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CLINICAL STUDY

Ribotypes in isolates and testing algorithm of *C. difficile* infections in the studied sample

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ABSTRACT

BACKGROUND: *Clostridium (Clostridioides) difficile* is the most common pathogen of nosocomial and antibiotic-related diarrhea in health-care facilities. The aim of the analysis was to show the testing algorithm and to identify hypervirulent strains (suspected RT 027).

METHODS: The retrospective analysis of patient samples suspected on CDI was carried out by a two-step algorithm. Biological specimens were analysed by GDH or culture, immunoenzymatic assay on toxins A/B and selected samples also by a real-time PCR.

RESULTS: In 1006 specimen suspected on CDI, 202 specimens were evaluated as positive in the two-step algorithm. Conflicting results (64 *C. difficile* isolates) were tested in a three-step algorithm by a real-time PCR and revealed 59 toxigenic and non RT 027 ribotypes. Statistically significant dependence among the independent variables, such as: diagnostic parameters and length of hospitalization (p = 0.175) and *C. difficile* (suspected RT027) ribotypes was not found.

CONCLUSION: The results of PCR ribotyping showed a high prevalence of hypervirulent and toxigenic ribotypes in the studied sample. A resistance to vancomycin was found in one isolate. The PCR method contributed to the rapid laboratory diagnosis and thus treatment of high risk patients or was used as a third step in in the case of unclear results of standard diagnostic methods

(Tab. 1, Fig. 4, Ref. 18). Text in PDF *www.elis.sk.*

KEY WORDS: toxigenic *C. difficile*, diagnostics, real-time PCR, ribotyping.

Introduction

Toxigenic strains of *C. difficile* are among the most common inducers of diarrhea acquired in the hospital environment and are considered to be the cause of a large proportion of antibiotic-related nosocomial diarrhea. Over the last two decades, there has been an increase in the incidence of CDI cases and severity of CDI infections, and new highly virulent *C. difficile* strains (e.g. ribotype 027) emerged (1).

An accurate and fast diagnostics of CDI is essential for an optimal patient care and to prevent the spread of infection (2).

C. difficile produce 2 large toxins. Toxin A is an enterotoxin and toxin B is a cytotoxin. *C. difficile* can produce a third toxin, referred to as the "binary toxin" (3). However, there are strains of *C. difficile,* which do not produce any of the toxins and are unable to induce the disease (4).

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In order to manage *C. difficile* infections effectively, a rapid and accurate diagnostics is essential to guide the treatment and to prevent its transmission.

The diagnosis of CDI is based on a combination of symptoms confirmed by a microbiological evidence of toxins produced by *C. difficile* or toxigenic strains of *C. difficile* in faeces, respectively colonoscopic evidence, or in the absence of other causes (5).

The optimal diagnostic approach to CDI is still under discussion. According to ESCMID (European society for clinical microbiology), the use of one standalone CDI test is not recommended due to the low positive predictive value at low CDI prevalence (2).

Due to the different sensitivity of the different methods used, according to current recommendations, a combination of at least two different tests is preferred. Therefore, at least 2 step algorithms are currently used to optimize CDI diagnostics (6).

In 2013, 48 % of hospitals in 20 European countries were using an optimised algorithm for laboratory diagnosis of CDI. Two-stage algorithms using a glutamate dehydrogenase enzyme immunoassay (EIA) or NAAT (e.g. real-time PCR) followed by a toxin detection have been adopted in the UK (1)

Testing algorithm initiated by PCR method followed by toxin (EIA), e.g. ELISA method is not yet a part of routine practice in many countries (7).

Diagnostic methods for identification of different target regions determine the presence of free toxins or toxigenic strains.

Methods that determine the presence of free faeces toxins are: immunoenzymatic assay (EIA) and cytotoxicity and neutralisation assay (CTNA). Methods that detect the presence of *C. difficile* include: EIA assay for evidence of glutamate dehydrogenase enzyme (GDH) and anaerobic culture and methods that detect the presence of a toxigenic *C. difficile* include: toxigenic culture (TC) with a high clinical sensitivity or NAAT (e.g. „real-time" PCR) method. A standard test for the detection of toxins (evidence of cytopathic effect on cell cultures and neutralization assay – CTNA or CCCNA) and toxigenic culture are used as the reference methods for laboratory confirmation of CDI (2).

