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REVIEW

Overview of current detection methods and microRNA potential in *Clostridioides difficile* infection screening

Marco Bocchetti, Maria Grazia Ferraro, Federica Melisi, Piera Grisolia, Marianna Scrima, Alessia Maria Cossu, Tung On Yau

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Marco Bocchetti, Federica Melisi, Piera Grisolia, Alessia Maria Cossu, Department of Precision Medicine, University of Campania "Luigi Vanvitelli," Naples 80138, Italy

Marco Bocchetti, Federica Melisi, Piera Grisolia, Marianna Scrima, Alessia Maria Cossu, Department of Molecular Oncology, Precision Medicine Laboratory and COVID19 Laboratory, Biogem Scarl, Ariano Irpino 83031, Italy

Maria Grazia Ferraro, School of Infection and Immunity, College of Medical Veterinary and Life Sciences, University of Glasgow, Glasgow G12 8QQ, United Kingdom

Maria Grazia Ferraro, Department of Pharmacy, School of Medicine and Surgery, University of Naples "Federico II," Naples 80131, Italy

Tung On Yau, School of Science and Technology, Nottingham Trent University, Nottingham NG11 8NS, United Kingdom

Tung On Yau, Department of Rural Land Use, Scotland's Rural College, Aberdeen AB21 9YA, Scotland, United Kingdom

Tung On Yau, Department of Health Science, University of the People, Pasadena, CA 9110112, United States

Corresponding author: Tung On Yau, BSc, MSc, PhD, Instructor, Lecturer, Researcher, School of Science and Technology, Nottingham Trent University, Clifton Lane, Clifton, Nottingham NG11 8NS, United Kingdom. payton.yau@ntu.ac.uk

Abstract

Clostridioides difficile (formerly called *Clostridium difficile*, *C. difficile*) infection (CDI) is listed as an urgent threat on the 2019 antibiotic resistance threats report in the United States by the Centers for Disease Control and Prevention. Early detection and appropriate disease management appear to be essential. Meanwhile, although the majority of cases are hospital-acquired CDI, community-acquired CDI cases are also on the rise, and this vulnerability is not limited to immunocompromised patients. Gastrointestinal treatments and/or gastrointestinal tract surgeries may be required for patients diagnosed with digestive diseases. Such treatments could suppress or interfere with the patient's immune system and disrupt gut flora homeostasis, creating a suitable microecosystem for *C. difficile* overgrowth. Currently, stool-based non-invasive screening is the first-line approach to CDI

diagnosis, but the accuracy is varied due to different clinical microbiology detection methods; therefore, improving reliability is clearly required. In this review, we briefly summarised the life cycle and toxicity of *C. difficile*, and we examined existing diagnostic approaches with an emphasis on novel biomarkers such as microRNAs. These biomarkers can be easily detected through non-invasive liquid biopsy and can yield crucial information about ongoing pathological phenomena, particularly in CDI.

Key Words: *Clostridioides difficile*; microRNA; Diagnostic; Prognostic; Biomarker

Core Tip: *Clostridioides difficile* infection (CDI) is listed as an urgent threat, and early detection and appropriate disease management from hospital-acquired or community-acquired CDI appear to be essential. Currently, stool-based non-invasive screening is the first-line approach to CDI diagnosis, but the accuracy is varied due to different clinical microbiology detection methods. Therefore, improving reliability is clearly required. This review summarised the life cycle and toxicity of *Clostridioides difficile* and examined existing diagnostic potentials on microRNA as novel biomarkers. MicroRNAs can be easily detected through non-invasive liquid biopsy and can yield crucial information about ongoing pathological phenomena, particularly in CDI.

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INTRODUCTION

Clostridioides difficile (formerly called *Clostridium difficile*, *C. difficile*) infection (CDI) was classified as an urgent threat in the 2019 antibiotic resistance report in the United States by the Centers for Disease Control and Prevention. Early detection and disease management are urgently needed to reduce public health spending[1]. In addition, of those infected with a medically-related CDI, 1 in 11 people over the age of 65 dies within a month[2]. The acute inflammation caused by the bacteria triggers cytokine production, neutrophil recruitment, mucosal permeability and fluid secretion, leading to colonic tissue damage, nasal diarrhoea and colitis[3,4], which is similar to inflammatory bowel disease[5].

