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MOLECULAR DIAGNOSTICS IN MICROBIOLOGY

Molecular methods for detecting and typing of *Clostridium difficile*

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Summary

Since the early 2000s, *Clostridium difficile* has emerged as a major international pathogen. Recently, strains of *C. difficile* in circulation appear to be changing, with greater diversity, leading to challenges for diagnostics and surveillance. Currently molecular diagnostic methods are favoured for their high sensitivity and rapid processing times; however, a number of issues still exist with molecular tests, in particular high cost, low clinical specificity and failure to detect some variant *C. difficile* strains. Molecular typing methods are used to determine the continually evolving epidemiology of *C. difficile* infection. Typing methods including PCR ribotyping and pulsed field gel electrophoresis are currently popular in Europe and North America, respectively, while high-throughput next-generation sequencing is likely to become more widely used in years to come. This review discusses current molecular detection and typing techniques for *C. difficile*.

Key words: Clostridium difficile, diagnostics, molecular testing, PCR, ribotyping, sequencing, surveillance, typing.

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INTRODUCTION

Following widespread use of broad spectrum cephalosporin antimicrobials during the 1980s and 1990s, *Clostridium difficile* infection (CDI) became one of the most common causes of nosocomial diarrhoea in the world.[1] Diarrhoea is a common complaint among hospital patients and can result from underlying disease or as a side effect of treatment with various drugs, particularly antimicrobials. *Clostridium difficile* causes 20–25% of cases of antimicrobial-associated diarrhoea in hospital patients.[2,3] In recent years, community-acquired CDI has been increasing.[4,5] Rapid diagnosis of CDI is desirable to allow early isolation and treatment of patients, reducing potential patient-to-patient transmission and length of hospital stay for those affected. In addition, *C. difficile* strain typing can identify outbreaks within a hospital or the wider community. Over the past 30 years, significant advances have been made in molecular techniques for *C. difficile* detection and typing.

C. difficile pathogenicity

The main virulence factors of *C. difficile* are toxin A (TcdA/enterotoxin) and toxin B (TcdB/cytotoxin). These are encoded by the genes *tcdA* and *tcdB* on the pathogenicity locus (PaLoc), which contains three other genes that regulate expression of *tcdA* and *tcdB* and promote toxin release (Fig. 1).[6] Toxigenic strains always produce toxin B, often produce toxin A also, and cause disease. Non-toxigenic strains do not produce toxin A or B.[6]

Variant *C. difficile* strains differ from the reference strain (VPI 10463) in restriction sites and length of *tcdA* and *tcdB*, and other PaLoc regions. Many groups of variant strains have been identified and can be defined by a series of overlapping polymerase chain reactions (PCRs) spanning the PaLoc, allowing strains to be assigned to different toxinotypes I-XXXI.[7]

A third toxin produced by some strains of *C. difficile* is binary toxin (*C. difficile* transferase; CDT). The genes *cdtA* and *cdtB* encode its two components, CDTa (enzymatic component) and CDTb (binding component), and are found on the CDT locus (CdtLoc), which is carried independently of the PaLoc (Fig. 1).[8] While the role of binary toxin in disease is not well understood, binary toxin is produced by several strains such as NAP1/BI/027 which emerged as a major epidemic strain in the early 2000s.[9–11]

C. DIFFICILE DETECTION METHODS

Until the early 1980s the most commonly used test for CDI laboratory diagnosis was detection of toxin B in stool by cell-culture cytotoxicity,[12] however, due to its complexity and long turnaround time, rapid enzyme immunoassay (EIA) kits became favoured. Initially, these kits were designed to detect toxin A, for two reasons. First, it was wrongly assumed that *C. difficile* produced either both toxin A and toxin B, or neither toxin and, second, toxin A was far more immunogenic and it was easier to raise antibodies against it. The discovery of toxin A negative strains of *C. difficile* shifted attention to EIA kits that detected both toxins, although even these still have greater avidity for toxin A.[13] During the process of developing one of the early toxin A kits, one manufacturer inadvertently produced a test that detected glutamate dehydrogenase (GDH), an enzyme produced by *C. difficile, C. botulinum* and *C. sporogenes*, as well as a few anaerobic cocci.[14] These EIAs detect the presence of toxins or GDH using antibodies linked to a chromogenic marker. Detection of GDH remains a useful screening test because of its high sensitivity but detection of *C. difficile* toxin is still considered as necessary for confirmation of CDI.[15]

Culture of *C. difficile* takes several days and does not differentiate asymptomatic carriers from those with disease (i.e., it lacks specificity), or toxigenic from non-toxigenic strains. However, isolation of the bacterium allows a broader array of tests to be performed, including determination of the organism's toxigenic status. So-called toxigenic culture has become the gold standard against which all new tests should be assessed. The low sensitivity of EIAs, averaging around 60%,[16] has driven the continuing search for highly

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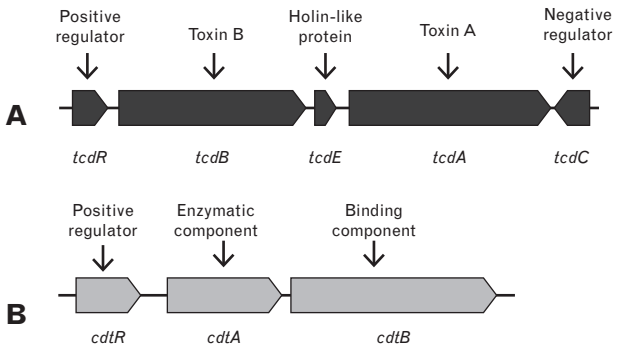