The first test should have a high negative predictive value of NPV (i.e. a highly sensitive test that reliably excludes patients without CDI. This may be an immunoenzyme assay for glutamate dehydrogenase or a PCR assay. In the positive result, the second test with a high positive predictive value, i.e. a highly specific test, such as A / B toxin detection is carried out (5). If the GDH test was performed as the first test in sequence, it is possible to carry out PCR screening as the third step. EIA for toxins – immunoenzymatic methods based on the detection of toxin A/B are considered to be less sensitive at the detection of toxigenic *C. difficile* than the PCR method (6). The importance of the real-time PCR method lies in the high sensitivity and specificity of the testing method and therefore can be used to accelerate the exclusion of *C. difficile* infection (7). The testing algorithm represents potential cost savings for laboratories. Each laboratory must evaluate and assess a multi-stage algorithm for its patient population (6).

Materials and methods

The samples were analysed in the Laboratory in Žilina (Klinicka biochemia). The data were analysed retrospectively from 1.1.2015 – 30.6.2016 by the following laboratory methods. The patient´s specimens were tested by direct diagnostic methods for *C. difficile* using immunochromatographic assay for the detection of Glutamate dehydrogenase enzyme (CERTEST *Clostridium difficile* GDH), toxins A or B (CERTEST *Clostridium difficile* Toxin A / B), and immunoenzymatic methods ELISA (ProSpectT *C.diffcile* Toxin A / B Microplate assay) to determine toxins A and B. Selected biological samples were analyzed also by multiplex real-time PCR (GeneXpert, Cepheid). The anaerobic cultivation on taurocholate-cycloserine-cefoxitin-fructose agar was established to confirm positive *C. difficile* assays. The MALDI TOF method was used to identify *C. difficile* on species und subspecies level.

C. difficile culture and identification by MALDI-TOF MS

The samples were directly cultured on selective media. The samples were inoculated into cycloserin-cefoxitin-fructose agar, supplemented with taurocholate and incubated under anaerobic conditions at 37 °C for 72 hours. *C. difficile* was identified by Matrix Assisted Laser Desorption/Ionisation Time-of-Flight Mass Spectrometry (MALDI-TOF MS) with the use of MALDI Biotyper v 3.0 system (Brucker Daltonics).

Antibiotic susceptibility testing of C. difficile isolates

C. difficile isolates were tested to vancomycin and metronidazole using E-test (BioMerieux) on blood agar (Oxoid). The minimum inhibitory concentration (MIC – minimal concentration that stops the growth of the bacterium) breakpoints for metronidazole and vancomycin were applied according to European Committee on Antimicrobial Susceptibility testing (EUCAST).

The detection of toxins was carried out by multiplex „real-time" PCR method (*GeneXpert C. difficile* Epi PCR Cepheid, Inc., CA) – enables the detection of the B toxin gene (*tcdB*), the binary toxin genes *(cdtA a cdt B)*, and deletion of tcdC gene on nucleotide 117 was carried out, which is suspicious for RT 027. Capillary electrophoresis PCR ribotyping is needed to be performed by amplification of ribosomal DNA and their separation.

Patient file results were processed using Excel (Microsoft), then the selected file was exported to SPSS, a 2-tailed t-test was used for the normal distribution of metric data. The Chi square test as well as the Fisher test for smaller file sizes were applied.

Results

The group of 1006 samples suspected for CDI was analysed from January 2015 to June 2016 by different testing methods. The number of patients´ samples tested positively for CDI from a total number of 1006 samples was 148. The samples from patients evaluated positively in immunoenzymatic assay for A / B toxins and culture/GDH and the total number of positively tested samples by the 2-step algorithm are shown in Figure 1.

148 patients´ samples were in 43 % from male and 57 % were from female patients. The majority of the positively tested samples were from patients over 65 years (76 %) and 24 % of the samples were from patients under 65 years.

