 24 hours of incubation on the new *C. difficile* agar from bioMérieux (France). Incubation for an additional 24 hours increased the sensitivity for recovery of *C. difficile* to 99%, but reduced the specificity (increased number of non-*C. difficile* isolates appearing as grey or black colonies). Heat and alcohol shock methods to kill vegetative bacteria present in stool samples, whilst allowing the clostridial spores to survive, have been applied to further improve culture selectivity [Carroll, 2011].

CCCNA:

CCCNA methodology is complex, with a number of steps. Briefly it requires culture of *C. difficile* on agar, followed by incubation of the cultured colonies in an enrichment broth. Supernatants are then added to cell culture monolayers, one with *C. sordelli* antitoxin and one without. Following incubation, rounding of cells only in the culture without the antitoxin, is interpreted as positive. Many different cell lines can be used to detect the toxin, but these have varying sensitivities [Cohen, 2010]. CCCNAs are costly, technically demanding, and require the

maintenance of cell lines (not routine in many microbiology laboratories). CCCNAs have a turnaround time of greater than 3 days.

Cytotoxin (CYT) Assays:

For CYT assays, stool is diluted and centrifuged. The supernatants are added to cell cultures, as for the CCCNAs discussed above. The testing methodology for CYT assays is not standardized. A number of factors, including pre-processing of samples, choice of cell line and interpretive end points used, may impact on the assay performance [Eastwood, 2009].

NAATs:

Molecular assays for the detection of the *tcd*A, *tcdB* or *tcdC* gene have been developed. Recently NAATs are increasingly being used in clinical laboratories for diagnosis of CDI. Commercial real-time PCR assays include the BD GeneOhm Cdiff (BD-GeneOhm, San Diego, CA), ProdGastro Cd (Gen-Probe, Inc. San Diego, CA) and Xpert *C. difficile* (Cepheid, Sunnyvale, CA), that target the *tcdB* gene [Lalande, 2011]. These assays are FDA approved. The *illumi*gene *C. difficile* assay (Meridian Biosciences, Cincinnati, OH) is based on loop-mediated isothermal amplification (LAMP) technology and targets a conserved sequence in the *tcdA* gene. In addition, a number of in-house PCR assays have been developed and evaluated [Larson, 2010; Knetsch, 2011; de Jong, 2012]. The presence of a toxin gene as detected by NAATs, does not necessarily equate to the expression of the gene *in vivo*. A study looking at the utility of a real-time PCR assay on unformed stool samples for the diagnosis of CDI demonstrated that there was good

correlation between the PCR result and the clinical condition of the patient [Eastwood, 2009]. Reported sensitivities and specificities of these assays range from 75% to 99%, and 93% to 99%, respectively, depending on the assay, the patient population and the gold standard used in the evaluation [Carroll, 2011]. Low specificities of some NAATs result in poor positive predictive values (PPVs). Whilst providing rapid results (within 1-4 hours), these assays are expensive. In general implementation of these assays is not feasible for smaller laboratories that lack the expertise and technology required for molecular diagnostics. The 'hands-on' time varies depending on the assay and this together with level of expertise required, may impact on actual turnaround times. The Xpert *C. difficile* assay is run on the GeneXpert instrument and requires little technical expertise [Carroll, 2011]. The *illumi*gene *C. difficile* assay does not require expensive equipment as the technology is isothermal. Reasonable 'hands-on' time and ease of performance is also reported for this assay [Lalande 2011; Nore′n, 2011].

Immunoassays:

Many commercial IA toxin detection kits are available. These IAs are widely utilized in routine microbiology laboratories for the diagnosis of CDI. The assays are easy to perform, provide rapid results and are relatively inexpensive. The toxin IAs are either a well-type or a membrane-type. Implementation in the laboratory of the membrane-type (lateral flow or immunochromatographic assays) may be easier than that of the well-type assays. However, the sensitivity of these assays is poor [Barbut, 2011]. A comparison of nine commercial *C. difficile* toxin detection IAs with cytotoxigenic culture, showed a sensitivity ranging from 60.0-87.6%. The

specificity of these nine assays ranged from 91.4-99.4%. The resulting PPV calculated at a 10% prevalence of CDI (expected prevalence rate in hospital setting) ranged from 47.0-88.7% (mean of 68.7%) [Eastwood, 2009]. Planche, et al. in their systematic review of commonly used toxin IAs, defined 'an acceptable test as one that would have a sensitivity of at least 90% and a false positive rate of 3% or less'. They found that no assay met the specified performance criteria and concluded that use of an IA as a standalone test is not recommended [2008]. Crobach, et al. in their evaluation of thirteen commercial toxin IAs, did not find any significant differences in the performance of the well-type as compared to the membrane-type assays [2009]. A recently published study demonstrated that patients with CDI diagnosed by NAATs, but with negative IA results, do not differ in terms of severity of disease or shedding of spores onto their skin or into their surroundings, from those patients whose stool samples test both NAAT and IA positive [Guerrero, 2011]. Hence false negative IA results can impact both on the outcomes of infected patients, and on *C. difficile* transmission to other patients.

GDH:

GDH is an enzyme produced by *C. difficile*. Rapid detection of GDH directly from stool samples using IAs is possible. The use of monoclonal antibodies to *C. difficile*-specific GDH allows for a positive GDH result to be used as a marker for the presence of *C. difficile* [Shetty, 2011]. The reported sensitivity of the more recently developed commercial assays is high [Cohen, 2010; Carroll, 2011]. However further testing to determine whether a toxigenic strain is present is required as both toxin- and non-toxin producing strains will show GDH positivity.

GDH is frequently used as a screening test. Due to the high negative predictive value (NPV) of this test, stools that test negative are regarded as negative for CDI. Samples that test GDH positive are tested with a second assay to confirm toxin production. The C. Diff Quik Chek Complete (TechLab, Blacksburg, VA) assay is a rapid immunochromatographic test that allows for the detection of GDH and toxin. Recently published evaluations have shown a sensitivity of 100% for the GDH component of the test and lower sensitivity (61-78%) for the toxin component [Carroll, 2011].

