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RIGA STRADINS UNIVERSITY

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Clostridium difficile Clinical and Molecular Epidemiology in Latvia

Summary of the Doctoral Thesis

Speciality – Clinical Microbiology

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Contents

1. Abbreviations

AGE - gel electrophoresis method

C.difficile - *Clostridium difficile*

CDAD - *Clostridium difficile* associated disease

CDI - *C.difficile* infection

MLST - Multilocus sequence typing

PaLoc - Pathogenicity locus

PMC - Pseudomembranous colitis

PCR - Polymerase chain reaction

ST-3 - sequence type - 3

tcdA - toxin A gene

tcdB - toxin B gene

TM - toxic megacolon

TT-1 - toxinotype - I

2. Introduction

Clostridium difficile is a Gram positive, anaerobic, spore forming, rod shaped and motile bacterium. It is considered to be one of the most frequent infectious agents causing gastrointestinal infections. In 1978 *C.difficile* was recognized as the main cause of pseudomembranous colitis. It may induce manifestations ranging from asymptomatic colonization of the gastrointestinal tract to severe diarrhea, pseudomembranous colitis (PMC), toxic megacolon (TM), intestinal perforation and death. All these severity levels are included in the term Clostridium difficile associated disease (CDAD). A patient at risk for *C.difficile* is an elderly person with a severe principal disease, who receives long - term inpatient treatment, receives antibiotics, antacid and antiulcer agents for a long time, who has had a gastrointestinal surgical intervention or any organ transplantation, which is always related to immune suppression.

CDAD rarely occurs without current or recent anamnestic antibacterial therapy. Almost all antibiotics are related to CDAD development. More frequently CDAD develops in patients, who have been prescribed ampicillin, amoxicillin, cephalosporins, clindamycin for treatment, less frequently – erythromycin, tetracycline, sulfanilamides, trimethoprim, fluorquinolones. Rarely CDAD is induced by vancomycin, metronidazole and parenterally administered aminoglycoside.

Strains of human disease inducing *C.difficile* produce two toxins – A and B, which causes damage to the intestinal mucous membrane, and simultaneously colitis develops. *TcdB* is a potent cytotoxin, but unlike TcdA, which is an enterotoxin, it does not cause damage to cell membranes. It is recognized that toxin A is the main virulence factor, but toxin B synergetically cooperates with it and is not too dangerous itself. The pathogenicity locus of *C.difficile* also comprises regulatory genes – *tcdD*, *tcdE* and *tcdC* which play their own role in the maintenance of both viability of bacteria and toxigenicity.

For the last two decades the incidence of these infections has been tending to grow. This increase is explained by better opportunities of diagnostics, extending consumption of antibiotics and increasingly higher contamination of health - care facilities with *C.difficile* spores. Especially toxic bacterial strains, able to produce several folds more toxins A and B that it had been observed earlier, have emerged.

Since 2002 special attention has been attracted to a hypervirulent strain of *C.difficile* which belongs to toxinotype III (ribotype 027) and initially spread in the USA and Canada, but now has already caused hospital outbreaks in Ireland, Belgium, France, Denmark, Austria and other countries. Patients infected with ribotype 027 *C.difficile* were forced to stay in hospital on average by 10.7 days longer and the mortality rate among them is significantly higher.

Taking into consideration that a very pathogenic *C.difficile* ribotype 027 causing very serious disease with incidence of 80 to 200 cases per 10 000 inpatients is currently spreading in the USA and Canada, also in Latvia the research of this microbe becomes very topical in order to perform not just diagnostics, but also ribotyping of this microorganism to start adequate therapy in case of necessity and perform infection control measures.

2.1. Objective of the Research

The objective of the given research is to determine the molecularly - genetic type of *C.difficile* circulating in Latvia, its role in the development of infectious diarrhoea in hospitals of different profiles and in patients of different age groups.

2.2. Task of the Research

1. To perform the phylogenetic analysis of *C.difficile* in Latvia, thus creating the phylogenetic tree of the strain.

2. To define and to describe the toxinotype and sequence type of *C.difficile* characteristic for conditions in Latvia.

3. To determine the presence of vancomycin resistance gene in the isolated *C.difficile* cultures.

4. To adjust the toxinogenic bacteria detection methods, methods of ribotype and toxinotype determination to the laboratory conditions, by the development of the testing algorithm.

5. To perform the clinical and epidemiological data analysis for CDAD patients with laboratory proved diagnosis.

6. To determine the role of *C.difficile* for diarrhoeas development for children stationed with diarrhoea and / or hemocolitis.

7. To develop the strain of isolated *C.difficile* stock culture and DNA criobanks of bacteria.

3. Materials and methods

3.1. Groups of the Research

The research consists of retrospective chapter, which includes the performed analysis of data obtained from histories of illness, and prospective chapter, which includes both the performed analysis of histories of illness and the performed sample analysis in laboratory.

Figure 1. Reflects the structure of the research.

