1 **A Biomarker Based Peptide Immunoassay for *Clostridioides difficile*: "Insights from
2 Central India"**

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19 ABBREVIATIONS

CDI	*Clostridioides difficile* infection
ELISA	enzyme-linked immunosorbent assay
CIIMS	Central India Institute Of Medical Sciences
OPD	outpatient department
CCFA	Cycloserine Cefoxitin Fructose Agar
MALDI TOF	matrix-assisted laser desorption/ionization image-time of flight mass spectrometry
GDH	Glutamate dehydrogenase
WCL	WHOLE CELL LYSATE
SDS-PAGE	Sodium Dodecyl sulphate- polyacrylamide gel electrophoresis
LC-MS/MS	Liquid chromatography–mass spectrometry
QC	quality control
NCBI	National Centre for Biotechnology Information
BLAST	Basic Local Alignment Search Tool
PBS	phosphate buffered saline
DMSO	dimethyl sulfoxide
PBST	phosphate-buffered Saline with Tween20

IgG	Immunoglobulin G
IgM	Immunoglobulin M
HRP	horseradish peroxidase
TMB	3,3′,5,5′-tetramethylbenzidine
ANOVA	Analysis of Variance
ROC	Receiver Operation
DMIMS	Datta Meghe Institute of Medical Sciences
HPLC	High Performance Liquid Chromatography
PCR	Polymerase chain reaction
FMT	Fecal microbiota transplantation

20 **Abstract:**

21 **Introduction:** *Clostridioides difficile* infection (CDI) is a major healthcare challenge due to
22 its virulence factors, Toxins A and B. Current diagnostic methods like NAAT and EIA face
23 limitations, including overdiagnosis and cultural resistance to stool sample collection,
24 particularly in India. This study explores blood-based diagnostics, focusing on detecting anti-
25 toxin antibodies through advanced proteomics and immunoassays. These innovative
26 approaches aim to improve diagnostic sensitivity, specificity, and patient accessibility,
27 addressing both clinical and cultural barriers.

28 **Methods:** This prospective observational study was conducted at the Advanced Research
29 Centre of the Central India Institute of Medical Sciences (CIIMS) in Nagpur. The study
30 enrolled 350 patients aged 18–70 years with clinical manifestations of diarrhea. This research
31 focused on methodologies including microbial isolation of *Clostridiodes difficile*, isolating
32 and analyzing novel proteins through LC-MS/MS, designing and synthesizing antigenic
33 peptides, and standardizing peptide ELISA.

34 **Results:** The study successfully isolated and analyzed toxins A and B from *C. difficile*. The
35 toxins were visualized using a 10% SDS-PAGE gel matrix, followed by peptide design and
36 analysis. The developed immunoassay was tested on 350 serum samples, revealing a higher
37 prevalence of toxin A than toxin B in the central Indian population.

38 **Conclusions:** The peptide-based immunoassay developed in this study marks a notable
39 improvement in diagnosing *Clostridioides difficile* infection, especially in contexts where
40 stool sample testing is impractical or culturally sensitive. Offering rapid, sensitive, and
41 patient-friendly detection of anti-toxin antibodies, this method shows potential for enhancing
42 CDI management and controlling its spread. However, additional refinement and validation
43 are necessary to confirm its standalone diagnostic utility. The findings also underscore the
44 intricate relationship between bacterial virulence, host immunity, and clinical outcomes,
45 opening avenues for personalized treatments.

46 **INTRODUCTION:**

47 *Clostridioides difficile* infection (CDI) represents a significant healthcare burden, particularly
48 due to its virulence factors, Toxins A and B. The existing diagnostic methods, such as
49 Nucleic Acid Amplification Test (NAAT) and Enzyme Immunoassay's (EIA), present
50 challenges of over diagnosis and limited specificity (Kraft C. et al., 2019) (Humphries R. et
51 al., 2012). This study addresses a key gap by focusing on antibody-based blood diagnostics,
52 overcoming the cultural barriers to stool sample collection in India. We highlight the
53 prevalence of CDI in Central India, emphasizing the need for innovative, patient-friendly
54 diagnostic approaches.

55 *Clostridioides difficile* infection is a leading cause of healthcare-associated diarrhea and
56 colitis, presenting a significant challenge to patient care and the healthcare system worldwide
57 (Balsells et al. 2019). A previous study suggested that *C.difficile* was detected in 6.2%, 4.8%,
58 and 0.5% in urban inpatient, urban outpatient, and rural populations tested, respectively. The
59 study also mentioned that the toxigenic *C. difficile* is an important but neglected aetiologic
60 cause of infective diarrhoea in India (Biswas et al. 2023, Kannambath R et al. 2021). Rapid
61 and accurate diagnosis of CDI is critical for timely initiation of appropriate treatment and
62 infection control measures. In the quest of effective diagnostic strategy, biomarkers have
63 emerged as effective promising tools for identifying and monitoring various infectious
64 diseases (Bodaghi et al. 2023). This biomarker encompasses various molecules, including
65 toxins, enzymes, and host response markers, which reflect the presence or activity of CDI in
66 the gastrointestinal tract during the host immune response to infection. The diagnosis of CDI
67 revolves around detecting its two primary virulence factors, Toxin A and Toxin B, which are
68 responsible for the clinical manifestations of the disease. Current diagnostic modalities vary
69 in sensitivity, specificity, and clinical utility. The Nucleic Acid Amplification Test (NAAT)
70 is a molecular method that detects the presence of *C. difficile* DNA, specifically targeting the
71 genes responsible for toxin production. NAAT boasts high sensitivity and can detect even
72 low levels of bacterial DNA. However, its major limitation is the potential for over diagnosis,
73 as it cannot differentiate between active infection and asymptomatic colonization, leading to
74 false-positive results (Kraft C. et al., 2019) (Humphries R. et al., 2012). Enzyme
75 Immunoassays (EIA), on the other hand, directly detect Toxin A and Toxin B in stool
76 samples. Its rapid and cost-effective and is considered a valuable tool because it confirms the
77 production of active toxins, a key marker of symptomatic infection.

78 In the realm of biomarker discovery, the development of enzyme-linked immunosorbent
79 assay (ELISA) technology has revolutionized the detection of proteins with unparallel
80 specificity, sensitivity, and scalability for biomarker analysis across diverse clinical and
81 research settings. The process of biomarker discovery for *Clostridioides difficile* diagnosis
82 often involves screening novel targets associated with CDI, such as specific antigens and
83 metabolites (Burnham and Carroll, 2013). Through advanced proteomics techniques,
84 researchers have identified potential biomarkers that offered enhanced sensitivity and
85 specificity compared with traditional methods (Islam Khan et al. 2022; Hudler et al. 2014).
86 Once identified, the validated biomarkers could be translated into diagnostic assays.

87 *Clostridioides difficile* proteins/toxins have become a standard diagnostic tool in clinical
88 microbiology laboratories, offering a scalable and cost-effective solution for high-throughput
89 testing.

90 Despite advancements in biomarker discovery and ELISA technology, challenges remain in
91 optimizing diagnostic assays for CDI, including the need for improved sensitivity, specificity,
92 cost-effectiveness, and shorter turnaround times (Markantonis et al. 2024; Biswas et al.
93 2023). Furthermore, the emergence of hypervirulent strains and antimicrobial resistance
94 underscores the importance of continuous surveillance and innovation in CDI diagnostics
95 (Cookson 2007).