According to the algorithm, 63 % of samples (633 samples) were evaluated as negative by GDH test or culture, which excluded the presence of *C. difficile* in specimen (Additionally, detection of toxins A/B was also negative). 373 samples were detected for GDH enzyme with the positive result and according to the 2-step algorithm were analysed further for detection of toxins A/B. 18 % of samples (183 samples) were evaluated positively for toxins A/B by EIA, with a high probability of CDI.

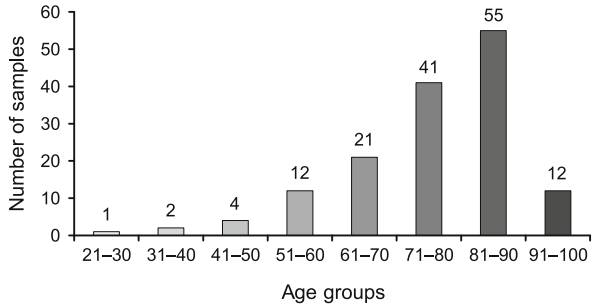

Fig. 1. Positive patients´ samples by GDH/culture and toxins A/B (EIA) according to the age groups in 2015–2016.

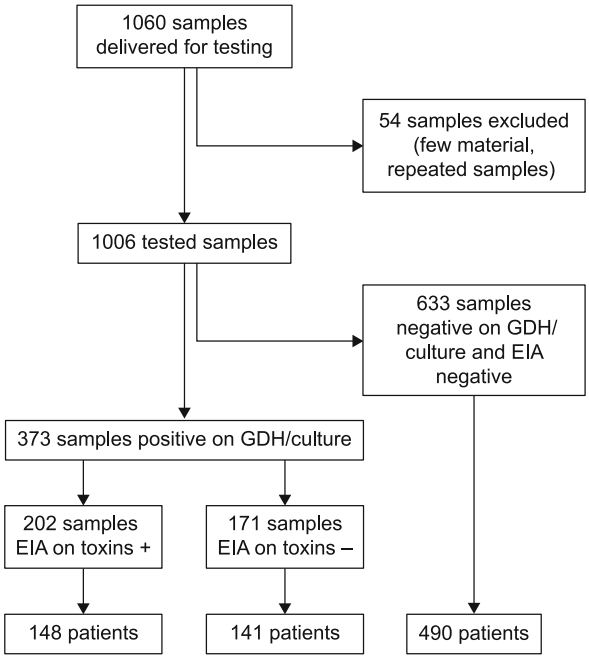

Fig. 2. Testing algorithm on CDI in the laboratory in 2015–2016.

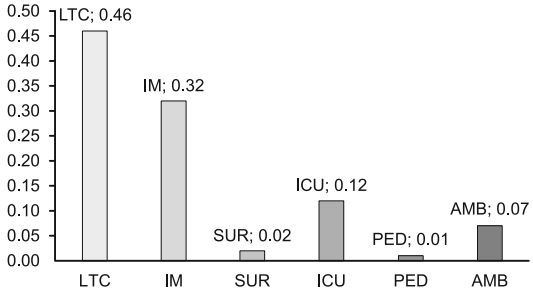

Fig. 3. Total number of positive samples in % of total samples from patients within departments (2-step algorithm, GDH test or anaerobic culture and ELISA test).

19 % of samples (191) were detected as GDH positive and toxin A/B negative by EIA test. This could be carriers of toxigenic strain/s or the amount of toxin was not sufficient amount to be clearly detected in an immunoenzymatic test. The preanalytic phase conditions (toxins are termolabile) could also play the role (Fig. 2).

From 171 samples, 64 samples with conflicting results and with clinical signs of suspected CDI were evaluated by the real-time PCR. Real-time PCR was carried out as the third step in the diagnostic algorithm.

The samples positively tested were from hospitalised patients (– 93 % from internal departments and departments of long term care and from non-hospitalised patients – 7 %) (Fig. 3).

