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THREE-STEP DIAGNOSTIC ALGORITHM IN DIAGNOSING PATIENTS SUSPECTED OF *CLOSTRIDIUM DIFFICILE* –ASSOCIATED DIARRHEA

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ABSTRACT

Clostridium difficile is a predominant etiological agent of healthcare-associated infectious diarrhea. Immunoenzymatic tests for detecting toxins A/B from faecal samples are still used in routine diagnosis of Clostridium difficile-associated diseases in a number of healthcare centers in Poland. Recently, however, new diagnostic tests were introduced which allow for detecting toxigenic strains of C. difficile in a more effective and precise manner. It is of importance, especially in the light of hypervirulent strain occurrence.

AIM. The aim of the present paper was to evaluate the efficacy of three-step algorithm in the diagnosis of Clostridium difficile-associated diseases (CDAD), considering the occurrence of false negative test results for toxins while using exclusively immunoenzymatic tests.

MATERIALS AND METHODS. In the present study, faecal samples collected from patients presenting diarrhea were tested. Immunoenzymatic tests were used for detecting glutamate dehydrogenase (GDH) and toxins A/B. Culture and RT-PCR were also employed.

RESULTS. Of 615 study participants, toxigenic strains GDH (+) TOX (+) were identified in 108 patients while for 67 patients, test results remained unspecified GDH (+) TOX (-). Further analysis of unspecified samples revealed 32 patients infected with toxigenic strains, i.e. 22.9% of all positive test results (n=140).

CONCLUSION. Three-step diagnostic algorithm is an effective and reliable tool for diagnosing C.difficile-associated diseases.

Key words: *Clostridium difficile-associated disease, three-step diagnostic algorithm for CDAD, PCR - ribotyping*

INTRODUCTION

Clostridium difficile (CD) is a predominant etiological agent of healthcare-associated infectious diarrhea worldwide. A list of the most important risk factors of C.difficile-associated disease includes: antibiotic therapy, long-term hospitalization and advanced age (older than 65 years). Disease may affect patients in all age groups (1,2). According to Barlett et al., 15-25% of antibiotic-associated diarrheas (AAD) and nearly 100% of pseudomembranous colitis (PMC) cases are attributed to C.difficile (3).

Recently, an increasing tendency in the prevalence of CDAD is observed worldwide. In the United States, Canada and Europe, a 4-fold increase in the number of CDAD cases was reported. Furthermore, the number of CDAD cases of severe course also increased. It may result from the emergence of new, virulent C. difficile strains (4-8). An estimated 10-30% of adult patients are colonized by C. difficile. Not all of them, however, would present diarrhea (9).

Accurate diagnosis of healthcare-associated infectious diarrhea is essential in identification of patients infected with C.difficile. Consequently, it could reduce the risk of transmission of potentially virulent strains. Currently, rapid and simple tests for detecting the markers of infection with toxigenic C.difficile strains directly from faecal samples are available. These tests differ in terms of sensitivity, specificity, duration and costs borne by hospitals (10-15).

This paper aimed at evaluating the efficacy of three-step algorithm in diagnosis of Clostridium difficile-associated diseases (CDAD). CDAD may not be identified if tests for detection of toxins are exclusively used in routine diagnosis.

MATERIAL AND METHODS

A total of 615 diarrheal faecal samples collected from adults hospitalized in the University Hospital of Lord's Transfiguration in Poznań between January 2011 and the end of February 2013 were tested. Study participants were hospitalized in the following departments: Vascular Surgery, Surgical Oncology, Cardiology, Cardiac Surgery, Anaesthetics and Critical Care, Haematology with Transplantation Section, Pulmonology, Chemotherapy, Internal Medicine and Palliative Medicine. An analysis of diagnostic management of faecal samples was performed, using three-step algorithm (number of faecal samples tested was equal to the number of patients). Figure 1 presents diagnostic algorithm employed.

For laboratory testing, immunoenzymatic tests by TechLab (Blacksburg, USA, VA 24060) for detecting glutamate dehydrogenase (GDH), i.e. C. difficile somatic antigen and toxins A/B were used. In the phase I, the following tests were applied: C.DIFF CHEK60, C.DIFF CHEK TOX A/B (to the end of November 2012). Since the launch of new test, which is more rapid and of higher sensitivity (December 2012), C.DIFF QUIK CHEK, C.DIFF QUIK TOX A/B, C.DIFF QUIK COMPLETE were used. Provided test results were unspecified GDH (+), toxins A/B (TOX A/B) (-), faecal samples were cultured on solid media and/or molecular testing was performed using Xpert CD, Cepheid, Sunnyvale, CA, USA. Media by bioMérieux SA, Marcy l'Etole, France were used for culture.

