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CLINICAL - EPIDEMIOLOGICAL IMPACT OF VACCINATION IN ROTAVIRAL INFECTION IN INFANTS

322.01 – Pediatrics and neonatology

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THE RESEARCH CONCEPTUAL FRAMEWORK

Actuality and significance of the research topic

Rotaviruses (RV) are the most common causes of acute gastroenteritis (AGE) worldwide, affecting 95,0% of children under the age of five. Globally, RV infection is estimated to cause 3,6 million episodes of AGE per year [1, 2]. Prior to the worldwide rotavirus vaccine implementation, more than 2 million children with rotavirus-related AGE were hospitalized annually [2, 3].

By the age of five, almost every child has suffered from rotavirus infection (RVI), which is the leading cause of severe diarrhea and dehydration in infants worldwide. In low-income countries, the mean age of primary RV infection occurs in infants aged 6 - 9 months (in 80,0% of infants under 1 year), whereas in high-income countries the first episode commonly occurs at the age of 2-5 years, the most affected being the infants (in 65,0% of cases) [3, 4].

Despite considerable progress, diarrheal disease remains the fourth most common cause of mortality and the second most common cause of morbidity worldwide, in children under the age of 5 years. Rotaviruses are associated with approximately one third of all severe diarrheal diseases in young children, whereas the annual RV-related mortality rate has been recently estimated to range from 453,000 (2008), 197,000 (2010) to 173,000 (2011) [1, 3, 5].

Since 2009, the World Health Organization (WHO) has recommended that rotavirus vaccines should be included in all national immunization programs of each country, being considered a public health priority [2, 4, 6].

The morbidity assessment of children under one year shows an increased incidence rate of the most common infections, thus influencing the health of "future generations"- society. In cases of acute gastroenteritis, primary rotaviruses are acquired by contact with a diseased person [1, 4, 7]. Rotaviruses are also found in fecal filtrates of children with gastroenterocolitis of unknown etiology, which have a major role in identifying severe gastrointestinal infections, due to difficulty in establishing the disease etiology [3, 5].

The rotavirus infection has been a current issue throughout the last decades, since the virus discovery, which exhibits an increasing incidence, particularly among children under 5 years old [8, 9]. Each child can bear from one to several disease episodes, most commonly in the first 5 years of life, characterized by a high incidence of severe cases and followed by complications, if no treatment has been applied. The clinical features of rotavirus infections includes intestinal and non-intestinal disorders, involving not only the lining of the gastrointestinal tract, but the other systems, as well. In infants, the RV severity degree, particularly, depends on the genotype and phenotype of health condition, which also determines the severity of dehydration and toxic syndromes [9, 10].

The present study was aimed at establishing clinical and evolutionary impact of rotavirus infection (RVI) in vaccinated and unvaccinated infants, as well as the genotype and phenotype variety within the Republic of Moldova.

The major scientific works that have studied the aspects of rotavirus infections in children have been carried out by Konstantinos Karampatsas, 2018; Nigel A. Cunliffe, 2010; Hip I. A., 2014; Vesikari T. et al, 2014. However, there are relatively few sufficient literature data regarding both the clinical and evolutionary impact, as well as the genotype and phenotype variety of rotavirus infections among infants [5, 26, 27].

Defining the current research situation and relevant issues. The acute diarrheal disease (ADD) features in the top 5 causes of death in children under the age of 5 worldwide. Therefore, the World Health Organization (WHO) created a program to combat diarrheal diseases at this age [4].

Diarrheal disease in infants, characterized by acute enteritis and gastroenteritis, severe forms of acute dehydration syndrome (ADS) and toxic shock syndrome (TSS), has commonly an unknown etiology in more than a third of cases.

According to World Health Organization data, gastrointestinal tract infections cause approximately 10027 deaths per year across Europe, particularly in children up to 5 years old. Thus, the total number of children suffering from rotavirus infections is 2,8 million annually, of which more than 2000 children die and 87000 are hospitalized within European countries [5, 6].

The purpose of the present study: estimating the clinical-epidemiological impact of infant vaccination against rotaviral infection depending on the genotypes of rotaviruses identified in the Republic of Moldova.

The general objectives:

1. To study and identify clinical-epidemiological and evolutionary features of rotaviral infection in vaccinated and unimmunized infants depending on rotavirus genotypes.
2. Identification with the help of evaluation of the intestinal microbiota particularities and the severity of the disease in infants depending on the application of the rotavirus vaccine.
3. To study and establish the correlation between the hydroelectrolytic changes of the anionic gap and the clinical manifestations in the infants in the study.
4. To evaluate the impact of the implementation of sentinel surveillance measures on the evolution of the clinical-epidemiological features of rotaviral infection in infants.

The research hypothesis is that the genotype and phenotype of rotaviruses determine the severity of gastroenteritis in infants by the presence of dehydration and toxiinfectious syndrome of varying degrees.

The general research methodology is based on the study and identification of the clinical and epidemiological, as well as on RVI evolutionary features in infants with different genotypes. It aims at highlighting the intestinal flora and studying the correlation between the hydro-electrolytic alterations of the anion gap and the pH values in biological media among infants with rotavirus infections. Children with acute diarrhea (defined as ≥3 loose stools over a 24-hour period and the disease onset <7 days before admission) were monitored, according to the protocols recommended by WHO.

A prior parents' written consent was carried out, followed by anamnesis, clinical examination and paraclinical investigations of the children. The immunization records are stored and provided by the health centers or at the family doctor's offices where the vaccine are commonly administered. The immunization records were identified according to the names, gender and birthdate of the children included in the study. The investigation methods used within this study were as follows: the virological examination of rotavirus infections via the serological ELISA test (Enzyme-Linked-Immunosorbent-Assay), the PCR gene amplification reaction was used for genotyping, the microbiological examination of the intestinal flora and antimicrobial susceptibility testing was carried out to determine the disease-causing agents. Stool specimen were collected within 48 hours of admission. The samples were stored at 2° C - 8° C before being transferred to the microbiological laboratory of the National Agency for Public Health on a weekly basis. The RV test was performed using a commercially available immunoenzyme assay

(ProSpecT; Oxoid, Cambridge, UK). Statistical data processing was performed via the following programs: SPSS Statistics for Windows, version 20 and Microsoft Excel 2010, and application of *SPSS statistical software set v18.0, QUANTO v1.2, Review Manager (RevMan) v5.1, GMDR Beta software 0.9.*

The scientific novelty.

- Investigated the multilateral features of rotavirus by applying the systemic principles in acute diarrheal disease among children under one year of age.
- Developed an early and differential diagnosis method, based on Vesikari score system and metabolic acidosis, in different RV genotypes and phenotypes among infants.
- Evaluated the severity of the rotavirus infection caused by different genotypes both in immunized and non-immunized infants.
- Improved and conducted the sentinel surveillance system for rotavirus infections among infants, together with other subdivisions (the microbiological laboratory and the Department of Epidemiological Surveillance of Foodborne and Waterborne Diseases) regarding the clinical features of the studied issue.

The scientific issue solved within this thesis:

- We've made a partnership with the Microbiological Laboratory of the National Agency for Public Health and the Department of Epidemiological Surveillance of Foodborne and Waterborne Diseases and joined the sentinel surveillance system, monitored by the regional office of the World Health Organization, referring to our clinical activity and relevant biomaterial collection. The study used molecular biology tests to identify the genotype and phenotype of rotavirus infection in infants, within the Reference Laboratory of the World Health Organization in Minsk.
- The present research determined the clinical and evolutionary characteristics, as well as the disease severity based on Vesikari score system regarding the genotype and phenotype variety of viruses.
- The clinical and paraclinical investigations of patients with different genotypes and phenotypes, resulted in developing early and differential methods for diagnosing rotavirus infection based on Vesikari score and metabolic acidosis in infants with rotavirus infection (Patent No. 9365, dated 04.07.2019 and Certificate of Innovator no. 5716, dated 10.06. 2019).

Theoretical significance of the research.

The theoretical significance of the research consists of the theoretical argumentation of the clinical-epidemiological impact following the vaccination of infants against rotaviral infection, etiologically caused by different genotypes.

The systemic approach used in the study of rotavirus infection among infants was improved by early and differential diagnosis methods, by determining the clinical and evolutionary particularities of the disease in immunized /non-immunized children, according to the genotype and phenotype variety, as well as by implementing the sentinel surveillance system for this infection.

The applicative value of the research:

- Contributions to the strengthening of pediatric instructions in the problems of approaching intestinal rotavirus infection in children under one.
- Development of an algorithm for early and differential diagnosis of rotaviral infection in infants, based on the study and evaluation of clinical and epidemiological features.

- Preparation and implementation of the new national clinical protocol "Rotavirus infection in infants" (Scientific Seminar on Pediatrics and Neonatology of Nicolae Testemitanu SUMPh PI, dated from 05.03.2020).
- Strengthening IRV prophylaxis through objective evidence of information about an effective vaccination clinic and the need for prophylactic vaccination with the Rotarix rotaviral vaccine in the country's National Immunization Program.
- Use of data obtained in the pre-university and university training process, of students and medical staff in accordance with the issue addressed.

The main scientific findings forwarded to PhD. thesis defense:

1. This present study assessed the situation regarding the variety of genotype and phenotype in terms of clinical and evolutionary patterns, early diagnosis and differential features of RV-related acute diarrheal disease in immunized and non-immunized infants.
2. It has highlighted the importance of research findings on the RV genotype and phenotype variety in acute diarrheal disease among infants.
3. The data, obtained via molecular biology tests, were used for determining the appropriate choice of anti- rotavirus vaccine among infants.
4. This study has completed the epidemiological measures of the sentinel surveillance system for rotavirus infections in infants, as being referred to clinical competences.

Implementation of research findings:

I Preparation and implementation of the Clinical National Protocol "Rotaviral Infection in Infants" (Scientific Seminar on Pediatrics and Neonatology of Nicolae Testemitanu SUMPh PI, dated from 05.03.2020).

II The study has implemented the early diagnosis method based on Vesikari score system for RV-diseased infants with acute diarrheal disease (Innovator Certificate no. 5716 from 10.06.2019) within the Department of Acute Diarrheal Diseases in infants and at the Pediatrics Department of „Nicolae Testemitanu“ SUMPH.

III The practical approach to differential diagnosis was carried out, based on the assessment of the acid-base balance and the anion gap measurement in RVI infants at the Department of Acute Diarrheal Diseases in infants , the Department of Resuscitation and Intensive Care , and the Pediatrics Department Clinic of Nicolae Testemitanu " SUMPh (Positive Decision of the Patent No. 9365 dated from 04.07.2019).