The samples most frequently positively tested were from internal departments, representing up 32 % of the total samples tested. Positive samples from the long-term care departments comprised 46 % of the total samples tested, followed by positive samples from anesthesiology – resuscitation department 12 % and samples from surgery departments 3 %, and ambulant patients 7 % (Fig. 3).

Genotypic and phenotypic characteristics of C. difficile in the studied sample

We analysed a set of 64 samples on toxins by immunochromatography for toxins A/B and GDH, real-time PCR, enzyme immunoassay, and culture. 59 samples were tested positive and 5 negative. The mean age was 77 years, 75 years for male and 79 for women. 17 isolates were tested by culture. The patients´ samples with the need of rapid testing or with conflicting results on GDH and toxins A/B (GDH positive and toxins A/B negative or slightly positive) were tested with the „real-time" PCR method in order to exclude the presence of toxigenic strain.

64 samples were from internal department patients (34 samples), department of long term care – 24 and surgery department – 1, intensive care unit –1 and from ambulant patients – 4. The length of hospitalization of the patients was on average 46 days.

From 64 *C. difficile* isolates included in the studied sample – 16 carried the B toxin gene (evaluated as toxigenic), 43 isolates carried both the B toxin gene and the binary toxin genes, and the deletion of nucleotide 117 on the tcdC regulatory gene for B toxin (hypervirulent or supertoxigenic) and 5 *C. difficile* isolates were negative (Tab. 1). In further surveillance procedure, the samples were sent for capillary electrophoresis PCR ribotyping detection.

Representation of ribotypes within the sample were as follows: suspected ribotypes RT027, and toxigenic ribotypes (non RT027). Within internal department, toxigenic ribotypes (non RT027) comprised – 8 isolates, suspected RT027 – 24 isolates, 2 were negative. Department of long-term care: suspected RT027 (16) and toxigenic ribotypes (non RT027) (7), 1 sample was negative. Surgery department – 1 isolate was non RT027, Intensive care unit (ICU) – 1 negative isolate, ambulant – 2 isolates suspected RT027, 1 isolate nonRT027, 1 negative.

Meanwhile, these ribotypes were present in patients´ specimen from in-patient departments (internal departments, departments of long-term care) but also in outpatients, which had a history of prior hospitalization. It shows the circulation of the toxigenic and hypervirulent ribotypes among the departments. Most of the patients were hospitalised several times with chronical diseases and they were further treated at the long-term care department.

The *C. difficile* isolates were tested on antimicrobial susceptibility on vancomycin and metronidazole. Minimal inhibition concentration (MIC 8–32 µg/ml) breakpoints were evaluated according to the standards of the European committee for susceptibility testing (EUCAST). The importance of anaerobic cultivation resides in the determination of *C. difficile* susceptibility on antimicrobial agents. The isolates *C. difficile* in the studied sample were susceptible to metronidazole (MTZ) and vancomycin (VAN). One *C. difficile* isolate was resistent to vancomycin (Tab. 1).

In the selected sample of patients, further indicators (creatinine, albumin, CRP, length of hospitalization) were compared between the patients with different ribotypes (RT027 presumptive positive and non RT027) in samples. There was no statistically significant

Tab. 1. Genotype and fenotype characteristics of *C. difficile* isolates in the studied samples.

Isolates of *C. difficile*	Fenotype characteristics of *C. difficile isolates*		Genotype characteristics of *C. difficile isolates*			
Number of isolates	MIC µg/ml (VAN)	MIC µg/ml (MTZ)	gene for B toxin (tcdB)	genes cdtA, cdtB for binary toxin	deletion of nt 117 in tcdC gene (susp. RT 027)	PCR ribotypes Presumptive RT027
15	sensitive	sensitive	positive	negative	negative	negative
1	resistant	sensitive	positive	negative	negative	negative
43	sensitive	sensitive	positive	positive	positive	positive
5	sensitive	sensitive	negative	negative	negative	negative

dependence among the independent variables, such as creatinine (p = 0.524), albumin (p = 0.682), CRP (p = 0.295) and the length of hospitalization (p = 0.175) among PCR ribotypes (non RT027 suspected) (e.g.RT001,etc.) and ribotypes (presumptive RT027 – e.g. RT176).

