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A Diagnostic Algorithm for the Detection of *Clostridium difficile*-Associated Diarrhea

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Background: *Clostridium difficile* is a common cause of hospital-acquired diarrhea, which is usually associated with previous antibiotic use. The clinical manifestations of *C. difficile* infection (CDI) may range from mild diarrhea to fulminant colitis. *Clostridium difficile* should be considered in diarrhea cases with a history of antibiotic use within the last 8 weeks (community-associated CDI) or with a hospital stay of at least 3 days, regardless of the duration of antibiotic use (hospital-acquired CDI).
Aims: This study investigated the frequency of CDI in diarrheic patients and evaluated the efficacy of the triple diagnostic algorithm that is proposed here for C. difficile detection.
Study Design: Cross-sectional study.
Methods: In this study, we compared three methods currently employed for *C. difficile* detection using 95 patient stool samples: an enzyme immunoassay (EIA) for toxin A/B (C. diff Toxin A+B; Diagnostic Automation Inc.; Calabasas, CA, USA), an EIA for glutamate dehydrogenase (GDH) (*C. DIFF* CHEK-60TM, TechLab Inc.; Blacksburg, VA, USA), and a polymerase chain reaction (PCR)-based assay (GeneXpert® *C. difficile*; Cepheid, Sunnyvale, CA, USA) that detects *C. difficile* toxin genes and conventional methods as well. In this study, 50.5% of the patients were male, 50 patients were outpatients, 32 were from inpatient clinics and 13 patients were from the intensive care unit.
Results: Of the 95 stool samples tested for GDH, 28 were positive. Six samples were positive by PCR, while nine samples were positive for toxin A/B. The hypervirulent strain NAP-1 and binary toxin was not detected. The rate of occurrence of toxigenic *C. difficile* was 5.1% in the samples. Cefaclor, ampicillin-sulbactam, ertapenem, and piperacillin-tazobactam were the most commonly used antibiotics by patients preceding the onset of diarrhea. Among the patients who were hospitalized in an intensive care unit for more than 7 days, 83.3% were positive for CDI by PCR screening. If the PCR test is accepted as the reference: *C. difficile* Toxin A/B ELISA sensitivity and specificity were 67% and 94%, respectively, and GDH sensitivity and specificity were 100% and 75%, respectively.
Conclusion: Tests targeting *C. difficile* toxins are frequently applied for the purpose of diagnosing CDI in a clinical setting. However, changes in the temperature and reductant composition of the feces may affect toxin stability, potentially yielding false-negative test results. Therefore, employment of a GDH EIA, which has high sensitivity, as a screening test for the detection of toxigenic strains, may prevent false-negative results, and its adoption as part of a multistep diagnostic algorithm may increase accuracy in the diagnosis of CDIs.
Keywords: *Clostridium difficile*, antibiotic-associated diarrhea, toxin A/B, glutamate dehydrogenase, PCR assay (GeneXpert®)

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Clostridium difficile colonization can vary in severity from being asymptomatic to causing self-limiting diarrhea or pseudomembranous colitis. *Clostridium difficile* isolates are able to survive on abiotic surfaces in a healthcare setting through endospore formation, leading to difficulties in eradication and increased transmission. Upon increased colonization of the gut, toxigenic strains of *C. difficile* can cause infections. Hospital-acquired *C. difficile* epidemics can spread among patients, who become infected by healthcare professionals or contaminated instruments. In addition to healthcare-associated cases, community-acquired infections have recently increased in number. Toxigenic strains of *C. difficile* produce exotoxin A (TcdA) and exotoxin B (TcdB). A binary toxin (CDT) is frequently observed in hypervirulent *C. difficile* strains associated with the increased severity of *C. difficile* infection (CDI) such as the NAP-1 strain. CDT belongs to a family of binary ADP-ribosylating toxins consisting of two separate toxin components: CDTa, an ADP-ribosyltransferase that modifies actin, and CDTb, which binds to host cells and translocates CDTa into the cytosol. The prevalence of the binary toxin in CDI is 1.6–20.8% (1). While the *C. difficile* carriage rate in healthy adults is 1–3%, hospitalization is an important risk factor that increases the risk of colonization. After hospitalization, the frequency of asymptomatic colonization increases to 20–30%, especially in elderly patients (2-4).