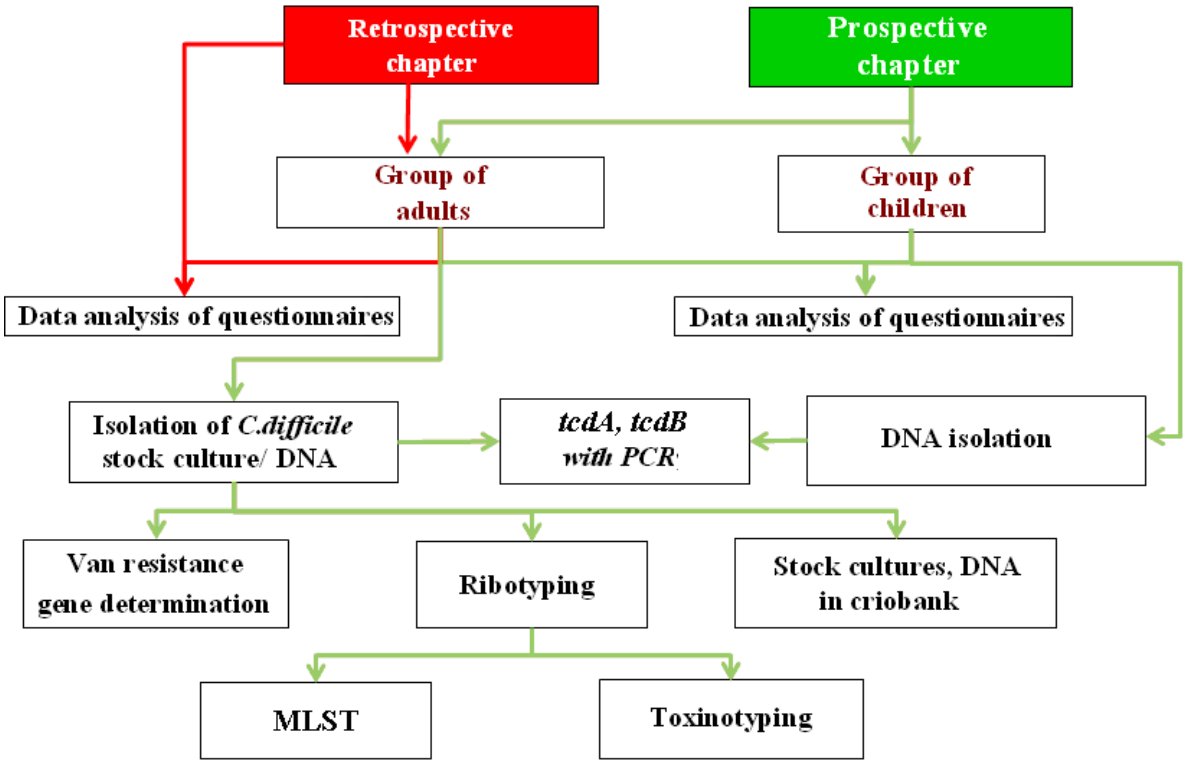

Figure 1. The structure of the research.

3.1.1. Criteria of children group selection

The objective of the work is to determine the prevalence of *C.difficile* for children treated in the Children's Clinical University Hospital (CCUH). A prospective descriptive research was realized in the period of time from October 2007 till March 2008.

An identification number was conferred to each child after his inclusion in the research. By questionnaire the data about the child's illness case record, demographic data and information about the history of antibacterial therapy. The research was made as a prospective and the data analysis based on Excel programme.

3.1.2. Criteria of adult group selection

For the patients data analysis the questionnaire was made, including the data about the patients, who were treated in the departments of P. Stradina CUH in the period of time from August 2006 up to the end of 2009. The survey was applied for previously approved cases, patients with *C.difficile* infection. The data about the illness case-record, demographical data, and information about the risk factors of illness development described in the literature, the detailed history of antibacterial therapy, and the sequence data of laboratory examination as well as the period of stay in the hospital and the manipulations performed in there. In the result 175 questionnaires were filled in and EpiInfo programme was used for data summarising and analysis.

During survey, basing on the methods described in the literature, the methods were developed and applied for the current laboratory conditions. In order to obtain the truthful result, which is suitable for the analysis, several methods of diagnostics, which were developed and applied simultaneously with sample collection and selection, were used during process for the analysis of one sample. The majority of samples were tested by three methods and the test material was obtained by two different methods.

500 coprocultures were tested within the survey and were sent to the laboratory from CUH "Gailezers", "Hospital of Traumatology and Orthopaedics" and P. Stradina

CUH with suspects of *C.difficile* infection. During period of time from August 2006 and end of 2009, basing on PCR and microbiological tests, 95 positive samples, which were ribotyped, were included in the survey. MLST detection as well as toxinotyping was used for the strains with equal ribotype by choice order. All the positive *C.difficile* strains were included in criobank. The survey is made as retrospectively – prospective.

4. Results

4.1. Children group

4.1.1. Demographic data analysis

187 children, treated in CCUH from October 2007 till March 2008 were included in the research. The age of patients varied from 3 days up to 16 years and 7 months with the average age of 2 years and 11 months. The majority of patients were aged from 12 months to 24 months – 28.9% (n=54) and from 0 to12 months – 25.1% (n=47).

4.1.2. Variations of diagnosis for sending to hospital and clinical diagnosis:

For 59.9% (n=112) of patients the diagnosis for sending to the hospital was acute gastroenteritis, for 9.1% (n=17) – the diagnosis of acute gastroenterocolitis, for 11.2 % (n=21) – acute enteritis, for 10.2% (n=19) – acute enterocolitis, for 8.0% (n=15) – functional digestive disorders, but 1.6% (n=3) had the other sending diagnosis with acute intestinal infection. 90.9% (n=170) of patients were sent to the hospital with the diagnosis of infection.

After the performed examination in the hospital and the made clinical diagnosis, the subjects of the research were divided as follows – 55.6% (n=104) of children had acute gastroenteritis, 13.4% (n=25) – acute gastroenterocolitis, 5.3% (n=10) – acute enteritis, 9.1 % (n=17) acute enterocolitis, 11.8% (n=22) – functional digestive disorders, but 4.8 % (n=9) had other principal diagnosis not related to acute intestinal infections. Hemocolitis was determined only for 9.6% (n=18) of patients.

After the confirmation of the clinical diagnosis the patients were treated in CCUH for approximately 4.1 bed - days (the minimum ratio was 1 day, the maximum – 90 days).

4.1.3. Evaluation of the progress of disease for stationary patients:

1.1% (n=2) of patients were stationed without any signs of exsiccosis, 79.1% (n=148) had exsiccosis of Stage I, 18.2% (n=34) – exsiccosis of Stage II, and 1.6% (n=3) of patients were stationed with exsiccosis of Stage III.

If we analyse the general condition of patients at the moment of stationing, we can say that the condition of 1.1% (n=2) of patients during stationing was easy, 91.4% (n=171) of patients were in medium severe condition, but 7.5% (n=13) of patients were stationed in generally severe health condition.

4.1.4. Evaluation of patients' clinical and laboratory data:

- The level of C - reactive protein (CRP) was determined for 97.3% (n=182) of patients and the mean CRP value for these patients was 22.2 mm/L (minimal - 0.1 mg/L, maximal - 187.8 mg/L). According to the analyses of the CRP value distribution by intervals, it is evident that the CRP value for 74.3% (n=139) of patients fits within interval of 0 to 25.0 mg/L.
- The total count of white blood cells was determined for 97.3% (n=182) of patients and the mean value was 12 553 x 10^6/L with minimal value of 1700 x 10^6/L and maximal value of 40 030 x 10^6/L. For the majority of patients – 21.9% (n=41) – the count of white blood cells fits within interval of 10 000 to 12 500 x 10^6/L.
- Rotavirus was determined in faeces for 96.2% (n=180) of patients and for 29.4 % (n=53) it was positive.
- Antigens of other viruses were determined in faeces for 11.8% (n=22) of patients, noroviruss was proven for 8 patients, but adenovirus was proven for 1 patient.
- Faecal bacterial inoculation was performed for 94.1% (n=176) of patients. In 86.6% (n=162) of cases the faecal inoculation was negative, in 6.4% (n=12) of cases *Salmonella enteritidis* was isolated, in one case - *Salmonella typhimurium* and in one case - *Shigella sonnei.* In three cases mixed infection was observed - *Salmonella*

enteritidis was isolated from faeces and rotavirus antigen was proven. In total, for 41.1% (n=77) of patients some of pathogens causing diarrhoea was proven.