In the first period (to a half of 2012), Columbia agar with 5% sheep blood with a mixture of antibiotics: cycloserine – 100 mg/L, cefoxitin - 8 mg/L and amphotericin B - mg/L (CLO) and Columbia agar with 5% sheep blood were employed. To enhance the accuracy of method used, faecal samples were exposed to ethanol (equal volume of faeces and ethanol 96%) for one hour. Consequently, it limited the growth of other non-endospore-forming bacteria. In this period, isolation of C. difficile strains raised difficulties. Since the launch of chromogenic medium - ChromID C.difficile (CDIF, bioMérieux SA, Marcy l'Etole, France) in a second half of 2012, containing i.a. sodium taurocholate, the frequency of CD isolation considerably increased. Media were incubated under anaerobic conditions for 48–72 hours (CCA medium) and 24 hours (chromogenic medium) at temperature of 37ºC. Strains isolated were identified on a basis of characteristic growth, para-cresol odour, assessment of preparation with Gram's method and biochemical tests (ANC, bioMérieux SA, Marcy l'Etole, France).

Toxigenicity of strains was analyzed using ELISA for toxin detection (C.DIFFICILE TOX A/B II) or molecular tests - RT-PCR (Xpert CD, Cepheid, Sunnyvale, CA, USA) for detecting C. difficile toxins B gene fragment, binary toxin and specific deletion at position 117 of tcdC gene acting as negative regulator. Selection of diagnostic test was dependent on patient's symptoms and general health.

Isolated C. difficile strains were subject to PCR-ribotyping. PCR-ribotyping was conducted in the Anaerobe Laboratory of the Department of Medical Microbiology at Warsaw Medical University. Reference strains were obtained from the Cardiff-ECDC collection. Strains which could not be classified to any PCR ribotype strains were sent to the reference centre in Leiden (Leiden University Medical Center, Leiden, the Netherlands).

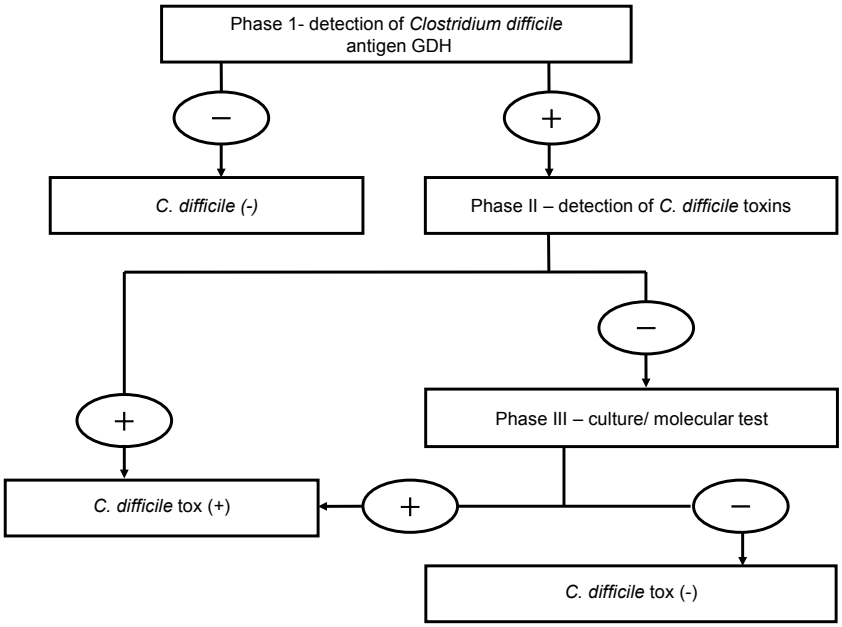

Fig. 1. **Phase I** – Detection of antigen from faecal sample tested – glutamate dehydrogenase (GDH) produced by both toxigenic and nontoxigenic *C. difficile* strains; negative test result excluded *C. difficile* infection.
Phase II – Testing of faecal sample where GDH for *C.difficile* toxins A and/or B was detected; positive test result was indicative of toxigenic strain infection.
Phase III – Evidence for toxigenicity of *C. difficile* strain isolated from faecal sample using immunoenzymatic test or molecular test for detecting the *C. difficile* toxins gene fragment.

RESULTS

In the period between 1st January 2011 and 28th February 2013, a total of 615 faecal samples collected from patients presenting diarrhea and intestinal obstruction, hospitalized in different departments, were tested (data were included in Table I).