Clostridium difficile diagnostic testing employs different clinical microbiology methods with varying degrees of sensitivity, including molecular methods, chromatographic techniques, and assays for the presence of TcdA/B using culture-cytotoxicity or immunological methods. A final diagnosis of CDI is based on the presence of free toxin in patient stool samples and the detection of associated cytopathic effects in cell culture. However, many of these methods are technically demanding and time-consuming; thus, many laboratories prefer enzyme-based immunological methods that detect glutamate dehydrogenase (GDH) or antigens and toxins. However, the latter approach has much lower sensitivity. Polymerase chain reaction (PCR)-based methods that identify the agent directly from clinical samples have recently been introduced and are currently in use in many laboratories (5).

The most widely used laboratory test is still the identification of toxins A and B via an enzyme-linked immunosorbent assay (ELISA) (6). However, difficulties arise in the standardization of screening methods due to the range and dissimilarity of the diagnostic tests used in laboratories. This study aimed to identify the presence of *C. difficile* in samples from patients with diarrhea using multiple techniques in an effort to suggest a screening algorithm with different approaches and diagnostic tests.

MATERIALS AND METHODS

Study, subjects, and participation

In this cross-sectional study, stool samples from 95 patients admitted to a university hospital were used. Each patient had been prescribed antibiotics within 8 weeks preceding the study and was admitted with a complaint of diarrhea. The study population was comprised of 45 inpatients and 50 outpatients. Ethics committee approval was received for this study from the ethics committee of Afyon Kocatepe University Faculty of Medicine and written informed consent was obtained from patients who participated in this study.

Methods

Based on macroscopic examinations, 95 soft, watery, bloody and/or mucous-containing stool samples were tested for *C. difficile*. Shaped and solid stool samples were not included in the study. For the purpose of direct microscopic examination, specimens were prepared using physiological saline and placed on slides with cover slips. The samples were then evaluated for the presence of erythrocytes, leukocytes, parasitic cysts, and eggs as well as yeast cells and/or pseudohyphae formation. To test for common enteropathogenic bacteria, all samples were plated onto eosin methylene blue agar and Salmonella Shigella (SS) agar and inoculated into selenite F medium. After 4 h, the samples were subcultured on SS agar and incubated at 37°C in an aerobic environment for 24–48 h.

Isolation and detection of *C. difficile*

The culture of *C. difficile* from the stool samples was performed using cycloserine-cefoxitin fructose agar selective solid media. The plate was incubated in Hi gas-pak jar at 37°C for 48 h using BD GasPak EZ Anaerobe container system with indicator or in individual sachets (BD GasPak™ EZ Gas Generating Pouch Systems; New Jersey, USA). On CCFA, circular, yellow, fimbriate colonies that were 4mm in size or larger, of Gram-positive bacilli with subterminal oval spores and a horse stable odor were presumptively identified as *C. difficile*.

Colonies morphologically resembling the organism were tested by latex agglutination with the Oxoid *C. difficile* Test Kit (DR 1107A), UK according to the manufacturer's instructions. Briefly, saline suspension of the suspected colony was mixed with Oxoid *C. difficile* Latex Reagent on the reaction card. Appearance of agglutination was examined for a maximum of two minutes, employing appropriate negative and positive controls.

GDH, Toxin Testing by EIA and PCR

The presence of GDH, a membrane-associated enzyme of *C. difficile*, in the fecal samples was determined using an ELI-

SA (C. DIFF CHEK-60™, TechLab Inc.; Blacksburg, VA). To identify the presence of TcdA and B in the fecal samples, an ELISA kit for toxin A/B (*C. diff* Toxin A+B; Diagnostic Automation Inc., Calabasas, CA) and a *C. difficile* PCR kit (GeneXpert® *C. difficile*; Cepheid, Sunnyvale, CA) were used. Following amplification by real-time PCR, DNA extraction and PCR were performed using a single-use cartridge. This system detected sequences specific to toxin B (*tcdB*), binary toxin (*cdt*), and *tcdC* Δ117. It also allowed for the determination of toxin-producing *C. difficile* and the NAP-1 strain. The PCR test was adopted as a reference assay for tests investigating toxins. Positive and negative control samples were included for each of the parameters tested.

Statistical analysis

The data were analyzed using SPSS 17.0 for Windows (SPSS Inc.; Chicago, IL, USA). A Chi-square test was applied to assess the statistical significance of the data.

RESULTS

Of the samples tested, 50.5% were obtained from male patients while 49.5% were from female patients. In total, 12% of the patients were 2–17 years old, 21% were 18–39 years old, 27.3% were 40–64 years old, and 44.2% were >65 years old.