- Blood culturing was performed for 3.7% (n=7) of patients. In one case the blood culture was positive - *Lactococcus lactis* was isolated.

4.1.5. Evaluation of acute intestinal infection risk factors

- 11.2% (n=21) of children had previous stationing in their case-record, but 12.8% (n=24) received antibacterial therapy at home prior to being sent to CCUH. The other family members were previously also stationed for one patient. 36.4% (n=68) of children attended preschool educational establishment or school.
- During stationary stage 82.9% (n=155) of patients did not receive antibacterial therapy, 10.7 % (n=20) of patients received antibacterial therapy with one antibacterial agent, 3.2% (n=6) had therapy with two antibacterial agents, 2.1% (n=4) received three antibacterial agents, but five and six antibacterial agents were received by one patient, respectively.

4.1.6. *C.difficile* prevalence for stationary children with clinical symptoms of diarrhoea:

DNA was extracted from faeces samples (n=248) of patients and the presence of the *C.difficile* toxin genes *tcdA/tcdB* was stated, using PCR gel electrophoresis method.

Relation of *C.difficile* to the other diagnosed causative agents, demographic data and clinical diagnoses:

- The *C.difficile* toxin A and B genes were simultaneously stated for 3.2% (n=8) of patients, but the *C. difficile* toxin B gene was stated for 1.2% (n=3) of patients (Figure 2).

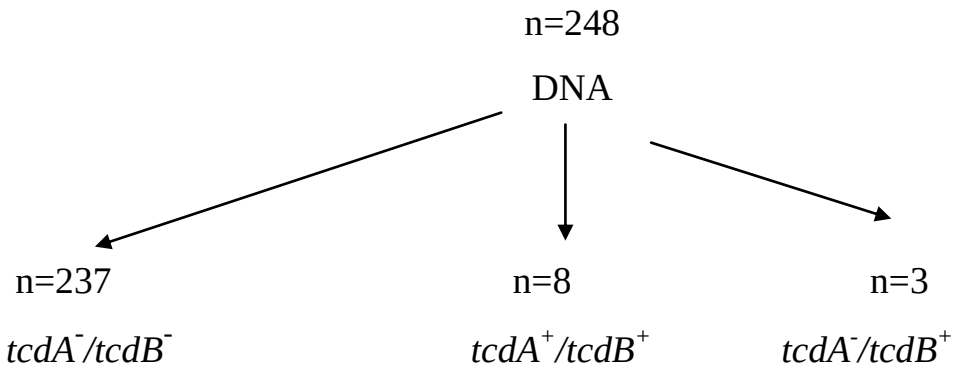

Figure 2. Number of the analyzed samples and distribution of positive finds

- In one case the simultaneous evidence of rotavirus antigen and presence of the *C.difficile* toxin A and B genes was observed, but in one case rotavirus and presence of the *C.difficile* toxin B gene were simultaneously proved. The other causative agents of intestinal infections were not proven for the other nine patients with the positive *C.difficile*.
- The average age of patients, for whom the toxin A and B genes were stated, is 31 month or 2 years and 7 months. On average they spent 2.6 bed-days in the hospital. Only one of these patients had received 5 day long antibacterial therapy with amoxycillin and 5 days - with cefadroxil before stationing. During stationary period the antibacterial therapy was received only by one patient – 1 day long therapy with ampicillin.
- The clinical diagnosis for 9 patients was acute gastroenteritis, the diagnosis of acute gastroenterocolitis was made for 1 patient and the infectious mononucleosis syndrome with functional digestive disorders was stated for 1 child. All these patients were stationed in a medium severe general condition.
- For patients with the positive *C.difficile* laboratory find the mean count of white blood cells in blood was 14 524 x 10^6/L and the mean CRP values were 17.42 mg/L. Comparing the total count of white blood cells and the CRP values for patients, no statistically credible differences between groups of patients with positive *tcdA/tcdB* and the group of patients with negative *tcdA/tcdB* ($p>0.05$) were found..
- Analyzing the positiveness of *C.difficile tcdA/tcdB* in relation to the age of patients, attending of the education establishments by a child, the previous stationing

and the stationing of the other family members, antibacterial therapy prior to the stationing, stationing and clinical diagnosis, severity of condition at the moment of stationing, the days spent in the hospital, the antibacterial therapy received in the hospital and its length, as well as the antibacterial agents used in Spirman range correlation analysis no statistically credible interconnections were stated ($p>0.05$).

- There is close relation of *C.difficile tcdA* and *tcdB* in this research (correlation coefficient is $rs=0.81$, $p<0.01$).

4.2. Analysis of adult group

4.2.1. Improvement of diagnostic methods of P.Stradins CUH CL microbiology department from 2005 to 2009

C.difficile detection methods used in P. Stradins CUH CL has changed during years, because the range of offered laboratory diagnostics methods is improved, thus allowing to choose the most precise and specific.

In year 2005 *C.difficile* antigen detection rapid test (Rapid pour *C.difficile*, France) was used for *C.difficile* identification in P. Stradins CUH CL. During the year 78 positive *C.difficile* samples were detected by this test. At the end of 2006, the antigen detection test was replaced by toxin A detection rapid test (*C.difficile* toxin A test, Germany).

Beginning this survey in August 2006, the advantages of this method were evaluated and therefore the comparison of two tests was performed. 50 sequent samples of faeces, which were sent to the laboratory with suspicion of *C.difficile* infection, were analysed by the two methods:

Method 1 - rapid test, Rapid pour *C.difficile*, by which *C.difficile* antigen was detected in faeces of patient.

Method 2 - based on PCR, by which *tcdA* and *tcdB* were detected.

In the result with rapid test, antigen for *C.difficile* was detected in 4 samples and toxinogens in 3 samples, the other samples were negative. Detection of toxinogens evidences about the presence of bacterium in the body and is more informative method as detection of antigen, which is the product, appeared in the result of bacterium

activity. PCR has high specifity, because virulence factors *tcdA* and *tcdB* were not detected for one sample, in which the antigen was found.

Method of *C.difficile* isolation was implemented in 2008, by cultivating on specific culture medium, which has preserved its priority up to the moment. During period of time from 2005 to 2009 several tests have been replaced and the current have improved in the range of *C.difficile* detection methods. The number of positive answers is closely related to the test principle, it can be observed in the period of time between 2005 and 2006, when antigen detection rapid test was replaced by toxin A detection test and the number of positive answers sharply reduced from 78 in 2005 to 22 in 2006. However after the implementation of stock test and PCR test, the number of positive answers increased. Figure 3 shows the dynamics of positive answer number in P. Stradina CUH CL microbiological laboratory from 2005 to 2009.