Research findings approval. The research findings were presented and discussed at various national and international scientific events such as: The annual scientific conference of Nicolae Testemitanu SUMPh PI , Chisinau, October 19-21, 2016; National Conference with International Participation The Days of Pediatrics in Iasi “N.N.Trifan”, October 20-22, Iasi 2016, XXIX edition and October 5-7, Iasi 2017, XXXI Edition; International Pediatrics Conference dedicated to the Year of Nicolae Testemitanu , organized by the Pediatrics Society of Moldova in collaboration with the Romanian Pediatrics Society, September 9-10, Chisinau 2016 (thesis); The 8th International Medical Congress for Students and Young Doctors, “MedEspera”, May 3-5, Chisinau 2018.

The study was carried out on the basis of the Ph.D. topic approval at the Meeting of the Department of Pediatrics, „Nicolae Testemitanu” SUMPh PI, dated from 21.12.2016, minutes no. 7, at the Meeting of the Scientific Seminar on Pediatrics and Neonatology, „Nicolae Testemitanu” SUMPh PI, dated from 15.03.2017, minutes no. 02, and at the Scientific Council of „Nicolae Testemitanu” SUMPh PI, dated from 15.03.2017, minutes no. 4 / 7.10.

The thesis was discussed, approved and recommended for defense at the meeting of the Department of Pediatrics of "Nicolae Testemitanu" State University of Medicine and Pharmacy, from the Republic of Moldova (minutes no. 4 of 18.12.2019) and at the Scientific Seminar on Pediatrics and Neonatology of Nicolae Testemitanu SUMPh PI, dated from 4.03.2020, minutes no. 2.

Publications related to PhD thesis. The obtained scientific results were published in 6 complete articles and 10 theses, 7 posters, 1 innovator certificate and 1 patent.

Volume and structure of the thesis. The thesis was carried out according to the traditional complex framework, including 106 pages with introduction, 4 chapters, with 3 to 5 sub-chapters, general conclusions, recommendations, and 137 bibliographic sources. The thesis comprises 13 annexes rendered on 11 pages. Iconography includes 23 tables and 41 figures, amounting for 22 pages (25% of the basic volume of the Ph.D. thesis).

Keywords: rotavirus infection, infants, genotype, impact, immunization

The thesis obtained the positive opinion of the Research Ethics Committee (minutes No. 54 of February 13, 2017).

THE CONTENT OF THE PHD THESIS

1. CONTEMPORARY ASPECTS ON ROTAVIRUS INFECTIONS IN INFANTS

Rotaviruses (RV) are the most common causes of acute gastroenteritis (AGE) worldwide, affecting 95.0% of children under the age of five. Globally, RV infection is estimated to cause 3.6 million episodes of AGE per year [5]. Prior to the worldwide rotavirus vaccine implementation, more than 2 million children with rotavirus-related AGE were hospitalized annually [6].

Most children have suffered from rotavirus infection (RVI), which is the leading cause of severe diarrhea and dehydration in infants worldwide. In low-income countries, the mean age of primary RV infection occurs in infants aged 6- 9 months (in 80.0% of infants under 1 year), whereas in high-income countries the first episode commonly occurs at the age of 2-5 years, the most affected being the infants (in 65.0% of cases) [3, 4, 7, 10].

Prior to anti-rotavirus immunization (implemented in 2008), the World Health Organization (WHO), there were estimated approximately 453,000 deaths among children with rotavirus gastroenteritis (RVGE) worldwide. This index made up around 5.0% of the reported childhood deaths, with a specific mortality rate of 86 deaths per 100,000 children aged under 5 years old [9]. About 90% of rotavirus deaths occur across Africa and Asia, being closely associated with poor healthcare services. At country level, the highest mortality rate ranges from 474 / 100,000 (Afghanistan) to less than 1 / 10,000 (in 63 countries); whereas in 4 countries, as Afghanistan, Burundi, Somalia and Chad, the mortality rate is about 300 / 100,000 [8].

A recent report on the RV-related sentinel surveillance system, performed by hospitals within 35 countries, showed that among the most important 6 regions with various economic levels, the mean number of hospitalized children, aged < 5 years, was 40.0% cases with RV-related diarrhea [9]. Very few studies have described the epidemiology of RVGE across the Central and Eastern Europe. The RVGE ratio of all AGEs reported in Romania during the last decade ranged between 15.0-50.0%, the highest incidence being reported in January and May. The highest incidence of RVGE has been recorded in children aged 6-12 months [10, 11].

Rotavirus infection is also known as infantile diarrhea (since almost all children are infected in the first years of life), or winter diarrhea (since in the United States, the disease occurs most frequently in winter, while the most common months are November - May). It is an extremely contagious and unpredictable disease and can lead to severe dehydration and even death. Rotavirus is an endemic disease worldwide, the infection being associated with high rates of global morbidity and high mortality rates within the developing countries. In the United States, rotavirus infection might lead to about 3 million cases of diarrhea and about 55,000 hospitalizations of diarrheal diseases and dehydration in children under 5 years old, though it is relatively rarely fatal. In developing countries, however, rotavirus gastroenteritis accounts for more than 800,000 childhood deaths per year due to poor nutrition and lack of healthcare services [7, 12].

The severe forms of rotavirus infection occurs during the first 2 years of life, of which more than half of the rotavirus-related hospitalizations occur in the first year of life, whereas 91.0% of the same cases have been reported in children under the age of 2 years. We should mention that only 14.0% of hospitalizations occur before the age of 6 months. Therefore, an immunization program administered before the first 16 weeks of life might prevent most of the severe rotavirus cases. Studies have also reported that, although, in China, there are natural and temporal variations of circulating rotavirus strains, the predominant local strains are similar to those that are dominant worldwide [13, 14].

The EuroRotaNet (European Rotavirus Network) surveillance network was launched in 2007, including 16 countries: Austria, Belgium, Bulgaria, Denmark, Finland, France, Germany, Greece, Hungary, Italy, Lithuania, Holland, Slovenia, Spain, Sweden and the Kingdom. United [15, 28]. The main objectives of this project were to develop specific methods and algorithms for efficient assessment of G and P rotaviruses; to describe in details the molecular epidemiology of rotavirus infections in Europe; to monitor the effectiveness of current genotyping methods and evolutionary changes associated with genetic modifications; to monitor the occurrence and spread of new rotavirus strains across Europe [28].

According to the surveillance network, a wide variety of rotavirus strains are circulating across Europe. Thus, the G1 P genotype [8] was the most widespread in Europe by 2014-2015, whereas in 2016-2017 its prevalence decreased. In the period of 2016-2017, G2 P [4] was the predominant type of rotavirus (35.0%), as well as the most commonly detected in 4 countries out of the 16 supervised countries [16, 28].

In Finland, the decrease of G1 P strains [8] was not substituted with another type, but rather with a greater range of general strains. In 2016, G3 P had the lowest reported rate of strains found in Spain and Germany [8] [15].

Our country does not exhibit similar data, which gave rise to this study.

2. THE RESEARCH MATERIAL AND METHODS

In order to achieve the research purpose, a prospective cohort analytical study was conducted on a large group of patients with rotavirus infection during their hospital stay. Patient surveillance was performed in different times, which is a specific criterion of the research called "prospective observation", as well as of the diagnosis and treatment methods [17].

The prospective cohort study was conducted during 2012 -2016, which included 193 children under the age of 1 year, diagnosed with rotavirus infection, and who underwent treatment within the Department of Acute Diarrheal Diseases, at the IMPH Municipal Children's

Clinical Hospital no 1. The patients were divided into the main group - 72 immunized children with rotavirus infection, and the control group - 121 of non-immunized children with rotavirus infection vs. rotavirus genotype and phenotype.

The clinical study was carried out within a multicenter research project initiated by the WHO Regional Mission in the Republic of Moldova, as part of World Health Organization data on RVI genotyping and phenotyping in infants.

During the study, all the admission requirements were followed according to the Government Decision of the Republic of Moldova (Order of the Ministry of Health no. 232 of 20.06.2008 and Order no. 985 of 19.12.2011, on the implementation of the epidemiological sentinel surveillance system for rotavirus infection in the Republic of Moldova), within the national project for implementation the sentinel surveillance system in rotavirus-infected infants. The medical data records included the assessment of anamnestic data, medical history, clinical examination, the laboratory findings of biochemical and bacteriological tests, as well as virological, immunoenzyme and molecular biology investigations [18].

The children with acute diarrhea were followed up according to the order of the MH and the institutional protocol on "*Acute diarrheal disease in infants*" of the IMPH Municipal Children's Clinical Hospital no. 1. The research took into consideration the age particularities of the immune status, thus the infants were considered the main group for developing rotavirus infection, which commonly result in severe complications [18, 19].

The expected sample size (193 RVI-diseased infants) included within the study exceeded the necessary amount of patients, which proves a positive impact on reliability of the obtained outcomes. This cohort study shows that population consists of individuals who are classified as exposed or non- exposed to a particular factor (comparison groups). A cohort study is commonly developed, based on a research hypothesis and on the relationship that is expected to be established between the study groups and the relevant health problem. Thus, the two study groups involved the main group and the control group, depending on the immunization status of infants [17, 18].

The required number of RVI patients was estimated, based on the following formula [17, 20]:

$$n = \frac{1}{(1-f)} \times \frac{2(Z_\alpha + Z_\beta)^2 xP(1-P)}{(P_o - P_1)^2}$$

$$n = \frac{1}{(1-0.1)} \times \frac{2(1.96 + 0.84)^2 \times 0.625 \times 0.375}{(0.50 - 0.75)^2} = 65$$

whereas:

P_o = According to the bibliographic data [21], the successful treatment of non-immunized patients with rotavirus infection should account for about 50,0% (P_0=0,50).

P_1 = the successful treatment of the study group, including immunized patients with rotavirus infection, should account for 75,0%

(P_1=0,75)

P = (P_0 + P_1)/2=0,625

Zα – table value, when the statistical significance is 95,0%, whereas the coefficient is Zα =1,96

Z_β– table value, when the statistically significant difference is 80,0%, whereas the coefficient is Z_β= 0,84

f = The number of subjects expected to abandon the study for other reasons than the investigated issue q = 1/(1-f), f=10,0% (0,1).

Therefore, the L1 study group should consist of at least 65 immunized RV patients and the L0 control group should include no less than 65 non-immunized RV patients.

After determining the immunization status of each patient, the study group was divided into the main group, which included 72 immunized RV infants, and the control group, including 121 non-immunized RV infants.

The inclusion criteria for this research were established according to the framework case included in the RBGE Guidelines for Supervision of Children (WHO 2012) [19], being as follows:

1. The written consent of a child's parent or tutor.
2. The age ranging between 2 - 12 months.
3. Presence of diarrhea with at least 3 loose stools during last 24 hours, though not exceeding 7 days.
4. Patients, who were assessed within the first 24 hours after hospital admission.