96 The majority of diagnostic approaches for CDI are based on stool sample analysis, with a
97 predominant focus on stool-based testing kits. These diagnostic tools typically target the
98 detection of *C. difficile* toxins, particularly toxins A and B, which are key virulence factors
99 responsible for the clinical manifestations of the infection. However, blood-based assays, such
100 as those aimed at detecting the host's immune response to *C. difficile*, including antibody
101 production, have not gained widespread adoption. This may be attributed to the limited
102 sensitivity and specificity of antibody detection in the context of acute infection, as well as the
103 challenges in correlating systemic antibody levels with local toxin production in the gut.
104 Despite these challenges, research into antibody-based diagnostics could offer complementary
105 insights, particularly in understanding patient immune responses.

106 *Clostridioides difficile* infection (CDI) presents a significant global health challenge,
107 particularly due to its high morbidity, recurrence rates, and potential for severe
108 complications. Current diagnostic methods, including stool-based nucleic acid amplification
109 tests (NAATs) and enzyme immunoassays (EIAs), are limited by issues such as over
110 diagnosis, low specificity, and patient reluctance to provide stool samples due to cultural
111 barriers—particularly in India.

112 This study addresses these gaps by exploring an alternative, non-invasive blood-based
113 diagnostic approach for CDI. By leveraging advanced proteomics and immunoassay
114 techniques, this research focuses on detecting anti-toxin antibodies in serum samples,
115 allowing for a more accessible, sensitive, and specific diagnostic method. The development
116 of a peptide-based ELISA offers a promising solution to overcome the limitations of existing
117 stool-based tests, particularly in regions where cultural norms hinder stool sample collection.

118 The rationale behind this study is driven by the need for an affordable, patient-friendly
119 diagnostic tool that can improve CDI detection and management. The findings have potential
120 implications for clinical decision-making, disease monitoring, and epidemiological
121 surveillance. Additionally, the study contributes to biomarker-based diagnostics, providing
122 insights into host immune responses to *C. difficile* infection and paving the way for future
123 innovations in CDI diagnosis and treatment.

124

125 **MATERIALS AND METHODS:**

126 **Study design:**

127 This prospective study was conducted between January 2020 and January 2024. The study
128 was set in the "Central India Institute Of Medical Sciences," Nagpur. All stool samples and
129 blood samples collected for the study were obtained from subjects recruited to the Central
130 India Institute of Medical Sciences (CIIMS), Nagpur. Patients within the age groups of 18
131 and 70 years were included in the study on the basis of clinical manifestations such as ≥3
132 loose stools in 24 hours along with additional gastrointestinal symptoms such as nausea,
133 vomiting, abdominal cramps, bloody stools, or fever (oral temperature ≥38°C). Every
134 participant in the *C. difficile*-infected group experienced diarrhea and tested positive for the
135 toxin. However, those who had an established non-infectious cause of diarrhea, such as
136 inflammatory bowel illness, were excluded from the study, such as those who were unable to
137 produce feces and were not able to submit a stool sample. Those who had cancer and were
138 undergoing chemotherapy, taking prednisolone (>5 mg/d), immunomodulators (calcineurin
139 inhibitor, methotrexate, azathioprine), or any biologics were considered as
140 immunosuppressed were found ineligible for the study.

141 In the present study, sample size was determined using Raosoft sample size estimation. As
142 per the data available from a previous study which mentioned the prevalence of CDI in
143 Nagpur region (Monaghan TM et al. 2022) of around (12%) with population size of 0.25
144 million and confidence interval of 90%, with margin of error of 5%. Estimated samples size
145 was around 350. Therefore, these samples were collected to evaluate the performance of the
146 assay for the diagnosis of *C.difficile* infection. Patients were categorized into four distinct
147 groups, namely, confirmed cases (n=100 stool+ blood samples), suspected cases (n=150
148 stool+ blood samples), infectious cases (n=50 stool+ blood samples) and healthy controls
149 (n=50 stool+ blood samples). The criteria for each groups are detailed below: The confirmed
150 cases included recruited patients with laboratory-confirmed CDI based on positive stool toxin
151 assays, culture, or molecular testing. Symptoms included diarrhea (≥3 unformed stools in 24
152 hours) and clinical evidence of CDI (e.g., abdominal pain, fever). The suspected cases were
153 patients presented with symptoms suggestive of CDI, but without laboratory confirmation at
154 the time of sample collection. Stool samples from these patients were tested during the study
155 to determine assay performance in suspected cases. The infectious controls were patients
156 diagnosed with gastrointestinal infections caused by other pathogens, including *Salmonella*
157 *spp*., *Shigella spp*., *Escherichia coli* (EHEC/ETEC), *Norovirus*, or *Rotavirus.* These patients
158 were confirmed cases of non-*C. difficile* diarrhea and were intended to evaluate the assay
159 specificity and cross-reactivity. Pathogen confirmation was performed using standard
160 diagnostic assays appropriate for each pathogen. The healthy controls were individuals
161 without symptoms of gastrointestinal distress or any known recent exposure to
162 gastrointestinal pathogens. Stool samples were collected from healthy volunteers after
163 obtaining informed consent, ensuring that they had no antibiotic use in the past three months.

164 **Microbial culturing of *Clostridiodes difficile*:**

165 A loopful of each sample from the confirmed and the suspected groups were added to
166 thioglycolate broth. After 24 h of incubation under strict anaerobic conditions in the
167 anaerobic Chamber (Don Whitley, DG250), tubes showing bacterial growth were (if any) was
168 streaked onto Cycloserine Cefoxitin Fructose Agar (CCFA) plates and incubated at 37 °C
169 under anaerobic conditions. After 72 h of incubation, colonies suggestive of *C.difficile* were
170 further confirmed through matrix-assisted laser desorption/ionization image-time of flight
171 mass spectrometry (MALDI-TOF). For whole cell lysate extraction, the confirmed *C.difficile*
172 colonies were subcultured in CCFA broth and incubated under anaerobic conditions.

173 **WHOLE CELL LYSATE (WCL) preparation:**

174 Approximately, 30mL of pure bacterial culture of *C.difficile* from each confirmed sample was
175 transferred to a screw capped tube and spun in a cooling centrifuge at 24000 g for 10 min at 4
176 °C. To the pelleted bacterial cells, 05 mL of cell lysis buffer (Tris HCl, Sucrose, MgCl2.
177 6H2O, Triton X-100) was added, and mixed uniformly. After incubation for 30 min at 37 °C,
178 the tubes were centrifuged at 24000 g for 10 min at 4 °C to collect the proteins in the
179 supernatant. Next, protein solubilizing buffer (7M Urea, 2M thiourea, and 4% 3-((3-
180 cholamidopropyl) dimethylammonio)-1-propanesulfonate) was added to the collected
181 supernatant and incubated at 37 ˚C for 02 hours. The supernatant was then collected, and a
182 protein purifying reagent (99.5% acetone) was added 4X times the volume of the collected
183 supernatant. Following an overnight incubation at -20 °C, the tubes were centrifuged at
184 24000 g for 10 min at 4 °C to harvest the pure whole cell lysate, which was then run in a
185 polyacrylamide gel electrophoresis (PAGE) for identifying the different proteins present in
186 the whole cell lysate (Bhartiya N et al. 2020).

187 **One-dimensional electrophoresis:**

188 The WCL of all the isolates were subjected to electrophoresis. For electrophoresis, the
189 sample was prepared by mixing WCL containing the mixture of bacterial proteins with 10 µL
190 of the tracking dye. One-dimensional sodium dodecyl sulfate polyacrylamide gel
191 electrophoresis (SDS-PAGE) was performed with a vertical slab gel electrophoresis system
192 (Broviga, India) using the standard Laemmali method with 5% stacking and 10% running gel.
193 Electrophoresis was performed at 150V. After electrophoresis, the gel was developed using
194 Coomassie brilliant blue to observe the protein profile. The stained protein bands were then
195 excised and processed for mass spectrometry (MS) analysis to check the nature and type of
196 protein extracted.

