

Association between ribotype and clinical form of enterocolitis with *C. difficile* in Western Romania: A 2-year study

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Abstract. During the last decade, several changes in the epidemiology, clinical symptoms, and evolution of *Clostridium difficile* infection (CDI) have been reported. The number of diagnosed cases has increased, especially in individuals over 60 years of age. There has also been an increase in the share of severe forms of the disease, the number of patients with recurrent infections and the lethality caused by this condition. The aim of the present study was to determine the incidence of CDI over the past few years; to monitor the bacterial toxin by ribotyping; to observe the migration of circulating toxins; to correlate the ribotyping with the clinical form of the disease; and to correlate the treatment with the ribotyping and the clinical form. Therefore, we performed an observational retrospective study regarding the incidence of CDI at 'Victor Babeș' Hospital of Infectious Diseases and Pneumophysiology of Timișoara, between January 2016 and December 2017. The results revealed that 210 patients were hospitalized with a diagnosis of acute enterocolitis with *C. difficile*. All patients tested showed *C. difficile* toxin A/B positivity. In 28 cases, the ribotyping was positive with binary toxin and hypervirulent strain 027. The tested patients were discharged or transferred from various medical/surgical clinics. In the study, we also ascertained the correlation between the clinical form of the disease and comorbidities and pre-hospital treatments as risk factors in the occurrence of the infection.

In accordance with the rise of CDI during the last decade, it was possible to observe the increase in the number of infectious recurrences. From this point of view, highlighting of the ribotype in this pathology becomes a primary aspect both by increasing the success rate in curing the disease (with a reduction in the risk of recurrence) and by targeted and non-empirical administration of the antibiotic.

Introduction

Over the last two decades, there has been a dramatic change in the epidemiology of *Clostridium difficile* infection (CDI). A disease considered to be a side effect of antibiotic use, easy to treat, is now associated with outbreaks, with increased mortality and morbidity. The incidence of CDI in hospitalized patients has increased dramatically with more than 300,000 reported cases per year, with an increased mortality rate. In the USA, more than 12,800 deaths were reported in 2017 due to this pathology, adding CDI to the list of diseases of the century we live in (1).

The new epidemic of CDI has been defined by community outbreaks affecting people considered to be at low risk of developing the disease. Initially, until the year 2000, it was considered that ribotype BI/NAP1/027 did not represent a risk of toxicity until the Quebec epidemic. Subsequently, several US hospitals reported outbreaks of CDI ribotype BI/NAP1/027, with fulminant clinical symptoms (2). The BI/NAP1/027 ribotype has certain unique characteristics that could explain why the severity associated with this strain has increased (3). From the ribotype point of view, CDI is a real 'chameleon' of infectious pathology; however, unfortunately, the overall power of knowledge about circulating toxins in different sections in the Western part of Romania today is an enigma due to the additional cost of the specificity test.

Another essential problem in this pathology is the fact that the *C. difficile* strain carries several mobile genetic elements (4), which are able to confer resistance genes to antibacterial medication. The use of broad-spectrum and ultra-broad-spectrum antibiotics has allowed the development of hypervirulent strains with a rate of therapeutic failure, mortality and recurrence. Generally, most circulating strains are sensitive to metronidazole and vancomycin, although

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Abbreviations: CDI, *Clostridium difficile* infection; IDSA, Infectious Diseases Society of America

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antimicrobial susceptibility testing has shown, in several international studies (5), lower susceptibility to metronidazole in infection with CD 027/BI/NAP1 strains, as well as with ribotypes 106 and 001. Albeit the current protocols used in our country recommend this type of therapy in case of recurrence (6); the importance of ribotyping and targeted antibiotic therapy based on the antibiogram is thus emphasized once again.

A study conducted in 2013-2014, a collaboration among the Clinical Hospital for Infectious Diseases of Timisoara, the Laboratory of Microbiology, Timisoara County Emergency Clinical Hospital and the Discipline of Epidemiology, 'Victor Babeș' University of Medicine and Pharmacy of Timisoara, also demonstrated the prevalence of CDI endemicity in the western part of Romania, with an incidence of 20.57/15.70 per 1,000 patients discharged in 2013/2014 or 17.73/14.04 per 10,000 patients per day. As a result of this research, the incidence of the pathology could be estimated and the risk factors were investigated in relapses and cases with fulminant symptoms (7).

The objectives of this study were to determine the incidence of *C. difficile* infection within the past few years, to monitor bacterial toxin by ribotyping, to test circulating toxins, to correlate the ribotyping with the clinical form of the disease and to correlate the treatment with ribotyping and clinical form.