In total, 52.6%, 33.7%, and 13.7% of the samples were collected from inpatients, outpatients, and intensive care unit patients, respectively. The presence of common enteropathogenic agents such as *Salmonella* sp., *Shigella* sp., *Campylobacter* sp., and *E. coli* O157 could not be determined in these patients using conventional bacterial identification methods. Any patient who tested positive for these pathogens was excluded from the study.

Positive PCR results for GDH were obtained in all of the toxigenic *C. difficile*-positive patients, but the Tcd ELISA yielded negative results in two patients. Similarly, two patients who tested positive for both GDH and toxins in their respective ELISA assays, showed negative results according to the PCR-based method. There were also three patients who tested negative for GDH and had negative PCR results but who showed the presence of toxins according to the ELISA. The proportions of positive results from the GDH test and the toxin screens are shown in Table 1.

In this study, toxigenic *C. difficile* was identified by PCR in 6.8% of the patients. Whereas the toxin ELISA had sensitivity, specificity, positive predictive, and negative predictive values of 67%, 94%, 21.4% and 95.5%, respectively, GDH had 100%, 75%, 21.4%, and 100% values, respectively, compared to the PCR-based method.

Of those patients who tested positive for toxigenic *C. difficile* by PCR, two had previously been treated with piperacillin-tazobactam, one with ertapenem and ampicillin+sulbactam, one had a history of ertapenem and colistin use, one had a history of cefaclor use, and one had been treated with ampicillin+sulbactam (each for 10–14 days). The statistical significance of the effects of antibiotic use on future CDI was investigated using a chi-square test (p=0.582). Demographic data for the toxigenic *C. difficile*-positive patients identified by PCR are given in Table 2.

In our study, hospitalized patients had a higher rate of *C. difficile* colonization in all of the tests we applied. The distribution of positive results by patient history at the time of hospitalization is shown in Table 3. PCR screening of the patient samples did not reveal any NAP-1- or binary toxin-positive strains in this study.

TABLE 1. A comparison of positive rate of GDH and the other tests for *C. difficile* diarrhea

	Toxin ELISA		PCR		Culture for *C. difficile*		
	Positive	Negative	Positive	Negative	Positive	Negative	Total
GDH Positive	6	22	6	22	10	18	28
GDH Negative	3	64	0	67	0	67	67
Total	9	86	6	89	10	85	95

GDH: glutamate dehydrogenase; PCR: polymerase chain reaction

TABLE 2. The epidemiological data of toxigenic *C. difficile* PCR-positive patients

Sex	Age	Comorbidities	Length of stay in hospital	Antibiotics used	Duration of antibiotic use	*C. difficile* toxin ELISA	GDH
Women	30	Thoracic intramedullary mass + pregnancy	30 days	Ampicillin/Sulbactam	8 days	+	+
Women	29	bloodstream infection	21	Sefaclor	10 days	-	+
Man	82	Epilepsy + Pneumonia + Acute Renal Failure	37 days	Ampicillin/Sulbactam Ertapenem	14 days 10 days	+	+
Man	83	Acute Renal Failure + Cerebro vascular stroke	58 days	Ertapenem Colistine	14 days 3 days	+	+
Women	51	Gastrointestinal bleeding	10 days	Piperacillin/tazobactam	10 days	+	+
Man	73	Brucellosis	7 days	Piperacillin/tazobactam	7 days	+	+

GDH: glutamate dehydrogenase; PCR: polymerase chain reaction

TABLE 3. The distribution of positive rate between inpatient and community associated *C. difficile* diarrhea

	GDH %	PCR %	Toxin ELISA %
Inpatient (n: 50)	46.7	11.1	17.8
Community associated *C. difficile* diarrhea (n: 45)	14	2	2
Total (n: 95)	29.5	6.3	9.5

GDH: glutamate dehydrogenase; PCR: polymerase chain reaction

DISCUSSION

The current gold standard approach for the diagnosis of CDIs is cell culture, cytotoxicity tests, and neutralization tests (7). These methods require experienced personnel and equipment, are often time-consuming, difficult to standardize, and have a high incidence of false-positives due to the presence of proteases in stool. As such, new screening methods are required (8). The algorithm best suited for a rapid and accurate diagnosis is controversial, and many laboratories use tests that look for toxins. Tests such as ELISAs and the card test, which are used to search for toxins A and B, have low sensitivity, although they are rapid and inexpensive. However, these toxins can also be inactivated by changes in temperature and fecal proteases, resulting in a potential increase in false-negatives (9,10).