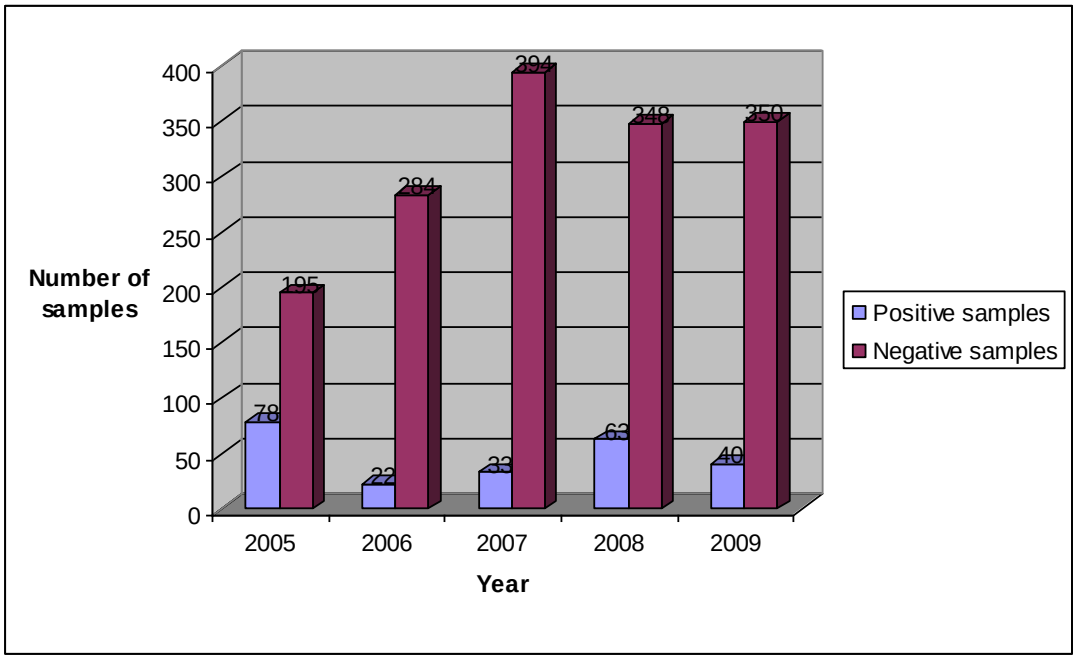

Figure 3. Relation of positive answers to negative answers from 2005 to 2009.

4.2.2. Demographic data analysis for patients with *C.difficile* infection

- From all the stationed patients women compiled 58.5% (n=103), men – 41.1% (n=72).
- The average age of patient is 60.12 years, minimal age – 20 years, maximal age – 90 years.
- Minimal time of patient stay in hospital is two days, but maximal – 90 days.
- The treatment in hospital has ended with discharge for 162 patients, but 12 have died.

4.2.3. Prevalence of *C.difficile* infection patients in P. Stradina CUH departments

The results are based on the questionnaire data analysis, which is processed by EpInfo program. The questionnaire is presented in addendum. 175 patients with laboratory proven *C.difficile* infection were included in the research during period of time from August 2006 up to the end of 2009.

In the relative patient division by the department profile, it was discovered that *C.difficile* as the originator of CDAD was proven in 43% cases of nephrology department patients.

4.2.4. Evaluation of possible risk factors for *C.difficile* infection patients

- The previous hospitalization was for 47.4% (n=83) of patients.
- Outpatient treatment with antibiotics was performed for 9.1% (n=16) of patients.
- Manipulations of gastrointestinal canal before the infection exacerbation were performed for 16.6% of patients (n=29).

- 4.6% of patients (n=8) *C.difficile* infection was already in case-record.
- Before the development of infection 35.4% (n=62) of patients (n=113) used antacids.
- As the other risk factors were detected chemical therapy n=1, imunosupression n=6, peritoneal dialysis n=12, haemodialysis n=3, chronic stomach ulcer n=1, ileus n=1
- 154 of 175 surveyed patients had the antibacterial therapy applied in hospital before the sample taking to *C.difficile* infection.
- 30.8% of patients (n=54) had antibacterial therapy with one and more antibiotics before CDAD symptom occurrence.
- In hospital treatment most often ciprofloxacin was applied (n=74). Ciprofloxacin is hinolon, which is effective *in vitro* against large amount of gram-negative aerobic bacteria as well as some gram-positive organisms.

4.2.5. Presentation of clinical symptoms for *C.difficile* infection patients

When analysing the clinical symptoms:

- Fever was observed in 56.6% cases (n=99) with average duration of 5 days.
- The main symptom for this infection is diarrhoea and it was observed for 93.1% of patients (n=163), pseudomembranous colitis was proven in 8.6% of cases (n=15), toxic megacolon – in 0.6% of cases (n=2).
- The other symptoms, like stomach ache, intoxication, nausea and sickness were observed in 9.7% of cases (n=17).

4.2.6. The analysis of bed days spent by CDAD patients in hospital

The amount of patients treated in P. Stradina CUH within the year is approximately 48000 and on average 5 bed days are spent in the hospital, which is two times less than on average in Latvia. CDAD patient on average spends 26 bed days in hospital, which is 4 times more than the average in P. Stradina CUH.

4.2.7. The analysis of therapy applied for *C.difficile* infection patients

- After the reception of positive result regarding *C.difficile* infection, the previous therapy was stopped in 15.5% of cases (n=27), changed in 35.4% (n=62) cases and was not changed in 49.1% of cases (n=86).
- In 97 cases after the reception of positive result regarding *C.difficile* therapy, metronidazole was ordered in 61.8% (n=97) and vancomycin in 18.5% (n=29).
- Type of medicine administration was oral for 7.6% of patients (n=12), intramuscularly for 74.5% of patients (n=117) and intravenously for 17.8% of patients (n=28).
- Prebiotic treatment was applied in 35.4% of cases (n=62). Yoghurt capsules, enterol, acidophilus bacteria and linex were used.

4.2.8. Analysis of patients, for whom *C.difficile* re-infection was developed

- *C.difficile* was detected in each time of hospitalization within several months for 5 patients.
- All patients had severe kidney affection and stayed in hospital often and for long time.
- Two patients died in the last hospitalization.