Presently, although most cases are hospital-acquired CDI, community-acquired CDI cases are rising in frequency[6]. Exposure to *C. difficile* in the community might come from various factors, including pets, water, soil, livestock, farms, food processing and production[7]. Previous studies focused on the hospitalisation length and indicated that environmental exposure to animals would be associated with a greater possibility of *C. difficile* colonisation[8]. A population-based study in India found that the composition of the gut microbiota was primarily associated with several geographical factors rather than body mass index and that these changes extended to circulating immunometabolic profiles such as serum N-glycans, immunoglobulins and short-chain fatty acid profiles[9]. These factors may also affect the infection rate.

Once patients have been diagnosed with digestive diseases, gastrointestinal treatments, including gastric-acid suppressing agents, broad-spectrum antibiotics, chemotherapy and/or gastrointestinal tract surgery may be required[10,11]. Such therapies could suppress or interfere with the patient's immune system and disrupt gut flora homeostasis, creating a suitable microecosystem for *C. difficile* overgrowth [12]. Antibiotics such as metronidazole, vancomycin and fidaxomicin have been approved for treatment of patients with CDI, while *C. difficile* strains resistant to various antibiotics have been reported that do not respond to the treatments[13-15]. Faecal microbiota transplantation (FMT) is an alternative treatment strategy for CDI patients; however, it is still in clinical trials because of the treatment safety concern[16].

To date, there are several diagnostic methods for CDI in medical laboratories. However, due to technical limitations and the difficulty of distinguishing symptomatic infections from asymptomatic *C. difficile* colonisation, the accuracy and turnaround time of the test varies[17]. As a result, searching for better tools may increase the accuracy of the detection. In this light, microRNA (miRNA) expression profiling can be helpful for prognosis and diagnosis.

This article reviewed this proposition by briefly presenting the *C. difficile* life cycle and describing the toxins produced. We then described the current laboratory-based diagnostic tools for CDI patient screening. We discussed the potential use of miRNA to monitor and improve the management of CDI patients.

C. DIFFICILE LIFE CYCLE AND TOXINS

C. difficile is a Gram-positive, anaerobic bacterium belonging to the phylum Firmicutes. There are three main stages that define the *C. difficile* life cycle: spore formation; germination; and growth (vegetative). At the spore stage (dormant phase), it is typically harmless in a balanced gut flora microenvironment. *C. difficile* can resist oxygen, heat and many other environmental insults, including ethanol-based disinfectants (Figure 1)[18].

Once set in a stable environment, spores germinate rapidly and produce two major toxins, toxin A and toxin B encoded by *TcdA* and *TcdB*, which are located at the 19.6 kbp long pathogenicity locus region (Figure 2)[19]. Toxin A and toxin B trigger cytosol translocation of target host cells and inactivate small GTP-binding proteins (such as CDC42, Rho and Rac) through monoglucosylation, leading to actin condensation, cytoskeleton disintegration, cell rounding and apoptosis[20]. tcdR is an RNA polymerase sigma factor that initiates tcdA and tcdB translation *via* its two tandem promoters[21,22] and is involved in the final stages of flagellar assembly[23]. Some *C. difficile* strains, such as ribotype 027 and ribotype 078 are able to produce *C. difficile* transferase toxin, an actin-specific ADP-ribosyltransferase homologous to iota-toxin from *Clostridium perfringens* and *Clostridium spiroforme* toxin, and potentially enhances *C. difficile* virulence and disease severity[24,25].

CURRENT DIAGNOSTIC TOOLS FOR IDENTIFYING PATIENTS WITH *C. DIFFICILE* INFECTION

Similar to colorectal cancer screening, CDI can be detected by examining the colon through flexible sigmoidoscopy or colonoscopy to look for pseudomembranes and inflamed areas. X-ray abdominal imaging or computerised tomography scan can also be applied on a case-by-case basis. In addition to the invasive approaches, the cost effective, non-invasive faecal-based screening is the first-line approach for CDI diagnosis. Still, it varies widely due to different clinical microbiology methods and their different accuracy and variance[26,27]. These methods include cell cytotoxicity neutralisation assay, toxigenic culture (TC), enzyme immunoassay (EIAs) [including toxins and glutamate dehydrogenase (GDH)] and nucleic acid amplification test (NAAT) and each of the methods has advantages and disadvantages in terms of turn-around time and the screening performance (Table 1).