197 **LIQUID CHROMATOGRAPHY MASS SPECTROMETRY (LC-MS/MS) analysis and**
198 **gel digestion:**

199 To partially purify the whole cell lysate proteins, the bands were excised and soaked in an
200 elution buffer (0.15 M phosphate-buffered saline (PBS), pH 7.4). The gel was subsequently
201 electro-eluted using a complete gel eluter system (Biotech India, New Delhi, India) for one
202 hour at 30 V. Following harvesting, the eluted proteins were dissolved in PBS, and a BioLab

kit was used to measure the protein concentration. SDS was used to further separate this partially purified material, and Coomassie Blue was used to stain the gel. The purified bands were then sent for LC-MS/MS analysis to Kendrick Laboratories (Madison, Wisconsin, USA). To characterize these proteins, Kendrick Labs followed this protocol: the gel pieces were destained using a 50% methanol and 10% acetic acid solution followed by 50% CH3CN/0.1 M Tris–HCl, pH 8.0, wash, and in-gel digestion. Prior to LC-MS/MS analysis, the protein bands were removed, trypsin-digested, and treated with reducing and alkylation agents (100 mM iodoacetamide). For roughly four hours, in-gel digestion was carried out in 50 mM NH4HCO4 buffer (pH 8.5) at 37°C. Typically, 20–50 µL of digestion buffer was added, depending on the size of the gel fragment. Depending on the size of the gel piece and the anticipated protein content, different amounts of proteolytic enzyme (Promega trypsin, modified, sequencing grade) were utilized; typically, 200 ng to 1 µg per gel band was used. Acetonitrile (CH3CN) was added to the digestion solution to extract the peptides. The samples were then centrifuged at high speed for five minutes. The supernatant was collected and dried on medium heat using SpeedVac. Alkylation agents (100 mM IAA) were introduced prior to the LC-MS/MS analysis for the reduction step. For the LC-MS/MS analysis, the dried material was reconstituted in 0.5% acetic acid. Finnigan (ThermoFinnigan, San Jose, CA, USA) LCQ ion trap MS combined with an HPLC system was used for LC-MS/MS analysis. An in-house constructed, 75 µm (ID) × 10 cm length, 3 µm packing C18 capillary column was used with a nanospray device that could electrospray steadily at flow rates between 100 and 1500 nl min−1. Solvent A (2% CH3CN, 97.9% H2O, 0.1% formic acid) and Solvent B (90% CH3CN, 9.9% H2O, 0.1% formic acid) were used as mobile phases. When the AGC was turned on, the ion trap MS was run in data-dependent mode. After comparing the MS/MS data to internal quality control (QC) criteria, the ProtQuest search engine was used to search for the most recent non-redundant protein database. The search results were analyzed manually. The digested proteins were identified as Toxin A (Supplementary data 1) and Toxin B (Supplementary data 2) which were found to be present in very high magnitude after the process. This information was further taken for peptide designing.

Peptide designing and selection:

Immunogenic antigens from *C.difficile*, such as toxins A and B, were targeted for the creation of antigenic peptides. Chromosome selection was performed using the National Centre for Biotechnology Information (NCBI) server UniProtKB/Swiss-Prot, and sequences of the relevant bacterial proteins were retrieved. Antigenic peptides were identified using the web tool "Molecular Immunology Foundation- Bioinformatics" based on Kolaskar and Tongaonkar techniques. The created peptide sequences were then subjected to multiple sequence alignment to determine whether they shared any homology with other animals and to compare them with non-redundant protein database sequences from various Clostridium species using NCBI BLAST (Basic Local Alignment Search Tool).

Peptides synthesis:

243 The designed synthetic peptides were custom-synthesized by GenicBio Limited (Shanghai, 244 China) with 95% purity and 14 mg quantity each. The peptides were finally dissolved to a 245 stock concentration of 1 mg/mL in phosphate buffered saline (PBS) and dimethyl sulfoxide 246 (DMSO), yielding a pH of 7.4.

247 **ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA):**

248 The peptides were standardized by coating with four different concentrations of synthetic 249 peptides. The different concentrations used for the study were 05 ng, 10ng, 20ng & 40ng of 250 each peptide per well. 100µL of different concentration of the peptides were diluted in PBS, 251 coated in different wells of the microtiter plates, and incubated overnight at 4°C. The wells 252 were then blocked with 0.5% Bovine Serum Albumin (BSA) and incubated for 2 h at 37°C. 253 The plate was washed thrice after overnight incubation with phosphate buffered saline-254 Tween 20 (PBST) and 100 µL serum samples collected under the 04 categories mentioned 255 above were added to the respective coated wells at four dilutions (1:100, 1:200, 1:400, and 256 1:800) and incubated for 1 h at 37°C. After washing three times with PBST, 100 µL of 257 secondary antibody (Goat anti-human IgG/IgM horseradish peroxidase (HRP) conjugate 258 Bangalore, Genei) with a dilution of 1:10000 was added and incubated for 45 min at 37°C. 259 The wells were washed again with PBST and 100 µL of 3,3′,5,5′-tetramethylbenzidine 260 (TMB)/H2O2 substrate solution was added, and the plate was incubated for 10 min for a 261 color reaction to develop. The reaction was stopped by adding 100 µL of 2.5N H2SO4 to 262 each well. The absorbance of the color developed in each well was measured at 450 nm 263 wavelength. The data of the optical densities (OD) were further analyzed using statistical 264 methodologies as compared to the positive and negative control.

265 **Statistical analysis:**

266 Statistical analysis was performed using MedCalc and GraphPad Prism software. Descriptive 267 statistics were reported as frequencies and proportions for categorical variables. The Kappa 268 statics was used to find the significance of the reproducibility of the data. The Receiver 269 Operating Characteristic (ROC) curve analysis was performed to determine the optimal cut-270 off value for assessing the sensitivity and specificity of the designed peptides. The P value of 271 < 0 05 were considered statistically significant for all the analysis.

272 **Ethical consideration:**

273 The present study has been approved by The Datta Meghe Institute of Higher Education & 274 Research (Deemed to be University) Institutional Ethics Committee with Ref no: 275 DMIMS(DU)/IEC/2020-21/8838 dated 16.06.2020 and by the institutional Ethics Committee 276 of Dr. G.M.Taori Central Indian Institute of medical sciences with Ref no: 277 02/11/19/PhD/IEC/CIIMS dated 14.11.2019. These approvals allowed the collection of stool, 278 blood and other body fluids of diseased and healthy control group for experimental analysis. 279 Informed consent of the participants was obtained before the study. Participants were assured 280 of the confidentiality of the information.

281 **RESULTS:**

282 To analyze the protein expression patterns in *Clostridioides difficile* isolates, whole-cell
283 lysates from 10 culture-positive samples out of the 100 confirmed cases were subjected to
284 SDS-PAGE. These selected samples procured the best Colony forming units (CFU) and so
285 was selected for further analysis. As shown in Figure 1, distinct protein bands were observed,
286 with notable differences in molecular weight. Lanes 1, 2, and 7 displayed two prominent
287 high-molecular-weight protein bands at approximately 250 kDa and 310 kDa. In contrast,
288 lanes 3, 4, 5, 6, 8, 9, and 10 exhibited an upregulated band at ~310 kDa. Additionally, lower
289 molecular weight bands (~150 kDa) were identified as auto-proteolytic fragments of larger
290 toxins due to enzymatic digestion or partial processing of the whole-cell lysates (Jank T et al.,
291 2008).