The exclusion criteria were as following:

1. RV-diseased patients with severe comorbidities.
2. Lack of a written consent from the child parent or tutor.
3. Patients younger than 2 months and older than 12 months.
4. Patients with diarrhea that had less than 3 loose stools during the last 24 hours.

The children's mean age from the control group was 7,2 ± 2,91 months, of which 71,9% were boys and 28,1% girls. The children's mean age from the main group was 6,8 ± 2,79 months, of which 51,2% were boys and 48,6% girls.

In order to assess the RVI interrelations, the groups were subsequently studied, based on their age and gender. Male infants prevailed in both study groups. Of the 121 non-immunized children with RV from the control group, 71,9% were boys and 28,0% were girls. The main group (72 children) included 51,3% of male patients and 48,6% of female patients, diagnosed with rotavirus infection.

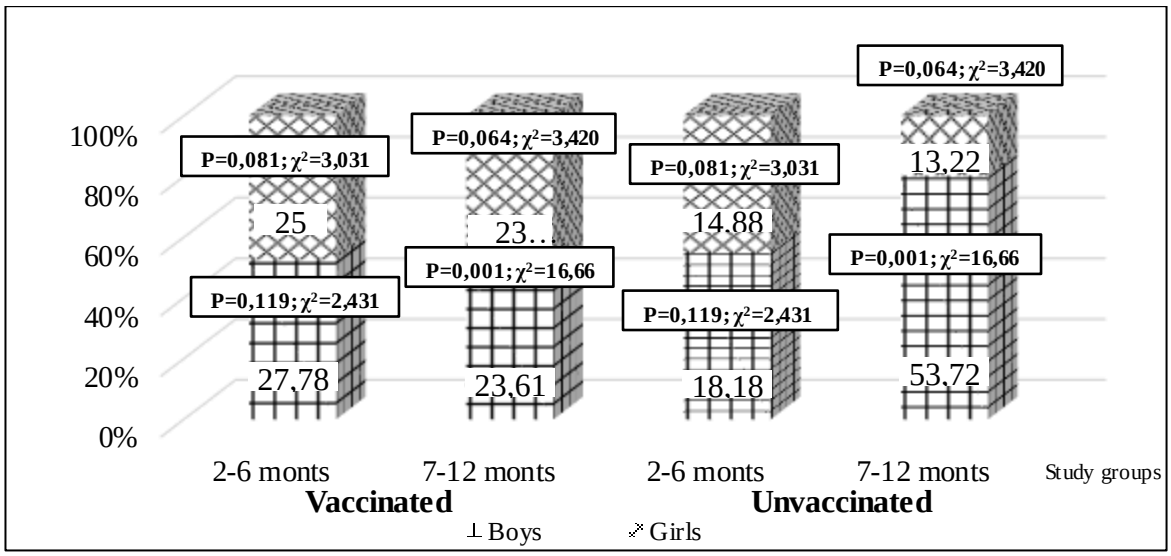


Figure 1. **Children distribution in both study groups based on their age and gender**

It should be noted that, the number of boys exceeded the number of girls from the control group viz. 71,9% vs. 28,0% ($p = 0.004$, $\chi 2 = 8,23$), whereas the main group exhibited a gender difference of only 2,7%, boys prevailed.

According to age distribution, the infants from both groups were divided into groups aged between 2 - 6 months and between 7 - 12 months. The control group involved predominantly children older than 6 months viz. 66,9%, compared to those under the age of 6 months, which

made up 33,0%. The main group sowed a higher percentage of children under 6 months – 52,7%, compared to those over 6 months with 47,2%.

The immunization scheme implies the administration of two doses of vaccine, 1,5 ml each. The first dose is given starting from the age of 6 to 8 weeks of the child's life. There should be an interval of at least 4 weeks between doses. The immunization scheme is recommended to be carried out in children under 16 weeks, however, it should be completed before the age of 24 weeks. Rotarix is administered per oral only.

3 CLINICAL-EPIDEMIOLOGICAL AND PARACLINICAL FEATURES IN ROTAVIRUS INFECTION IN INFANTS

Epidemiological aspects of rotavirus infection in infants from the Republic of Moldova.

In the Republic of Moldova, the seasonal RVI activity reached its peak in the cold months of the year, thus in February 2012-2016, the disease incidence was 22,3% in the main group and 22,2% in the control group. In January, February and March, the incidence ranged between about 19,8, 22,3 and 21,4%, respectively, whereas in April and May, the incidence showed a lower percentage of 9,9 and 9,0% in the total number of children from the control group. The minimum number of RVI-diseased children was registered during the summer period (0 – 2,5%).

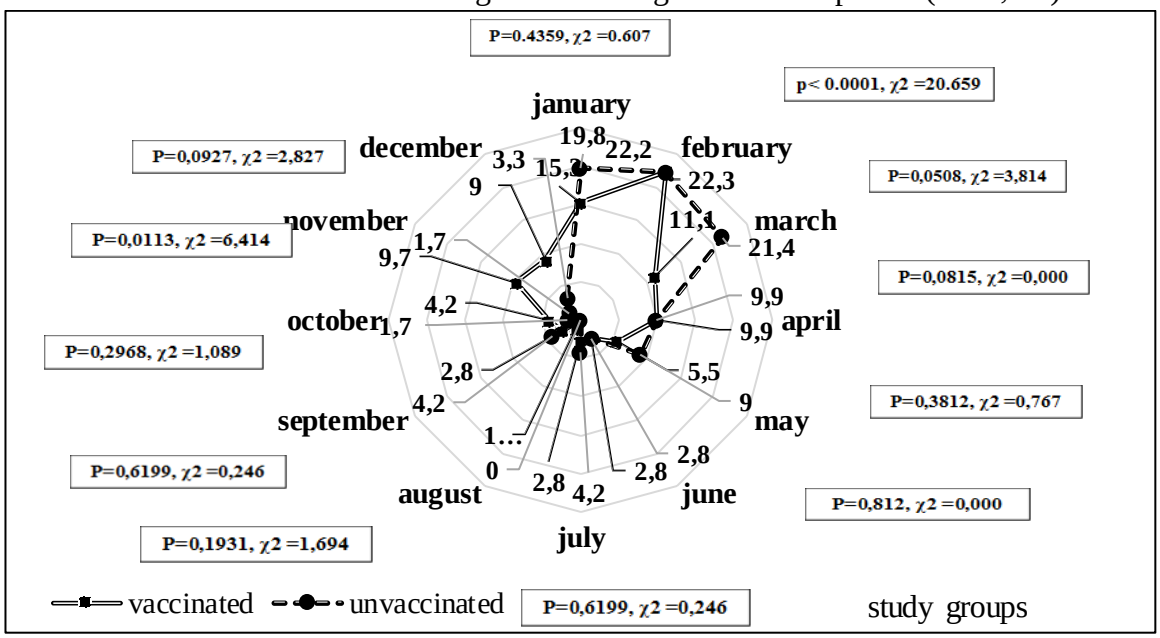


Figure 2. **Seasonal distribution of RVI indices (%)**

The seasonal RVI activity in the main group exhibited the highest occurrence in winter, particularly in January and February, viz. 15,3% and 22,2%, respectively; in November and December – 9,7% and 9,0 %. In summer, RVI activity was reported in 1,4% - 2,8% of cases.

In regions, characterized by a continental climate, RVI occurs more frequently during the cold season. In the Republic of Moldova, the highest number of RVI morbidity cases and outbreaks were recorded between Octobers - May. In the tropics, regardless of the season, the incidence of RVI cases increases, due to a decrease in temperature [18].

Clinical features of rotavirus infection in infants

The severe condition of children during their hospital admission was assessed by presence of repeated vomiting episodes, frequent loose stools and high fever, resulting in fast dehydration, especially in infants. Clinical signs of impaired gastrointestinal system were present

at patient's admission. Thus, non-immunized patients suffered from nausea in 49,0%, compared with immunized ones, who had nausea in 16,0% of cases (Figure 3, 4).

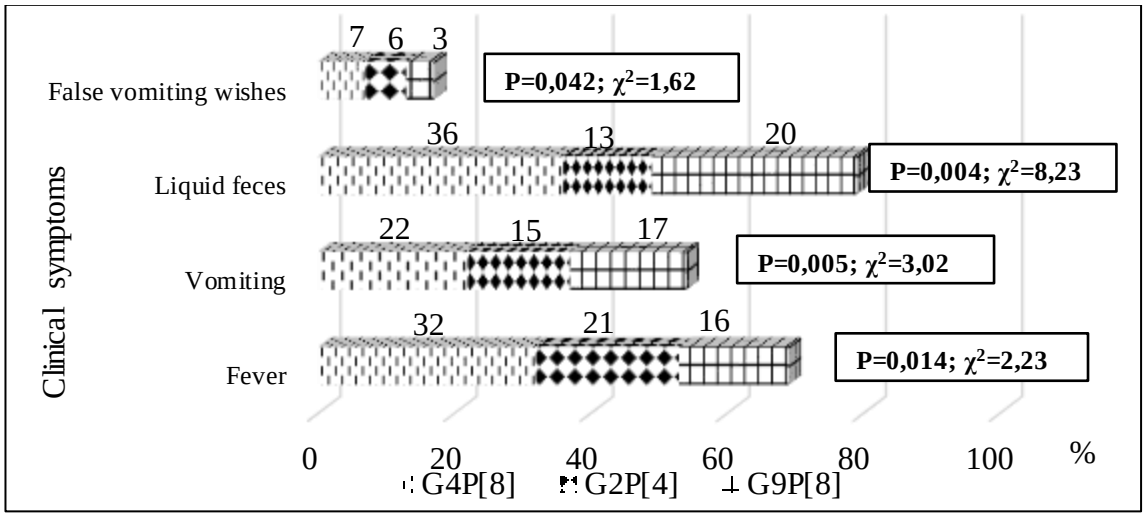


Figure 3. **The clinical symptom indices based on the genotypes found in RVI –diseased children on the day of admission**

Vomiting was found in 62,0% cases in non-immunized children vs 54,0% in immunized ones. The control group reported a 10,0-15,0% higher frequency of fever, loose stools and other symptoms, compared to children from the main group.

The variety of clinical features determines a delay in the etiological diagnosis of gastroenteritis, which can be both viral and bacterial, as well as mixed one.