An ideal laboratory test for CDI would be a sensitive and specific assay that provides rapid results. Additionally such an assay would have to be easy to implement in a routine diagnostic laboratory and be reasonably priced. Due to the lack of a single test method that fulfils all the above criteria, some laboratories have implemented testing algorithms [Fang, 2010]. These algorithms involve stepwise sample testing with different assays, with the aim of improving diagnostic accuracy, turnaround time and/or cost-effectiveness. A number of studies have compared the feasibility and utility of a single test method to multistep testing algorithms [Reller 2007; Gilligan, 2008; Wren, 2009; Cohen, 2010; Doing, 2010; Orellana, 2012]. No such evaluations in the South African setting have been published. An evaluation of a three-step algorithm for detection of CDI in a clinical microbiology laboratory in Washington, found that sequential testing of stool samples with a GDH IA, followed by a toxin IA for GDH-positive samples, and lastly PCR testing of GDH-positive, toxin IA-negative samples, provide a rapid and convenient alternative to PCR testing of all samples [Larson, 2010].

Assays with a poor sensitivity can result in missed diagnoses and poor patient outcomes. Also increased cross transmission of *C. difficile* due to inadequate infection control measures may occur. Suboptimal specificity of an assay coupled with a low prevalence of CDI can result in the reporting of false positive results. Even in the presence of a nosocomial outbreak of CDI, *C. difficile* will only be responsible for about 30% of antibiotic-associated diarrhoea cases [Cohen, 2010]. This has a number of implications for the patient. Stopping of antibiotic therapy that is prescribed for an infection at another site, unnecessary treatment for the suspected CDI, cohorting of the patient with other patients with CDI (thus increasing the risk of the patient acquiring CDI), and lack of investigation for the true cause of the diarrhoea, may result [Eastwood, 2009].

Cost consciousness with regards to laboratory testing is critically important . In an era of very constrained healthcare budgets, laboratories need to offer tests that provide rapid and reliable results at as low-a-cost as possible. This facilitates the initiation of prompt and appropriate therapy in patients with CDI. Published analyses of the costs of algorithm-based CDI laboratory testing show considerable potential savings. These studies show a significant difference in the costs of PCR-based testing of all stool samples compared to GDH and toxin IA testing followed by PCR testing only of GDH positive, toxin negative samples [Carroll, 2011]. The healthcare expense associated with delayed or inappropriate patient management, and with nosocomial infections is substantial. Infection control resources too are costly, and targeted use of these resources based on results of accurate laboratory testing is required.

1.1.5 CDI diagnosis at CMJAH

Currently the Charlotte Maxeke Johannesburg Hospital (CMJAH) microbiology laboratory offers a toxin IA, the Meridian Immuno *Card*™ Toxins A & B assay (Meridian Bioscience, Cincinnati, OH), for *C. difficile* toxin detection. The test is easy to perform with a built-in control. The testing process takes 35 minutes. Batch testing of samples is not required. 24-hour testing, 7 days a week is offered, with a guaranteed turnaround time of 4 hours. The suboptimal sensitivity and specificity of the assay is known to some clinicians. When the suspicion of CDI is high, a second sample is often submitted for IA testing if the initial test results are negative. However this is not recommended as repeat testing rarely increases the yield of positive results [Drees, 2008; Bartlett, 2010; Carroll, 2011]. Also patients with suspected CDI are sometimes treated for the suspected infection, despite negative IA results. Enteric precautions are not practiced for all hospitalized patients with diarrhoea. Consequently with false negative IA results, appropriate infection control measures are frequently not instituted. This contributes to ongoing nosocomial transmission of *C. difficile*.

Recently a real-time PCR assay, for diagnosis of CDI directly from stool samples, was introduced at the Infection Control Services Laboratory. This testing requires expensive equipment and is performed by trained scientists. The accuracy of the PCR-based testing is superior to the IA testing. However the cost of the PCR is double that of the IA. Samples are batched and run once per day resulting in an up to 24-hour turnaround time.

Clearly accurate lab diagnosis of CDI is crucial for the various reasons cited above. However the optimal test or testing algorithm for the laboratory diagnosis of CDI remains controversial [Fang, 2010; Freeman, 2010]. The aim of this study was to establish the optimal test or testing algorithm for CMJAH, taking into account the technology and resources available. In addition valuable epidemiological information regarding CDI rates at CMJAH would be established. The baseline data provided by this study could facilitate future surveillance efforts and more detailed epidemiological studies, to improve the understanding of *C. difficile* in our setting.

1.2 Objectives

1.2.1 Primary Objectives

(1) To compare the performance of a real-time PCR assay and two immunoassays to toxigenic culture for direct detection of toxin-producing *C. difficile* in clinical samples.

-Compare the relative sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of the various methods

(2) Establish the optimal stand alone test or testing algorithm for Charlotte Maxeke Johannesburg Academic Hospital microbiology laboratory.

-determine whether a testing algorithm, using a combination of different tests in a step-wise manner, provides superior results in terms of accuracy and cost-effectiveness, without increasing turnaround time excessively

1.2.2 Secondary objectives

(1) Establish the burden of laboratory-confirmed *C. difficile* disease at Charlotte Maxeke Johannesburg Academic Hospital

-use Charlotte Maxeke Johannesburg Academic Hospital patient admission numbers during the study period to estimate burden of disease at this healthcare facility

2.0 Materials and Methods

2.1 Sample selection

A convenience sample of 190 consecutive soft and liquid stool samples submitted between 1 September 2011 and 30 September 2011 to the CMJAH microbiology laboratory and Infection Control Services laboratory for routine *C. difficile* testing were used in the evaluation. Duplicate samples received from the same patient within 7 days were excluded.

2.2 Testing Protocol

For each sample the following testing protocol was adhered to.

- All stools were tested using the in-use Meridian Immuno *Card* Toxins A & B IA, and secondly using the TechLab C. Diff Quik Chek Complete IA (detects both GDH and Toxins A and B). Stools were tested within 24 hours of receipt of the stool sample in the lab.
- PCR-based direct detection of the *tcdC* gene from stool samples, using the Roche assay, was done within 72 hours of sample receipt.
- All stool samples were cultured (on bioMe′rieux chromID *C. difficile* chromogenic agar and in a selective broth) within 24 hours of receipt in the lab.

2.2.1 Immuno *Card* Toxins A & B IA

The Immuno *Card* Toxins A & B is a qualitative horizontal-flow enzyme IA. Testing with the Immuno *Card* Toxins A & B assay was performed according to the manufacturer's instructions.