We can conclude that the laboratory results in patients with ribotype RT027 and patients with non ribotype RT027 did not show a significant difference. We explain this by the size of the sample (limited size of patients) and patients with multiple co-morbidities, such as: cardiovascular diseases, lung diseases, renal diseases and oncological diseases.

In the analysed procedure, we tested the samples for GDH and toxins A/B (EIA) – two-step algorithm. The samples GDH (EIA) positive and toxins A/B negative underwent testing for ELISA test and subsequent PCR testing was performed as the third step, as shown in the Figure 4.

Interpretation of the test results – if GDH (immunoenzymatically) or PCR positive and toxin ELISA positive (PPV = 91.4 %), then *C. difficile* is most likely present. The result should be compulsorily reported. If GDH (immunoenzymatically) or PCR is positive and the toxin ELISA negative, then *C. difficile* may be present, i.e. potentially a carrier of *C. difficile* – results are not included in the mandatory report. If GDH (immunoenzymatically) or PCR negative toxin by ELISA is negative (NPV = 98.9 %), then *C. difficile* is very unlikely to be present (7).

Discussion

C. difficile is an anaerobic gram-positive spore-forming bacillus. Colonization rates in healthy humans in the community range from 0.8 % to 13 % and are higher in long-term care facility residents (8) This frequency is higher in hospitalised patients (20–30 %). Colonization with *C. difficile* does not automatically lead to development of symptomatic CDI (2).

Transmission is by oral-fecal route. Intestinal dysmicrobia (most commonly associated with antibiotic therapy) may be considered to be the most important factor of infection, which may result in a loss of colonisation resistance to *C. difficile.*

The immune response of the host may partially explain how colonisation with *C. difficile* results in a wide spectrum of outcomes (8), such as diarrhea, colitis or pseudomembranous colitis with complications. Risk factors for severe CDI are: age over 65 years, treatment with broad spectrum antibiotics, long-term hospitalization (longer than 3 weeks), major underlying disease (4)

It has been shown that a rapid diagnostics positively impacts the patient´s care by reducing delays in the initiation of the isolation and treatment for the confirmed CDI cases (2).

The incidence of CDI in Slovakia has a growing trend, with incidence 19.5/10,000 patients in 2016. In addition to the fact that the incidence of CDI in the SR is increasing, it is mainly due to better reporting and surveillance that helps to monitor CDI cases.

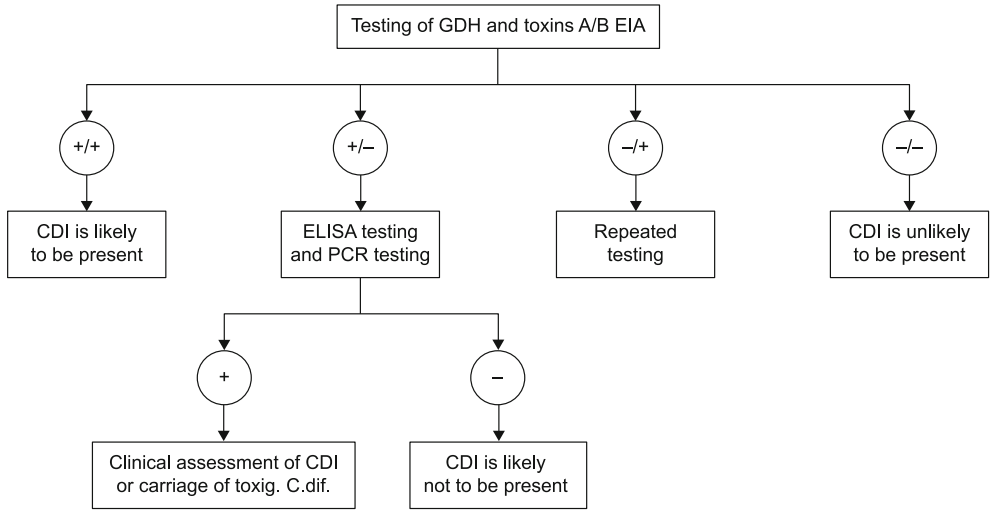

Fig.4. Testing protocol (algorithmic approach) adopted in the laboratory practice (own processing according (2) and (5).