Fig. 2 presents the results of three-step diagnostic algorithm employed.

C.difficile toxins and/or toxigenic strains were identified in 140 faecal samples collected from patients with diarrhea, i.e. 22.7% of all tests performed. Of them, 26.4% (n=37) and 73.6% (n=103) were females aged 26-87 years (average-60 years, median-63 years) and males aged 21-88 years (average-62 years, median-63 years), respectively. In the group analyzed (n=140), hospitalization ranged from 1 to 76 days (average-14 days, median-10 days). In the majority of patients (131/140), diarrhea occurred between 3 and 76 days of hospitalization. Patients were hospitalized due to heart diseases, heart defects, vascular diseases, myeloproliferative disorders and respiratory diseases.

Screening using test for detecting glutamate dehydrogenase revealed that GDH was present in 175 (28.45%) samples. Such marker was not identified in 440 (71.54%) samples. Based on negative test result for the presence of GDH, infection with C.difficile was excluded. Samples, where GDH was detected (n=175), were further analyzed using ELISA for detecting C. difficile toxins. C.difficile toxins A/B were detected in 106 (17.23%) faecal samples while 67 samples were toxin-free. Due to severe general health of patients, RT-PCR was performed for 2 faecal samples which revealed the presence of C. difficile toxin B gene fragment (tcdB). No fragments of binary toxin were detected. As many as 67 samples, which were toxin-free in the phase II, were subject to further testing. A total of 59 faecal samples were cultured. Due to the general health of patients and the need for rapid test result, molecular test - RT-PCR was performed for 8 samples. Having used RT-PCR, toxin gene fragments tcdB, cdtA and/or cdtB and dele-

Table I. Number of faecal samples tested with positive test results for *C.difficile* toxins A and B in patients hospitalized in the University Hospital of Lord's Transfiguration in Poznań.

Departments	Number of faecal samples tested	Number/percentage of positive faecal samples
Anaesthetics and Critical Care	84	13/15.47%
General and Vascular Surgery	153	48/31.37%
Cardiac Surgery with Postoperative Section	75	29/19.33%
Cardiology	45	9/20%
Internal Medicine	2	1/50%
Haematology with Transplantation Section	207	30/14.49%
Pulmonology	6	2/33.33%
Palliative Medicine	31	9/29.03%
Chemotherapy, Surgical Oncology and Gynaecologic Oncology	13	0

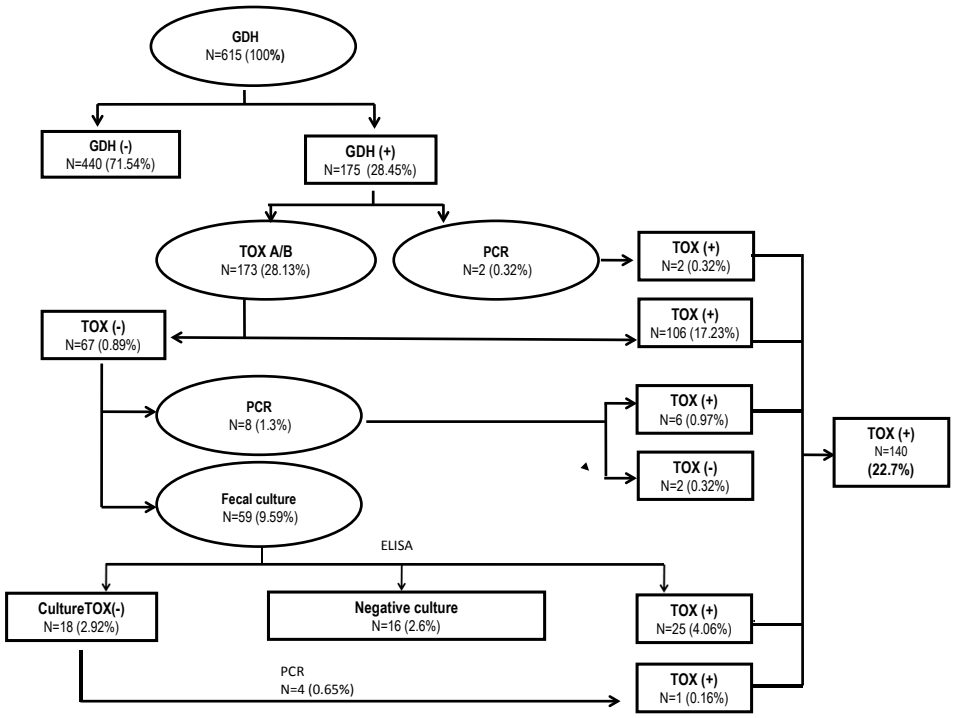

Fig. 2. Results of three-step diagnostic algorithm in patients suspected of *C. difficile*–associated diarrhea between 1st January 2011 and 28th February 2013.