4.2.9. The analysis of vancomycin resistance gens

8 patients were discovered within the survey after filling in the questionnaire part about the applied treatment of *C.difficille*, for whom the therapy of vancomycin was changed to metronidazole within CDAD treatment process. Such change of treatment tactics is turned against *C.difficile* strains resistance to vancomycin. In order to obtain the laboratory assertion about the circulation of strains resistant to vancomycin in the hospital, six *C.difficile* strain from the patients with suspicion about *C.difficile* bacterium resistance to vancomycin were chosen. PCR with specific *van* primer pairs and the sequent specific electrophoresis of resistant gene fragments was performed for all six samples. Specific *van* gene was detected for five samples. Enterococcus also has *van* gene, like *C.difficille* bacterium, because they have plasmids, which can pass this gene resistant to vancomycin to the other Gr+ bacteria – clostridium, staphylococcus. It made to clarify if the sample of clostridium has genes of enterococcus. The test with specific primers on enterococcus detected that all the clostridium positive samples have enterococcus gene signals in all six samples.

4.2.10. Phylogenetic analysis of *C.difficile* strains

Basing on PCR and microbiological tests, 95 toxinogen *C.difficile* strains were included in the survey. Ribosomal gen location in the certain strain was determined for them.

The results of bacteria electrophoresis were input in BioNumerics software, in which the tree of ribotypes was located (Figure 4).

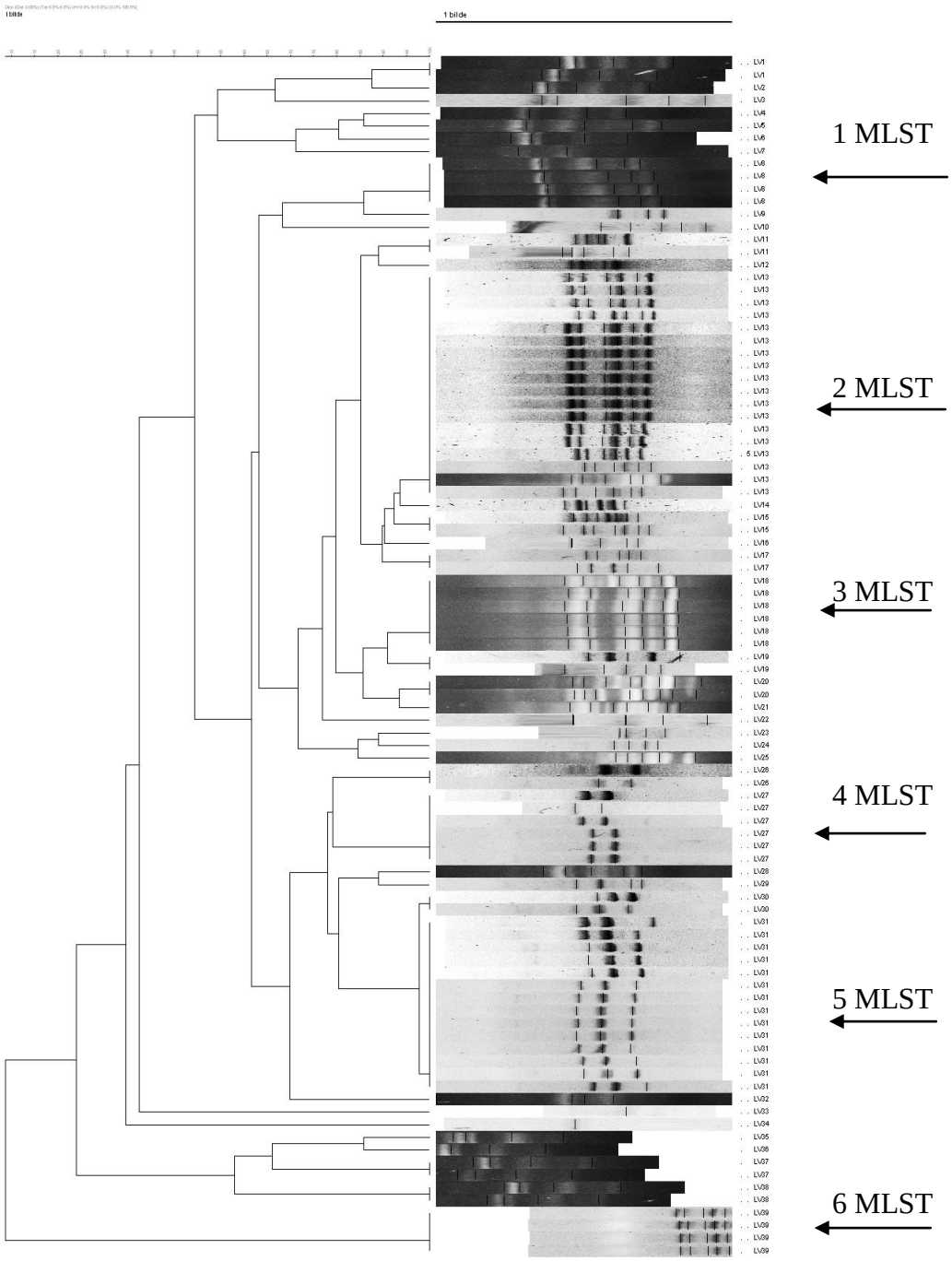

Figure 4. Filogenetic tree of *C.difficile* isolates.

39 ribotypes, which united 95 *C.difficile* strains, were discovered in Latvia. 6 main ribotype groups were marked and the largest group unite 18 strains. This group of ribotype has the code LV13.

4.2.11. MLST typing

MLST typing was applied to six selectively chosen cultures from the biggest groups of opposite branches of the filogenetic tree acquired as a result of ribotyping. On MLST scheme all six samples from 7 genes have identical allele combination and they belong to one sequence type ST - 3 (Table 1).

Table 1. MLST results for *C.difficile* Latvian isolates.

sample	*aroE*	*ddL*	*dutA*	*gmK*	*recA*	*solA*	*spI*	ST
140	2	2	2	1	1	5	1	3
107	2	2	2	1	1	5	1	3
177	2	2	2	1	1	5	1	3
189	2	2	2	1	1	5	1	3
190	2	2	2	1	1	5	1	3
192	2	2	2	1	1	5	1	3

4.2.12. Analysis of toxinotype variations

Amplification and restriction with specific primer pairs and sequent gel electrophoresis of genome fragments PL1, PL2, PL3, PL4, A3 and B1 was performed for six cultures selectively chosen from the largest groups of the last branches of phylogenetic tree obtained in the result of ribotyping. Figure 5 presents the sample of toxinotyping electrophoresis for one sample.