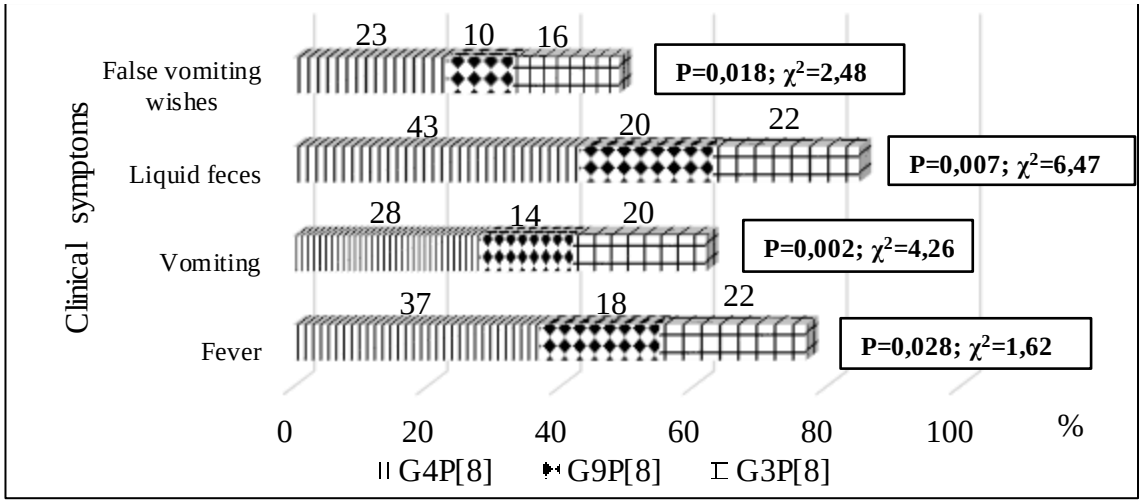


Figure 4**. The clinical symptom indices based on the virus genotypes in unvaccinated children**

The incidence of dehydration was considerably higher in the control group. Thus, according to the basic clinical diagnosis, rotavirus infection developed more complications in non-immunized children. 46,4% children from the control group had AGE with moderate dehydration vs. in 23,6% of immunized children ($\chi 2$ = 9,91, P = 0,0016). A 4-fold higher incidence of severe dehydration was found in the control group compared to the main group, accounting for 4,1% of cases in non-immunized vs. 1,4% in immunized children ($\chi 2$ = 1,09, P = 0,2960). Acute enteritis, characterized by moderate dehydration made up 5,6% in vaccinated children, thus being with 2,3% higher than in the main group. The indices of acute diarrheal disease of rotavirus etiology following the basic diagnosis are shown in figure 5.

At the onset of the disease, children who vomited 12 times were present only in the non-immunized group (1,4%) vs. immunized children without vomiting episodes. The group of

children who had 8 vomits at the onset, reported a 6-fold higher incidence, compared to the main group (12,0% vs. 2,6%).

The main group included 28,0% children with 3 vomiting episodes, while the control group exhibited a more severe onset, with an average of 4 vomits in 26,7% of cases; the maximum index was 12 vomiting episodes.

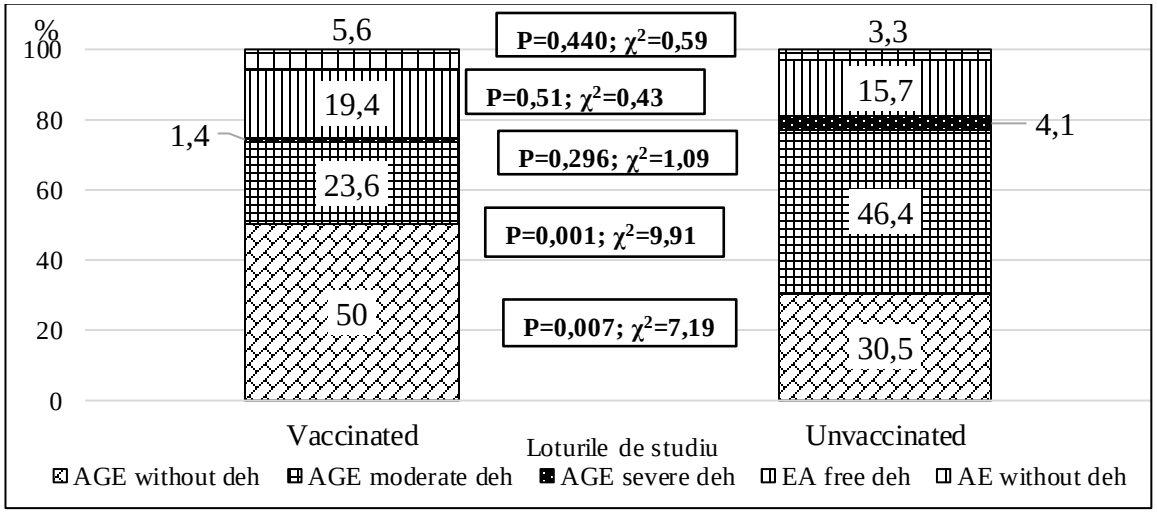


Figure 5. **The indices of rotavirus-related acute diarrheal disease following the main diagnosis**

RVI-diseased infants from the study groups were reported with high fever, vomiting and diarrheal episodes, abdominal pain and loss of appetite. Loose stools were diagnosed in 100% cases from both groups, whereas vomiting more frequently occurred in non-immunized children, with an incidence of 80,0%, compared to immunized ones with 69,4% of cases. Fever was a common symptom, being equally present in both groups (76,9% - in non-immunized patients compared to 75,0%-immunized patients). Abdominal pain was found in all non-immunized patients. Abdominal pain was diagnosed in 2/3 of immunized cases. Loose stools were reported in 95,0% of non-immunized and 83,3% immunized children. Semi-regular stools were recorded in both main and control groups, showing no significant difference (2,5% compared to 16,7%) and only 2,5% of non-immunized children were diagnosed with foamy loose stool. The clinical characteristics of the study groups are shown in Figure 6.

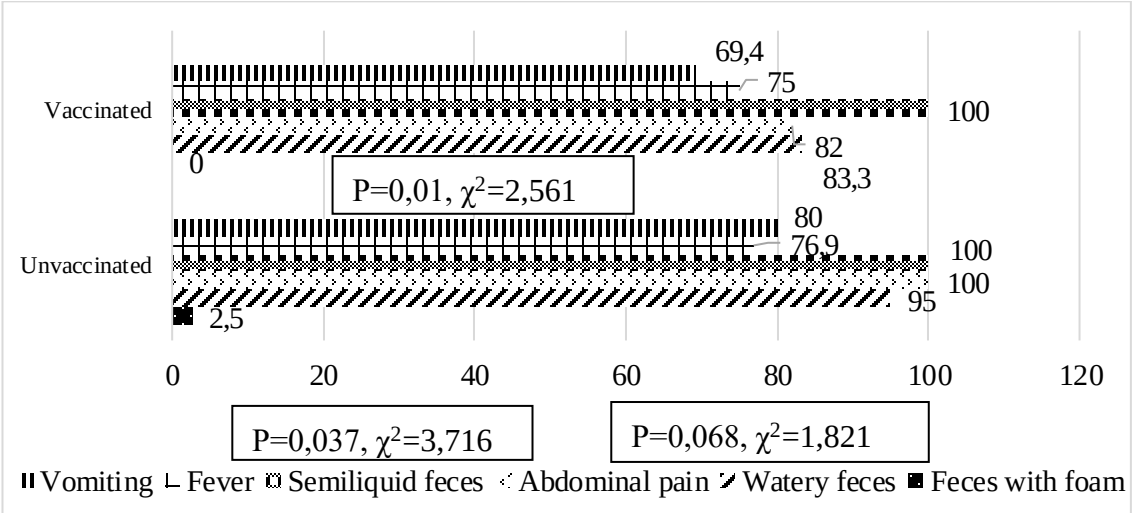


Figure 6. **Clinical features of the study groups**

The Vesikari Scoring System [19] for assessing the clinical severity of rotavirus infection is currently recognized as the most accurate system for studying rotavirus within various regions.

Moreover, it is a complex measurement system that considers all the clinical manifestations of rotavirus in order to identify severe episodes. The system includes seven parameters, including the major symptoms: diarrhea, vomiting, fever, dehydration, duration of diarrhea and vomiting episodes, rehydration. Vesikari score provides three levels of dehydration as one parameter. The use of Vesikari score to determine the severity of rotavirus infection in infants enrolled within the study was as following: [19] (Table 1).

Table 1. **The severity of clinical characteristics among infants, included within the study, based on Vesikari Scoring System**

Parameter	Unvaccinated		Vaccinated		Unvaccinated	Vaccinated	Unvaccinated	Vaccinated
Nr. of feces / day	0	1-3		0	4-5		≥6	
	-	24	8	-	33	15	64	49
Duration of diarrhea (days)	0	1-4		0	5		≥6	
	-	77	38	-	33	27	11	7
Nr. of vomiting / day	0	1		0	2-4		≥5	
	33	19	11	40	42	18	20	10
Duration of vomiting (days)	0	1		0	2		≥3	
	33	46	24	40	20	10	15	6
Fever (° C)	0	37,1-38,4		0	38,5-38,9		≥39	
	28	65	31	33	14	7	9	6
Degree of dehydration	0	light		0	medium		sever	
	31	56	20	-	61	20	4	1
Parenteral rehydration (hours)	0	24 hours		0	48 hours		≥72 hours	
	29	20	18	35	31	15	35	10

Tables 1 and 2 show a significant difference in the indices obtained in the main group compared to the control group, indicating a higher severity of symptoms in non-immunized children. Thus, according to the Vesikari Scoring System, a higher severity score was registered in 35,0% of non-immunized children vs. 13,0% of immunized children. The mean severity score was found in 45,0% of children from the control group and 34,0% of children from the main group. A low severity level was reported in more than half (53,0%) of the children in group I, compared to 20,0% of children from group II. The score results showed that non-immunized children suffered from RVI more severely (35,0%) and 2,6 times more frequently than immunized children (13,0%).

Table 2. **Vesikari scores**

Vesikari score	<7	7-10	>11	X^2	P
Unvaccinated	24(20,0 %)	55(45,0 %)	42(35,0 %)	3,503	0,002
Vaccinated	38(53,0 %)	24(34,0 %)	10(13,0 %)	2,932	0,071

The maximum hospital stay of the patients, included in the study, was 10 days, that is 5,0% in the control group vs. 4,2% in the main group, whereas 6,6% of the non-immunized children, and 5.5% of immunized children required 9 days of inpatient treatment. Similar situation was confirmed for treatment duration, which was 8 days for 9,7% of patients from group I and 11,6% from group II. Children who had only a 4-day hospital stay (8,3% -vaccinated vs. 1,7% -unvaccinated infants) showed a more significant difference of this parameter.