In 2005, our previously study (11) investigated the presence of toxigenic *C. difficile* in stool samples collected from 91 patients, of which 45 were outpatients and 46 were inpatients, as well as from hospital staff (7 intensive care and 20 food service personnel). In that study, culture and latex agglutination tests with specific antisera were performed and the presence of either toxin A or B was investigated using an ELISA or the latex agglutination assay. Based on their ELISA results, 15.5% of the outpatients and 17.1% of the inpatients were positive for CDI, while no hospital personnel tested positive. When the toxin ELISA results were compared to those of the culture-latex agglutination test, the toxin A latex method had a sensitivity of 30.7% compared to 100% for the toxin ELISA. In that study, the number of cases with *C. difficile* toxin present was comparable to that in other studies of hospital- and community-acquired cases. Hospitalization is an important risk factor for CDI. In present study, toxin ELISA positivity was higher in the hospitalized patient group.

Another important risk factor for CDI is advanced age (>65 years) (9). In our study, 66.7% of the toxigenic *C. difficile*-positive patients were older than 55 years of age. Consistently in the literature, the most frequently used antibiotics in patients with CDI are beta-lactams. In our study, this was not the case: the most frequently used antibiotics in diarrhea cases were from different antibiotic families (p=0.582).

Since the sensitivity of the ELISA and card test methods used to investigate the presence of toxins A and B is low, a GDH ELISA is often involved in diagnostic algorithms. In addition to the increased sensitivity of the assay, GDH antigens appear both in toxigenic and non-toxigenic strains, allowing for the detection of both variants in patient samples. Upon the identification of antigen in stool samples using a GDH ELISA, the presence of *C. difficile* should be confirmed using an alternate method because it has low specificity despite its high sensitivity (12).

The Society for Healthcare Epidemiology of America and the Infectious Diseases Society of America recommend testing amorphous stool samples from suspected *C. difficile* cases only for patients suffering from diarrhea, excluding cases of ileus. Testing fecal samples from asymptomatic patients is not recommended. It is believed that although fecal culture is important and sensitive for the purpose of epidemiological studies, it is not suitable for use in clinical practice because it is time-consuming and the identification of toxigenic strains requires specialized experience and equipment. Toxin ELISAs are rapid but have low sensitivity; therefore, they are suboptimal diagnostic tools. Latex agglutination kits identifying GDH have a sensitivity of 58–68% and specificity of 94–98%, while ELISA-based kits have 85–95% sensitivity and 89–99% specificity. The use of GDH in two-step diagnostic algorithms together with confirmatory tests is an adoptable strategy. It is recommended to interpret the result as *C. difficile*-negative if GDH assays are negative, and to proceed with a confirmation test if GDH is detected. Since PCR-based tests are fast, sensitive, and specific, they can be adopted as a new diagnostic approach, although additional data are required for these tests before they are put into routine clinical use (13).

In a study of 114 stool samples performed by LaSala et al. (14), 24 (12%) were positive for CDI using a GDH test, a PCR-based test, and a toxin-based ELISA, 22 (19%) were positive using GDH- and PCR-based tests, 7 (33%) were positive using a GDH-based test, and 1 was positive according to a GDH test and toxin-based ELISA. The authors concluded that using only a stand-alone toxin test may give false-negative results. Fenner et al. (15) made use of a two-step *C. difficile* diagnostic algorithm for 1468 stool samples and used PCR to resolve any inconsistencies. They reported 187 (12.7%) GDH-positive samples and 69 (36.9%) toxin A/B positive-samples. Of the GDH-negative patients, ten had toxin positivity, and a slight bacterial burden was identified in five patients using PCR. In total, 52.9% of the GDH- and toxin-positive patients had a positive toxigenic culture, although the culture sensitivity was reported to be low.

Crobach et al. (6) investigated 18 different diagnostic methods from 1991–2008; 13 different ELISAs in 34 toxin A/B studies, 4 GDH methods in 14 ELISA GDH studies, and 1 PCR method in 4 PCR studies. In 85% of the studies, the gold

standard cytotoxicity test served as the reference test. Using a two-step algorithm, a 5% prevalence of CDI was calculated for 10000 patients. Among the patients, 9541 were truly negative, 432 were truly positive, 68 had a false-negative result, and 49 had a false-positive result. Application of the high-sensitivity GDH method as the initial screening test with a high-specificity toxin test as confirmation, and screening for the presence of *C. difficile* in diarrhea cases with no presence of other enteropathogenic agents is recommended. Williamson et al. (16) analyzed stool samples from 7106 patients, allocating 3100 of the samples to the GDH assay group and 4006 samples to a two-step procedure using PCR together with a GDH assay. According to their results, 4.7% of the samples showed the presence of *C. difficile* based on testing with only the GDH assay. This number increased to 9.9% when the two-step algorithm was used. According to our results, utilization of a GDH test with high sensitivity as a rapid screening test appears to be an important step in preventing false-negatives. Babady et al. (17) compared PCR and GDH culture cytotoxicity using 560 stool samples and found that the sensitivity of GDH culture cytotoxicity and PCR combined (100%) was higher than that of GDH-positive culture cytotoxicity assays alone (57%).