Materials and methods

Study population. We performed an observational retrospective study regarding the incidence of CDI at 'Victor Babeș' Hospital of Infectious Diseases and Pneumophthisiology of Timisoara, between January 2016 and December 2017.

Upon admission, the patients signed the standardized informed consent by which they consented to their data being used for research purposes. The study was approved by the Ethics Committee of 'Victor Babeș' Hospital of Infectious Diseases and Pneumophthisiology of Timisoara.

'Victor Babeș' Hospital of Infectious Diseases and Pneumophthisiology of Timisoara is composed of two sections of infectious diseases, with a case rate of 2,076 cases in 2016 and 2,048 cases in 2017 (patients over 18 years of age).

Methods. We only included laboratory confirmed cases in patients aged 18 or older, subdivided in 6 age groups, regardless of sex, personal history, and probable infection source nosocomial or community acquired.

We collected demographic data, the presence of various comorbidities (malignancy, diabetes, chronic renal failure, cardiac, pulmonary, mild/moderate/severe liver pathology, or peripheral vascular, cerebrovascular, hematological diseases, dementia, gastro-duodenal ulcer, presence of concomitant infections); possible causes of immunosuppression during the last 2 months prior to the onset (chemotherapy, radiotherapy, corticosteroids, chronic dialysis, surgery including the type of intervention); other risk factors (inflammatory bowel disease, colorectal cancer, previous exposure to antimicrobial agents, enteral/parenteral nutrition); and clinical data.

The stool samples were collected through regular methods, using sterile recipients.

The etiology was confirmed by VIDAS® *C. difficile* Toxin A & B (bioMérieux) test, an ELFA (enzyme-linked fluorescent assay) that detects toxins A and B in fresh stool samples with a sensitivity of 88.3% and a specificity of 99.8%. Ribotyping was performed using *C. difficile* assay polymerase chain reaction, which allowed the differentiation between toxin B and binary toxin as well as the presumptive detection of strain 027/NAP/BI with a sensitivity of 93.49% and a specificity of 94.02%.

Statistical analysis. Data analysis was performed using the Statistical Package for Social Sciences (SPSS) v.25 (IBM Corp.). We presented continuous variables as mean and standard deviation and categorical variables as frequency and percentages. For the evaluation of the potential connection between ribotype and clinical form of CDI, we employed the χ^2 test. In order to determine the association between the treatment, clinical form and ribotype, a regression model and the Hosmer-Lemeshow test was used. P-value <0.05 was considered to indicate a statistically significant difference.

Results

Between January 2016 and December 2017, 210 patients were hospitalized with a diagnosis of acute enterocolitis with *C. difficile*. All patients tested showed *C. difficile* toxin A/B positivity.

In 2016, 95 patients with the diagnosis of *C. difficile* enterocolitis were hospitalized. If we refer to 2017, we can see an increase in 20 hospitalized cases with the same pathology, amounting to 115 reported cases (Table I).

The incidence of patients with *C. difficile* in the period of 2016-2017 was $I_{\text{total}}=4.801164$. In 2016, the incidence was $I_{2016}=4.190751$, and in 2017, $I_{2017}=5.419922$, there was an increase in the incidence of CDI comparing the years 2016 and 2017.

In this study, we followed the evolution of 210 patients, aged between 28 and 90 years, mean age 67.99 ± 17.50 years ($P < 0.0001$). Regarding the distribution by sex, both in 2016 and in 2017, the female sex predominated, with a total number of 121 cases, divided into 53 cases (56%), recorded in 2016 vs. 68 cases (59%) in 2017 ($P < 0.0001$). Conversely, the male sex maintained a linear pattern with 42 cases (44%) reported in 2016 vs. 47 cases (41%) in 2017 ($P < 0.0001$) (Fig. 1).

Urban-rural distribution was found to have the same increased incidence (59%) in urban areas (Utot) in 2016/2017, thus reporting 124 cases out of the total ($P = 0.0091$). Regarding the rural environment (Rtot), it was found to have an equally increasing trend, with a percentage of 41% of the cases (86 patients), explainable due to easy access to the urban health system and the lack of territorial care systems (Table II).

The incidence of these infections was significantly higher in the age group over 61 years (totaling over half of the cases, $P < 0.0001$), followed by adults (40-60 years, over 20% of cases, $P < 0.0001$). Although it was the prerogative of the study to research the elderly in the period of the study, there were also cases of young individuals under 40 (9.47% in 2016, 5.20% in 2017, $P < 0.0001$) (Table III).