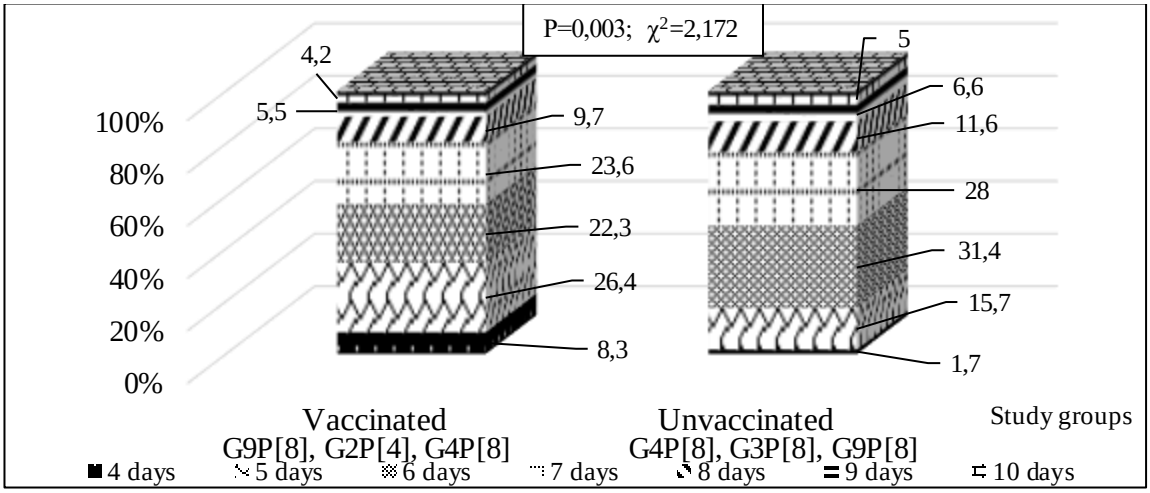


Figure 7. **The RVI severity depending on the hospital stay length within both study groups**

The study groups exhibited a wide range of comorbidities. In most cases, the patients presented diseases of the respiratory system, viz. upper and lower respiratory tract disorders (laryngotracheitis, simple acute bronchitis, acute obstructive bronchitis, pneumonia, and bronchopneumonia) (Figure 8). 61,1% cases of respiratory diseases were present in the control group and 52,7% cases -in the main group.

Gastrointestinal disorders were found in 57,8% cases of non-immunized children and in 37,5% of immunized ones. Metabolic disorders were commonly diagnosed, predominantly in the control group, which was 1,4 times (38,8%,) higher than in children from the main group – 27,7% cases.

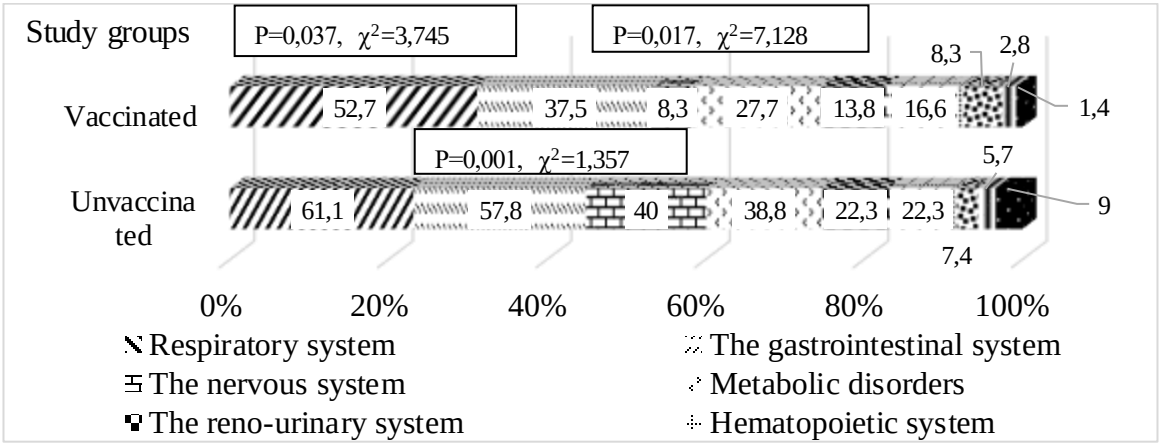


Figure 8. **The structure of RVI-related comorbidities**

Central nervous system impairment associated with rotavirus infection in non-immunized children was found in 40,0% cases (p = 0,001, $\chi 2$ = 1,357), featured by febrile seizures, toxic encephalopathy, hypoxic-ischemic encephalopathy, occurred 4 times more often than in immunized children (8,3%).

Kidney disorders (urinary infections) and hematopoietic disorders (especially, anemia) have been detected in almost equal numbers of patients with rotavirus infection. Thus, the incidence of kidney and hematopoietic diseases in non-immunized children with a 10-day hospital stay, was 22,3%, being characterized by post-infectious anemia, whereas the incidence of these diseases in immunized children was 13,8% and 16,6% of cases (p = 0,037, $\chi 2$ = 3,745).

Allergic dermatitis – 8,3% vs. 7,4%, malnutrition – 2,8% vs. 5,7%, and ENT (otolaryngology) disorders – 1,4% vs. 9,0% (p = 0,017, $\chi 2$ = 7,128) were more rarely recorded.

The characteristics of the paraclinical changes and imaging patterns

The hematological changes in RVI children were studied via hemolymphogram, by assessing the level of hemoglobin, leukocytes, and ESR (erythrocyte sedimentation rate) during the clinical manifestations of gastrointestinal infection.

The hemolymphogram and the assessment of hemoglobin and RBs showed the presence of a deficiency anemia (Hb <110 g / l), which was 1,4 times more often (27,3%) in the group of unvaccinated children, compared to the main group, where anemia cases accounted for 19,4% (p = 0,004, $\chi 2$ = 1,253). This indicates a much more severe development of rotavirus infection in the control group, resulting in loss and / or poor absorption of nutritional components required for infants.

The mean hemoglobin level in the group of non-immunized children was 118,54 ± 1,07 g/l, whereas RBC was 4,22 ± 0,14x10^{12}/l. The mean hemoglobin level in the group of immunized infants was 120,25 ± 1,43 g/l and RBC- 4,0x10^{12}/l (p>0,05).

An increased level of leukocytes (> 15x10^{9}/l) in non-immunized children was found in 8,3% cases, and in 5,5% of immunized infants. This determines the association between the bacterial infectious process and a higher frequency in the control group (p = 0,014, $\chi 2$ = 2,781).

Erythrocyte sedimentation rate (ESR) was at highest among non-immunized children, being 50 mm/h, whereas immunized children reported 44 mm/h. ESR proves the severity and complexity of the pathological inflammatory process, a fact confirmed by the clinical data of the investigated cases.

The mean WBC assessed in non-immunized children was 7,47 ± 0,82x10^{9}/l, whereas the immunized children showed no significant differences – 7,8 ± 1,24x10^{9}/l (P>0,05). Both study groups of non-immunized and immunized children exhibited normal WBC levels.

The urinalysis revealed leukocyturia in non-immunized children, the highest value -500, the mean value – 170,7 ± 3,3. The mean value of leukocyturia in immunized infants was 70,3 ± 2,5. Ketone bodies (acetonuria) were also determined, which denoted the presence of metabolic disorders. The control group showed a marked acetonuria (150 mg/dl) that was 1,7 times less than in the main group, viz. 0,8% cases vs. 1,4% in main group (p = 0,682, χ^2 = 1,164). A value of 15 mg/dL was found in non-immunized children, which was 1,4 times more often (19,8%) compared to 13,8% in immunized children (p = 0,295, χ^2 = 1,115).

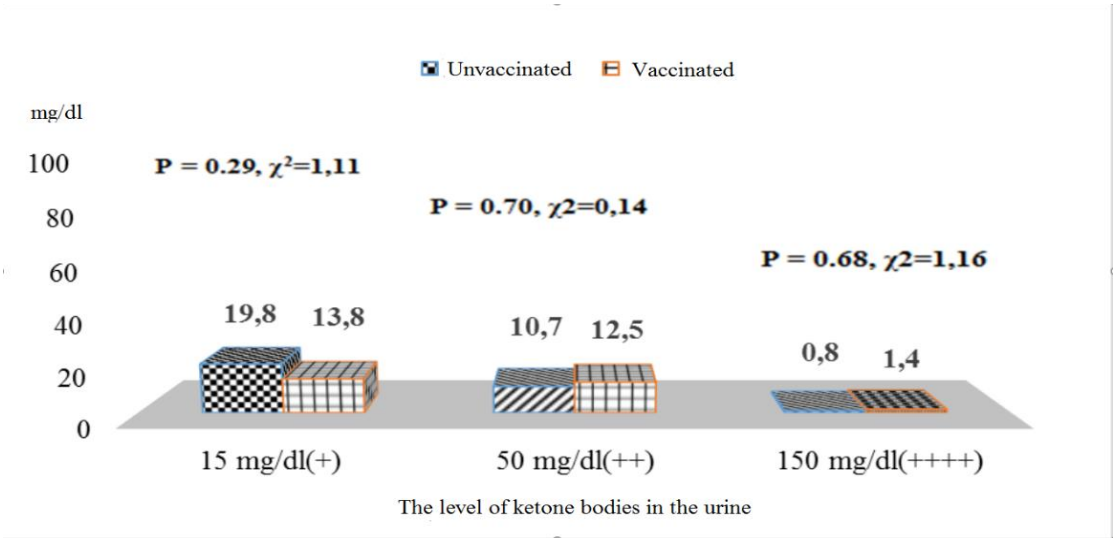


Figure 9. **Distribution of acetonuria-related cases depending on the patients' vaccine status with rotavirus diarrhea**

Ketoneuria resulted from an excessive accumulation of ketone bodies within the body, being excreted with urine. Non-diabetic ketoacidosis occurs when the body consumes the energy reserves from burning fat instead of glucose, which is the common energy source. Ketoacidosis indicates an impaired pancreas that might occur as a result of gastroenterocolitis [19, 20]. The metabolic disorders, characterized by ketoneuria in RVI patients are shown in Figure 9.

A marked ketoacidosis more commonly occurred in immunized patients, whereas non-immunized children revealed no significant acetonuria.

The biochemical assessment of RVI children showed a slight increase in the mean level of ALT, particularly in non-immunized infants compared to immunized ones, viz. 38 ± 0,89 U/L (N = 33 U/L) vs 31,63 ± 1,90 U/L (N = 33 U/L), respectively. Additionally, a low mean calcium level of 2,00 ± 0,02 mmol/l (N = 2,20-2,60 mmol/l was recorded in both groups. The glucose index was slightly lower in non-immunized children compared to immunized ones, viz. 3,38 ± 0,18 vs. 4,67 ± 0,42, respectively.

Due to the short length of hospital stay, the general protein level suffered no significant changes, viz. 59,9 g/l vs. 60,53 g/l), whereas the mean calcium level was the same in both groups, being lower than the minimum normal count.

The blood biochemical tests revealed elevated transaminase levels (57,8%) in more than half of the children from the control group, which indicated a transient liver impairment, resulting from RVI- associated complications (intoxication, dehydration syndrome), compared with the main group, where the liver enzyme level increased in 37,5% of cases.