The detection of toxins via latex agglutination tests may produce false-positive results due to cross-reactions between toxigenic and/or nontoxigenic strains of *C. difficile* and *Peptostreptococcus anaerobius* and *Bacteroides asaccharolyticus* (18). The lower sensitivity and specificity of the toxin-based ELISA and its inconsistencies with the GDH method observed in this study point to PCR as a beneficial test for the confirmation of GDH-positive patients. GDH-positive, toxin-negative, PCR-positive results should be correlated with the clinical symptoms of the patient. Other causes of diarrhea should be ruled out before seeking a *C. difficile* diagnosis. However, positive PCR results cannot distinguish between asymptomatic colonization and a true CDI. A potential triple diagnostic algorithm is illustrated in Figure 1. Table 4 provides a summary of the *C. difficile* test results.

An additional study investigated current *C. difficile* diagnostic algorithms and determined the number of positive tests in 170 hospitals in the United Kingdom between 2009 and 2010. The study determined that the majority of hospital laboratories (70%) use toxin A/B tests (6% use the card test and 64% use ELISAs), while 3.6% of hospitals use cell cytotoxicity neutralization tests. Only one laboratory used PCR as a screening method. Moreover, 19% of the laboratories were reported to have adopted a two-step algorithm, only performing the second step if the results of the initial screen were positive. An additional 5% of laboratories used a triple algorithm comprised of a GDH test, toxin ELISA, and PCR, while 5% were using GDH and toxin A/B combination kits (19).

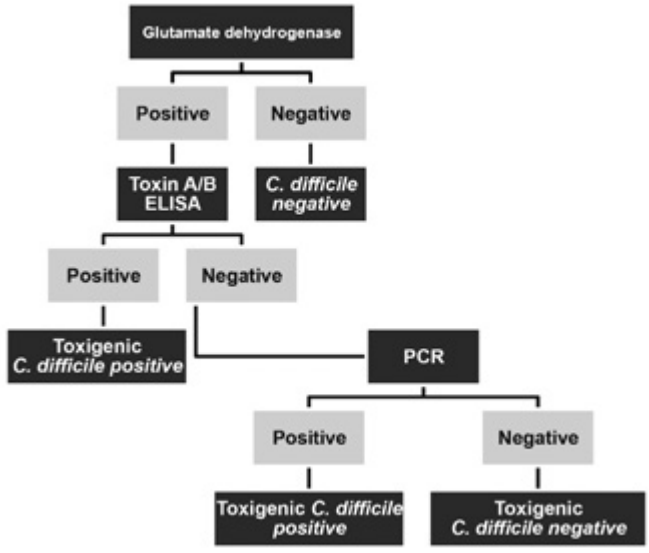

FIG. 1. Diagnostic algorithm of *Clostridium difficile*

TABLE 4. Interpretation of *C. difficile* tests results

GDH/NAAT	Toxin ELISA	Comments
Positive	Positive	*C. difficile* Positive
Positive	Negative	Potential carriers of *C. difficile*
Negative	Negative	There is no *C. difficile* (Probably other pathogens)

GDH: glutamate dehydrogenase; NAAT: nucleic acid amplification testing

C. difficile infection is increasing in prevalence and severity. Major risk factors include previous antibiotic use, advanced age, stays in a hospital or healthcare setting, and (possibly) the use of proton pump inhibitors. Most cases are related to antibiotic use, but sporadic cases can occur in otherwise healthy individuals with no known risk factors (20). Korac et al. (21) demonstrated that fluoroquinolones and third-generation cephalosporins were used most often among CDI patients who had been treated previously with antibiotics. Until recently, fluoroquinolones were widely used for surgical prophylaxis in Serbia, which may explain why more than 50% of the patients in our study had received them before CDI occurred. In this study, several CDI patients identified by PCR had previously been treated with antibiotics: two with piperacillin-tazobactam, one with ertapenem and ampicillin+sulbactam, one with ertapenem and colistin, one with cefaclor, and one with ampicillin+sulbactam, each for a duration of 10–14 days (Table 2).