All test cards and reagents were brought to room temperature. 200µl of sample diluent was added to a test tube. To this 150µl of enzyme conjugate was added, followed by 25µl of the stool sample. The sample mixture was vortexed for 10 seconds, and then left to stand for 5 minutes. The sample mixture was vortexed again for 10 seconds, before adding 150µl of the mixture to each of the two sample ports on the test card. Following 5 minutes of incubation, 3 drops of the wash reagent was added to each of the reaction ports. Three drops of the substrate reagent was then dispensed in each reaction port. An incubation period of 5 minutes was allowed, after which the results were read.

A blue colour in the test and control reaction ports was interpreted as positive. A negative test result was indicated by a blue colour in the control reagent port only.

Quality Control

Internal: Each test card has an internal control. The appearance of a blue colour on the control reaction port confirms that the reagents were active at the time of use, that the sample was added and that there was adequate migration of the sample.

External: Upon opening of a new kit the reagents were tested using the supplied positive control and negative control (diluent). Correct results obtained with the positive control indicate that the test was performed correctly, the antibodies embedded in the membrane and the enzyme conjugate are active, and that the membrane supports adequate sample flow. In addition, testing with the negative control was done to demonstrate that non-specific reactivity of the reagents was absent.

2.2.2 C. Diff Quik Chek Complete IA

The C. Diff Quik Chek Complete assay is a lateral-flow membrane enzyme IA. Testing with this assay was performed according to the manufacturer's instructions. Reagents and test devices were brought to room temperature. 750µl of diluent was added to a test tube. To this 1 drop of conjugate was added, followed by 25µl of the stool sample. The sample mixture was vortexed for 10 seconds. 500µl of the sample mixture was transferred to sample well. This was followed by a 15 minute incubation period. 300µl of wash buffer was then added to the reaction well. Once this was absorbed, 2 drops of the substrate reagent was added. The test was allowed to incubate for 10 minutes before reading the results.

The appearance of any control dots was taken to represent a valid internal control. The presence of a blue antigen line and the dotted control line was interpreted as a positive GDH result. A positive GDH and toxin result was recorded when a blue antigen line, a blue toxin line and the control line were visible. The presence of only the dotted control line was interpreted as a negative result.

Quality Control

Internal: Each sampling device has a built-in internal control. The appearance of the blue control dots confirms that the samples and reagents were added correctly, that the reagents were active at the time of performing the assay, and that the sample migrated properly through the membrane device.

External: On opening of each new kit testing with the supplied positive control and negative control (diluent) was performed. The positive control confirms that the reagents are working. The negative control is used to exclude non-specific reactivity of the kit reagents.

2.2.3 *tcdC* gene real-time PCR

Manual Extraction of DNA:

A manual DNA extraction using the Roche High Pure PCR Template was done. A cotton swab was inserted into each stool sample multiple times and swirled around. The swab was then placed in 1 ml of sterile water and the suspension was allowed to settle. 200µl of supernatant was aspirated for use as the sample.

200µl of PBS and 5µl of lysozyme (10mg/ml) was added to the sample. The mixture was incubated at 37° C for 15 minutes. Then 200µl of binding buffer and 40µl of proteinase K was added. Following a 10 minute incubation at 70° C, 100µl of isopropanol was added. The fluid was applied to a spin column in a 2ml collection tube and then centrifuged for 1 min at 8 000 x g. The flow-through was discarded and the column placed in new collection tube. 500µl of inhibitor removal buffer was added, followed by centrifugation for 1 minute at 8 000 x g. The flow-through was discarded and the column inserted in another collection tube. 500µl of wash buffer was added and the column was centrifuged at 8 000 x g for 1 minute. The flow-through was discarded and the wash buffer-centrifugation step repeated once. The flow-through was discarded. The spin column was centrifuged at full speed for 10 seconds and the placed in a 1.5ml tube. 200µl of elution buffer was added and the column centrifuged for 1 minute at 8 000x g. The elutant containing the DNA was used as sample in the PCR reaction.

PCR:

The Roche LightMix kit for *C. difficile* was used. Primers for a 176 base pair (bp) fragment of the *C. difficile tcdC* gene and a 158 bp fragment of the 18 bp deletion mutant *C. difficile* del. (ribotype 027) were used.

Preparation of LightCycler reagents

66µl of PCR-grade water was added to both the reagent mix green cap vial and the internal control white cap vial.

Preparation of Control DNA (supplied with kit)

40µl of PCR-grade water was added to the positive control colourless cap vials.

Preparation of the LightCycler reaction mix

Three controls were run with each sample or batch of samples- a negative control and a *C. difficile* and *C. difficile del*. positive control. In a cooled reaction tube, the reaction mix was prepared. Example of reaction mix calculation:

Component	1 Reaction (µl)	Master mix for 1 sample (plus 3 controls) = 5X mix
PCR-grade water	2.6µl	13µl
Mg2+ solution 25mM	2.4µl	12µl
Reagent mix	4µl	20µl
Internal control mix	4µl	20µl
Roche Master	2µl	10µl
Template DNA	5µl	
Total	20µl	

The reaction mixture was briefly vortexed and then centrifuged to settle the fluid. 15µl of reaction mix was aliquoted per LightCycler capillary. The DNA template, positive controls and blank (negative control) were added to the appropriately labelled tubes. The capillaries were capped and centrifuged for 10 seconds at 700x g. The capillaries were loaded in the LightCycler and the run started.

Data analysis

The internal control data was viewed in channel 705 and was used to check for possible PCR inhibition. The negative control and low-concentrated *C. difficile* DNA samples (10 – 1 000 copies) should show an amplification curve for the internal control with a crossing point at approximately cycle 30.

The *C. difficile* del. DNA exhibits a melting temperature (Tm*) of 65° C. *C. difficile* shows a melting profile between a Tm of 55° C and 65° C.

The PCR products were analyzed with hybridization probes labelled with LightCycler red 640. The PCR products were identified with melt curve analysis.

Interpretation of results

The negative control must show no signal.

The positive controls must give an amplification signal.

Test samples were analyzed according to the table below.