292 To identify these proteins, the high-molecular-weight bands were excised via electro-elution
293 and analyzed using LC-MS/MS. The results confirmed that the ~310 kDa bands corresponded
294 to Toxin A, while the ~250 kDa bands were identified as Toxin B—both recognized as major
295 virulence factors of *C. difficile*. Notably, Toxin A was detected in all cases (100%), whereas
296 Toxin B was present in only 30% of cases, always co-occurring with Toxin A. No cases were
297 found where Toxin B was detected without Toxin A, indicating Toxin A's predominant role
298 in the studied population.

299 Given their immunogenic potential, Toxin A and Toxin B were selected for antigenic peptide
300 design. Using UniProtKB/Swiss-Prot and antigenicity prediction techniques (Kolaskar and
301 Tongaonkar method), 18 antigenic peptides (11 for Toxin A and 7 for Toxin B) were
302 designed as illustrated in figure 2 and 3. These peptides underwent multiple sequence
303 alignment and were screened for similarity with other Clostridium species using NCBI
304 BLAST. High-performance liquid chromatography (HPLC) analysis confirmed the peptides'
305 purity at 95% (GenicBio Limited, Shanghai, China).

306 To evaluate the antigenicity of these peptides, an indirect ELISA assay was optimized. A
307 toxin-positive serum sample with the highest IgG titer was selected for testing. Peptide
308 screening using a checkerboard approach as shown in table 01, identified SLFYFDP as the
309 most effective for detecting Toxin A, yielding maximum absorbance at 450 nm when coated
310 at 10 ng/µL. Similarly, IVQIGVF was identified as the optimal peptide for detecting Toxin B,
311 achieving peak absorbance at 5 ng/µL. To establish diagnostic accuracy, a Receiver
312 Operating Characteristic (ROC) analysis was performed on serum samples from 5 confirmed
313 positive cases and 5 healthy controls. The results determined cut-off absorbance values of
314 >0.387 for SLFYFDP (Toxin A) and >0.679 for IVQIGVF (Toxin B), demonstrating strong
315 discriminatory power as mentioned in figure 4.

316

317 Subsequently, an Enzyme-Linked Immunosorbent Assay (ELISA) procedure was then carried
318 out following the established protocol to check the reproducibility of the developed assay.
319 The resulting data revealed a cut off value of greater than 0.64, which demonstrated a
320 sensitivity and specificity of 100% each as mentioned in table 02. The statistical table 02

321 presented summarizes diagnostic test performance for detecting a condition, likely in the
322 context of a study. The test demonstrates high accuracy (99.43%) with excellent sensitivity
323 (95.24%) and perfect specificity (100%), indicating its strong ability to correctly identify both
324 positive and negative cases. The positive predictive value (PPV) is 100%, suggesting that all
325 positive test results are true positives, while the negative predictive value (NPV) is also high
326 (99.35%), meaning most negative results are true negatives. The negative likelihood ratio
327 (0.05) indicates that a negative result significantly reduces the probability of disease
328 presence. Given a disease prevalence of 12%, these metrics suggest the test is highly reliable
329 for diagnosing the condition as mentioned in table 03.

330 For assay validation, an ELISA was performed using a characterized set of 20 positives and
331 10 negative samples, confirmed via the Quick Check Complete *C. difficile* analysis kit. The
332 test showed high reproducibility, with a Cohen's Kappa coefficient of 0.927, indicating
333 almost perfect agreement between independent operators. The assay demonstrated an overall
334 agreement of 97% for Toxin A detection and 93% for Toxin B detection, with high sensitivity
335 and specificity (100%).

336 To assess the real-world applicability of the developed immunoassay, 350 serum samples
337 from clinically diagnosed cases were tested. As shown in Figure 5, confirmed cases (n=100)
338 exhibited high antibody titers against Toxin A, well above the cut-off value. Suspected cases
339 (n=150) displayed a range of titers, with some individuals exceeding the cut-off, suggesting
340 potential exposure. In contrast, infectious controls (n=50) and healthy controls (n=50) had
341 low antibody titers, consistently below the cut-off, indicating no detectable infection.

342 Similarly, Figure 6 presents the antibody distribution for Toxin B. Confirmed cases exhibited
343 the highest titers, while suspected cases showed intermediate levels, suggesting possible early
344 or subclinical infection. Infectious controls had slightly elevated titers compared to healthy
345 controls, implying past exposure but no active infection. These findings highlight the
346 effectiveness of the developed ELISA in distinguishing infected individuals from non-
347 infected ones. The test demonstrated high sensitivity in detecting antibodies even in
348 suspected cases, making it a promising tool for both diagnostic and epidemiological
349 applications.

350

351 **DISCUSSION:**

352 *Clostridioides difficile* infection (CDI) is a significant health concern due to its potential to
353 cause severe complications, including fulminant colitis, chronic illness, and pain, which can
354 severely impact the quality of life (Bouza, 2012; Rajack et al., 2023). Persistent diarrhoea and
355 recurrent CDI often lead to dehydration, electrolyte imbalances, and nutritional deficits, and
356 in severe cases, can escalate to life-threatening conditions like toxic megacolon, intestinal
357 perforation, sepsis, and multi-organ failure. If left untreated, CDI not only increases
358 healthcare utilization and costs but also facilitates its spread in healthcare and community

settings, potentially causing outbreaks among vulnerable populations (Bhattacharyya et al., 2020). Early detection and timely treatment are therefore critical to mitigating these risks and reducing transmission (Martinez et al., 2012; Cofini et al.2021).

The current standard preferable tests for CDI diagnosis primarily involves stool-based testing methods such as ElA, nucleic acid amplification tests (NAATs), and glutamate dehydrogenase (GDH) assays (Kraft et al., 2019). While these tools provide direct toxin detection, they come with a few limitations. For example, the *C. diff* Quik Chek Complete kit, commonly used in resource-limited settings, suffers from suboptimal sensitivity and specificity (Krutova et al., 2019). More advanced molecular techniques like NAATs offer improved accuracy but are costly and less user-friendly. Furthermore, cultural and ethical barriers in countries like India limit the willingness of patients to provide stool samples, posing challenges for effective diagnosis and management of CDI.

To address these barriers, this study explored the development of a peptide-based immunoassay to detect antibodies against *Clostridioides difficile* toxins A and B in blood samples. This approach not only circumvents the reluctance associated with stool-based testing but also enhances diagnostic potential by targeting the host immune response to *C. difficile* toxins.

Development of the Peptide-Based Immunoassay

Stool and blood samples were collected from patients presenting with classic CDI symptoms. Stool samples underwent initial screening using the *C. diff* Quik Chek Complete kit, with toxin-positive samples preserved for further analysis. To isolate *C. difficile*, a two-step enrichment culturing protocol followed by plating on CCFA was employed. Confirmed isolates were subjected to protein extraction. Subsequent SDS-PAGE analysis revealed distinct high-molecular- weight protein bands, corresponding to the enterotoxins, Toxin A (-310 kDa) and Toxin B (~250 kDa), which were identified via LC-MS. (Notably, Toxin A was detected in all cases, while Toxin B co-occurred with Toxin A in 30% of samples, underscoring the predominance of Toxin A in this patient cohorts. High serum levels of Toxin A usually correlates with high disease severity as well (Granata G. Et al, 2021).
Using these insights, antigenic peptides were designed to target Toxin A and Toxin B. Bioinformatics tools were employed to identify immunogenic regions, ensuring specificity and minimal cross-reactivity with other Clostridium species. Checkerboard titration assays optimized peptide and serum dilutions, enabling the development of a prototype ELISA. Receiver operating characteristic (ROC) curve analysis validated the assay, with nearly perfect agreement between operators (Cohen's Kappa: 0.927 for both toxins). The assay demonstrated high sensitivity and specificity, providing results within 2 hours, a time frame acceptable for clinical settings.