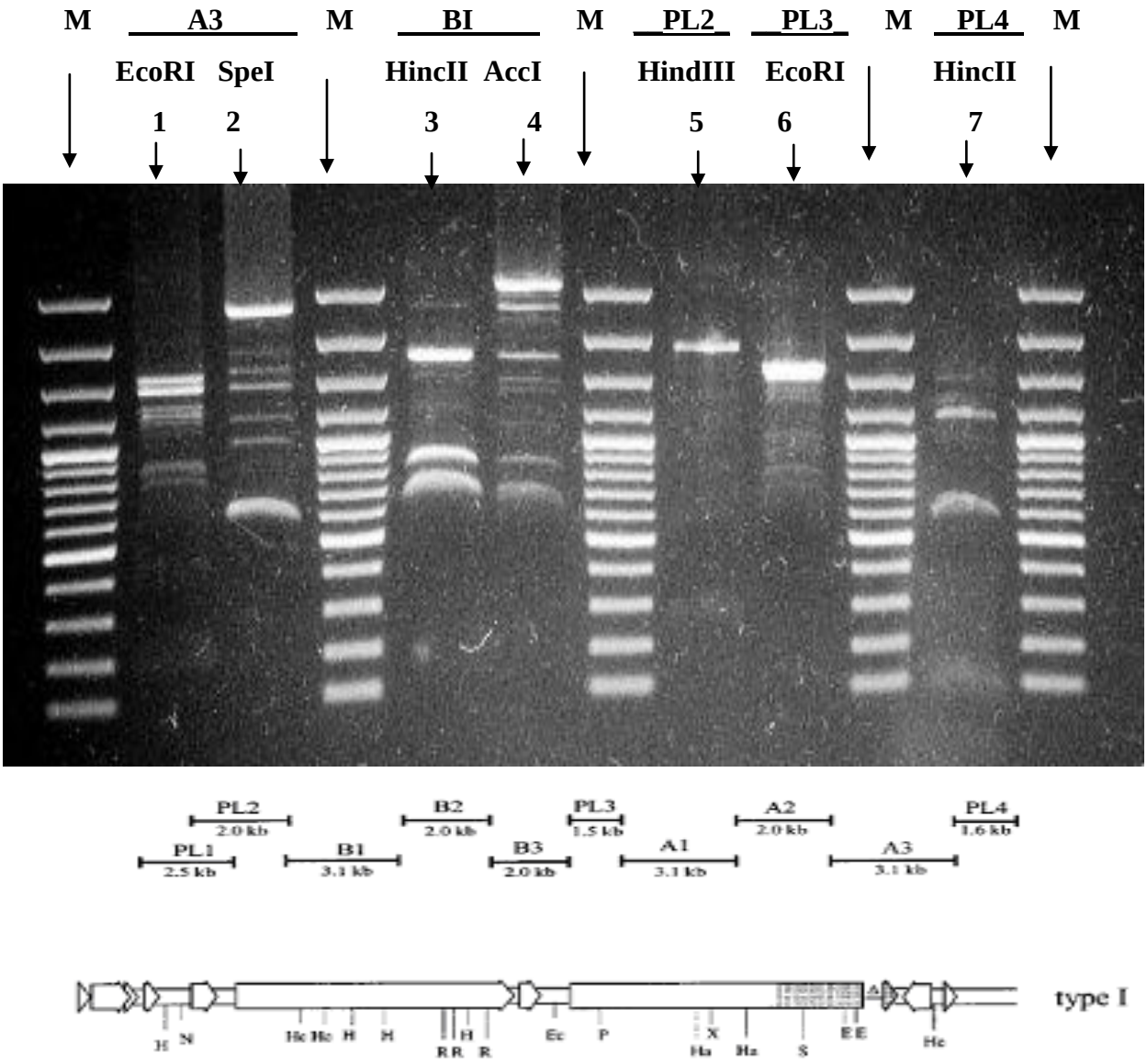

Figure 5. M – marker, 1-7 genome fragments after restriction with the relevant restrictase, A3, BI, PL2, PL3, PL4 – genome fragments, EcoRI, SpeI, HincII, AccI, HindIII - restrictases.

Linking the data of gel electrophoresis with the results available in literature, we obtain one sight of characteristic genome PaLoc, which complies with the first toxinotype. The characteristic genome has A3 fragment deletion, which corresponds to toxinotype I (TT - 1).

5. Guidelines for *C.difficile* testing

Regulations of material collection in the hospital and regulations of material delivery to the laboratory for the identification of *Clostridium dificille*

1. Material – faeces of patient, who is suspicious about diarrhoea caused by *C.dificille*.
2. Time of material delivery to the laboratory – 1 h after its collection or within the week, if the material is stored in the freezer.
3. Form of material delivery to the laboratory – container with brown cap and spoon of 400 mg – two full spoons.
4. The container must have the identification code of the patient.

Algorithm of *C.difficile* microbiological and molecularly biological test

Algorithm is developed, basing on the laboratory diagnostic methods developed during the work, and adapted to the possibilities of P. Stradina CUH CL Microbiology and Molecular biology and genetics departments.