Cell cytotoxicity neutralisation assay and C. difficile culture

The cell cytotoxicity neutralisation assay is the first-line faecal-based diagnostic test for CDI[28,29]. It requires multiple steps in order to isolate *C. difficile* toxins from faeces and at least 24 h of cell culture (mainly human fibroblasts). The cytopathic effect is characterised by rounding and morphologic changes of the cultured cells[29,30], and the phenomena must also be reverted by *C. difficile* or *Clostridium sordellii* anti-toxin used as a control to prove that the cytopathic effect is not related to nonspecific substances in the faeces. In contrast, toxigenic culture requires faeces inoculation to selective cycloserine-cefoxitin-fructose, chromogenic or similar agar plates for a specific incubation period; suspicious colonies are selected for further bacteria culture and/or *C. difficile* toxin test to confirm the finding (Figure 3)[31-33]. It is important to remember that there is no standard to eliminate or reduce nonspecific bacteria colonisation from *C. difficile* culture. Heat shock and alcohol shock are the traditional preculture approaches, while a culture media containing bacteria "suppressors" such as antibiotics may also be applied[34-38]. Both culture-based assays are time-consuming, labour-intensive and require a certain level of laboratory skill. As such, it is unlikely to be used for first-line clinical screening and is commonly used as the reference method for research and outbreak investigations, even though it is considered the gold standard[31,32,38].

Nucleic acid-based detection assays

The nucleic acid amplification test is a quantitative real-time (qRT)-PCR-based diagnostic assay able to rapidly detect *C. difficile* toxin genes such as *tcdA, tcdB, tcdBv, cdt* and/or *tcdC*Δ117 at the DNA level. There is a wide range of United States Food and Drug Administration-approved detection assays, from *C. difficile* specific point-of-care testing to high-volume, high-throughput multiple gastrointestinal pathogens laboratory tests. The majority of PCR detection approaches for *C. difficile* screening are probe-based qRT-PCR (Table 2). The use of probes increases amplification specificity during the PCR cycle since the additional sequence of the probe is specific for and binds to the *C. difficile* DNA sequence. Additionally, it is possible to perform multiplex qRT-PCR by using different fluorescent dyes[39]. The

Table 1 Summary of *Clostridioides difficile* diagnostic tests

Test	Abbreviation	Sensitivity	Specificity	Turn-around time	Target substance
Culture-based					
Cell cytotoxicity neutralisation assay	CCTN	High	High	< 24 h	Toxins
Toxigenic culture	TC	High	Low[1]	> 3 d	*C. difficile* vegetative cells or spores
DNA-based					
Nucleic acid amplification test[2]	NAAT	High	Low/moderate	< 4 h	Toxin genes
Protein-based					
Glutamate dehydrogenase	GDH-EIA	High	Low[1]	< 2 h	*C. difficile* antigens
Toxin A and B enzyme immunoassays	EIA	Low	Moderate	< 2 h	Toxins

[1]Nucleic acid amplification test, mainly using the PCR technique.
[2]Must be combined with a toxin test.
C. difficile: *Clostridioides difficile*; TC: Toxigenic culture; NAAT: Nucleic acid amplification test; CCTN: Cell cytotoxicity neutralisation assay; GDH: Glutamate dehydrogenase; EIA: Enzyme immunoassay.

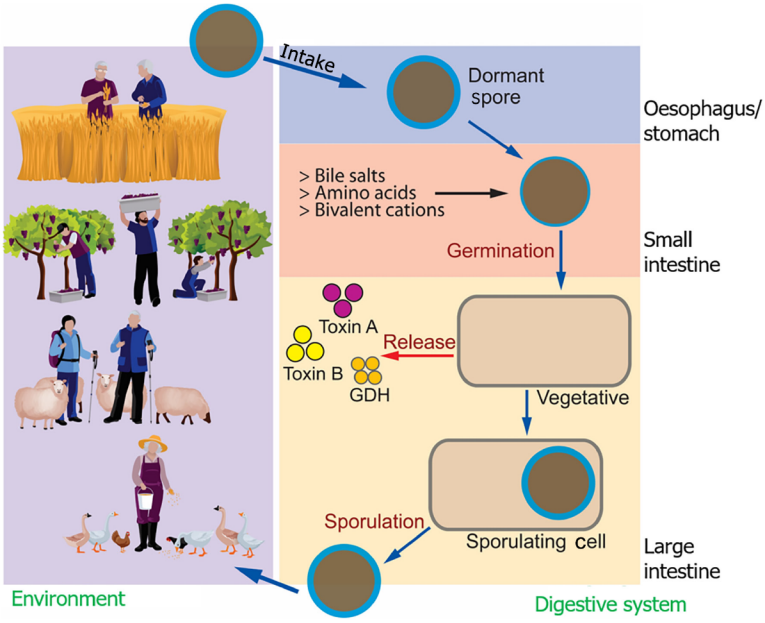

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Figure 1 *Clostridioides difficile* life cycle. *Clostridioides difficile* (*C. difficile*) can be found in soil and is transmitted to humans (as well as animals) through different daily activities. Besides hospital-acquired *C. difficile* infections, community-acquired *C. difficile* infections are also known, with agriculture being one of the main sources, from the spore stage into the human digestive system. Once stable, *C. difficile* may repopulate and release toxins that can affect intestinal health. *C. difficile* may go back to the environment and repeat the life cycle. GDH: Glutamate dehydrogenase.