Advantages and Clinical Implications

401 The developed immunoassay addresses a critical gap in CDI diagnostics by offering a blood-
402 based alternative to stool testing. This approach not only aligns with patient preferences but
403 also provides quantitative insights into host immune responses, aiding in clinical decision-
404 making. For instance, the observed prevalence of Toxin A over Toxin B in this study
405 suggested that Toxin A plays a more significant role in CDI pathogenesis. The finding has
406 implications for treatment strategies, as therapies such as monoclonal antibodies (e.g.
407 bezlotoxumab) and fecal microbiota transplantation (FMT) may yield variable efficacy
408 depending on the toxin profile (Kuehne et al. 2011; Bagdasarian et al. 2015).

409 Moreover, the detection of anti-toxin antibodies offers potential for monitoring immunity
410 against CDI, particularly in populations frequently exposed to *C. difficile*, such as healthcare
411 workers or patients with recurrent infections. Although the presence of antibodies does not
412 guarantee complete immunity, it provides valuable information for risk stratification and
413 vaccine development.

414 **Limitations and Future Directions**

415 Despite its promise, the developed immunoassay has limitations that warrant further
416 investigation. First of all, the current study focused solely on IgG detection. Incorporating
417 IgM analysis could enhance the assay's ability to differentiate between acute and past
418 infections, providing a more comprehensive diagnostic tool. Secondly, the assay was
419 developed and validated using samples collected from central India. Given the regional
420 variability in gut microbiota and *C. difficile* strains, additional studies in diverse geographical
421 settings are necessary to generalize these findings. Additionally, the assay's reliance on Toxin
422 A and B detection may overlook other virulence factors or non-toxigenic strains of *C.*
423 *difficile*.

424 Future research should aim to expand the biomarker panel to capture a broader spectrum of
425 pathogenic and commensal strains. Even though the assay offers high sensitivity and
426 specificity, its performance must be compared directly with molecular techniques like
427 NAATs and cytotoxicity assays in larger, multicenter studies to establish its clinical utility.

428 **CONCLUSION:**

429 The peptide-based immunoassay developed in the present study represents a significant
430 advancement in the diagnosis of *C. difficile* infection, particularly in settings where stool
431 sample testing is infeasible or culturally unacceptable. By enabling rapid, sensitive, and
432 patient- friendly detection of anti-toxin antibodies, this approach holds promise for improving
433 CDI management and reducing transmission. However, further refinement and validation are
434 required to establish its role as a standalone diagnostic tool. Insights from this study also
435 highlight the complex interplay between bacterial virulence factors, host immune responses,
436 and clinical outcomes, paving the way for personalized therapeutic interventions.

437

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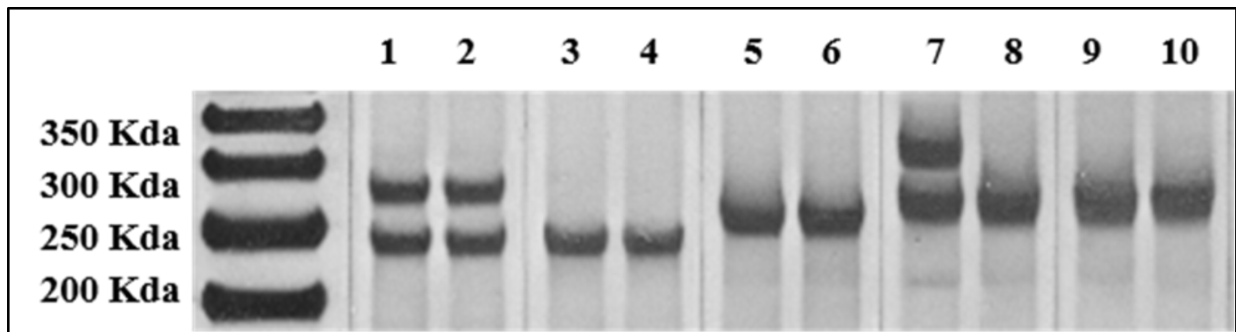

Figure 1: The figure shows the presence of upregulated bands on a 10% SDS-PAGE gel matrix extracted from 10 randomly selected whole cell lysates isolated from the microbial culture of CDI positive patients. A 350 KDa protein marker was run. A 1mg/ml concentration of whole cell lysate extracted from 10 different patients was loaded in Lane 1 to Lane 10. Lane no. 1,2 & 7 appeared to show presence of 2 protein bands at ~250KDa & ~300KDa. However, Lane no. 3,4,5,6,8,9 & 10 show a prominent band at ~300KDa.

TOXIN A Predicted Peptides

Predicted peptides:					
Sr No	No.	Start	End	Peptide	Length
1	2	79	85	LKEVILI	7
2	15	431	437	HDSLFNS	7
3	18	494	500	ASDLIEF	7
4	21	594	600	EATCNLF	7
5	37	920	926	FSKYSEH	7
6	47	1166	1172	LGTCNIL	7
7	55	1480	1486	KSIIHYK	7
8	67	1839	1845	SLFYFDP	7
9	75	2186	2192	QAILYQN	7
10	77	2244	2250	YFSYDGI	7
11	81	2521	2527	QIGVFKG	7