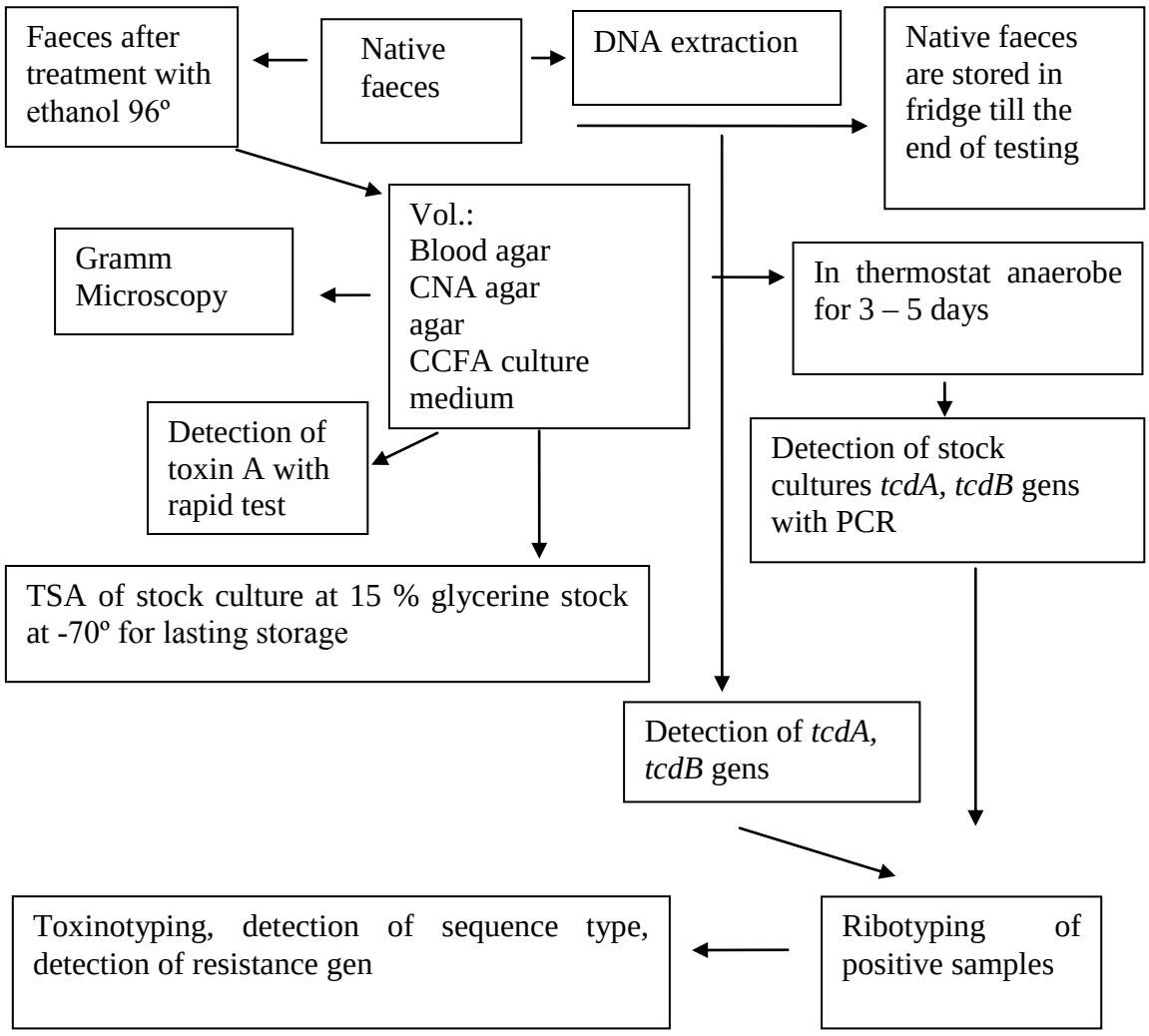

Figure 6. Algorithm of *C.difficile* microbiological and molecularly biological test.

6. Discussion

The data about the *C.difficile* role in diarrhoea development for children stationed in CCUH from October 2007 to March 2008 were analyzed in the research. The *C.difficile* toxin A and B genes were simultaneously stated for 3.2% (n=8) of patients, but the *C.difficile* toxin B gene was stated for 1.2% (n=3) of patients. These data differ from the literary data about the frequency of the *C.difficile* diarrhoea cases in hospitals, which is possibly related to the various research population; it is considered that 80% of all the *C.difficile* diarrhoea cases has happened in the age group above 65.

Studying the children population we can find that there are children, for whom the *C.difficile* toxin genes were found, but when consulting with their attending doctors, it is obvious that mild clinical diarrhoea, which is treated symptomatically and does not request more radical therapy, dominates in their clinical presentation. It is also important that the children have only the toxin B gene and have no toxin A gene. Basing on the previous scientific researches it is known that the toxin B causes complications only in synergistic cooperation with the toxin A, but if the toxin A is not present then it explains why the children do not have *C.difficile* infection in their clinical presentation. According to the literary data it is known that the children under two years can be *C.difficile* carriers.

The average age of patients with the positive *C.difficile* toxin A and B genes find was 31 month, which slightly differs from the data provided by other authors – the average age of patients with the proven *C.difficile* B toxin in the research of Klein et al. was 18 months.

In the literature CDAD expressions are described as very variable, beginning with medium severe to severe diarrhoea with tiredness, stomach aches, nausea and vomiting. According to the results of our research it is hard to expand on the clinical symptoms of CDAD. The group of the *C.difficile* patients is small and obviously these patients were stationed with other clinical presentation of acute intestinal infection, but the proven *C.difficile* may possibly be considered as a normal representative of microflora. Leukocytosis can be the evidence of the *C.difficile* infection in paraclinical parameters. *Bauza* et al. mention that leukocytosis of higher than 15 000 is the

additional sign of the *C.difficile* infection. Among the research patients with the proven positive *C.difficile* toxin A and B genes the mean count of white blood cells was 14 524 x 10^6/L, and no statistically credible relation between the total count of white blood cells and positiveness of the *C.difficile* toxins was stated ($p>0.05$) using Spearman range correlation analysis.

Antibacterial therapy is considered to be the one of the most important risk factors of the *C.difficile* infection. In the group of patients with the positive *C.difficile* toxin genes, only 1 of 11 patients received the antibacterial therapy prior to stationing, whereas the antibacterial therapy was prescribed only to one stationary patient. Analyzing the relation between the positiveness of the *C.difficile* toxins and the antibacterial therapy prior to stationing with Spirman range correlation analysis technique, no statistically credible relation was stated ($p>0.05$). In the research of Polish authors, who analyzed the positiveness of the *C.difficile* A toxin in children with acute diarrhoea, also no open relation between the previous antibacterial therapy and the proven *C.difficile* toxins was stated.

Analyzing the results obtained in this research no unambiguous conclusions about the occurrence of the *C.difficile* diarrhoea for children can be made. Evaluating the occurrence of the *C.difficile* toxin A and B genes for children the possibility of asymptomatic carriage, which is most frequent for infants – 40 – 60% of cases, it reduces after age of one, but is also possible for adults – its frequency varies in the researches of different authors, from 2% in Sweden to 15% in Japan, should be also taken into account.

During the analysis of adult patient data by rapid test, *C.difficile* antigen was detected in 4 samples and toxinogen was detected in 3 samples. The other samples were negative. Detection of toxinogen certifies about the presence of bacterium in the organism and is more informative method than the detection of antigen, which is the product, appeared in the result of bacterium and is maintained even after the elimination of gen from the organism.

The average age of the patient was 60.12 year, the minimal age – 20 years and the maximal age – 90 years. In *Lowy et al* (2010) survey the eldest patient was 101 year old and the average age was 64 years.

