savyonDIAGNOSTICS
member of the Gamida Diagnostics Division

A novel concept in diagnosis of *Clostridium difficile* - rapid functional testing of toxigenic activity in ELISA format

Zvi Greenberg*, Ephraim Fass, Oded Babai, Amit Hananel, Shimon Gross

Department of Research and Development, Savyon Diagnostics Ltd., Ashdod, Israel

Abstract

Objectives. *Clostridium difficile* is the leading cause of nosocomial enteric infections. Pathogenicity is directly attributed to the toxigenic effects of toxins A and B produced by the bacterium. Diagnosis of *C. difficile* relies often on detecting the presence of these toxins in stools. However, limited performance in currently-available diagnostic tests yielded a plethora of testing algorithms consisting multiple targets and methodologies. The ultimate gold standard in this context is the cytotoxin assay which detects not only the presence of toxins but their activity. However this test is lengthy (days) and requires maintenance of cell culture. Savyon Diagnostics has developed a novel rapid functional assay in which *C. difficile* toxigenic activity is detected in ELISA format within less than 2.5 hours. Two alternative approaches have been demonstrated: (a) Rac-Tox assay relying on glucosyltransferase activity of toxins A/B, which catalyzes monoglucosylation of small GTPases (i.e Rac1). Here toxigenic activity is detected by an antibody which recognizes Rac1 only in its glucosylated form. (b) Gox-Tox assay, which relies on detection of toxin A/B-dependent release of free glucose following UDP-glucose hydrolysis. Here, Glucosyl oxidase (Gox) – dependent generation of peroxide is measured and is directly correlated to free glucose, and hence to toxigenic activity.

Methods. In the Rac-Tox assay, toxin positive or negative samples were added to soluble His-Rac1 in the presence of UDP-glucose. Following incubation, His-Rac1 was selectively immobilized on Ni^{2+} plates. Detection was achieved using a recombinant antibody selected from a combinatorial library for its ability to specifically recognize glucosylated Rac1 but not non-glucosylated polypeptide. In the Gox-Tox assay positive or negative samples were added to immobilized anti-toxin antibodies in the presence of UDP-glucose as the substrate. Detection of peroxide generation was achieved following Gox activity using HRP/TMB system.

Results. Rac-Tox assay detects activity of less than 1 ng toxin within 2.5 hours. Gox-Tox assay detects activity of less than 10 ng toxin within 2 hours. Both values are of clinical relevance.

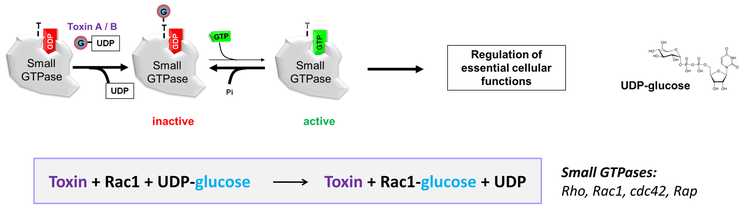
Conclusions. The two alternative approaches have been shown to have the capability to reliably detect *C. difficile* toxigenic activity in a rapid ELISA configuration, offering the potential to be used as a stand-alone confirmatory assay for diagnosis of *C. difficile*.