>WP_021377821.1 glycosylating toxin TcdA [Clostridiodes difficile]
MSLISKEELIKLAYSIRPRENEYKTILTNLDEYNKLTTNNNENKYLQLKKLNESIDVFMNKYKTSSRNRA
LSNLKKDI**LKEVILI**KNSNTSPVEKNLHFVWIGGEVSDIALEYIKQWADINAEYNIKLWYDSEAFLVNTL
KKAIVESSTTEALQLLEEEIQNPQFDNMKFYKKRMEFIYDRQKRFINYYKSQINKPTVPTIDDIIKSHLV
SEYNRDETVLESYRTNSLRKINSNHGIDIRANSLFTEQELLNIYSQELLNRGNLAAASDIVRLLALKNFG
GVYLDVDMLPGIHSDLFKTISRPSSIGLDRWEMIKLEAIMKYKKYINNYTSENFDKLDQQLKDNFKLIIE
SKSEKSEIFSKLENLNVSDLEIKIAFALGSVINQALISKQGSYLTNLVIEQVKNRYQFLNQHLNPAIESD
NNFTDTTKIF**HDSLFNS**ATAENSMFLTKIAPYLQVGFMPEARSTISLSGPGAYASAYYDFINLQENTIEK
TLK**ASDLIEF**KFPENNLSQLTEQEINSLWSFDQASAKYQFEKYVRDYTGGSLSEDNGVDFNKNTALDKNY
LLNNKIPSNNVEEAGSKNYVHYIIQLQGDDISY**EATCNLF**SKNPKNSIIIQRNMNESAKSYFLSDDGESI
LELNKYRIPERLKNKEKVKVTFIGHGKDEFNTSEFARLSVDSLSNEISSFLDTIKLDISPKNVEVNLLGC
NMFSYDFNVEETYPGKLLLSIMDKITSTLPDVNKNSITIGANQYEVRINSEGRKELLAHSGKWINKEEAI
MSDLSSKEYIFFDSIDNKLKAKSKNIPGLASISEDIKTLLLDASVSPDTKFILNNLKLNIESSIGDYIYY
EKLEPVKNIIHNSIDDLIDEFNLLENVSDELYELKKLNNLDEKYLISFEDISKNNSTYSVRFINKSNGES
VYVETEKEI**FSKYSEH**ITKEISTIKNSIITDVNGNLLDNIQLDHTSQVNTLNAAFFIQSLIDYSSNKDVL
NDLSTSVKVQLYAQLFSTGLNTIYDSIQLVNLISNAVNDTINVLPTITEGIPIVSTILDGINLGAAIKEL
LDEHDPLLKKELEAKVGVLAINMSLSIAATVASIVGIGAEVTIFLLPIAGISAGIPSLVNNELILHDKAT
SVVNYFNHLSESKKYGPLKTEDDKILVPIDDLVISEIDFNNNSIK**LGTCNIL**AMEGGSGHTVTGNIDHFF
SSPSISSHIPSLSIYSAIGIETENLDFSKKIMMLPNAPSRVFWWETGAVPGLRSLENDGTRLLDSIRDLY
PGKFYWRFYAFFDYAITTLKPVYEDTNIKIKLDKDTRNFIMPTITTNEIRNKLSYSFDGAGGTYSLLLSS
YPISTNINLSKDDLWIFNIDNEVREISIENGTIKKGKLIKDVLSKIDINKNKLIIGNQTIDFSGDIDNKD
RYIFLTCELDDKISLIIEINLVAKSYSLLLSGDKNYLISNLSNTIEKINTLGLDSKNIAYNYTDESNNKY
FGAISKTSQ**KSIIHYK**KDSKNILEFYNDSTLEFNSKDFIAEDINVFMKDDINTITGKYYVDNNTDKSIDF
SISLVSKNQVKVNGLYLNESVYSSYLDFVKNSDGHHNTSNFMNLFLDNISFWKLFGFENINFVIDKYFTL
VGKTNLGYVEFICDNNKNIDIYFGEWKTSSSKSTIFSGNGRNVVVEPIYNPDTGEDISTSLDFSYEPLYG
IDRYINKVLIAPDLYTSLININTNYYSNEYYPEIIVLNPNTFHKKVNINLDSSSFEYKWSTEGSDFILVR
YLEESNKKILQKIRIKGILSNTQSFNKMSIDFKDIKKLSLGYIMSNFKSFNSENELDRDHLGFKIIDNKT
YYYDEDSKLVKGLININN**SLFYFDP**IEFNLVTGWQTINGKKYYFDINTGAALTSYKIINGKHFYFNNDGV
MQLGVFKGPDGFEYFAPANTQNNNIEGQAIVYQSKFLTLNGKKYYFDNDSKAVTGWRIINNEKYYFNPNN
AIAAVGLQVIDNNKYYFNPDTAIISKGWQTVNGSRYYFDTDTAIAFNGYKTIDGKHFYFDSDCVVKIGVF
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WQTIDGKKYYFNTNTAEAATGWQTIDGKKYYFNTNTAIASTGYTIINGKHFYFNTDGIM**QIGVFKG**PNGF
EYFAPANTDANNIEG**QAILYQN**EFLTLNGKKYYFGSDSKAVTGWRIINNKKYYFNPNNAIAAIHLCTINN
DKY**YFSYDGI**LQNGYITIERNNFYFDANNESKMVTGVFKGPNGFEYFAPANTHNNNIEGQAIVYQNKFLT
LNGKKYYFDNDSKAVTGWQTIDGKKYYFNLNTAEAATGWQTIDGKKYYFNLNTAEAATGWQTIDGKKYYF
NTNTFIASTGYTSINGKHFYFNTDGIMQIGVFKGPNGFEYFAPANTHNNNIEGQAILYQNKFLTLNGKKY
YFGSDSKAVTGLRTIDGKKYYFNTNTAVAVTGWQTINGKKYYFNTNTSIASTGYTIISGKHFYFNTDGIM
QIGVFKGPDGFEYFAPANTDANNIEGQAIRYQNRFLYLHDNIYYFGNNSKAATGWVTIDGNRYYFEPNTA
MGANGYKTIDNKNFYFRNGLPQIGVFKGSNGFEYFAPANTDANNIEGQAIRYQNRFLHLLGKIYYFGNNS
KAVTGWQTINGKVYYFMPDTAMAAAGGLFEIDGVIYFFGVDGVKAPGIYG

Figure 2: The figure illustrates the entire sequence coverage for *C. difficile* Toxin A using the BLAST analysis. The highlighted segments are the selected peptides from the entire protein. A total of 11 peptides were selected, which showed no similarity with any other species and were novel.

TOXIN B Predicted Peptides

Predicted peptides:					
Sr No.	No.	Start	End	Peptide	Length
1	35	1066	1072	IMAVNLT	7
2	43	1218	1224	DLMVLPN	7
3	47	1340	1346	ESDVWII	7
4	60	1632	1638	QPYFIKF	7
5	69	2073	2079	FTAVVGW	7
6	72	2156	2162	IVQIGVF	7
7	73	2168	2174	YKYFAPA	7