Table II. Genotyping results using PCR – ribotyping of C. difficile strains isolated from faecal samples GDH (+) TOX (-).

No.	Strain No.	Isolation date	Department	PCR-ribotype
1.	2835/11	12 Feb 2011	Cardiac Surgery	176
2.	9954/11	01 June 2011	Cardiac Surgery	176
3.	13904/11	31 July 2011	Anaesthetics and Critical Care	027
4.	14672/11	15 Aug 2011	General and Vascular Surgery	005
5.	19034/11	18 Oct 2011	General and Vascular Surgery	027
6.	19930/11	30 Oct 2011	General and Vascular Surgery	027
7.	3318/12	12 Feb 2012	General and Vascular Surgery	027
8.	6857/12	22 Mar 2012	General and Vascular Surgery	027
9.	12421/12	04 June 2012	Internal Medicine	027
10.	14859/12	09 July 2012	Cardiac Surgery Section	027
11.	16221/12	26 July 2012	Cardiac Surgery	027
12.	18759/12	02 Sep 2012	Haematology	012
13.	19757/12	12 Sep 2012	Haematology	002
14.	21283/12	02 Oct 2012	Haematology	087
15.	21307/12	04 Oct 2012	Cardiac Surgery Section	005
16.	23383/12	05 Nov 2012	Anaesthetics and Critical Care	015
17.	766/13	09 Feb 2013	Haematology	014
18.	4735/13	27 Feb 2013	Haematology	014
19.	3837/12	17 Feb 2013	Cardiology	027

tion at position 117 of tcdC gene were detected in 3 samples. In 3 samples, exclusively toxin gene fragments tcdB were identified. C.difficile strains were isolated from 43 faecal samples. Toxigenicity was confirmed for 25 strains using ELISA for detecting toxins. Having considered the symptoms and risk factors of CDI, for 4 out of 18 potentially nontoxigenic strains in ELISA, RT-PCR was additionally performed. Gene fragment tcdB was identified in one strain. Isolated strains for whose the following test result was obtained - GDH(+) TOX(-) were subject to PCR–ribotyping. It was determined that 8 strains belonged to hypervirulent PCR-ribotype 027 and the next two – to PCR-ribotype 176 which is genetically related to PCR-ribotype 027. The remaining strains belonged to other ribotypes, i.e. 002 (n=1), 005 (n=2), 012 (n=1), 014 (n=2), 015 (n=1), and 087 (n=1). Results of ribotyping were presented in Table II. All C. difficile strains subject to PCR-ribotyping were toxigenic which was confirmed by proper tests for detecting toxins A/B.

DISCUSSION

Occurrence of symptoms and detection of toxins A/B in gastrointestinal tract of patients serve as a basis for diagnosis of C.difficile infection. A number of laboratories use rapid commercial immunoenzymatic tests for detecting C. difficile toxins A and/or B. Sensitivity of these methods, however, is not sufficient enough for identification of C.difficile infection in all patients. Application of proper diagnostic algorithm seems to be a solution to this problem.

In 2009, the experts of the European Society of Clinical Microbiology and Infectious Diseases (ESCMID) and a year later, the experts of the Society for Healthcare Epidemiology of America (SHEA), Infectious Diseases Society of America (IDSA) and American Society of Microbiology formulated the guidance for diagnosing Clostridium difficile infection (16,17,18). Screening tests for detection of glutamate dehyrogenase (GDH) were recommended. In case of positive test result, it was advocated to perform confirmatory test for detecting toxins A/B using ELISA or molecular test. High sensitivity (ranging from 97.6% to 100%) and negative predictive value (NPV 99%) are typical of tests for detecting somatic antigen GDH. It may be assumed that negative test result excludes the presence of C.difficile in faecal sample analyzed. It should not be forgotten that detection of antigen GDH does not allow for differentiating between infection with toxigenic and nontoxigenic strains (11,14,17,19,20).