CDAD risk factors described in the literature were included in the evaluation as the risk factors for our patients. The analysis show: 47.4% (n=83) of patients had the

previous stationing. Outpatient treatment with antibiotics was performed for 9.1% (n=16) of patients. Manipulations of gastrointestinal canal before the infection exacerbation were performed for 16.6% of patients (n=29). 4.6% of patients (n=8) *C.difficile* infection was already in case-record. Before the development of infection 35.4% (n=62) of patients (n=113) used antacids. As the other risk factors were detected chemical therapy n=1, imunosupression n=6, peritoneal dialysis n=12, haemodialysis n=3, chronic stomach ulcer n=1, ileus n=1. 154 of 175 surveyed patients had the antibacterial therapy applied in hospital before the sample taking to *C.difficile* infection. 30.8% of patients (n=54) had antibacterial therapy with one and more antibiotics before CDAD symptom occurrence. In hospital treatment most often ciprofloxacin was applied (n=74). Ciprofloxacin is hinolon, which is effective *in vitro* against large amount of gram-negative aerobic bacteria as well as some gram-positive organisms. Ciprofloxacin has characteristic of rapid bactericide impact, when inhibiting DNA gyrase, in the result of which DNA synthesis inhibiting occur. Ciprofloxacin is rapidly and effectively absorbed after internal administration. There is linear correlation between the dose and its concentration in plasma. Empiric treatment with wide range of antibiotics (fluoroquinolone) and potential further treatment of 10 – 14 days, basing on the urine culture and sensitivity is applied for patients with severe infections of urinary tract (UTI). The precondition in order to avoid the failure during the treatment and occurrence of resistance is the adequate reaction of patient and dosing. Literature data also approves that the patient of *C.difficile* is a man in years with severe principal illnesses and variable range of applied medicines during treatment. *O'Connor et al* (2004) performed the study in Minster region, geriatric clinic from 1992 to 2002 and proved that the intravenous usage of cephalosporin is the largest risk for CDAD development for elder people and recommended to change the policy of antibacterial therapy already in the beginning. The author recommended to use piperacillin – tazobactam intravenously or moxifloxacin orally for treatment of pneumonia and glomerulonephritis.

Minimal time of patient stay in hospital is 2 days, but maximal – 90 days. *Barbut et al*., 1996 mentions that the stationing, which is longer than 1 week is the main reason of CDAD infection development. The average stationing time should be reduced in P. Stradina CUH departments, because as longer the patient stays in the hospital environment as greater is the risk to be infected by *C.difficile* spores. The isolation

principle of infected patients should be observed, because the possibility to be infected from patient to patient is very big. The analysis by departments clearly demonstrates that the biggest number of confirmed *C.difficile* patients is in the Nephrology Department. All the principles of hygiene and isolation of patients were observed in this department and no infection exacerbation was detected, but all the cases were individual in time. *C.difficile* possibly has the larger prevalence also in the other departments, but the analysis is required rarely and the diagnosis passes by. *Barbut et al*., in 1996 mentioned that the test on *C.difficile* in hospitals of France is required rarely and according to authors consideration it is related to the lack of knowledge for staff regarding this infection.

Prebiotic treatment was applied in 35.4% of cases (n=62). Yoghurt capsules, enterol, acidophilus bacteria and linex were used. Nowadays, *C.difficile* bacterium is resistant against all the antibiotics except metronidazole and vancomycin, but as its genome is capable to accumulate the mutations rapidly and is capable to change, it may not be excluded that the bacterium may become resistant against these antibiotics.

Within the survey six *C.difficile* samples were tested with enterococcus specific *van* resistant coding primers and these genes were detected in *C.difficile* samples. The test with specific primers on enterococcus detected that all the clostridium positive samples have enterococcus gene signals in all six samples. Genome of *C.difficile* bacterium contains enterococcus *van* resistance gens. The question is – if it is contracted in the process of infection or it is *C.difficile* gen, which does not give the positive result for patients, who are treated with vancomycin. Literature data testifies that the resistance of *C.difficile* strain against vancomycin is not proven and the resistance against metronidazole occurs rarely. Performing the survey in the clinic in Germany enterococcus gens resistant to vancomycin were detected in 17 samples from 164 faeces samples positive to *C.difficile*. As enterococcus have plasmids, which can pass the resistance genes both to staphylococcus and clostridiums, then it is possible that in the result of gene administration, the clostridium clones resistant to vancomycin may appear.

39 *C.difficile* ribotype strains with one characteristic ST are detected in Latvia. 6 main ribotype groups are marked and the largest group unites 18 strains and had the ribotype group code LV13. The names of ribotypes are formed by turns, placing LV - Latvian State before the number. In the world also the ribotype names are formed in

every laboratory according their own principles, because the united standardization may not be related to ribotyping in all laboratories. On the 1st of January 2010 www.pasteur.fr/cgi-bin/genopole/PF8/mlstdlent.plfile=Cdifficile2_isolates has summarized 57 ribotypes, including also the undifferentiated strains as ribotypes. ST - 3 is widespread in Latvia, but in the world range it takes the 6th place by prevalence. 0 toxinotype is the most widespread from the eleven known toxinotypes. TT - 1 is discovered in Latvia, but in the data basis of www .pasteur .fr/cgi-bin/ genopole/PF8/mlstdlent. plfile= Cdifficile2_isolates only one case is registered.

C.difficile infection monitoring as well as the national guidelines was developed in the Netherlands in 2005. 2758 samples were ribotyped from April 2005 till June 2009. In 2009 ribotype of 027 type reduced till 3.0%, but ribotype 001 expanded up to 27.5%, 014 type was in 9.3% of cases, but 078 ribotype expanded up to 9.1%.

All CDAD patients need individual approach, developing the most optimal and effective regime of therapy. As CDAD is severe infection illness and it requires the usage of many and different medicines: antibacterial, cardiologic, anaesthetics and resolvents, etc., the presence of clinical pharmacist during therapy process could significantly optimize the patient therapy, as well as to reduce the treatment costs.

7. Conclusion

1. 39 ribotypes, which united 95 *C.difficile* strains, were discovered in Latvia.

2. The third sequence type of bacterium (ST-3) and the first toxinotype (TT-1) is characteristic to the region of Latvia.

3. Patients with confirmed *C.difficile* infection, the DNA samples contain *E.faecalis* and *E.faecium* vancomycin resistance genes. Latvian has been found vancomycin resistant strains of *C.difficile*.

4. Algorithm of *C.difficile* diagnostics unites the latest methods, which are approbated to laboratory conditions.

5. *C.difficile* infections develop on the background of severe chronic illnesses in patients, who receive the antibiotics of fluoroquinolone or cephalosporin group.

6. The positiveness of *C.difficile tcdA/tcdB* for children in relation to the age of patients, attending of the education establishments by a child, the previous stationing and the stationing of the other family members, antibacterial therapy prior to the stationing, stationing and clinical diagnosis, severity of condition at the moment of stationing, the days spent in the hospital, the antibacterial therapy received in the hospital and its length, as well as the antibacterial agents used in Spirman range correlation analysis no statistically credible interconnections were stated ($p>0.05$).

7. Criobank of *C.difficile* provides the formation of strain stock culture and DNA collection in Latvia.

8. Theses relating to the reserch subject

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9. Publications relating to the reserch subjekt

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4. Bauer MP, Notermans DW, van Benthem BHB, Brazier JS, Wilcox MH, Rupnik M, Monnet DL, van Dissel JT and Kuijper EJ, for the ECDIS Study Group. Clostridium difficile infection in Europe: the first pan-European hospital-based survey. *The Lancet, 2010*; 377, No.9759:63-73.

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