The number of reported cases of CDI in medical institutions (HAI-CDI) in Slovakia reached within internal wards 88.58 %, followed by surgical wards, intensive care unit (ICU) and others (9).

These finding were also confirmed by our positively evaluated samples, which came mainly from hospitalised patients (93 %) (from which were 78 % internal departments and departments of long-term care) and non-hospitalised patients (7 %).

Our results also revealed that the majority of positively tested CDI cases are with the age over 65 years – 75 % of all cases.

The real-time PCR can help an early diagnostics and early recognition of patients with *C. difficile* before complications occur. The PCR tests are very sensitive to *C. difficile*, but do not distinguish between an active infection and asymptomatic colonisation as they determine the genes for production of toxins (6). Genetic evidence of the toxigenic strain does not automatically mean the toxigenic genes´ expression.

PCR detected a possible deletion of tcdC, a suspected ribotype 027, which was important from the epidemiological point of view, but also the turn-around time and thus early treatment. Not only would the patient benefit from this, but the possible cost of patient care in the event of complications would also be reduced (7).

Traditional typing methods used to identify the ribotype of a strain causing CDI required culture first and so were not timely. In this regard, a rapid „presumptive RT027" could be of value. The assay had a high NPV for „presumptive RT027, but did overcall the number of samples that truly contained PCR-ribotype 027 (69.9 %) (10).

Samples with conflicting results between the highly sensitive GDH assay and the A and B toxins that had a lower sensitivity but higher specificity were often discussed. Studies recommend using PCR for such discrepancies.

In cases suspected of CDI (GDH positive, toxine negative) – the real-time PCR method can confirm or exclude a presence of toxigenic strain, even if it does not differentiate between the symptomatic infection and colonisation. (7).

Notably, outcomes of the patients, who are NAAT positive and toxin negative, are indistinguishable from NAAT negative and toxine negative (10).

For patients, who are *C. difficile* positive by molecular-biological assay but, who are toxin negative by immunoenzymatic methods, e.g. ELISA method, may be referred to as "*C. difficile*" carriers, although they may be isolated but do not require treatment (7).

The hypervirulent PCR ribotype 027 is known worldwide. Some studies point to a more severe course of CDI disease, when this ribotype occurs. The hypervirulent strain is referred to as *C. difficile* BI / NAP1 / 027 (6). The strain BI / NAP1 / 027 contains a nucleotide mutation at position 117 on the tcdC gene that encodes the protein C, which causes the suppression of genes for A/B toxins (11).

Further analyzes showed that there are more ribotypes with similar properties (4).

Ribotype 176 is close to ribotype 027 and also encodes binary toxin and contains a nucleotide mutation at position 117 on the tcdC gene that encodes the protein C, that causes the suppression of genes for A/B toxins, and can be erroneously identified by commercial assays aimed at deletion of one base pair at nucleotide 117 in the *C. difficile* tcdC gene.

This ribotype persists in the Czech Republic and has been recorded in Poland in 2013 (12). According to studies (13) it is also present in Slovakia.

It is important to note, that there may be other attributes of the *C. difficile* genome that can significantly affect virulence (not only binary toxin and tcdC deletion) and hence the clinical course of the disease, which should be taken into account in treatment strategy management (14).

The ribotype or strain type is referred to as another possible cause of increased morbidity, severity of disease and higher CDI relapse rates. According to the Canadian study conducted by Miller, PCR ribotype 027 correlated with an increased disease severity in patients, in almost all age groups. However, toxin expression is related to CDI severity. Several studies report a high and uncontrolled toxin production for ribotype 027 (i.e. deletion of negative control tcdC gene).

The most frequently occurring ribotypes in our region according to the study published by Novaková et al. were RT176 (among hypervirulent ribotypes-presumptive RT027) and 001 among toxigenic ribotypes (non RT027) (13).

Among toxigenic ribotypes we distinguish 15 toxinotypes (I – XV) (15).