Cell cytotoxicity assay is considered to be a gold standard in detecting toxin B. It is a time-consuming, expensive method, requiring tissue culture and confirmation by neutralization test. Thus, it is not routinely executed (16,17,18). Studies conducted by the European scientific group revealed that the percentage of laboratories whose diagnostic methods of CDI are based on commercial tests for detecting toxins A/B from faecal samples amounts to 93%. Of them, approximately 80% analyze the toxigenicity using immunoenzymatic test while 41.6% apply both ELISA and culture (21). Tests based on immunoenzymatic reactions (ELISA) are easy-to-perform. Furthermore, test results can be provided quickly. ELISA is of very good specificity, however, it is not sensitive enough. Consequently, it may lead to underestimating the number of infections (22). It is also confirmed by the results of the present paper. Having adopted diagnostic algorithm, further analysis of 67 samples which were initially toxin-free by ELISA ensured detection of infection with potentially toxigenic C. difficile strain in the next 32 (22.9%) patients presenting symptoms indicative of infection. All of the strains isolated from patients, in whom exclusively antigen GDH was detected, were toxigenic. Similar results were obtained in the study by Nurzyńska et al. where two-step

algorithm was used for testing samples which initially were considered as negative GDH (+) TOX (-) (23).

Patients whose test results were false negative may be a potential source of cross infection, leading to hospital outbreaks. Lack of proper, effective microbiological diagnostic methods results in failure to diagnose patients which consequently triggers the consequences of clinical and epidemiological nature. Introduction of proper diagnostic methods may contribute to an increase in the number of detected infections.

Faecal culture for C. difficile may be useful as a supplementary method to immunoenzymatic tests in case of patients whose symptoms are indicative of C. difficile infection and if exclusively antigen GDH is identified in them. Culture is a sensitive method, however, it is time-consuming and requires confirmation of toxigenicity of isolated strain. Nevertheless, strains which are isolated in culture may be subject to a number of additional tests with an example being: determination of genes which allows for more precise analysis of epidemiology of C.difficile infection.

Testing of samples in the period between January 2011 and a half of 2012 raised difficulties consisting in failure to isolate C. difficile strain from faecal sample where antigen GDH was identified. No reasons of such situation were determined. It could result from the presence of agents inhibiting the growth of strain in faeces under in vitro conditions or lower sensitivity of culture medium which was used initially (24).

Molecular methods seems to be the prospects in the diagnosis of CDAD. Currently, tests based on Real -Time PCR are available. They allow for detection of toxin gene fragments: toxin B (tcdB), binary toxin (cdtA and cdtB) and specific deletion tcdC at position 117, occurring in strains important from epidemiological perspective, i.e. strains belonging to PCR-ribotype 027. It is a commercial method which was approved by the American Food and Drug Administration (FDA). From studies by Novak-Weekley et al. transpires that RT-PCR is of higher sensitivity (94.4%) and negative predictive value NPV (98.8%) compared to immunoenzymatic tests (83.1%) and cell cytotoxicity assay (55.6%) (25). Such method provides quickly (45 min) test results which are decisive for initially negative or inconclusive test results in case of the presence of specific symptoms and suspicion of C.difficile infection. It should not be forgotten, however, that detection of C.difficile toxin gene fragment is not indicative of toxin expression but it may suggest C.difficile carriage. Therefore, concomitant interpretation of molecular test result and symptoms if of importance.

In the present paper, genetic typing of isolated C.difficile strains, using PCR-ribotyping, was performed. Genetic diversity of C.difficile strains isolated from patients presenting diarrhea and intestinal obstruction was demonstrated. Of 8 different ribotypes identified, two belonged to PCR-ribotypes 027 and 176 being of high virulence. Detection of ribotype 176, closely related to ribotype NAP1/BI/027, which emerged in Poland at the turn of 2008 and 2009, should be highlighted (26). Increased virulence of C.difficile strains pose a threat for colonization of this pathogen in hospital settings, leading to higher risk of CDAD infections.

CONCLUSIONS

Diagnosis of patients infected with toxigenic C. difficile strains, using exclusively ELISA for detecting toxins A/B from faecal samples, is not sufficient enough. Results of the present paper confirm the usefulness of molecular methods and culture in diagnostic algorithm of CDAD.

Acknowledgements: The authors would like to thank Prof. J. Brazier (Anaerobe Reference Laboratory, Cardiff, the Great Britain) and Prof. Edward Kuijper (Leiden University Medical Center, Leiden, the Netherlands) for the provision of reference strains for PCR-ribotyping. We would like to thank Prof. E. Kuijper and Celina Hamanus for their assistance in PCR-ribotyping of selected strains. PCR- ribotyping, performed in the Anaerobe Laboratory of the Department of Medical Microbiology at Warsaw Medical University, was financed by the resources of the National Science Centre (NCN) in Cracow within 2011/01/B/NZ7/02720grant.

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Received: 14.05.2014
Accepted for publication: 29.09.2014

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