nucleic acid-based *C. difficile* toxin screening has over ten times higher sensitivity than a cytotoxin assay [40,41], but the screening specificity is relatively low due to high false positive cases from asymptomatic infection. As a result, optimisation is required, especially on PCR threshold cycle settings[42,43]. In addition, patient preselection based on clinical symptoms and intestinal inflammation biomarkers (*i.e.* faecal calprotectin, lactoferrin and cytokines) appears to be necessary to reduce the risk of a false positive[27,43-47].

Toxins and GDH EIA

EIAs utilise antibodies to detect the presence of antigens. It is still relatively common to detect toxin A, toxin B and/or GDH in faeces for CDI. There are several types of EIAs available for CDI, including microplates (enzyme-linked immunosorbent assay), membrane EIA, chemiluminescence immunoassay, enzyme-linked fluorescent assay and chromatographic immunoassay (Table 3). A meta-analysis

Table 2 Selected Food and Drug Administration-approved molecular assays

Assay name	Developer	Target	Method	Sensitivity/specificity (%)
AmpliVue *C. difficile* assay[70]	Quidel	*tcdA*	Isothermal nucleic acid amplification	93.6/94.1
ARIES *C. difficile* assay[71,72]	Luminex	*tcdA* and *tcdB*	qRT-PCR	90-98/92-98
ARTUS *C. difficile* QS-RGQ MDX Kit [73]	QIAGEN, GMBH	*tcdA* and *tcdB* (+ *tcdBv*)	qRT-PCR	100/90-100
BD Diagnostics BD MAX *C. diff* assay[71,74-77]	GeneOhm Sciences	*tcdB*	TaqMan probe-based qRT-PCR	86-98/89-100
BD GeneOhm *C. diff* assay[78-82]	BD Diagnostics/GeneOhm Sciences	*tcdB*	Beacon probe-based PCR	91-95/96-100
Cobas *C. diff* Nucleic Acid Test For Use On The Cobas Liat System[83, 84]	Roche	*tcdB*	TaqMan probe-based qRT-PCR	93/99
GenePOC *C. diff*[85]	GenePOC	*tcdB* of toxigenic C	TaqMan probe-based qRT-PCR	81/97
ICEPlex *C. diff* Kit[1][86]	PrimeraDx	*tcdB*	qRT-PCR + capillary electrophoresis-based detection	90/97
Illumigene *C. diff* DNA Amplification assay[77]	Meridian Bioscience	*tcdA*	Loop-mediated isothermal DNA amplification	82-100/94-100
IMDx *C. difficile* for Abbott m2000 [76,77]	Intelligent Medical Devices	*tcdA* and *tcdB* (+ *tcdBv*)	Probe-based qRT-PCR	62-84/94-99
Portrait Toxigenic *C. difficile* assay [87,88]	Great Basin Scientific	*tcdB*	Primer-mediated helicase-dependent amplification + chip-based detection	98.2/92.8
ProGastro Cd assay[81,89]	Prodesse	*tcdB*	Probe-based qRT-PCR	77-100/94-99
Quidel Molecular Direct *C. difficile* assay[70,90]	Quidel	tcdA and tcdB	TaqMan probe-based qRT-PCR	82-96/97-100
Simplexa *C. difficile* Universal Direct assay[70,91]	Focus Diagnostics	*tcdB*	qRT-PCR + bifunctional fluorescent primer-probes	87-98/99-100
Solana *C. difficile* assay[1][92]	Quidel	*tcdA*	Helicase-dependent amplification	93/99
Verigene *C. difficile* Nucleic acid Test [91,93,94]	Nanosphere	tcdA, tcdB, *cdt* and *tcdC*Δ117	PCR + nanoparticle-based array	94-96/96-98
X/Pert *C. difficile*/Epi[71,77,95,96]	Cepheid	tcdA, tcdB, *cdt* and *tcdC*Δ117	TaqMan probe-based qRT-PCR	90-100/93-99

[1]The sensitivity and specificity were provided by the manufacturer.

*tcdC*Δ117: The *tcdC* deletion nt 117; *tcdBv*: tcdB variant; *cdt*: Binary toxin; *C. difficile* or *C. diff*: *Clostridioides difficile*; qRT: Quantitative real-time.