>WP_009895693.1 glycosylating toxin TcdB [Clostridiodes difficile]
MSLVNRKQLEKMANVRFRTQEDEYVAILDALEEYHNMSENTVVEKYLKLKDINSLTDIYIDTYKKSGRNK
ALKKFKEYLVTEVLELKNNNLTPVEKNLHFVWIGGQINDTAINYINQWKDVNSDYNVNVFYDSNAFLINT
LKKTVVESAINDTLESFRENLNDPRFDYNKFFRKRMEIIYDKQKNFINYYKAQREENPELIIDDIVKTYL
SNEYSKEIDELNTYIEESLNKITQNSGNDVRNFEEFKNGESFNLYEQELVERWNLAAASDILRISALKEI
GGMYLDVDMLPGIQPDLFESIEKPSSVTVDFWEMTKLEAIMKYKEYIPEYTSEHFDMLDEEVQSSFESVL
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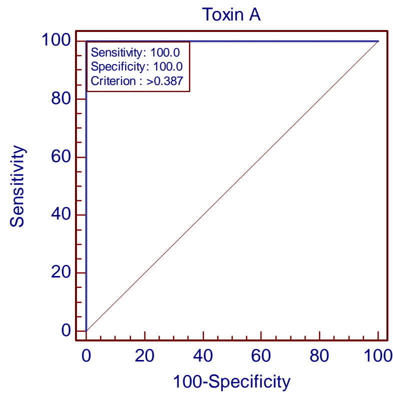
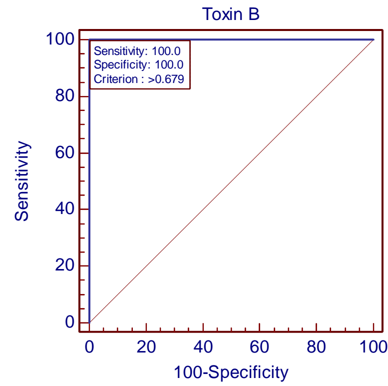
DNDFNTTTNTFIDSIMAEANADNGRFMMELGKYLRVGFFPDVKTTINLSGPEAYAAAYQDLLMFKEGSMN
IHLIEADLRNFEISKTNISQSTEQEMASLWSFDDARAKAQFEEYKRNYFEGSLGEDDNLDFSQNIVVDKE
YLLEKISSLARSSERGYIHYIVQLQGDKISYEAACNLFAKTPYDSVLFQKNIEDSEIAYYYNPGDGEIQE
IDKYKIPSIISDRPKIKLTFIGHGKDEFNTDIFAGFDVDSLSTEIEAAIDLAKEDISPKSIEINLLGCNM
FSYSINVEETYPGKLLLKVKDKISELMPSISQDSIIVSANQYEVRINSEGRRELLDHSGEWINKEESIIK
DISSKEYISFNPKENKITVKSKNLPELSTLLQEIRNNSNSSDIELEEKVMLTECEINVISNIDTQIVEER
IEEAKNLTSDSINYIKDEFKLIESISDALCDLKQQNELEDSHFISFEDISETDEGFSIRFINKETGESIF
VETEKTIFSEYANHITEEISKIKGTIFDTVNGKLVKKVNLDTTHEVNTLNAAFFIQSLIEYNSSKESLSN
LSVAMKVQVYAQLFSTGLNTITDAAKVVELVSTALDETIDLLPTLSEGLPIIATIIDGVSLGAAIKELSE
TSDPLLRQEIEAKIG**IMAVNLT**TATTAIITSSLGVASGFSILLVPLAGISAGIPSLVNNELVLRDKATKV
VDYFKHVSLVETEGVFTLLDDKIMMPQDDLVISEIDFNNNSIVLGKCEIWRMEGGSGHTVTDDIDHFFSA
PSITYREPHLSIYDVLEVQKEELDLSK**DLMVLPN**APNRVFAWETGWTPGLRSLENDGTKLLDRIRDNYEG
EFYWRYFAFIADALITTLKPRYEDTNIRINLDSNTRSFIVPIITTEYIREKLSYSFYGSGGTYALSLSQY
NMGINIELS**ESDVWII**DVDNVVRDVTIESDKIKKGDLIEGILSTLSIEENKIILNSHEINFSGEVNGSNG
FVSLTFSILEGINAIIEVDLLSKSYKLLISGELKILMLNSNHIQQKIDYIGFNSELQKNIPYSFVDSEGK
ENGFINGSTKEGLFVSELPDVVLISKVYMDDSKPSFGYYSNNLKDVKVITKDNVNILTGYYLKDDIKISL
SLTLQDEKTIKLNSVHLDESGVAEILKFMNRKGNTNTSDSLMSFLESMNIKSIFVNFLQSNIKFILDANF
IISGTTSIGQFEFICDENDNI**QPYFIKF**NTLETNYTLYVGNRQNMIVEPNYDLDDSGDISSTVINFSQKY
LYGIDSCVNKVVISPNIYTDEINITPVYETNNTYPEVIVLDANYINEKINVNINDLSIRYVWSNDGNDFI
LMSTSEENKVSQVKIRFVNVFKDKTLANKLSFNFSDKQDVPVSEIILSFTPSYYEDGLIGYDLGLVSLYN
EKFYINNFGMMVSGLIYINDSLYYFKPPVNNLITGFVTVGDDKYYFNPINGGAASIGETIIDDKNYYFNQ
SGVLQTGVFSTEDGFKYFAPANTLDENLEGEAIDFTGKLIIDENIYYFDDNYRGAVEWKELDGEMHYFSP
ETGKAFKGLNQIGDYKYYFNSDGVMQKGFVSINDNKHYFDDSGVMKVGYTEIDGKHFYFAENGEMQIGVF
NTEDGFKYFAHHNEDLGNEEGEEISYSGILNFNNKIYYFDDS**FTAVVGW**KDLEDGSKYYFDEDTAEAYIG
LSLINDGQYYFNDDGIMQVGFVTINDKVFYFSDSGIIESGVQNIDDNYFYIDDNG**IVQIGVF**DTSDGYKY
FAPANTVNDNIYGQAVEYSGLVRVGEDVYYFGETYTIETGWIYDMENESDKYYFNPETKKACKGINLIDD
IKYYFDEKGIMRTGLIS**YKYFAPA**FENNNYYFNENGEMQFGYINIEDKMFYFGEDGVMQIGVFNTPDGFKY
FAHQNTLDENFEGESINYTGWLDLDEKRYYFTDEYIAATGSVIIDGEEYYFDPDTAQLVISE

Figure 3: The figure illustrates the entire sequence coverage for *C. difficile* Toxin B using the BLAST analysis. The highlighted segments are the selected peptides from the entire protein. A total of 07 peptides were selected, which showed no similarity with any other species and were novel.

A



B



Protein	Peptide	AUC	Criterion	Sensitivity	95% CI	Specificity	95% CI
Toxin A	**SLFYFDP**	1	>0.387 *	100.00	83.0 - 100.0	100.00	69.0 - 100.0
Toxin B	**IVQIGVF**	1	>0.679 *	100.00	83.0 - 100.0	100.00	69.0 - 100.0

Figure 4: A) Receiver Operating Characteristic (ROC) curve analysis of the developed peptide ELISA protocol for detecting antibodies against *Clostridium difficile* toxins A in serum samples. The analysis was conducted at an optimized sample dilution of 1:100, with peptide concentrations of 10 ng for toxin A, utilizing *C. difficile* positive samples (n=20) and negative samples (n=10). (B) Receiver Operating Characteristic (ROC) curve analysis of the developed peptide ELISA protocol for detecting antibodies against *Clostridium difficile* toxins B in serum samples. The analysis was conducted at an optimized sample dilution of 1:100, with peptide concentrations of 05 ng for toxin B, utilizing *C. difficile* positive samples (n=20) and negative samples (n=10).