Introduction

Clostridium difficile is the leading cause of nosocomial enteric infections. Toxin production by the bacterium is essential for disease to occur. Only toxigenic strains of *C. difficile* produce clinical disease, but toxin production does not guarantee symptomatic progression. *C. difficile*'s primary virulence factors are toxins A (TcdA) and B (TcdB). Prompt diagnosis and treatment of *C. difficile* associated diarrhea (CDAD) is crucial, in order to prevent progression of the disease to moderate or severe state. Misdiagnosis may delay appropriate care and consequently may lead to possible clinical complications as well as increased risk of disease transmission. On the other hand, false positive results may lead to termination of needed broad-range antimicrobial therapy, isolating together with real cases and consequently increasing risk of acquiring true *C. difficile* infection (CDI). Currently, there are a variety of tests for diagnosis of *C. difficile*, mostly based on detecting the *C. difficile* GDH antigen or its toxins in stools of individuals with suspected disease. The currently available kits for detection of *C. difficile* have variable performances. To increase the accuracy of the results, multiple diagnostic algorithms, of two or three different types of tests have been established. Cell-culture-based Cytotoxin assays (CTA), which involve exposing cultured cells to fecal extracts in the absence or presence of an antagonizing anti-toxin antibody, are still regarded as the most accurate tests. The CTA detects not only the presence of toxins but also their activity. However, these tests have disadvantages in terms of time and the need for cell culture facility. Savyon Diagnostics has developed a novel rapid functional assay in which *C. difficile* toxigenic activity is detected in an ELISA format within 2.5 hours. Two alternative approaches have been demonstrated: (a) Rac-Tox assay relying on glucosyltransferase activity of toxins A/B, which catalyzes monoglucosylation of small GTPases (such as Rho, Rac, and Cdc42). Here toxigenic activity is detected by an antibody which recognizes Rac1 only in its glucosylated form. (b) Gox-Tox assay, which relies on detection of toxin A/B-dependent release of free glucose following UDP-glucose hydrolysis by the toxin. Here, Glucosyl oxidase (Gox) – dependent generation of peroxide is measured and is directly correlated to free glucose, and hence to toxigenic activity. These approaches enable convenient rapid and confirmative diagnosis as a stand-alone test.

Concept

Figure 1. Glucosylation of small GTPase proteins blocks their function



Toxin + Rac1 + UDP-glucose ⟶ Toxin + Rac1-glucose + UDP

Small GTPases:
Rho, Rac1, cdc42, Rap

Objective

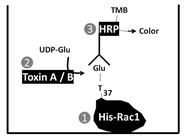
The aim of this work is to present a novel concept in diagnosis of CDI in which a toxin functional assay is exercised in a rapid cell-free configuration, thus providing a proper alternative to the currently applied testing algorithms for the purpose of screening as well as diagnosis

Methods

In the Rac-Tox assay, toxin positive or negative samples were added to soluble His-Rac1 in the presence of UDP-glucose. Following incubation, His-Rac1 was selectively immobilized on Ni^{2+} plates. Detection was achieved using a recombinant antibody selected from a combinatorial library for its ability to specifically recognize glucosylated Rac1 but not non-glucosylated polypeptide. In the Gox-Tox assay positive or negative samples were added to immobilized anti-toxin antibodies in the presence of UDP-glucose as the substrate. Detection of peroxide generation was achieved following Gox activity using HRP/TMB system.

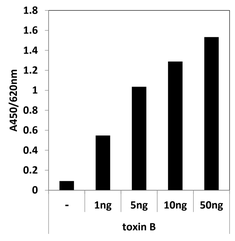
Results – Rac-Tox Approach

Figure 2. The functional Rac-Tox immunoassay



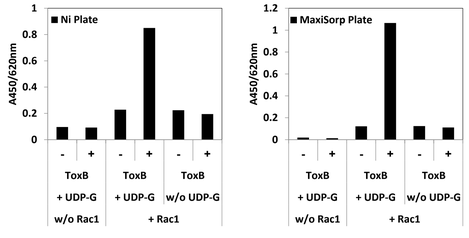
C. difficile toxin A or B mediate a glucosyltransferse reaction in which glucose is transferred from UDP-glucose to members of the Rho family of small GTPases e.g. Rac1. (1) The GTPase substrate Rac1 having a Thr35 residue is incubated with (2) a *C. difficile* toxin-containing sample in the presence of UDP-glucose and reaction buffer, and subsequently immobilized on an ELISA plate. (3) HRP-labeled recombinant antibodies specifically detect Rac1 which is monoglucosylated on residue Thr35

Figure 3. Activity of *C. difficile* toxin is detected at ng toxin levels



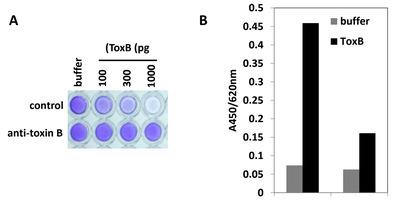
The recombinant antibody is able to detect toxin mediated glucosylated Rac1 protein in a dose dependent manner showing LOD of less than 1 ng toxin