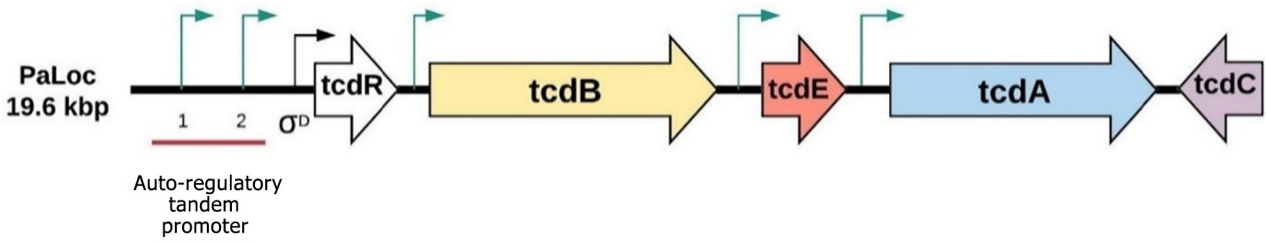

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Figure 2 Pathogenicity loci of toxicogenic *Clostridioides difficile*. The 19.6 kbp long pathogenicity loci in toxicogenic *Clostridioides difficile*, including *tcdC*, *tcdA*, *tcdE*, *tcdB* and *tcdR* coding genes. *tcdR* codes for an RNA polymerase sigma factor that controls the expression of the *tcdB* and *tcdA* genes and possibly *tcdE*. The transcription of *tcdR* can be regulated by three promoters. One is under the control of σ^0, and the other two are autoregulatory tandem promoters. Figure adopted and modified from Isidro *et al*[19].

published in 2016 evaluated the major commercially available *C. difficile* diagnosis assays compared to the gold standards. The pooled sensitivities were 83% [95% confidence interval (CI): 76%-88%] and 57% (95%CI: 51%-63%) compared to cell culture cytotoxicity assay and toxigenic bacterial culture,

Table 3 Major immunochromatographic membrane assays

Methods/assays	Target(s)	Developer	Cell cytotoxicity assay		Toxigenic culture	
			Sen, %	Spe, %	Sen, %	Spe, %
Microwell enzyme immunoassay (enzyme-linked immunosorbent assay)						
GA *C. diff* Antigen[97]	Toxin A + B	The Binding Site	76.8	90.0	68.8	91.4
Premier toxin A+B[97-106]	Toxin A + B	Meridian Bioscience	58-99	94-100	40-86	91-100
Ridascreen toxin A/B[97,107,108]	Toxin A + B	Ridascreen	57-67	95-97	52-60	96-98
ProSpecT toxin A/B[97,109]	Toxin A + B	Remel/Oxoid (Thermo Scientific)	90-91	93-97	82	93
Techlab *C. diff* Chek-60 (GDH)[53,97,110]	GDH	Techlab	92.0-93.5	94.0-98.0	88.0-93.0	94.0-97.0
TechLab toxin A/B II[81,97,100,109-111]	Toxin A + B	TechLab	72-91	87-100	58-85	96-99
Membrane enzyme immunoassay						
ImmunoCard toxins A/B[41,97,109,112,113]	Toxin A + B	Meridian Bioscience	85-96	97-99	41-69	93-99
Quick Chek Complete[40,105,112,114-117]	Toxin A + B	TechLab	50-73	100	29-79	89-100
Remel X/pect *C. diff* toxin A/B[97,113,118]	Toxin A + B	Remel	44-83	99-100	48-69	95-99
Tox A/B Quick Check[97,115,119-121]	Toxin A + B	TechLab	61-84	99	40-74	94-100
Chemiluminescence immunoassay						
Liaison *C. difficile* toxins A and B[122,123]	Toxin A + B	DiaSorin	88	95	69-88	95-99
Enzyme-linked fluorescent assay						
Vidas toxin A and B[97,116,123-128]	Toxin A + B	Vidas	53-98	99-100	44-80	95-99
Vidas GDH[40,129]	GDH	Vidas	97	87	56	100

Sen: Sensitivity; Sep: Specificity; GDH: Glutamate dehydrogenase; *C. difficile* or *C. diff*: *Clostridioides difficile*.