Hypervirulent ribotype 176 is also associated with a more severe course of the disease and may be misidentified by commercial assays aimed at deletion on base pair at nucleotide 117 in the tcdC regulatory gene for B toxin (4) We suppose this fact also in our study sample (13).

The new epidemic strains are less sensitive to antibiotics, e.g. resistance to fluoroquinolones. For many strains, metronidazole susceptibility decreases gradually, which can be demonstrated by an increasing MIC (minimal inhibitory concentration).

Epidemic ribotypes were connected with multiple antimicrobial resistance. Ribotypes 017, 018 a 356 showed a high level of antimicrobial resistance (16).

This was confirmed also in our studied sample, one isolate showed a resistance to vancomycin.

PCR ribotyping is useful for monitoring the spread of CDI, the course of disease, as well as the detection of resistance to antimicrobial agents. This method is performed to identify individual strains, to carry out surveillance of the *C. difficile* infection spreading.

Despite an increased virulence of certain ribotypes, the PCR ribotype value as a predictor of disease severity is limited because the ribotype involved in infection is not known until it is diagnosed. However, in PCR epidemics, the ribotype could be considered, when deciding on the choice of empirical treatments (17).

In the recent extensive study (18) it was shown that the effect of individual ribotypes on overall disease progression and mortality and biomarkers (especially those associated with inflammation) varied. In addition to *C. difficile* PCR ribotype 027, there are other strains that are associated with epidemics and a severe course of *C. difficile* infection.

It is necessary to provide a timely appropriate antibiotic therapy with an early selection of patients who are at high risk and to perform a rapid selection of the most appropriate therapy. Predictive markers associated with a poor prognosis of CDI (e.g. hemodialysis, intubation, etc.), laboratory predictive markers, such as: differential blood count, acute phase protein levels, procalcitonin, lactate level, calprotectin, which are significant predictive factors as well as parameters that comprehensively assess water – mineral metabolism (4).

At the same time, the interpretation of the results requires the knowledge of the possible limits of specific diagnostic methods and experience in the clinical assessment of the results of specific diagnostic methods in clinical microbiology.

Reliable data is also crucial for monitoring CDI incidence over time and comparing individual healthcare facilities.

The treatment and diagnostics of the disease laboratory findings should always be interpreted together with patient status and other laboratory results, diagnostic methods and the patient's clinical status as well as the current epidemiological situation (4).

Conclusion

C. difficile infection (CDI) is the most common pathogen of nosocomial and antibiotic-related diarrhea in health care facilities and a significant medical and economic burden in healthcare institutions. Accurate and fast diagnostics of CDI is essential for an optimal patient care and in order to prevent the spread of infection.

For the treatment and diagnostics of disease, microbiological laboratory diagnosis 2-step algorithm was applied involving a high sensitivity screening assay (GDH EIA), followed by a high specificity assay (EIA for toxins A/B) is important. The 3 – step algorithm was applied in the diagnostic procedure. Conflicting results were tested by the „real-time" PCR. The „real-time" PCR method can assist in the early diagnosis and early recognition of patients with *C. difficile* infection before complications occur. GDH and the „real-time" PCR test have a high NPV and can therefore be used to accelerate the exclusion of *C. difficile* infection.

PCR ribotyping identified 2 different PCR ribotypes (suspected RT027) in 43 isolates and 16 isolates (non RT027). The ribotype RT 176 is genetically close to ribotype 027, it has a deletion in nucleotide 117, in regulation gene (tcdC) for toxin B. The ribotype 001 and ribotype 176 with a high prevalence are the most present PCR ribotypes in our country.

C. difficile isolates were tested for susceptibility to metronidazole and vancomycin. One isolate (non RT027) was resistant to vancomycin. The retrospective analysis of patient samples suspected for CDI was carried out from January 2015 to July 2016.

The real-time PCR method contributes to the rapid diagnostics of high-risk patients in case of unclear results of standard phenotypic methods directed on exoenzyme (GDH EIA), and toxins (EIA). PCR ribotyping plays also an important role in CDI surveillance.

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