Analysis of PCR results as per Roche PCR package insert:

C difficile (sample)	Internal control (sample)	Positive control	Negative control	Result
640	705	640	640	
No amplification	Detectable	Amplification	Negative	Negative
Amplification	Irrelevant*	Amplification	Negative	Positive
No amplification	Not detectable	Amplification	Irrelevant*	PCR failure
Irrelevant*	Irrelevant*	No amplification	Irrelevant*	PCR failure
Irrelevant*	Irrelevant*	Irrelevant*	Positive	Contamination

* Results of control or sample amplification does not impact on overall interpretation of PCR results

2.2.4 Toxigenic culture

-Stool samples were cultured on chromID *C. difficile* agar and in a selective broth (Rob's meat broth with cycloserine 250mg/litre, cefoxitin 8mg/litre and lysozyme 5mg/litre). Samples were first treated with an alcohol shock procedure (1 ml of stool plus 1 ml of absolute alcohol agitated on a mixer for 1 hour). Samples were then centrifuged for ten minutes at 3 500 x g. The supernatant was discarded, and 0.2 ml of the sediment cultured on chromID *C. difficile* agar. The remainder of the sediment was added to the selective broth.

-The inoculated cultures were incubated anaerobically at 35 degrees Celsius.

-The culture plates were read after 24 hours of incubation. If colonies resembling *C. difficile* (grey to black colonies with an irregular or smooth border) were not isolated on the chromogenic agar, the broth was sub-cultured onto 10% blood agar and the chromID *C. difficile* agar. These agar plates were incubated anaerobically and read after 24 hours of incubation. From the 10% blood agar plates, non-haemolytic, greyish-transluscent, spreading colonies were picked off.

-All isolates resembling *C. difficile* on colony morphology (from initial chromogenic agar, and from sub-culture of the selective broth, had confirmatory identification done with gram staining (gram-positive to gram-variable bacilli with or without subterminal spores) and the bioMèrieux anaerobic API system (rapid ID 32 A).

-All isolates confirmed to be *C. difficile*, were tested for the presence of the *tcdC* gene using the Roche PCR assay. Three to five colonies were touched with a cotton swab. A manual extraction procedure, followed by the PCR was performed as described above (under tcdC gene real-time PCR).

Two different American Type Culture Collection (ATCC) *C. difficile* strains were inoculated onto the chromID *C. difficile* agar and into the selective broth and incubated anaerobically. The broths were subcultured onto 10% blood agar after 24 hours of incubation. Growth of the ATCC strains on the chromID *C. difficile* and 10% blood agars confirmed that the media and incubation conditions supported the growth of *C. difficile*.

2.3 Data Analysis

A specimen was considered to contain toxigenic *C. difficile* if *C. difficile* was cultured and the presence of the *tcdC* gene was confirmed with PCR testing of the culture.

- The performance of the
    - Immuno *Card* Toxins A & B IA ,
    - C. Diff Quik Chek Complete IA (the combined GDH plus Toxin A/B result defined as the following: GDH positive, Toxin A/B positive results were considered positive. GDH positive, Toxin A/B negative, and GDH negative, Toxin A/B negative results were interpreted as negative), and
    - the *tcdC* gene PCR assay for the direct detection of toxin-producing *C. difficile* from stool samples

was compared to toxigenic culture.

- Performance was further investigated by considering the results of the
    - GDH component of the C. Diff Quik Chek Complete,
    - Toxin component of the C. Diff Quik Chek Complete,
    - Immuno *Card* Toxins A & B IA and PCR results combined (initial testing of all samples with Immuno *Card* Toxins A & B IA; followed by PCR testing only of IA negative samples) versus toxigenic culture, and
    - C. Diff Quik Chek Complete IA and PCR results combined (initial testing of all samples with C. Diff Quik Chek Complete IA; followed by PCR testing only of GDH positive, toxin A/B negative samples) versus toxigenic culture.

Results were captured and analyzed using Microsoft Excel.

The sensitivity and specificity for each assay and algorithm was calculated.

-Sensitivity: (number of true-positive results)/(sum of true-positive and false-negative results)

-Specificity: (number of true-negative results)/(sum of true-negative and false-positive results)

95% Confidence intervals (CI) were calculated for the sensitivity and specificity for each of the assays and algorithms. The formula used: $p \pm 1.96 \times \sqrt{p(1-p)/n}$, where p is the sensitivity or specificity and n is the number of samples.

The sensitivity and specificity data were used to calculate the PPV and NPV for CDI.

-PPV: (number of true-positive results)/(sum of true-positive and false-positive results)

-NPV: (number of true-negative results)/(sum of true-negative and false-negative results)

The prevalence of CDI at CMJAH was estimated using the number of admissions for the month over which the 190 samples were submitted, and the number of samples testing toxigenic culture positive.

The PPV and NPV at different CDI prevalences of 2%, 3.3%, 5% and 10% were calculated using a web-based calculator (http://vassarstats.net/clin2.html), accessed on 9 July 2012. The prevalence, sensitivity and specificity data was input for the calculation.

The charge per sample for the various assays and testing algorithms was calculated based on the 2011 NHLS State pricing list [NHLS state pricing catalogue 2011/12]. In addition, turnaround times for the various assays and algorithms were compared.

3.0 Results

3.1 Results of assays and algorithms compared to toxigenic culture

One hundred and ninety stool samples were included in the evaluation. Of these, 43 samples were toxigenic culture positive and the remaining 147 were toxigenic culture negative.

One stool sample was *C. difficile* culture positive, but the *tcdC* gene PCR on the culture isolate was negative. Sequencing (16S rDNA) of the isolate confirmed it to be *C. difficile*. The *tcdC* gene PCR performed directly on the stool sample was negative, as were the Immuno *Card* Toxins A & B and the C. Diff Quik Chek Complete IAs results. The Xpert *C. difficile* PCR that targets the *tcdB* gene, performed on the stool sample was also negative. This sample was considered to be toxigenic culture negative.

None of the 43 isolates were found to harbour the *tcdC* gene deletion associated with ribotype 027.

Below are the results of the various assays and algorithms compared to the gold standard of toxigenic culture.

Table 1. Summary of results of 190 samples compared to toxigenic culture for the various assays and algorithms

	Immuno *Card* Toxins A & B	GDH component of C. Diff Quik Chek Complete	Toxin component of C. Diff Quik Chek Complete	C. Diff Quik Chek Complete*	Roche *tcdC* gene PCR	Immuno *Card* Toxins A & B plus Roche *tcdC* gene PCR#	C. Diff Quik Chek Complete plus Roche *tcdC* gene PCRδ
True Positive	15	38	22	22	33	34	33
False Positive	0	4	0	0	3	3	0
True Negative	148	144	148	148	145	145	148
False Negative	27	4	20	20	9	8	9

* TP= GDH positive plus toxin positive

Testing of Immuno *Card* negative samples with PCR

δ Testing of GDH positive, toxin negative samples with PCR

The calculated sensitivity, specificity, PPV and NPV data for the three assays and the two algorithms is summarized in Table 2 below.