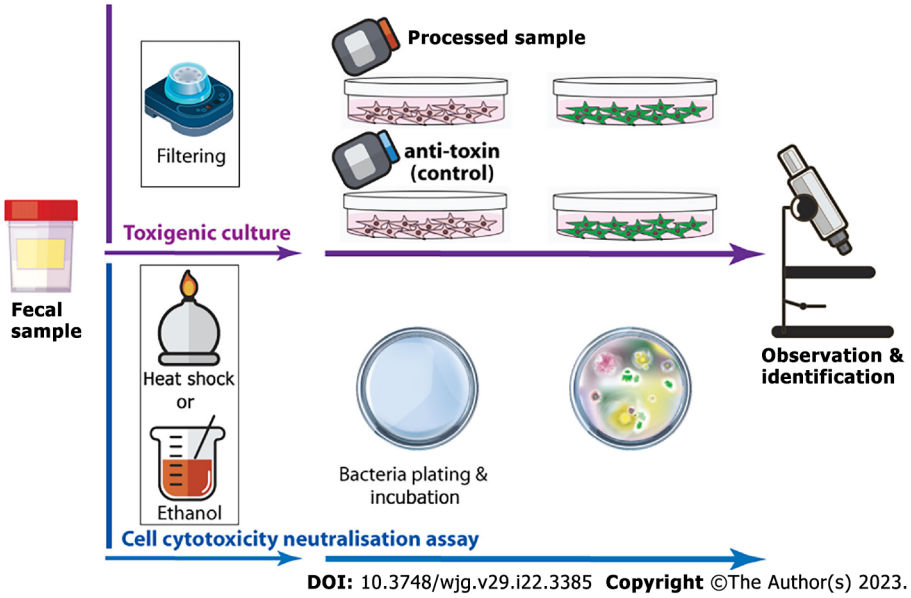

Figure 3 Idealised summary of toxigenic culture and cell cytotoxicity neutralisation assay for *Clostridioides difficile* infection identification. In toxigenic culture, the faecal sample is first exposed to heat shock or alcohol shock to kill *Clostridioides difficile* (*C. difficile*) and other microorganisms, and heat/alcohol resistant spores of *C. difficile* survive. The sample is then plated on selective agar and incubated anaerobically at 37 °C for at least 48 h to observe and confirm the colonisation of *C. difficile*. For the cell cytotoxicity neutralisation assay, the faecal sample is filtered and added to a toxin-sensitive cell line with/without antiserum. After 24-48 h of incubation, the cytopathological effect is then examined microscopically.

respectively, at a specificity of 99%[48]. This may be because these EIAs were developed in the early 21st century and are less sensitive to low toxin(s) levels, and immunocompromised CDI patients have a lower concentration trigger point[49]. This meta-analysis also indicated that GDH detection had a range

of 94% (95%CI: 86%-97%) for sensitivity and 96% (95%CI: 92%-98%) for specificity compared to toxigenic bacterial culture and cell culture cytotoxicity assay together[48]. However, a few studies reported sensitivities below 90% for GDH assays[38,50,51]. Due to the known limitations and relatively poor accuracy, stand-alone toxin or GDH EIA tests are not recommended by professional medical societies, including the European Society of Clinical Microbiology and Infectious Diseases and the Infectious Diseases Society of America[27,48,52].

As a result, several multiple-step diagnostic algorithms have been proposed to improve assay performance and detection accuracy. For example, a two-step algorithm initially detects GDH, and positive GDH cases are followed up with a toxin A and/or toxin B immunoassay. If the toxin immunoassay result is negative, a bacterial culture will be performed[53]. In addition, new protein-based assays such as lateral flow assay are under development and may potentially yield a result in 15 min[54].

MIRNA DYSREGULATION AND *C. DIFFICILE* INFECTION DETECTION AND MONITORING POTENTIAL

miRNAs are small single-stranded endogenous RNA molecules that are key regulators of gene expression and silencing at the post-transcriptional level. miRNA complex biosynthesis begins in the nucleus where primary miRNAs are produced by transcription of miRNAs from DNA sequences or miRNA genes. Precursor miRNAs are transcripts of approximately 60-110 nucleotides in length with a shorter stem-loop structure, produced from primary miRNAs by RNase type III enzymes (DROSHA) and undergo several maturation processes inside and outside the nucleus to form mature miRNAs, which are transported into the cytoplasm[55].