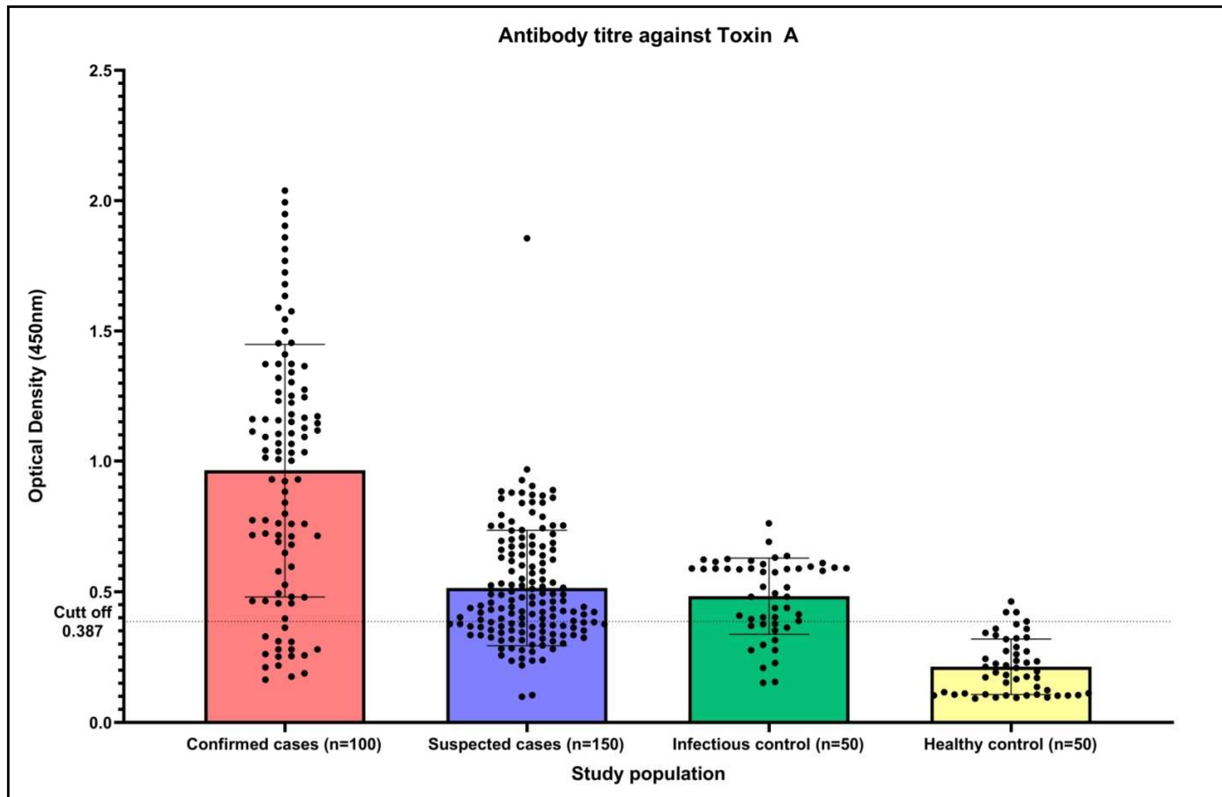


Figure 5: The figure demonstrates the presence of Toxin A-specific antibodies across different study populations. The red bar represents confirmed cases with significantly elevated antibody levels, while the blue bar shows suspected cases with variable antibody responses. The green bar indicates infectious controls with moderate antibody levels, and the yellow bar represents healthy controls with minimal antibody presence. The dotted line marks the positivity threshold (cut-off at OD = 0.387), highlighting the distinction between positive and negative responses.

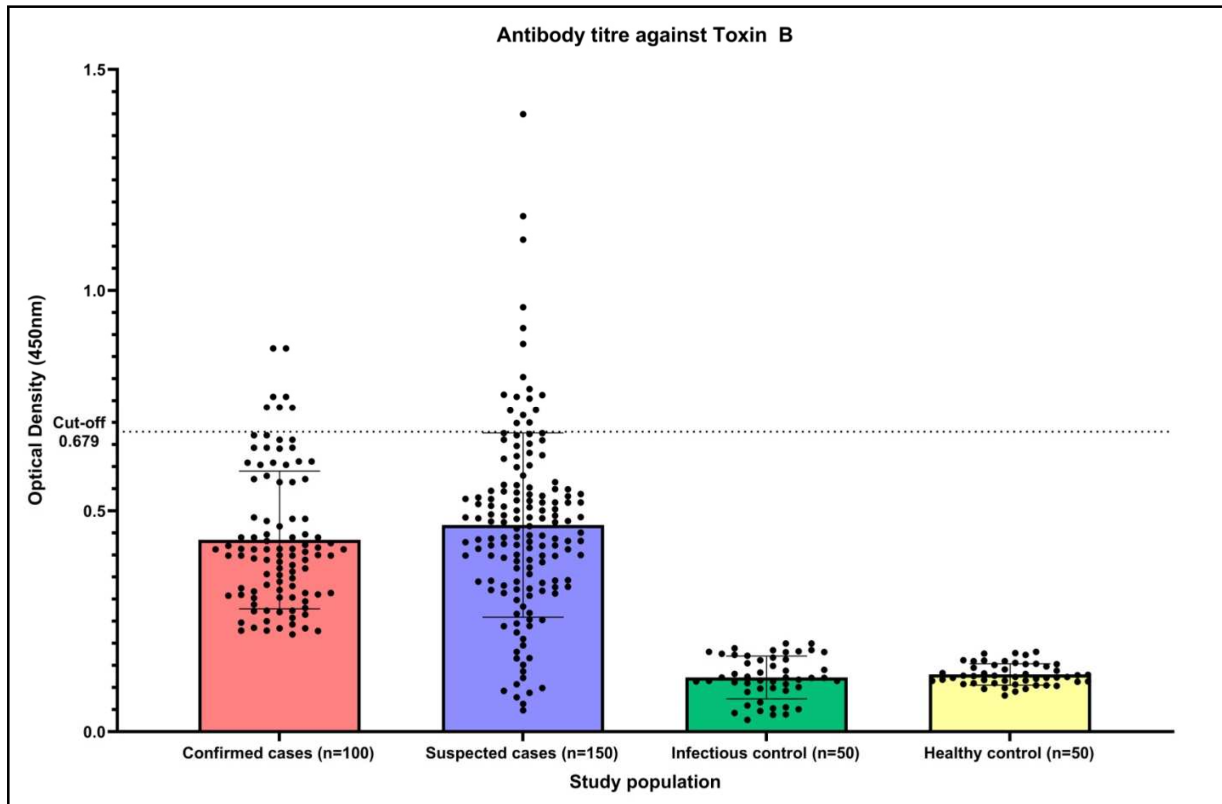


Figure 6: The figure demonstrates the presence of Toxin B-specific antibodies across different study populations. The red bar represents confirmed cases with significantly elevated antibody levels, while the blue bar shows suspected cases with variable antibody responses. The green bar indicates infectious controls with moderate antibody levels, and the yellow bar represents healthy controls with minimal antibody presence. The dotted line marks the positivity threshold (cut-off at OD = 0.679), highlighting the distinction between positive and negative responses.

Samples	Dilution	Coating Concentration			
		5ng	10ng	20ng	40ng
C. diff Toxin A peptide "SLFYFDP"					
Positive Control (n=1)	1:100	0.999	**1.221**	0.931	1.07
	1:200	0.979	0.972	0.984	0.986
	1:400	0.745	0.85	0.974	0.813
	1:800	0.569	0.653	0.711	0.668
Negative Control (n=1)	1:100	0.120	**0.115**	0.122	0.130
	1:200	0.102	0.105	0.110	0.108
	1:400	0.088	0.091	0.093	0.095
	1:800	0.071	0.074	0.076	0.078
C. diff Toxin B peptide "IVQIGVF"					
Positive Control (n=1)	1:100	**1.324**	1.115	1.126	1.094
	1:200	0.465	0.415	0.441	0.457
	1:400	0.27	0.237	0.279	0.286
	1:800	0.648	0.495	0.492	0.672
Negative Control (n=1)	1:100	**0.102**	0.098	0.107	0.113
	1:200	0.088	0.085	0.091	0.089
	1:400	0.076	0.073	0.078	0.080
	1:800	0.065	0.068	0.071	0.067

Table 01: Optimization of the peptide ELISA assay protocol for detecting *Clostridium difficile* in serum samples. Optical density (OD) values indicate the immunoreactivity between peptides targeting *C. difficile* toxins A and B, tested against positive and negative control samples. The checkerboard method was employed to determine optimal assay conditions.

		Operator 1		Agreements n (%)	Kappa value	95% CI
		Positive	Negative			
Toxin A Antibody ELISA						
Operator 2	**Positive**	19	0	29 (97%)	0.927	0.786- 1.000
	Negative	1	10			
Toxin B Antibody ELISA						
Operator 2	**Positive**	19	1	28 (93%)	0.850	0.649-1.000
	Negative	1	9			

Table 02: Assessment of reproducibility of the developed Peptide-Based ELISA Assay for Detecting *C. difficile* Infection by Two Independent Operators Using Kappa Statistics.

Statistic	Value	95% CI
Sensitivity	95.24%	76.18% to 99.88%
Specificity	100.00%	66.37% to 100.00%
Negative Likelihood Ratio	0.05	0.01 to 0.32
Disease prevalence (*)	12.00%	-
Positive Predictive Value (*)	100.00%	83.16% to 100.00%
Negative Predictive Value (*)	99.35%	95.79% to 99.90%
Accuracy (*)	99.43%	87.37% to 100.00%

Table No. 03: The table presents the statistical data achieved after testing the gathered data through Medcal.