Table 2. Comparison of assays and algorithms with results of toxigenic culture for the 190 samples

	Immuno *Card* Toxins A & B (95% CI)	GDH component of C. Diff Quik Chek Complete (95% CI)	Toxin component of C. Diff Quik Chek Complete (95% CI)	C. Diff Quik Chek Complete* (95% CI)	Roche *tcdC* gene PCR (95% CI)	Immuno *Card* Toxins A & B plus Roche *tcdC* gene PCR# (95% CI)	C. Diff Quik Chek Complete plus Roche *tcdC* gene PCRδ (95% CI)
Sensitivity %	38 (23-53)	90 (81-99)	52 (37-67)	52 (37-67)	79 (67-91)	81 (69-93)	79 (67-91)
Specificity %	100	97 (94-100)	100	100	98 (96-100)	98 (96-100)	100
PPV %	100	90	100	100	92	92	100
NPV %	85	97	88	88	94	95	94

95% confidence interval (95% CI) values are shown in parentheses

* TP= GDH positive plus toxin positive

Testing of Immuno *Card* negative samples with PCR

δ Testing of GDH positive, toxin negative samples with PCR

3.1.1 Sensitivity and specificity:

The 90% sensitivity of the GDH was the highest of the various assays evaluated. The Immuno *Card* Toxins A & B assay had the lowest sensitivity at 38%. The toxin IA component, the C. Diff Quik Chek Complete, showed a slightly better sensitivity of 52%. The sensitivity of the C. Diff Quik Chek assay overall was 52% (expected to be similar to sensitivity of toxin IA component). The 79 % sensitivity of the Roche *tcdC* gene PCR, was very similar to that of the Immuno *Card* Toxins A & B-, and the C. Diff Quik Chek Complete-based testing algorithms (81 and 79%, respectively). The Immuno *Card* Toxins A & B algorithm required 94 PCRs to be done compared to 13 for the C. Diff Quik Chek Complete algorithm. All the assays and testing algorithms had high specificities (97-100%).

3.1.2 PPV and NPV

All the evaluated assays and algorithms demonstrated a high PPV (92-100%). The NPV of the Immuno *Card* Toxins A & B and the C. Diff Quik Chek were 85% and 88%, respectively. The NPV for both the Roche *tcdC* gene PCR and the C. Diff Quik Chek Complete-based algorithm was 94%, whilst that of the Immuno Card Toxins A & B algorithm was 95%.

3.2 Detailed comparison of C. Diff Quik Chek Complete assay to toxigenic culture

Results of the C. Diff Quik Chek Complete results in comparison to toxigenic culture are summarized in table 3 below.

Table 3. Detailed comparison of C. Diff Quik Chek Complete assay to toxigenic culture results.

Toxigenic culture Results (190 samples)	**C. Diff Quik Chek Complete Results**
Toxigenic culture Positive (39 samples)	
22	GDH-positive, Toxin-positive
17	GDH-positive, Toxin-negative
Toxigenic culture Negative (151 samples)	
148	GDH-negative, Toxin-negative
3	GDH-positive, Toxin-negative

If results for samples testing GDH positive, Toxin A/B positive were sent out as positive, and results for samples testing GDH negative, Toxin A/B negative, were sent out as negative, 170 (22 + 148) results would be correctly reported. This represents 89% (170/190) of the samples.

3.3 Estimation of CDI prevalence at CMJAH

Number of admissions for one month period: 1240

Number of true positive *C. difficile* samples in same one month period: 42

42/1240= 3.3%

3.4 Positive- and negative predictive values of assays and algorithms at different prevalences

The calculated PPVs and NPVs at different CDI prevalence rates are summarized in Table 4 below.

Table 4. Positive- and negative predictive values of assays and algorithms at different prevalences

Prevalence	Immuno *Card* Toxins A & B		C. Diff Quik Chek Complete*		Roche *tcdC* gene PCR		Immuno *Card* Toxins A & B plus Roche *tcdC* gene PCR#		C. Diff Quik Chek Complete plus Roche *tcdC* gene PCRδ	
	PPV	NPV	PPV	NPV	PPV	NPV	PPV	NPV	PPV	NPV
2%	100	99	100	99	45	100	45	100	100	100
3.3%	100	98	100	98	57	99	58	99	100	99
5%	100	97	100	98	68	98	68	99	100	99
10%	100	94	100	95	81	98	82	98	100	98

PPV and NPV expressed as %

* TP= GDH positive plus toxin positive

Testing of Immuno *Card* negative samples with PCR

δ Testing of GDH positive, toxin negative samples with PCR

Overall (across the range of prevalences) the C. Diff Quik Chek Complete IA based testing algorithm, had the highest predictive values (PPV of 100% and NPV ranging from 98-100%).

At a prevalence of 2%, which is the estimated prevalence in the community (Eastwood, 2009), the two IAs, showed high PPV's (100% for both IAs) and NPV's (94-99% for the Immuno *Card* Toxins A & B assay, and 95-99% for the C. Diff Quik Check Complete assay). The PPV's (45%) for the Roche *tcdC* gene PCR and the Immuno *Card* Toxins A & B based algorithm were very low at the 2% prevalence.

At the calculated CDI prevalence for CMJAH of 3.3%, the IAs again demonstrated high predictive values.

The PPV's (68%) of the Roche *tcdC* gene PCR and the Immuno *Card* Toxins A & B IA-based algorithm remained low at a prevalence of 5%.

At a prevalence of 10%, the NPV's of the IAs decreased somewhat whilst the PPV's for the Roche *tcdC* gene PCR and the Immuno *Card* Toxins A & B based testing algorithm increased (81 and 82% respectively).

3.5 Cost and Turnaround Time analysis

The charge per sample and turnaround time (TAT) for the various assays and algorithms is summarized in the table 5 below:

Table 5. Comparison of charge per sample and TATs for the various assays and algorithms.