A major analysis of the correlation between the patient clinical diagnosis of RVI patients and the increase of liver transaminase level, revealed that 57,0% of children suffered from metabolic disorders and 52,5% - of respiratory diseases. Nearly same indices were found in children with disorders of the hematopoietic system (28,5%), kidney disorders (25,7%) and nervous system impairment. Patients with otitis (8,4%), allergic dermatitis and malnutrition (4,2%) were among children with increased hepatic transaminase levels.

All children with elevated hepatic transaminase levels were investigated, via specific serological tests for cytomegalovirus, herpes, and viral hepatitis B, C. The probable occurrence of these infections was excluded. Thus, out of the 27 vaccinated children, 24 (88,8%) infants did not exhibit CMV infection, herpes and viral hepatitis. Only three children were found with CMV infection.

65 children (92,8%), out of 70 non-immunized children with elevated hepatic transaminase level, did not show CMV, herpes and hepatitis infections. CMV was confirmed in 2 children, CMV and herpes -in one case, herpes -1 case and viral hepatitis B- one case.

The increase of transaminase levels in our study correlated with dehydration and electrolyte disorders. Thus, it was concluded that high transaminase indices are the pathognomonic signs for patients hospitalized with moderate to severe rotavirus gastroenteritis, complicated by acute dehydration and toxicity syndromes.

In order to assess the severe RVI-related changes of the internal organs, all children, included within the study, underwent the abdomen and kidney ultrasound exam. The statistical analysis of the obtained results revealed some differences between the studied groups.

Therefore, hepatomegaly was found in the control group, the incidence being of 18,1%, compared to 16.0% in the main group ($p = 0,023$, $\chi^2 = 0,452$). Nearly a 4-fold increase of liver reactive changes were observed in non-immunized rather than in immunized children, viz. 5,0% vs. 1,3% ($p = 0,201$, $\chi^2 = 0,391$). The number of cases, initially diagnosed with gall bladder

deformity, was 5,7% and 2,6% in children from the control group vs. the main group (p = 0,297, χ^2 = 0,621).

Both study groups showed almost equal values of reactive changes in the pancreas – 13,8% vs. 14,0% (p = 0,038, χ^2 = 1,280) and primarily detected pyeloectasis – 5,5% vs. 5,7% (p = 0,002, χ^2 = 3,721). Other diseases (splenomegaly, double kidney, single kidney, etc.) were found in 11,5% of non-immunized vs. 5,8% immunized children. More than half of the immunized children (55,0%) exhibited no changes on the abdominal and kidney ultrasound exam. Among non-immunized infants, this index accounted for 40,0% (p = 0,389, χ^2 = 1,048).

To determine steatorrhea, the quantitative analysis of lipids was assessed: neutral fats and fatty acids. Non-immunized RVI patients had predominantly low levels of NF (neutral fats) in 36,3% cases, moderate NF values – 47,1% cases and high levels- in 16,6%. The main group revealed the following indices: low NF levels -in 48,8%, mean values - in 40,2%, and large amounts – in 11,0% of infants.

The control group revealed low amount of fatty acids (FA) - in 91,7% of the studied children and mean values in 8,3% of cases. The study findings showed low indices – in 87,0% cases and mean indices in 13,0% of children from the main group.

Excessive leukocyte amounts in feces is a sign of an inflammatory process. The fecal leukocyte testing showed more than 10 leukocytes in 6,6% of non-immunized children vs. in 2,7% of immunized children. Thus, group II revealed a 3-fold increase in the number of cases with small intestine inflammatory response, resulting from a bacterial infection.

The amount of cellulose present in the stool tests of non-immunized children was moderate in 3,0% cases and low-in 97,0%. The mean value for the cellulose content of feces doubled to 6,0% in immunized children (Table 3).

Table 3. **The stool test indices in studied RVI infants**

Coprogram feature	Unvaccinated (n=121), %	Vaccinated (n=72), %	X^2	P
Cellulose: Low	97,0	93,0	2.163	0.0271
- Average	3,0	6,0	4.273	0.0037
- High	-	-	0.513	0.3287
Neutral fats: Low	36,3	48,8	1,271	0.2491
- Average	47,1	40,2	0.628	0.3186
- High	16,6	11,0	8.241	0.0024
Fatty acids: Low	91,7	87,0	3.528	0.0205
- Average	8,3	13,0	2.591	0.0528
- High	-	-	3.469	0.0003
Leukocytes (>10)	6,6	2,7	0.261	0.0251
Erythrocytes	4,1	1,4	8.295	0.0247
Yeast	10,7	8,3	3.418	0.0038

The molecular biology exam was carried out to confirm the rotavirus etiology of acute gastroenterocolitis, by using the ELISA immunoenzyme assay, as well as to determine the RV genotypes and phenotypes via chain polymerization reaction (PCR). The study groups were formed depending on the obtained results.

35 immunized children out of 72 and 77 non-immunized infants out of 121 underwent neurosonography. The exam findings differed in both groups. Thus, 60,2% cases of non-immunized children vs 41,4% of immunized children showed increased rates of cerebral vein pulsation. Ventriculomegaly was found 6 times more often in non-immunized vs. immunized children, viz. 12,9% vs. 2,8% (p = 0,016, χ^2 = 3,517). Other ultrasound exam findings (presence

of pseudocysts or other formations) revealed a double frequency in the main group that made up 20,0% compared to 10,3% of children from the control group (p = 0,142, χ^2 = 8,239). RVI-related central nervous system disorders did not occur in 16,6% of non-immunized children and in 35,8% of immunized patients (p = 0,001, χ^2 = 1,318).

Based on the recorded clinical features and Vesikari scoring system, we developed an early diagnostic algorithm for RVI (Figure 10). The investigations showed that infants with rotavirus infection had a severe Vesikari score (≥11 points), while the other infants showed a light to medium level of Vesikari scoring system (<7 and 7-10 points, respectively).

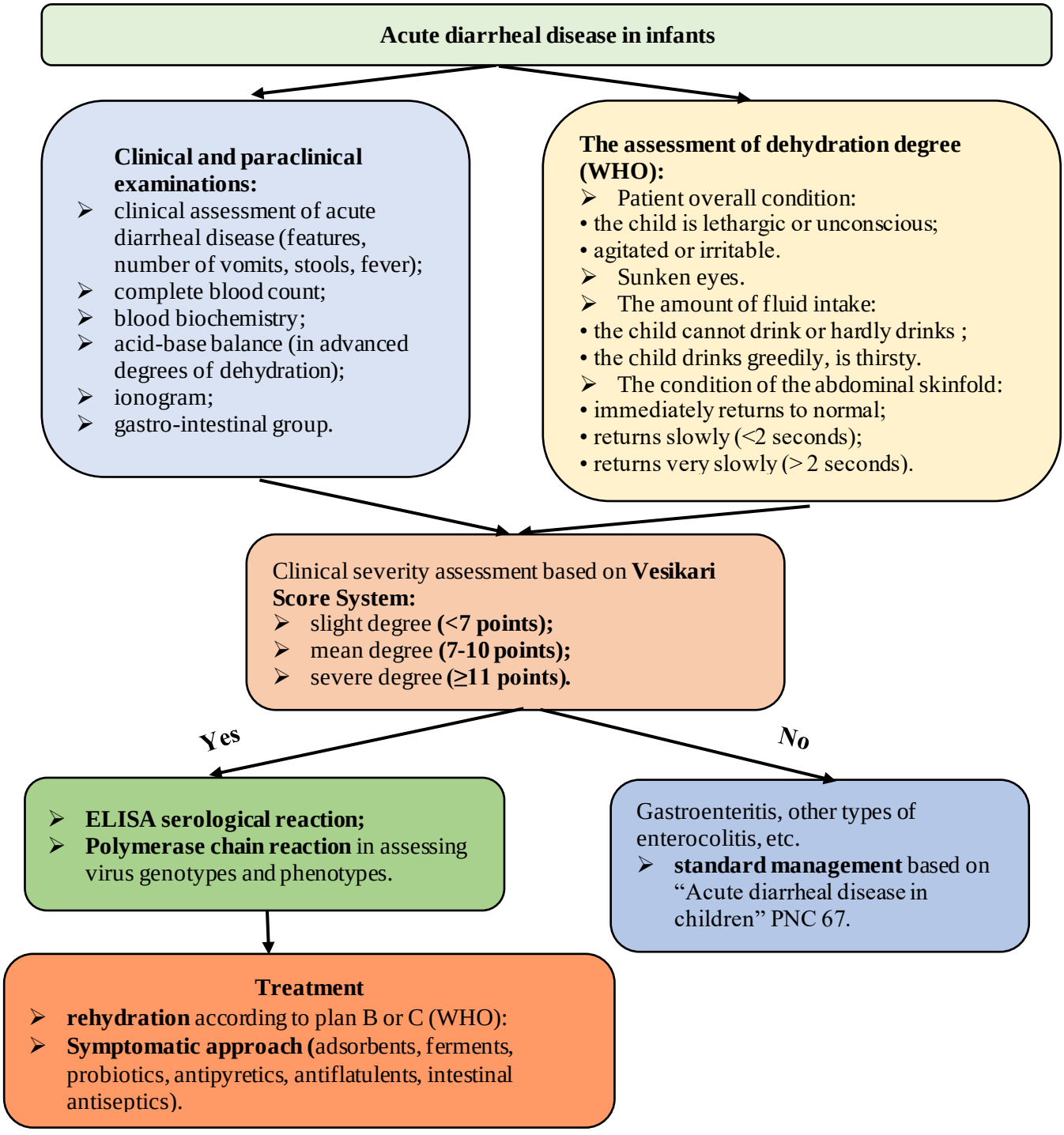


Figure 10. **The algorithm used in early diagnosis of rotavirus infection in infants**

Table 4. The patient RVGE "profile" depending on their immunization status

Vaccinated status	Unvaccinated	Vaccinated
Average age (months)	7,4±0,26	4,8±0,32
Gender	male	male
Month of diagnosis	february	february
Watery diarrhea	≥6 feces/day	4-5 feces/day
Vomiting	≥5 vomiting/day	2-4 vomiting/day
Fever	38,5-38,9º C	37,1-38,4º C
Acute dehydration syndrome	medium and severe	easy
Transaminases ↑	57,8%	37,5%
Acetonurie	easy(+)	medium(++)
Rehydration	≥72 hours	≤24 hours

The RVGE "profile" of non-immunized patients was as following: the mean age was 7,4 months with male prevalence, whereas the infection incidence peak in February (22,3%). Clinically, it was characterized by loose diarrhea with more than 6 stools per day, more than 5 vomits within 24 hours, febrile temperature and moderate to severe ADS. Paraclinically, it showed an increased hepatic transaminase level (in 57,8% cases), mild acetonuria, which required rehydration > 72 hours.