Fig. 1 The PaLoc (A) and the CdtLoc (B). The PaLoc encodes five genes. *tcdA* and *tcdB* encode toxins A and B respectively. *tcdC* is a polymorphic locus with many known mutations, which encodes TcdC. The TcdC protein is believed to negatively regulate production of toxins A and B. *tcdE* encodes a protein thought to play a role in toxin release from the cell. The CdtLoc is located downstream of the PaLoc. The *cdtA* and *cdtB* genes encode the proteins which comprise the enzymatic and binding subunits of binary toxin.

sensitive, rapid techniques for CDI laboratory diagnosis. Currently, molecular methods have gained popularity for their rapid turnaround time and high sensitivity. However, they are expensive and may in fact be too sensitive for the diagnosis of disease as opposed to detecting the organism.[17]

Molecular assays for *C. difficile* detection

Nucleic acid amplification tests (NAATs) for *C. difficile* have been in use since the early 1990s. These early PCR tests targeted various genes including *tcdA* and *tcdB*.[18–20] They were relatively labour intensive, requiring manual DNA extraction and visualisation of results using gel electrophoresis or Southern blot analysis. Some early primers also showed cross-reactivity with other clostridia.[19] Throughout the 1990s, DNA extraction kits and NAAT techniques improved, and the development of real-time PCR (rtPCR) techniques followed. A number of assays gained US Food and Drug Administration (FDA) approval for use within diagnostic laboratories, ushering in a new age of rapid diagnostic techniques.

The currently approved molecular assays for *C. difficile* detection are summarised in Table 1. The first NAAT to receive FDA approval was the BD GeneOhm Cdiff assay in 2009. This and other early assays required a manual DNA extraction step, while most of the newer assays are fully automated. In the automated assays a small amount of stool sample is placed into a closed cartridge or tube where the entire process (DNA extraction, amplification reaction and product detection) takes place. These automated assays are desirable due to their short hands-on processing time and reduced risk of cross-contamination of samples.

The majority of the assays employ rtPCR-based reactions, some with detection of products using molecular beacons (BD

Table 1 Current approved molecular assays for *C. difficile* detection

Assay name (Manufacturer)	Target	Reaction type	Extraction type	System	TAT (min)
ICEPlex *C. difficile* Kit (PrimeraDx)	*tcdB*	rtPCR	Manual	ICEPlex System	<480
IMDx *C. difficile* for Abbott *m*2000 (Intelligent Medical Devices)	*tcdA, tcdB, tcdB*-variant	Multiplex rtPCR	Automated	Abbott *m*2000 System	
BD Diagnostics BD Max Cdiff Assay (GeneOhm Sciences Canada)	*tcdB*	rtPCR	Automated	BD Max System	<180
Quidel Molecular Direct *C. difficile* Assay (Quidel Corporation)	*tcdA, tcdB*	Multiplex rtPCR	Automated	QuantStudio Dx, Applied Biosystems 7500 Fast Dx, Cepheid Smart Cycler II	<70
Verigene *C. difficile* Nucleic Acid Test (Nanosphere)	*tcdA, tcdB, tcdC*Δ117, *cdt*	Multiplex rtPCR	Automated	Verigene System	240
Portrait Toxigenic *C. difficile* Assay (Great Basin Scientific)	*tcdB*	Helicase-dependent multiplex amplification	Automated	Portrait Dx Analyzer	<90
Simplexa *C. difficile* Universal Direct Assay (Focus Diagnostics)	*tcdB*	rtPCR	Automated	3M Integrated Cycler	<90
Xpert *C. difficile/Epi* (Cepheid)	*tcdB, tcdC*Δ117, *cdt*	Multiplex rtPCR	Automated	GeneXpert Instrument	<60
Illumigene *C. difficile* DNA Amplification Assay (Meridian Bioscience)	*tcdA*	LAMP	Manual	Illumipro-10	<60
Xpert *C. difficile* (Cepheid)	*tcdB, tcdC*Δ117, *cdt*	Multiplex rtPCR	Automated	GeneXpert Instrument	45
ProGastro Cd Assay (Prodesse)	*tcdB*	rtPCR	Manual	Cepheid SmartCycler II	<180
BD GeneOhm C. diff Assay (BD Diagnostics/GeneOhm Sciences)	*tcdB*	rtPCR	Manual	Cepheid SmartCycler II	120
EasyScreen *C. difficile* Detection Kit (Genetic Signatures)	*tcdA, tcdB*	rtPCR	Manual	Roche LightCycler 480, Biorad CFX96, Agilent (Stratagene) MX3000, Qiagen Rotor-Gene Q, Cepheid SmartCycler	<180
EasyScreen *C. difficile* Reflex Kit (Genetic Signatures)	*tcdC*Δ117, *cdtA*, *gyrA* mutation	rtPCR	Manual	Roche LightCycler 480, Biorad CFX96, Agilent (Stratagene) MX3000, Qiagen Rotor-Gene Q, Cepheid SmartCycler	<180
Seeplex Diarrhea ACE (Seegene)	*tcdB*	Multiplex PCR	Manual	Calipher LabChip Dx, MCE-202 MultiNA, Agilent 2200TapeStation	<60
Faecal Pathogens C (16Plex) (AusDiagnostics)	*tcdB*	Multiplex PCR	Manual	High-Plex	<180

rtPCR, real-time polymerase chain reaction; TAT, turnaround time.