	Immuno *Card* Toxins A & B	C. Diff Quik Chek Complete*	Roche *tcdC* gene PCR	Immuno *Card* Toxins A & B plus Roche *tcdC* gene PCR #	C. Diff Quik Chek Complete plus Roche *tcdC* gene PCR δ
Charge per sample (Rands)	225	225	564	[(190x225)+ (175x564)] /190= 744	[(190x225)+ (20x564)] /190= 284
Maximum TAT	4 hours (35 minutes lab testing time)	4 hours (30 minutes lab testing time)	24 hours (batch run once per day)	4 hours for 15 (8%) samples. Other 175 (92%) samples require PCR	4 hours for 170 (89%) samples. Other 20 (11%) samples require PCR

* TP= GDH positive plus toxin positive

Testing of Immuno *Card* negative samples with PCR

δ Testing of GDH positive, toxin negative samples with PCR

It is important to note that the charge per sample, rather than the cost per sample processed has been used for these calculations.

As a result of the current higher cost of PCR testing, the charge for a *C. difficile* PCR is twice that for a *C. difficile* IA test. Due to the larger number of PCRs

required in the Immuno *Card* Toxins A & B- compared to the C. Diff Quik Chek Complete-based algorithm, the charge per sample for the former algorithm is more than double that of the latter. When testing all samples with the *C. difficile* PCR the charge per sample is twice that when compared to implementing the C Diff Quik Chek Complete testing algorithm.

The simplicity and little hands-on time required for the rapid IAs allows for a rapid TAT. The DNA extraction procedure and the setting up of the PCR is comparatively more tedious and complex, necessitating batch testing, and results in a longer TAT.

Discussion and Conclusions

4.1 Summary of results

Overall the sensitivity of the GDH assay was the highest, followed by the testing algorithms and *tcdC* gene PCR, and lastly, the toxin IAs. High specificities were demonstrated by all the assays. As a result all the assays and both algorithms showed high PPVs. The NPVs of the IAs were lower than that of the PCR and testing algorithms. Reporting of samples testing GDH positive, Toxin A/B positive as toxin positive, and samples testing GDH negative, Toxin A/B negative as toxin negative, allowed for close to 90% of results to be reported correctly.

The estimated CDI prevalence for CMJAH was calculated to be 3.3%. At this prevalence and across a prevalence range of 2% to 10%, the C Diff Quik Chek Complete IA based testing algorithm had the highest predictive values.

The charge per sample for the IAs is half that of the *tcdC* gene PCR testing. The C Diff Quik Chek Complete based algorithm required PCR testing of few samples only, and as a result the charge per sample was similar to that of the IAs. The TAT for the IAs is much quicker than the PCR TAT.

4.2 Detailed discussion of results

4.2.1 GDH

Swindells, et al. in their evaluation of the C. Diff Quik Chek Complete assay, found that the GDH component of the test had a sensitivity of 100% (82.4-100, 95% CI) and a specificity of 97% (92.5-98.8, 95% CI) when compared to toxigenic

culture. These authors comment on the utility of the GDH test component in excluding the presence of *C. difficile* in stool samples. They further add that due to the rapidity of this test, it can be used for screening in laboratories with a high volume of samples [2010].

Similarly, Peterson, et al. in their evaluation of 1000 stool samples, found that the sensitivity of the GDH component of the C. Diff Quik Chek assay was 'not inferior to toxigenic culture' [2011]. Other recent publications have likewise reported on the good sensitivity of GDH as a marker of the presence of *C. difficile* [Fenner, 2008; Sharpe, 2010; Kwada, 2011]. In their meta-analysis on the role of GDH in the detection of *C. difficile*, Shetty and Coen found that GDH has a 'greater than 90% sensitivity' and a 'false positivity rate (1- specificity) of less than or equal to 2%' when compared to culture of *C. difficile* from stool samples [2011]. As expected (because GDH is produced by toxin- and non-toxin-producing isolates), when compared to the standard of toxigenic culture, the specificity of GDH was between 80 and 100%. Our results showing a sensitivity of 90% and a specificity of 97% are comparable to the published data.

The study published by Rene′ et al. compared the performance of the C. Diff Quik Chek GDH assay for different *C. difficile* pulsed field gel electrophoresis types (PFGE) and found no impact of PFGE type on the sensitivity [2012]. Carmen et al. evaluated 104 *C. difficile* isolates, encompassing 77 different ribotypes, for the production of GDH using the C. Diff Chek-60, C. Diff Quik Chek and C. Diff

Quik Chek Complete assays [2012]. All 104 isolates showed *in vitro* (broth cultures) GDH production detectable by the three commercial GDH assays. This is in contrast to the findings of Tenover et al who demonstrated that the sensitivity of the GDH assay for strains other than the NAP1/027 strain was significantly lower, when compared to the Xpert *C. difficile* PCR assay [2010].

4.2.2 Toxin IAs

The Immuno *Card* Toxins A & B and the toxin component of the C. Diff Quik Chek Complete IAs showed poor sensitivities (38% and 52%, respectively). Peterson, et al. in their evaluation of laboratory tests for CDI, found that all of the 5 toxin IAs included in their study performed poorly when compared to broth enriched toxigenic culture. They further comment that the Eastwood study that reported sensitivities of 60% to 86%, did not include broth enrichment culture for the reference standard (resulting in a lower sensitivity of the reference standard), and as a result may have had falsely elevated sensitivities for the assays being evaluated [Eastwood, 2009; Peterson, 2011]. In their review of commercially available assays for CDI diagnosis, Crobach, et al. reported sensitivities ranging from 43% to 71% for eight toxin IAs when compared to toxigenic culture [2009]. Rene′ et al found slightly lower sensitivities (32.8% to 57.1%) in their comparison of six toxin IAs to toxigenic culture [2012]. Another recent publication similarly reported sensitivities ranging from 41.1% to 54.8% for three toxin IAs compared to toxigenic culture [Bruins, 2012].

The specificities of the toxin IAs tend to be higher than the sensitivities of these assays [Planche, 2008; Eastwood, 2009]. The specificities of the Immuno *Card* Toxins A & B and the toxin component of the C. Diff Quik Chek Complete assays in our evaluation were high at 100% . In keeping with this, the Crobach et al. review found specificities of 84% to 100% for the assays when compared to toxigenic culture [2009]. A number of studies have reported a high specificity for the toxin component of the C. Diff Quik Chek complete assay. Sharp et al., Kwada et al. and Swindells et al. found specificities of 99.2%, 96.9% and 100%, respectively [Sharp, 2010; Swindells, 2010; Kwada, 2011].