Clinically, the profile of immunized patients was more favorable, with 4-5 loose stools per day, 2-4 vomiting episodes over 24 hours, subfebrile temperature and mild ASD. Paraclinically, increased hepatic transaminase level accounted for only 37,5%, with a mean acetonuria and the need for a < 24 hour dehydration.

4. THE CHARACTERISTICS OF THE ACID-BASE BALANCE, ROTAVIRUS GENOTYPYING AND THE ETIOLOGICAL STRUCTURE OF MIXED ROTAVIRAS INFECTION

Acid-base changes in RVI-diseased infants

Monitoring of the acid-base balance in patients revealed the severity of RVI evolution and contributed to establishing the types of metabolic disorder and the need for rehydration therapy. 22 children from each of the two study groups were assessed for acid-base changes and severe forms or advanced dehydration degrees.

Non-immunized children were reported to have metabolic acidosis, having a mean blood pH level of 7,28 ± 0,87, whereas the immunized ones had a normal mean pH level of 7,42 ± 1,25 (Table 6).

Sodium is the major extracellular ion, which significantly contributes to determining serum osmolarity. Sodium, potassium and calcium are highly important for neuromuscular function, acid-base balance, cellular chemical reactions and for membrane fusion [20].

Table 6. **Acid-basic balance indices in both study groups (mean values)**

Indices	pH (7,35-7,45)	Na^+ mmol/l (132-144)	K^+ mmol/l (3,6-4,8)	Ca^{++} mmol/l (2,20-2,60)	Cl^- mmol/l (96-105)	$HCO3^-$ mmol/l (22-28)	BE^- mmol/l (0±2)	AnGap (mEq/l) (16)
Lot I	7,42±1,25	134±0,23	3,9±1,35	2,3±0,35	106±0,24	21,9±0,15	1,4±0,03	7,9±1,34
Lot II	7,28±0,87	137±0,12	7,28±0,87	137±0,12	7,28±0,87	137±0,12	7,28±0,87	137±0,12

The mean sodium value in children from both study groups was within the normal range, being 137 ± 0,12 mmol/l, in non-immunized children, and 134 ± 0,23 mmol/l -in immunized children. Normal potassium values were also reported in both groups, 3,8 ± 0,96 mmol/l - in non-immunized children, and 3,9 ± 1,35 mmol/l -in immunized ones. Due to carbohydrate malabsorption, viral gastroenteritis is accompanied by loss of hypotonic fluids, which leads to maintenance of a normal sodium concentration or its increase.

Calcium exhibited low values in the group of non-immunized children, the mean value being of 2,0 ± 1,32 mmol/l, whereas the mean calcium value in immunized children was 2,3 ± 0,35 mmol/l.

Bicarbonate (HCO3-) had low values of 17,8 ± 0,35 mmol/l in non-immunized children and 21,9 ± 0,15 mmol/l in immunized children.

BE (base excess) refers to base deficit, when the value is negative, or an excess of bases, when the value is positive. Thus, the study results of the acid-basic balance showed a base deficit in group II (-3,6 ± 0,06 mmol/l) and a base excess in group I (1,4 ± 0,03 mmol/l), whereas the normal values were-2 - 2 mmol/l.

The serum anion gap (AG) relates to the difference between cations and anions, being commonly determined in plasma of both study and control groups. A reduced index is caused by the hypoproteinemic states (dietary protein deficiency, low food absorption, protein synthesis deficiency or protein and electrolyte loss due to acute diarrhea). Therefore, this phenomenon might be detected in pediatric RVI-related acute diarrhea.

Metabolic disorders in RVI infants may develop with normal anionic gap, viz. up to 16 mEq/l or above 16 mEq/l. Normal anion gap metabolic disorders were reported in diarrhea and other pathological disorders. High anion gap-related metabolic disorders might occur due to the toxic syndrome and other types of intoxication [16].

For a more detailed analysis of the acid-base alteration, which is an essential factor in determining RVI-related complications, their correlation with the immunization status of RVI-diseased children has been assessed.

The measurement of the anion gap in acid-base balance determined the type of metabolic disorders in RVI-diseased infants, a fact that was subsequently used in the therapeutic management of dehydration among these children.

The mean value of the anion gap determined in non-immunized RVI –diseased infants was equal to 15,1 mEq/l, whereas in immunized infants, it was 7,9 mEq/l, viz. the control group revealed a 1,9-fold increase in the anion gap.

It should be noted that the highest value of the anion gap exceeded 16 mEq/l and was detected in 6,6% of non-immunized children, compared to the highest value of 10,2 mEq/l, found in 2,4% cases of immunized infants

The etiological structure of the mixed rotavirus infection in the study groups

37 children from the immunized group were found with viral-bacterial infections, the most commonly detected pathogens being *Staphylococcus aureus, Klebsiella pneumoniae, Proteus mirabilis and Citrobacter freundi.*

Klebsiella pneumoniae, Escherichia coli and other members of the Enterobacteriaceae family are common human disease-causing agents that have become resistant to a wide range antibiotics [21]. The negative impact of antibiotics on the normal gut microbiota allows these conditioned pathogens to spread within the colon, thus significantly increasing the risk of an increased blood flow and of a generalized inflammatory response.

The bacterial flora fromboth study groups showed a wide variety of pathogenic or conditioned pathogenic microbial strains *as Klebsiella pneumoniae, Proteus mirabilis, Staphylococcus aureus, Citrobacter freundii, Escherichia coli atypical h +, Klebsiella oxytoca.*

Thus, combinations of rotavirus with *Klebsiella pneumoniae* and *Klebsiella oxytoca* was found in 24,3% on-immunized children, whereas the same combination was found in 16,0% cases (p = 0,4780, χ^2 = 0,504) of immunized children.

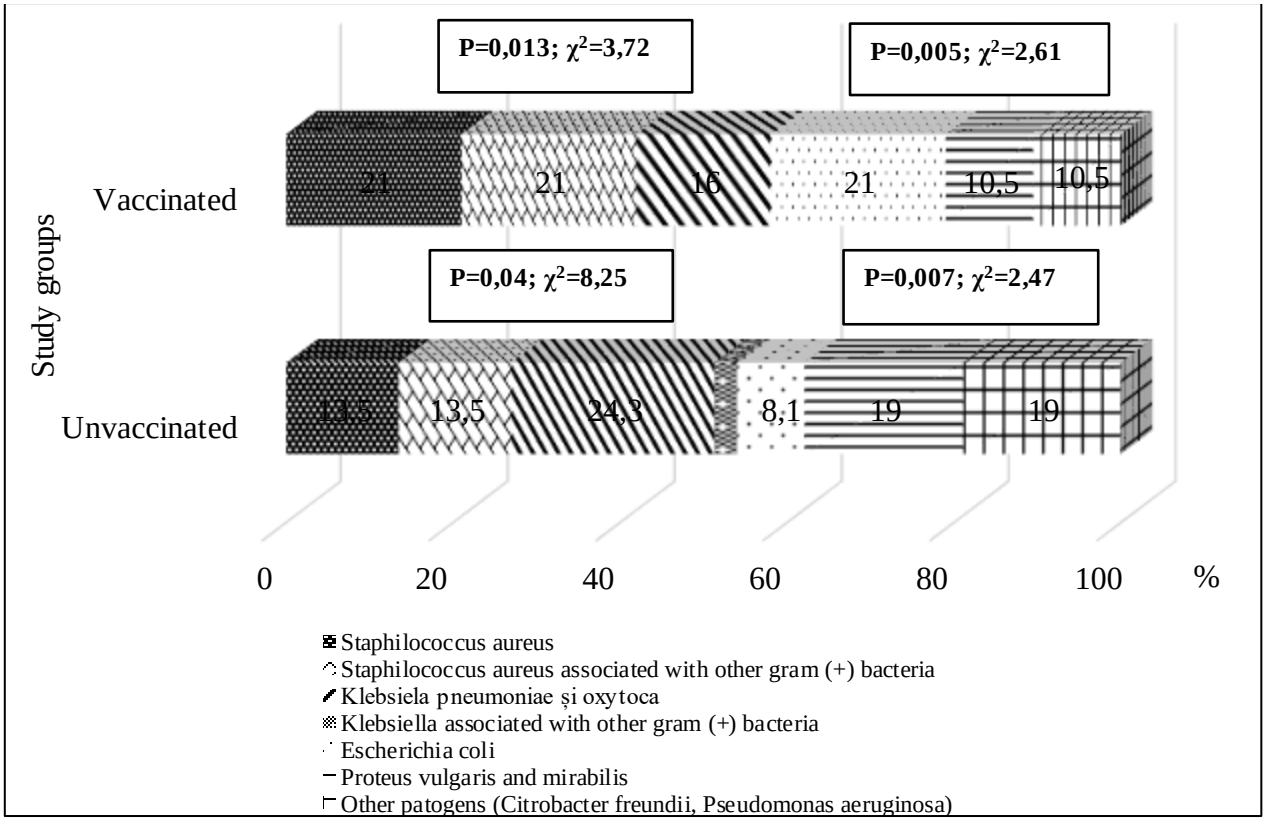


Figure 11. **Indices of viral-bacterial infection in patients from both study groups**

Staphylococcus aureus associated with other gram (+) bacteria had an equal percentage, and was ranked first within group I, whereas *Escherichia coli* made up 21,0% of cases. The most rare strain was *Klebsiella* associated with other gram (+) bacteria, was found in 2,7% of non-immunized children and in 0% of vaccinated children. The etiological structure of rotavirus infection is shown in Figure 11.

Combination of rotavirus + *Proteus mirabilis* and *Proteus vulgaris* was ranked second among other strains, viz. it was detected in 19,0% cases from group II and in 10,5% - group I (p = 0,4169, χ^2 = 0,659). *Staphylococcus aureus* associated with other gram (+) bacteria, found in group II, had equal percentage with rotavirus, thus being detected in 13,5% cases (p = 0,4730, χ^2 = 0,515), whereas *Escherichia coli* - in 8,1% of cases (p = 0,1705, χ^2 = 1,879). Other pathogens associated with rotavirus infection were found in 19,0% in non-immunized children and in 10,5% of immunized children (p = 0,4169, χ^2 = 0,659). The disease evolution in children with mixed infection occurred more severely, leading to toxic syndrome and being complicated by moderate and/or severe dehydration in most cases.

Genotypic spectrum of rotavirus infection in both immunized and non-immunized infants

The present research included molecular-genetic investigations via RNA denaturation, followed by reverse transcription PCR (RT-PCR), by using the "ProSpecT ROTAVIRUS Kit" (manufacturer - Zhejiang Orient Gene Biotech Co., LTD). As a result, the successful

identification and sequencing of viral RNA in RVI-diseased children was possible. That was carried out separately, depending on the immunization status of each RVI –diseased child and clinical manifestations of the disease.