Because most hospital-acquired CDI cases occur in elderly patients with an increased burden of chronic comorbidities

Table I. Incidence of *Clostridium difficile* infection (CDI) at 'Victor Babeș' hospital of infectious diseases and pneumophthisiology of Timisoara.

Year	Hospitalized patients	Relapses	New cases	Total Clinic I + Clinic II
2016	95	8	87	2,076
2017	115	4	111	2,048
Total	210	12	198	4,124

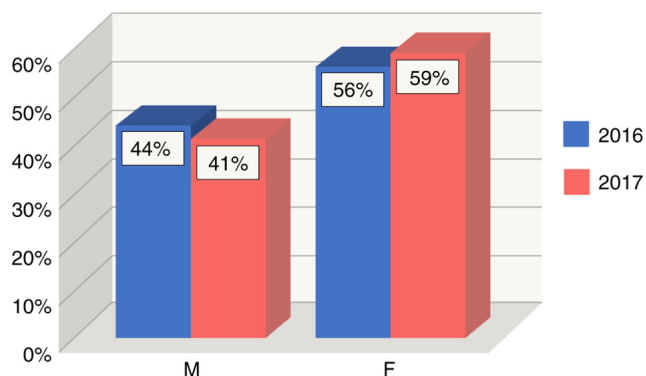


Figure 1. Sex distribution of the *Clostridium difficile* infection (CDI) cases. F, female; M, male.

affecting multiple organs and systems (8), multimorbidity itself may play a relevant role in defining CDI risk.

Comorbidity was well represented. Cardiovascular diseases were found to be a primary comorbidity (over 60% of cases reported in both years, $P=0.0018$), followed by renal pathology, metabolic diseases (diabetes), chronic lung disease and dementia (with a percentage of over 10% of cases in 2016, $P<0.0001$) (Table IV).

Because elderly patients are currently the ones who most frequently develop CDI, further studies are needed in order to explore the association between this infection and the areas of comorbidity, deficiencies and polymedication, which are intrinsic features of hospital-admitted geriatric patients.

The comorbidity score, Cumulative Illness Rating Scale (CIRS) can be a useful tool in CDI to estimate this additional risk, especially in patients undergoing long-term antibiotic treatment (9).

Until recently, septic shock was considered to be composed of three components: systemic hypotension, tissue hypoperfusion associated with organ dysfunction and hyperlactatemia (10,11).

According to the new definition, septic shock can be diagnosed in two conditions. The first condition is persistent hypotension after fluid correction; it requires vasopressors to maintain mean blood pressure >65 mmHg (12). The second condition is the level of serum lactate >2 mmol/l. Since heart rate, respiratory rate and other laboratory data are not included, the diagnosis and recognition of septic shock have become simplified (13). This very new definition implies that elevated serum lactate levels may represent tissue hypoperfusion associated with signs of organ dysfunction in critically

ill patients (14). In addition, it should be noted that the serum lactate level was decreased from 4 to 2 mmol/l.

The level of serum lactate as a clinical tool was described about half a century ago by Broder and Weil (15). At that time, a serum lactate level >4 mmol/l was associated with shock. Because serum lactate levels have been reduced to 2 mmol/l, serum lactate levels are a more sensitive marker for septic shock.

Lactate is an important source of energy, especially during diet (when the body has no other resources). Therefore, when lactate is not produced, humans cannot survive. Lactate also contributes to the acidic environment by converting to lactic acid. Next, lactate is converted to bicarbonate, and it becomes a major source of alkalization of the body under normal conditions. Lactate of 1.4-1.5 mmol/day consists of the reduction of pyruvate, which is largely generated by anaerobic glycolysis. In tissue hypoxia, lactate is overproduced by increasing anaerobic glycolysis (16). Usually, lactic acid clearance occurs in the liver (60%), followed by the kidneys (30%) and to a lesser extent by other organs (heart and skeletal muscle).

Lactic acid clearance cannot exceed lactate production and can be altered during the critical state. Septic shock with liver dysfunction and acute kidney damage increase lactate levels due to decreased clearance.

Some patients recovering from septic shock have normal serum lactate values, although vasopressors are still needed to maintain an average blood pressure of 65 mmHg or higher (17). In addition, low or normalized lactate levels are important signs of recovery from septic shock.