With regards to effect of strain type on the performance of toxin IAs, Reneʻ et al. found that the six assays evaluated showed a lower sensitivity for non-NAP1 isolates [2012]. Tenover, et al. reported that the sensitivity of the toxin IA's for ribotypes 027, 002 and 106 was reduced when compared to that of the Xpert *C. difficile* assay [2010]. De Jong et al. in their evaluation of the Immuno *Card* Toxin A & B IA, also analyzed the possible effect of ribotype on the performance of the toxin IA. They concluded that the low sensitivity (47%) of the IA could not be attributed to 'difficult-to-detect ribotypes' (as described by Tenover, et al.) as a minority of the false negatives were of these ribotypes [2012]. We do not have information on the strain types of our study samples.

4.2.3 C. Diff Quik Chek Complete assay overall

The sensitivity of the C. Diff Quik Chek Complete assay was only 52% when considering GDH positive, toxin A/B positive samples as positive and all toxin A/B

negative samples as negative. The real value of the C. Diff Quik Chek Complete assay is revealed if GDH positive plus toxin A/B positive results are reported as positive, and GDH negative plus toxin A/B negative results, are reported as negative. This would allow for the results of 89% of all samples to be reported correctly, rapidly and cost-effectively. A number of published evaluations comment on the utility of GHD and toxin IA testing as initial steps in a testing algorithm [Fenner, 2008; Larson, 2010; Reller, 2010; Selvaraju, 2011; Orendi, 2012]. Investigators report that results for between 81% and 92% of samples can be rapidly and conveniently finalized when using the above assays in a testing algorithm [Fenner, 2008; Reller, 2010; Swindells, 2010; Selvaraju, 2011].

4.2.4 PCR

Various evaluations of both in-house PCRs and commercially available PCRs for the diagnosis of CDI have been published [Sloan, 2008; Eastwood, 2009; Larson, 2010; Novak-Weekley, 2010; Swindells, 2010; Dubberke, 2011; Knetsch, 2011; Peterson, 2011; Selvaraju, 2011; Viala, 2012]. The patient populations and the gold standards used in these studies vary. Overall the reported sensitivities range between 88% and 100%, and are higher than that of the toxin IAs included in these comparative evaluations. The sensitivity of the Roche *tcdC* gene PCR we evaluated is somewhat lower at 79%. However it is difficult to compare our results to that of other evaluations due to differences in sample size, patient populations, clinicians' specimen submission practices, specimen transport issues, laboratory techniques (sampling technique, methods of nucleic acid extraction methods) and the reference gold standard used. The specificity of the Roche *tcdC* gene PCR was

98%. The reported specificities of the PCR assays in the literature range between 93% and 99%. Some authors comment on the lack of specificity of PCR assays in their evaluations [Dubberke, 2011; Knetsch, 2011 and Selvaraju, 2011]. Dubberke et al. found that the specificity of the evaluated PCR assays was 'significantly' lower when the reference standard used was positive toxigenic culture plus clinically significant diarrhoea compared to having four or more different *C. difficile* assays (assays included two toxin IAs, one GDH IA and three PCR assays and the Illumigene *C. difficile* assay) positive regardless of diarrhoea severity [2011]. In the Knetsch et al. study, the three in-house PCR assays had specificities ranging from 88-90.7%. Selvaraju et al. found that 73% of possible CDI cases detected by PCR only (toxigenic culture and CCNA negative), had alternative diagnoses confirmed. A number of authors cite clinicians' concerns regarding the fact that PCR detects *C. difficile* toxin genes and not actual toxin production [Kufelnicka, 2011; Wilcox, 2011; Guarner, 2012]. The possibility of asymptomatic colonization coupled with the high sensitivity of PCR, can result in reduced specificity of PCR for the diagnosis of CDI requiring treatment.

A recently published meta-analysis looking at published *C. difficile* PCR evaluations, spanning the period 1995 to 2010, found an overall mean sensitivity and specificity of 90% and 96%, respectively [Deshpande, 2011]. These investigators concluded that the sensitivity and specificity of PCR for the diagnosis of CDI is high, but that its overall utility was determined by the prevalence of CDI in the population being tested.

4.2.5 Algorithms

The performance in terms of sensitivity and specificity of the Immuno *Card* Toxins A & B- and the C. Diff Quik Chek Complete-based algorithms was comparable to that of the Roche *tcdC* gene PCR. However, the Immuno *Card* Toxins A & B algorithm necessitated many more PCRs than the C. Diff Quik Chek Complete algorithm. The sensitivity of the algorithm-based testing was clearly superior to that of the IA's alone.

Eastwood et al. in their comparison of nine *C. difficile* toxin IAs, a PCR assay and a GDH assay, concluded that 'diagnostic algorithms that optimize test combinations for the laboratory diagnosis of CDI need to be defined' [2009]. The European Society of Clinical Microbiology and Infectious Diseases (ESCMID) found that in CDI endemic populations, a negative result obtained using currently available assays is reliable. However, if a positive result is obtained, this needs to confirmed. They put forward two different algorithms and cite examples. One option is to confirm all positive results using a reference method, for example, GDH testing followed by CCCNA testing of GDH-positive samples. The other, a three step algorithm, involves testing of GDH-positive samples with a toxin IA or PCR assay, followed by testing of the toxin-negative subset with a reference method [Crobach, 2009].

Various testing algorithms have been evaluated and published. Some studies have found that PCR testing performed on all samples provides superior sensitivity to algorithmic approaches starting with GDH and toxin IA [Larson, 2010 and Novak-

Weekly, 2010]. Larson et al. did conclude however that the three-step algorithm provided a 'convenient and specific alternative with rapid results for the bulk of the samples tested. Sharp et al., Schmidt et al. and Swindells et al., found that the three-step algorithm performed favourably, providing rapid and reliable results [Schmidt, 2009; Swindells, 2009; Sharp, 2010].

The decision to implement a particular *C. difficile* assay or testing algorithm in a particular laboratory will be based on a number of factors including the available technology, staff numbers and skills (including night and weekend shifts), the ease of testing, the volume of work, cost-effectiveness analyses, the rapidity of results , the need to batch-test versus single sample testing and the performance of the assay or algorithm at the given local prevalence of CDI [Schmidt, 2009].