The risk factors for *C. difficile*-associated diarrhea are well-known and clinicians are recommended to evaluate patients according to these risk factors. Problems in making an accurate diagnosis may occur despite the availability of rapid and

reliable tests due to differences in the specificity of the various screens available (22).

In making a CDI diagnosis, stool samples may have blood present if severe colitis has occurred, but grossly bloody stools are unusual. Additionally, fecal leukocytes are present in about half of cases. Currently used stool assays for *C. difficile*, from the most to least sensitive are: stool culture, GDH enzyme immunoassays (EIAs), RT-PCR, toxin A/B EIAs, and latex agglutination assays (23,24).

The culture of *C. difficile* from stool is the most sensitive test, but it is labor-intensive; results may be delayed by 48–96 h. This method also often yields false-positives due to the presence of non-toxigenic strains. However, stool culture is important for epidemiological studies such as ribotyping and examinations of antibiotic susceptibility (25).

The GDH EIA is both a sensitive and specific test for CDI (85–100% and 87–98%, respectively). Latex agglutination assays are an alternative method for detecting *C. difficile* GDH; however, these methods are significantly less sensitive (48–59%) and less specific (95–96%) than EIAs.

In our study cohort, sensitivity, specificity, positive predictive value, and negative predictive value were 67%, 94%, 21.4% and 95.5% for toxin EIA and 100%, 75%, 21.4%, and 100% for GDH, respectively. The PPV of 21.4% for GDH alone confirmed the need to pursue additional testing of GDH-positive/toxin or PCR negative specimens to resolve these specimens as positive or negative for *C. Difficile* toxin. Zheng et al. (26) reported that the Techlab C. diff Chek-60 GDH assay had good sensitivity compared to CYT testing of 92%, but it had a low specificity of 89.1% and poor positive predictive value (PPV) of 57.7%.

RT-PCR, which is used to detect *C. difficile* toxin genes, could be considered an alternative gold standard test owing to its specificity, but it is a comparatively expensive assay to perform. Currently, cell culture toxin neutralization tests are the gold standard for identifying CDIs, but they are less sensitive than PCR or toxigenic culture in patients presenting with diarrhea. Costs, sensitivity and specificity of molecular diagnostic tests vary. The costs of material and labor for *C. difficile* Toxin A B, cytotoxin Assay, GDH, GDH/toxin 2 step algorithm and nucleic acid amplification testing (NAAT) were 6, 25, 5-10, 8-14, and 25-48 USD, respectively (25). Similarly, toxin EIAs are among the most commonly used screening methods in laboratories, in part due to their high specificity. However, the sensitivity of these assays is considerably lower (70–80%) than that of many of the other methods described here.

Many laboratories still use stand-alone toxin tests to diagnose CDI, and there is no standardization between healthcare facilities in terms of accepted diagnostic algorithms. In this study, we investigated three diagnostic tests to determine their respective sensitivities and specificities and proposed a new diagnostic algorithm for use in clinical settings. When the GDH EIA method is administered as a screening test with a toxin ELISA or card test as the confirmatory secondary screen, it increases the sensitivity and allows for the evaluation of possible false-positive results (27). The investigation of toxigenic *C. difficile* using PCR offers high sensitivity and specificity, but it is perhaps too expensive to be introduced widely into clinics. The simultaneous identification of 027/NAP-1 strains of *C. difficile* is an important advantage for epidemiological studies, particularly in countries where this hypervirulent strain is common. *Clostridium difficile* ribotype 176 may be misinterpreted as ribotype 027 by GeneXpert® *C. difficile* (Cepheid). Therefore, further molecular analysis such as ribotyping based on capillary electrophoresis is needed to better differentiate between *C. difficile* ribotypes 027 and 176 such that appropriate steps can be taken at the local and national levels to prevent outbreaks (28). In many places, *C. difficile* surveillance is not yet sufficient; thus, the number of outbreaks is increasing. Adopting a triple diagnostic algorithm and performing GDH tests and ELISAs with the confirmation of inconsistent results by PCR would enable clinicians to make a more accurate diagnosis.

Ethics Committee Approval: Ethics committee approval was received for this study from the ethics committee of Afyon Kocatepe University Faculty of Medicine**.**

Informed Consent: Written informed consent was obtained from patients who participated in this study.

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