The stability of miRNA-specific target mRNAs is disturbed when RNA-induced silencing complexes possess loaded miRNAs. A portion of the miRNA, the seed sequence, which is two to eight nucleotides long, pairs with a specific sequence on the target mRNA and is referred to as an miRNA response element that results in translational repression and degradation of the target mRNA due to the binding of miRNAs in the 3′ untranslated region. miRNAs primarily repress genes by inhibiting protein synthesis, preventing elongation and ribosome decline and disrupting mRNAs through the processes of demethylation and decap, resulting in their degradation[56].

miRNAs have the potential to act *via* molecular mechanisms at every step of CDI, inhibiting specific transcripts or inflammatory molecule transcription, thereby influencing the pathology grade. The imbalance of these biomarkers can be measured and exploited for diagnosis, coupled with standard methods to strengthen the results. In particular, miRNAs are easily detectable (through sequencing, RT-qPCR, *etc*) in body fluids such as saliva, blood and even faecal material, and their levels correlate with target transcript alterations or non-physiological events[57]. These biomarkers are contributing increasingly to the establishment of less invasive "liquid biopsies," which is important and appealing for patient compliance compared to normal, invasive biopsies that require a long time for results. Although, it cannot fully substitute canonical diagnosis methods in most cases currently, it will undoubtedly happen in the near future.

Numerous studies have shown that miRNAs can be used to detect diseases and for their management. This may apply to patients with CDI. However, studies of the relationship between CDI and host miRNA are limited. To the best of our knowledge, the first reported study utilised C57BL/6J wild-type mice with a CDI model and identified the induction of mmu-miR-146b, mmu-miR-1940 and mmu-miR-1298 expression and upregulated proinflammatory cytokine expression, such as monocyte chemoattractant protein-1, interleukin (IL)-6, IL-17 and IL-1β in colonic tissues[58]. The authors also showed that the miR-146b potentially targets nuclear receptor co-activator 4 (NCOA4), *CD36* and *GLUT4* mRNA expression levels were downregulated. *In silico* simulation predicts upregulation of mmu-miR-146b and IL-17 resulting in the downregulation of *NCOA4* and peroxisome proliferator-activated receptor gamma (*PPAR-γ*) in the CDI mice model. Null mice silenced for *PPAR-γ* in T cells following CDI presented more severe colonic disease activity, inflammatory lesions and inflammatory cytokine expression[58]. There is also a filed patent claiming that miR-27a-5p could modulate the inflammatory response induced by CDI[59]. The inventors claim that miR-27a-5p can be linked to a matrix and delivered by vectors and/or incorporated into particles. The miR-27a-5p-linked particle could be administered to infected subjects for the treatment[59]. In faeces, a small-scale study with only 8 CDI participants demonstrated that a higher level of faecal hsa-miR-1246 was found in human CDI patients compared to the control group[60].

FMT has been proven in the treatment of recurrent CDI[61,62]. It is intended to restore colonic microbiota through introducing "healthy" bacteria *via* colonoscopy, enema or oral capsules that contain bacteria in a powder form. However, the safety concerns of FMT could be an obstacle to extending the application as a regular treatment strategy[63,64]. To monitor treatment conditions, detecting a panel of miRNA markers in circulation could help physicians make a more accurate decision[65,66]. Another study showed that 71 different circulating miRNAs were found to be expressed in 126 sera from 42 patients at 4 wk and 12 wk after FMT treatment[65]. The authors used qRT-PCR and 3′ untranslated