4.2.6 Estimated CDI prevalence

The predictive value of the various *C. difficile* assays evaluated is dependent on the prevalence of CDI in the population we tested. The calculated estimated prevalence was 3.3%. However, our estimate is subject to some limitations. The estimate was calculated using patient admission numbers and samples submitted over a one month period. Ideally this should have been done over longer time period. In addition, the use of pure admission numbers in the estimate overinflates the denominator, and hence falsely lowers the calculated prevalence. The use of admission numbers rather than 'cases per 10,000 patient-days' was chosen due to ease of access to admission data. The calculated prevalence is also dependent on the clinicians' sample submission practices. This is influenced by the clinicians'

awareness of CDI as a cause of healthcare- or antibiotic-associated diarrhoea. Awareness of CDI at CMJAH is greater in the critical-care units compared to the other wards. Also the practice of treating patients with diarrhoea empirically and not submitting samples for laboratory investigations may have impacted on the estimated prevalence. As stated by Crobach et al, in an endemic setting the CDI prevalence is probably between 5% and 10% [Crobach, 2009].The actual prevalence at our institution is likely higher than the calculated 3.3%. Establishment of an ongoing surveillence programme for CDI at our site will provide data more reflective of the true prevalence.

4.2.7 Performance of assays and algorithms at different prevalences

At a low prevalence (2-5%) a diagnostic assay with a high specificity is required. Otherwise low PPVs as observed with the Roche PCR and the Immuno *Card* Toxins A & B plus Roche PCR algorithm will result. In the recently published meta-analysis on PCR for CDI diagnosis, at a prevalence of less than 10%, the PPV of PCR was found to be unacceptably low at 71% [Deshpande, 2011]. Crobach et al in their systematic review of laboratory diagnosis assays for CDI concluded that 'all currently available types are not suitable as stand-alone tests to diagnose CDI in endemic populations because of their low PPVs at these prevalences' [Crobach, 2009]. Due to the 100% specificity of the IAs and the low number of PCRs required for the C. Diff Quick Chek Complete-based algorithm, the PPVs for both assays in our evaluation was high, irrespective of prevalence.

At the lower CDI prevalences, despite the poor sensitivity of the IAs, the NPVs for these assays were high, and comparable to those of the Roche PCR (between 98% and 100%) and the algorithms (between 99 and 100%). Crobach, et al. too found that at low prevalences the NPVs of toxin and GDH IAs and PCR 'were very acceptable', allowing for these assays to be used for the exclusion of CDI [2009]. At the higher prevalence of 10% the Roche PCR showed a somewhat improved PPV and superior NPV to the IAs. Overall (based on a prevalence of 2%-10%) the C. Diff Quik Chek Complete-based algorithm showed the most favourable predictive values (PPV of 100% and NPV ranging from 98-100%).

4.2.8 Cost and Turnaround Time

Laboratories are under increasing pressure to provide accurate results rapidly and cost-effectively. Quick reporting of results facilitates prompt treatment of infected patients and implementation of appropriate infection control measures. This in turn can potentially reduce hospitalization costs overall. As discussed earlier use of the C. Diff Quik Chek Complete assay as the initial test in a testing algorithm, both in this evaluation and in published evaluations, allowed for rapid reporting of results for a large proportion of samples [Fenner, 2008; Reller, 2010; Swindells, 2010; Selvaraju, 2011]. PCR, depending on which platform is used can also provide rapid results. However, as in this evaluation, cost analyses in a number of publications have demonstrated reduced overall testing costs when using PCR in a testing algorithm rather than testing of all samples with PCR [Sharpe, 2010; Carroll, 2011; Selvaraju, 2011].

4.3 Study Limitations

Toxigenic culture, currently considered the gold standard, was used for this evaluation. However, "In general toxigenic culture has not been standardized" [Carroll, 2011]. The use of selective media, as well as spore enhancement and broth enrichment cultures, have been found to increase the culture yield. These methods were utilised in this evaluation. The detection of toxin from cultured isolates has not been standardized either. It is clear that the poor sensitivity of toxin IAs, preclude the use of these rapid assays as the sole method for toxin detection [She, 2009]. CCCNA or PCR is recommended, and a number of published studies have used one of these two methods [Sloan, 2008; Stamper, 2009; Swindells, 2010; Carroll, 2011; Rene, 2012]. Comparative studies looking at CCCNA versus PCR for detection toxin or toxin genes, are lacking. However, CCCNA performed directly from stool samples has been found to be less sensitive than PCR from clinical samples [Carroll, 2011]. For this evaluation real-time PCR was utilized for detection of the *tcdC* gene. Sloan et al. performed conventional PCR and real-time PCR (using the Roche LightCycler) for the detection of *tcdA*, *tcdB* and *tcdC* genes from *C..difficile* cultures [Sloan, 2008]. These authors found complete concordance between the two PCR methods, confirming the utility of the real-time *tcdC* gene PCR for use on cultured isolates.

With regards to the costing analysis, charge per sample (based on the NHLS pricing catalogue) rather than the cost per sample processed, was used. Costing of a test must factor in the kit, materials required but not supplied in the kit, equipment costs (rental, service contracts) and labour. Costing of a test needs to be

adjusted periodically to account for changes in cost of the above components. The NHLS pricing catalogue may not accurately reflect the current costs of the PCR and IAs.

There are a number of limitations in the calculated estimated CDI prevalence. These have been discussed above in 4.2.6.

4.4 Conclusions

With the rising incidence of CDI and the importance of this condition as a hospital-acquired infection, it has become imperative that laboratory assays that provide accurate and timeous results are implemented. The gold standard of toxigenic culture being labour-intensive and time-consuming is not practical for most routine microbiology laboratories. Many laboratories currently utilize *C. difficile* toxin IAs as stand-alone tests for the diagnosis of CDI. However the poor sensitivity of these assays results in many cases of CDI being missed. PCR-based testing for CDI diagnosis has demonstrated both higher sensitivities and more rapid turnaround times. The downside of this technology is the higher cost associated with it. In addition, depending on the PCR platform being used, the hands-on time required, and the local staffing situation, batch-testing of samples once a day may be necessary, resulting in lengthened turnaround times. Multistep testing algorithms may provide a solution by limiting costs, whilst providing good sensitivities and reasonable turnaround times. The aim of this evaluation was to determine the optimal test or testing algorithm for CDI at our site.

The performance of the C. Diff Quik Chek Complete-based algorithm in terms of diagnostic accuracy at the estimated local CDI prevalence rate, the ease and rapidity of the testing procedure, and the fewer number of samples requiring more time-consuming and costly PCR testing, suggests that currently this may be the optimal CDI laboratory testing strategy for our healthcare facility. This will require periodic re-evaluation as other diagnostic assays like the Xpert C Diff PCR (simple procedure, little hands-on time, and random access) become available to us, the costs of PCR reduce and our CDI prevalence evolves.

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