Within the case study in hospitalized patients in the period of 2016-2017, an increased prevalence of lactic acid was observed. A total of 105 cases (prevalence 0.5) showed a lactic acid value between 2-5 mmol/l. Nine cases (prevalence 0.04) out of the 201 presented a lactic acid with a value >5 mmol/l (Table V).

If we correlate the ribotype with the clinical form of the disease by applying the χ^2 test to see the association of the two variables, we obtained $\chi^2=5.9$ with $n=2$ degrees of freedom, and $P\text{-value}=0.0522>0.05$, which is why no conclusion can be drawn regarding the association of the two variables without the Cramer's correction $V=0.167$, which indicates a weak association. In conclusion, it can be stated that the severity of the disease depends on the form of the ribotype (Table VI).

In terms of treatment, metronidazole in the form of oral tablets should be limited only to mild cases of the disease. Oral vancomycin remains the recommended treatment for mild/moderate forms that do not respond to metronidazole treatment. Repeated courses of metronidazole should be avoided due to the increased risk of potentially irreversible cumulative neurotoxicity.

Table II. Distribution by urban vs. rural origin of the *Clostridium difficile* infection (CDI) cases for 2016 and 2017.

Urban (U) distribution	Utot=	0.585714	59%	U2016=	0.568421	57%	U2017=	0.6	60%
Rural (R) distribution	Rtot=	0.414286	41%	R2016=	0.431579	43%	R2017=	0.4	40%

Table III. Breakdown of the incidence of *Clostridium difficile* infection (CDI) by age group.

Year	Age group (years)					
	<40 (%)	(40-50) (%)	(50-60) (%)	(60-70) (%)	(70-80) (%)	(80-90) (%)
2016	9.47 (9 cases)	1.05 (1 case)	11.58 (11 cases)	17.90 (17 cases)	42.10 (40 cases)	17.90 (17 cases)
2017	5.20 (6 cases)	5.20 (6 cases)	13.05 (15 cases)	17.40 (20 cases)	46.10 (53 cases)	13.05 (15 cases)

Table IV. Comorbidities of the *Clostridium difficile* infection (CDI) cases.

Comorbidities	Year		
	2016 (%)	2017 (%)	Total (%)
Acute renal pathology	17.89 (17 cases)	29.57 (34 cases)	47.46 (51 cases)
Vascular cerebral pathology	10.53 (10 cases)	4.35 (5 cases)	14.88 (15 cases)
Malignant pathology	10.53 (10 cases)	10.43 (12 cases)	20.96 (22 cases)
Diabetes	28.42 (27 cases)	21.74 (25 cases)	50.16 (52 cases)
Chronic kidney failure	14.74 (14 cases)	28.70 (33 cases)	43.44 (47 cases)
Chronic heart disease	60.00 (57 cases)	65.22 (75 cases)	125.22 (132 cases)
Peripheral vascular disease	5.26 (5 cases)	5.22 (6 cases)	10.48 (11 cases)
Chronic lung disease	13.68 (13 cases)	6.96 (8 cases)	20.64 (21 cases)
Medium/severe hepatic pathology	7.37 (7 cases)	9.57 (11 cases)	16.94 (18 cases)
Dementia	12.63 (12 cases)	8.70 (10 cases)	21.33 (22 cases)

Table V. Lactic acid levels in the *Clostridium difficile* infection (CDI) cases.

Year	Lactic acid		
	<2 mmol/l	2-5 mmol/l	>5 mmol/l
2016	0.463157895	0.515789474	0.021052632
2017	0.452173913	0.486956522	0.060869565
Total	0.457142857	0.500000000	0.042857143

Although metronidazole may be associated with more common side effects and there has been a significant increase in treatment failure (especially in patients infected with emerging strain 027/BI/NAP1), oral metronidazole 500 three times a day for 10 days can be used to treat mild to moderate cases of CDI.

In current IDSA guidelines, metronidazole is used only in mild cases in patients without comorbidities or the risk of recurrence, as access to vancomycin or fidaxomicin is limited.

In a study published in 2015, a meta-analysis and systematic review was performed, comparing the efficacy and safety of metronidazole monotherapy with vancomycin monotherapy and combination therapy in patients with CDI. No statistically significant differences were found in the clinical cure rate between metronidazole and vancomycin for mild CDI form (4) or between monotherapy and combination therapy for CDI; however, the clinical cure rate was lower for metronidazole than for vancomycin for severe CDI.