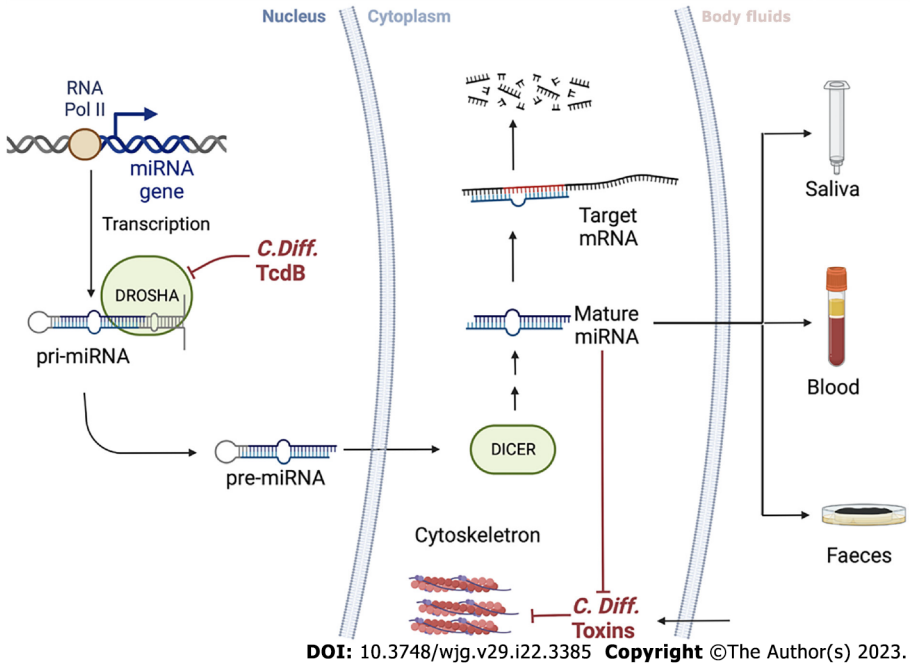

Figure 4 *Clostridioides difficile* and microRNAs cross-interact inside the cells. microRNA (miRNA) is a small single-stranded RNA that plays a key role in gene expression regulation. It starts in the cell nucleus, where primary miRNAs are produced by RNA Pol II through miRNA transcription from DNA sequences or miRNA genes. Precursor miRNA, around 60-110 nucleotides long transcripts with a shorter stem-loop structure, are produced from primary miRNAs by RNase type III (DROSHA) enzyme with several maturation processes and is then transported to the cytoplasm *via* DICER. Toxin B from *Clostridioides difficile* may interfere with DROSHA function. A fraction of miRNA binding specific sequences (2-8 nucleotides long) can pair with miRNA response elements in the 3' untranslated region of the target mRNA, causing translational repression and degradation of the target mRNA. Thus, miRNA expression patterns can indicate non-physiological events such as *Clostridioides difficile* infection. By detecting changes in miRNA patterns in body fluids, such as saliva, blood, and stool, the screening accuracy can be improved. miRNA: microRNA; pri-miRNA: Primary miRNA; pre-miRNA: Precursor miRNA; *C. difficile*: *Clostridioides difficile*.

region luciferase reporter assays to validate the top miRNA candidates and confirmed that hsa-miR-23a-3p, hsa-miR-150-5p, hsa-miR-26b-5p and hsa-miR-28-5p expression levels inversely correlated with the sera protein and cell-free circulating mRNA on several inflammation-related biomarkers, such as IL-12B, IL-18, FGF21 and TNFRSF9[65].

In a mouse model of relapsing CDI, qRT-PCR analyses of faecal and sera RNA extracts revealed inhibition of these miRNAs, while the FMT treatment enabled the recovery of their inhibitory effect. This study also showed that toxin B (TcdB) mediates the inhibitory effect of CDI on miRNA *via* DROSHA, based on the human colonoids and the mice colon models, where miR-23a and miR-150 were used to demonstrate the cytoprotective effects against TcdB[65]. A small, in-depth phenomics study of four adults treated with sequential FMT for severe or fulminant CDI found that miR-451a and miR-16 from the serum samples were upregulated in the responders *vs* the non-responders on average across all timepoints[66].

Using miRNAs as a diagnostic tool for CDI presents both opportunities and challenges. On one hand, as miRNAs play a role in controlling and influencing gene expression, changes in their activity and expression levels can be associated with different pathological events. This makes miRNA expression patterns a potentially powerful diagnostic tool, with the potential for use in therapeutic applications. Monitoring the response to FMT with miRNA can be an additional indicator for personalised treatment, and miRNA detection from non-invasive sources, such as blood, faeces, urine and saliva, can provide convenient and longitudinal measurements of miRNA levels for CDI. On the other hand, the fact that miRNA can be derived from blood cells released by different pathological events makes detecting CDI specifically a challenging task. Therefore, the use of miRNAs as a diagnostic tool for CDI requires further research and validation. Figure 4 provides a schematic summary of the intracellular mechanisms discussed and the potential use of miRNA profiling from a variety of body fluids, such as liquid biopsies, for diagnostic purposes as demonstrated in various research studies related to colorectal cancer screening[67-69].

CONCLUSION

To date, current laboratory-based CDI assays have varied in their detection accuracy. The expression of miRNAs has become increasingly important as novel biomarkers for assessment. The different signature profiles obtained through the differential expression of these small non-coding RNAs are essential for

early diagnosis and prediction of therapeutic response and disease management, these are also including cancer and infection. Notably, this can be accomplished by using liquid biopsy, enabling a non-invasive and low-cost screening approach. This approach may also be used to detect *C. difficile* infections, while further research and validation are clearly needed. These reported miRNA studies can be used in conjunction with current diagnostic tools to improve diagnostic accuracy aiding in patient management for both symptomatic and asymptomatic CDI patients.

FOOTNOTES

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Country/Territory of origin: United Kingdom

ORCID number: Marco Bocchetti 0000-0002-2143-6669; Tung On Yau 0000-0002-3283-0370.

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