Figure 4. Toxin B mediated glucosyltansferase activity in the presence or absence of UDP-G and Rac1 using Ni or MaxiSorp® microtiter plates



Rac1 Glucosylation is detected only when toxin B, His Rac-1 and UDP-glucsoe are present either when His-Rac1 is immobilized in a MaxiSorp® plate or in Ni coated plate, indicating that the immobilization platform does not affect the assay outcome

Figure 5. The antibody that antagonizes toxin B in the cytotoxin assay also inhibits the toxin mediated glucosylation of Rac1 in the assay



The specificity of the assay is demonstrated by using toxin B antagonizing antibodies that are typically employed in the cytotoxin assay. (A) Addition of toxin B results in cell death in a dose dependent manner, however, when cells are pretreated with anti-toxin B antibodies they remain viable even at toxin levels (1000 pg) that achieves full cell death in the control cells, an indication that this antibody antagonizes the activity of toxin B. (B) The antibody that antagonizes toxin B in the cytotoxin assay also inhibits the toxin mediated glucosylation of Rac1 in the assay

Conclusions

Rac-Tox approach

- The toxigenic activity of *C. difficile* can be detected by measuring both toxins A and B function as glucosyltransferases to glucosylate small GTPases
- ELISA detection settings of the Rac-Tox assay allow specific detection of less than 1 ng *C. difficile* toxin within 2.5 hours

Gox-Tox approach

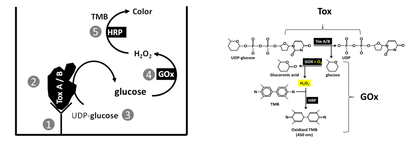
- The results demonstrate that the activity of toxin B could be measured in a simple, rapid and cost effective assay by detecting the glucose which derives solely from UDP-glucose due to the hydrolysis activity of the toxin
- Gox-Tox assay detects activity of less than 10 ng toxin within 2 hours

Overall

- Having the capability to detect efficiently and specifically *C. difficile* toxin activity in rapid configuration, both approaches offer a proper alternative to the laborious tissue culture dependent cytotoxic assay that requires 48 – 72 hours, and have the potential to be used as stand-alone confirmatory assays for diagnosis of *C. difficile*

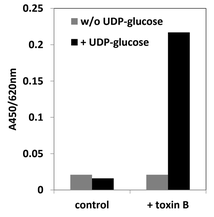
Results – GOx-Tox Approach

Figure 6. The functional GOx-Tox assay



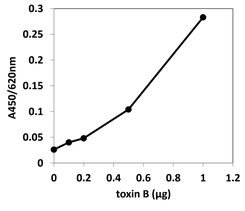
Anti-toxin A/B antibodies coated on ELISA plate capture *C. difficile* toxins from a stool sample (2). UDP-glucose is added into the wells and toxins A/B cleave UDP-glucose (3) to UDP and glucose. (4) Free glucose is oxidized by glucosyl oxidase (GOx) and H_2O_2 is released. (5) H_2O_2 serves as an HRP substrate to oxidize TMB thus resulting in a color change that is quantified at 450/620nm.

Figure 7. Production of free glucose after toxin B reacts with UDP-glucose



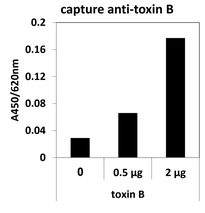
The source of the measured free glucose is from toxin B cleavage of UDP-glucose to UDP and glucose because free glucose is not detected in control samples that lack either toxin B or UDP-glucose

Figure 8. Production of free glucose as a function of toxin B



The free glucose levels increases as a function of the level of toxin B

Figure 9. Quantitative production of free glucose as a function of immobilized and extracted toxin B from a spiked sample



The assay was able to detect activity of toxin B that was extracted and immobilized