In the present study, applying the χ^2 test to see the association of the two variables (ribotype and treatment), we obtained $\chi^2=1.55$ with $n=2$ degrees of freedom, and $P=0.46>0.05$, so the two variables are independent. The conclusion being that there is no association between the two variables (Table VII).

In order to determine the association between the 3 variables (treatment, clinical form and ribotype), the regression model was used, resulting in: $\text{logit}(p)=-2.34-0.33x \text{ treatment} +0.68x \text{ form}$.

The Hosmer-Lemeshow test shows that the logistics model is the right one ($P=0.6859>0.05$) and that 86.67% of the cases were accurately predicted (Table VIII).

Table VI. Association between disease form and ribotype of the *Clostridium difficile* infection (CDI) cases.

Disease form/Ribotype	Mild form	Average form	Severe form	Total
0.27	16	8	4	28
Other	130	45	7	182
Total	146	53	11	210

Table VII. Association between ribotype and treatment of the *Clostridium difficile* infection (CDI) cases.

Treatment/Ribotype	Metronidazole	Vancomycine	Metronidazole + vancomycine	Total
0.27	15	8	5	28
Other	115	34	33	182
Total	130	42	38	210

Table VIII. Association of the ribotype with the clinical form of disease and treatment of the *Clostridium difficile* infection (CDI) cases.

Form/Treatment	Ribotype=0			Ribotype=1		
	Metronidazole	Vancomycine	Metronidazole + vancomycine	Metronidazole	Vancomycine	Metronidazole + vancomycine
Mild	95	21	14	13	2	0
Average	18	10	17	1	5	2
Severe	2	3	2	2	1	2

Discussion

The etiology of infectious diarrhea is very varied, involving numerous pathogenic germs of bacterial, viral, parasitic, and fungal origin. The higher frequency represented by intestinal bacteriosis in the official statistics in our country does not represent the real incidence of the etiologies involved, but rather the concerns and limits of the activity of the specialized laboratory, especially for determining these bacterial etiologies.

The endemic nature of CDI reported in recent years cannot exclude the presence of deficiencies in the development of the epidemiological surveillance process of the hospital. In the presence of diseases with potential to induce secondary immunodeficiencies, the appropriate therapeutic act for the disease in question must be accompanied by the application of measures aimed at limiting nosocomial infections, in our case of *Clostridium* infection.

A study published in 2015 reported the cases of CDIs in our hospital. The incidence of infections in 2013-2014 was 20.57/15.70 per 1,000 patients discharged. If we compare it with current data, we can see an increase in the incidence of about 2% per hospitalized patient. This seeks to highlight once again the importance of the emergence of *C. difficile* infections (7).

Age is considered a primary risk factor for common forms of CDI (especially in young people, there are fewer relapses), but also for severe forms.

A feature that deserves a more detailed analysis is the role of general health condition, including dietary deficiency (hypoproteinemia) and the risk of CDI. Dietary deficiency (hypoproteinemia), the expression of biological aging, increases susceptibility to a variety of adverse events. Deficiency also results in increased exposure to health systems, including encounters with emergency departments, hospitalization and institutionalization, which has led to increased exposure to antibiotics, the most important risk factor for CDI.

The clinical picture of CDI causes a wide range of clinical manifestations, ranging from asymptomatic colonization of the gastrointestinal tract to diarrhea and colitis, which has the potential to progress to severe forms with sepsis, hemodynamic instability, and toxic dilation of the colon. This evolutionary method is associated with an increased risk of spontaneous perforation. A review of existing observational data has shown that 3% of patients with CDI will progress to fulminant colitis, which is associated with an 80% mortality rate.

Knowing the many factors that can contribute to the increased incidence of *C. difficile* infections in hospitals, it is necessary to intensify active surveillance, to promote the proper use of protective equipment and disinfection techniques, to ensure isolation spaces, to conduct visitor traffic control, to review antibiotic use policy, and to monitor measures undertaken in the case of 'serial' diseases.

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Availability of data and materials

All data and materials supporting the results of the present study are available in the published article.

Authors' contributions

ARM and ML designed the study. VL, RL, VM, NN, CD and CO consulted the literature and collected the bibliographical data. ARM and ML wrote the paper. TGC and VL reviewed the data and edited the manuscript. All authors read and approved the final manuscript for publication.

Ethics approval and consent to participate

The study was approved by the Ethics Committee of 'Victor Babeş' Hospital of Infectious Diseases and Pneumophysiology of Timisoara, Romania (approval number 4536/2021).

Patient consent for publication

Not applicable.

Competing interest

The authors declare that they have no competing interests.

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