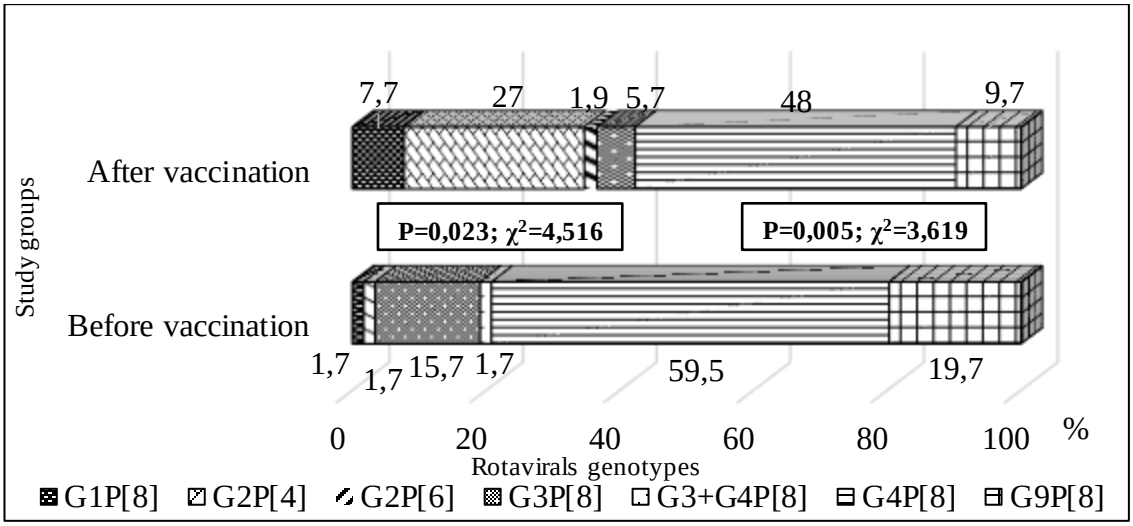


Figure 12. **The evolution of the frequency of genotype incidence among RVI patients before and after vaccination (%)**

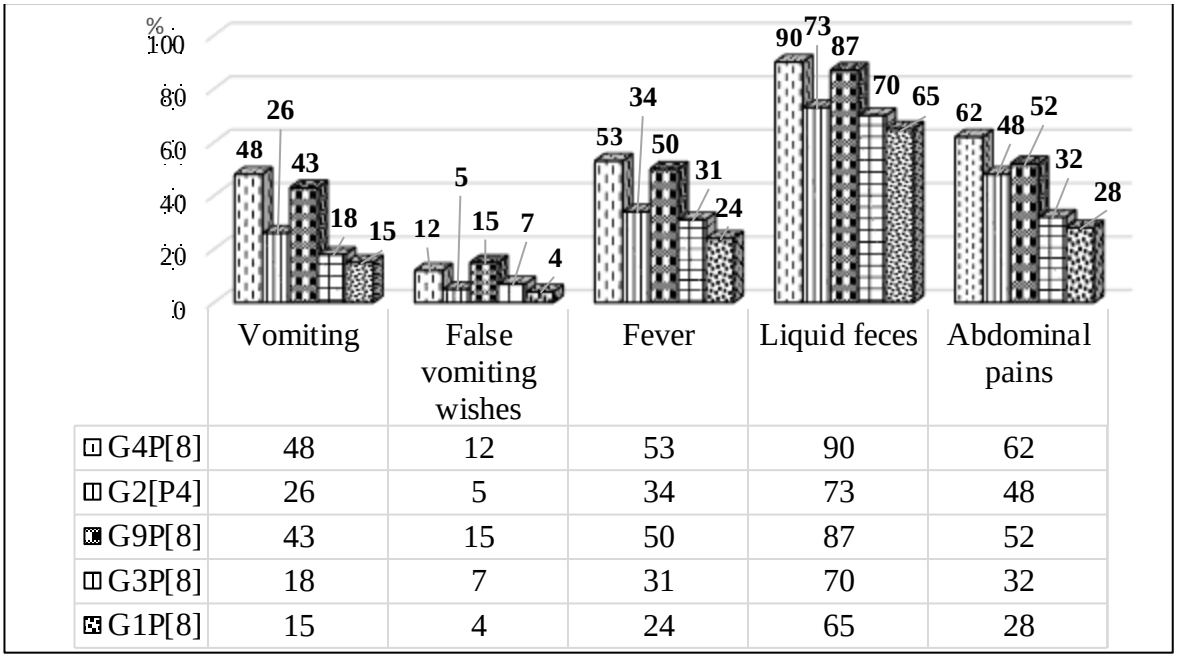
In group II, G4 genotype [P8] ranked first among other genotypes and made up 59,5% (p = 0,2310, χ^2 = 1,435), followed by G9P[8] -19,7% (p = 0,1453, χ^2=2,121) and then by G3P[8]-15.7% (p = 0,0962, χ^2 = 2,768). The other three genotypes found in unvaccinated children were G1P[8] (p = 0,1356, χ^2 = 2,228), G2P[4] (p = 0,0001, χ^2 = 14,525) and G3 + G4P[8] (p = 0,3886, χ^2 = 0,743), accounting for 1,7% of cases (p = 0,0001, χ^2 = 14,525).

Only the G4 genotype P[8] predominated in 48% cases of immunized children (p = 0,2310, χ^2 = 1,435), the other genotypes were reversed. Thus, G2 genotype P[4] was found in 27,0% cases (p = 0,0001, χ^2 = 14,525), G9P[8] – 9,7% (p = 0,1453, χ^2 = 2,121), G1P[8] was encountered in 7,7% (p = 0,1356, χ^2 = 2,228), and G3P[8] – 5,7% cases (p = 0,0962, χ^2 = 2,768).

Of the total number of genotype samples, the most common incidence for genotypes identified in patients with rotavirus infection during the pre-immunization period, was determined for G4P[8], G3P[8] and G9P[8]. After immunization, their frequency decreased, whereas G2P[4] and G4 P [8] were ranked first among the other genotypes. The incidence of genotypes identified in patients with rotavirus infection before and after vaccination is shown in Figure 1. Therefore, G4P[8], G9P[8], G3P[8] genotypes were predominantly found in non-immunized children and G4P[8], G2P[4] and G1P[8] genotypes in immunized ones.

The analysis of clinical manifestations of RVI-diseased patients, depending on the virus genotype, displayed a more severe evolution in children with G4P genotype [8], followed by G9P [8]. The severe condition of these patients was featured by loose stools in 90,0% cases, vomiting in almost 48,0% and fever in over 50.0% cases.

Depending on the severity degree, G2P[4] genotype ranked third, characterized by symptoms, high incidence of loose stools in 73,0% cases, followed by abdominal pain in 48,0% cases, and fever and vomiting in 34,0% and 26,0% cases, respectively.



	Vomiting	False vomiting wishes	Fever	Liquid feces	Abdominal pains
G4P[8]	48	12	53	90	62
G2[P4]	26	5	34	73	48
G9P[8]	43	15	50	87	52
G3P[8]	18	7	31	70	32
G1P[8]	15	4	24	65	28

Figure 13. **The characteristics of RVI genotype-related clinical symptoms**

Depending on rotavirus genotypes, acute dehydration syndrome occurred just in the same order. Of the 83 children with medium and severe dehydration, a maximum number of cases was found in children with the G4P[8] genotype, accounting for just over half of the cases – 52,0% ($p = 0,136$, $\chi^2 = 1,931$), whereas G9P [8] ranked second in 28,0% of cases ($p = 0,039$, $\chi^2 = 2,903$). These genotypes were followed by G2P[4] and G3P[8]- in 12,0% and – in 5,0% of cases, respectively ($p = 0,254$, $\chi^2 = 0,571$) (Figure 14).

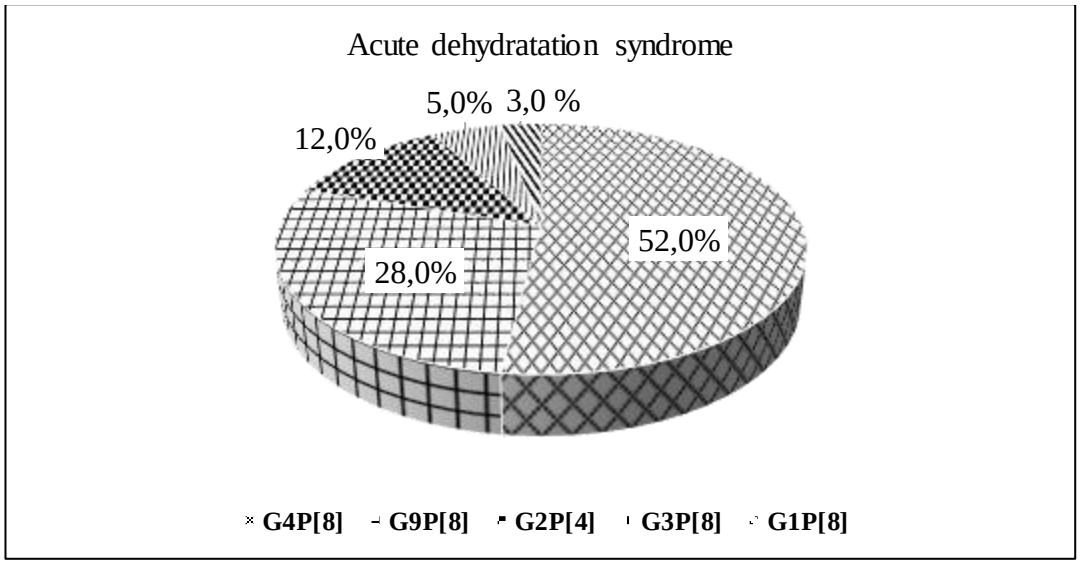


Figure 14. **Indices of acute dehydration syndrome related to RV genotypes among the studied children**

The study of RV genotype circulation for the 2012–2016 period, in the Republic of Moldova, showed that, in 2012 - 2014, the G4P[8] genotype ranked first among the other genotypes, followed by the G9P[8]. G3P[8] was recorded in 2013, however, it was not detected in 2014 - 2015, until it occurred again in 20,0% cases, in 2016. In case of non-immunized children, G4P[8] was found in more than 80,0%, whereas in immunized children this genotype showed a 2-fold decrease. In 2014, G2P[4] was registered in 20,0% cases, whereas in 2015, this

genotype exhibited a 3-fold increase, which then disappeared completely in 2016. Therefore, three genotypes were recorded in the pre-vaccination period: G1P[8], G9P[8] and G4P[8], the latter occurred in more than 80,0% cases, whereas after immunization, the genotype variety of RVI changed in 2012 (Figure 14).

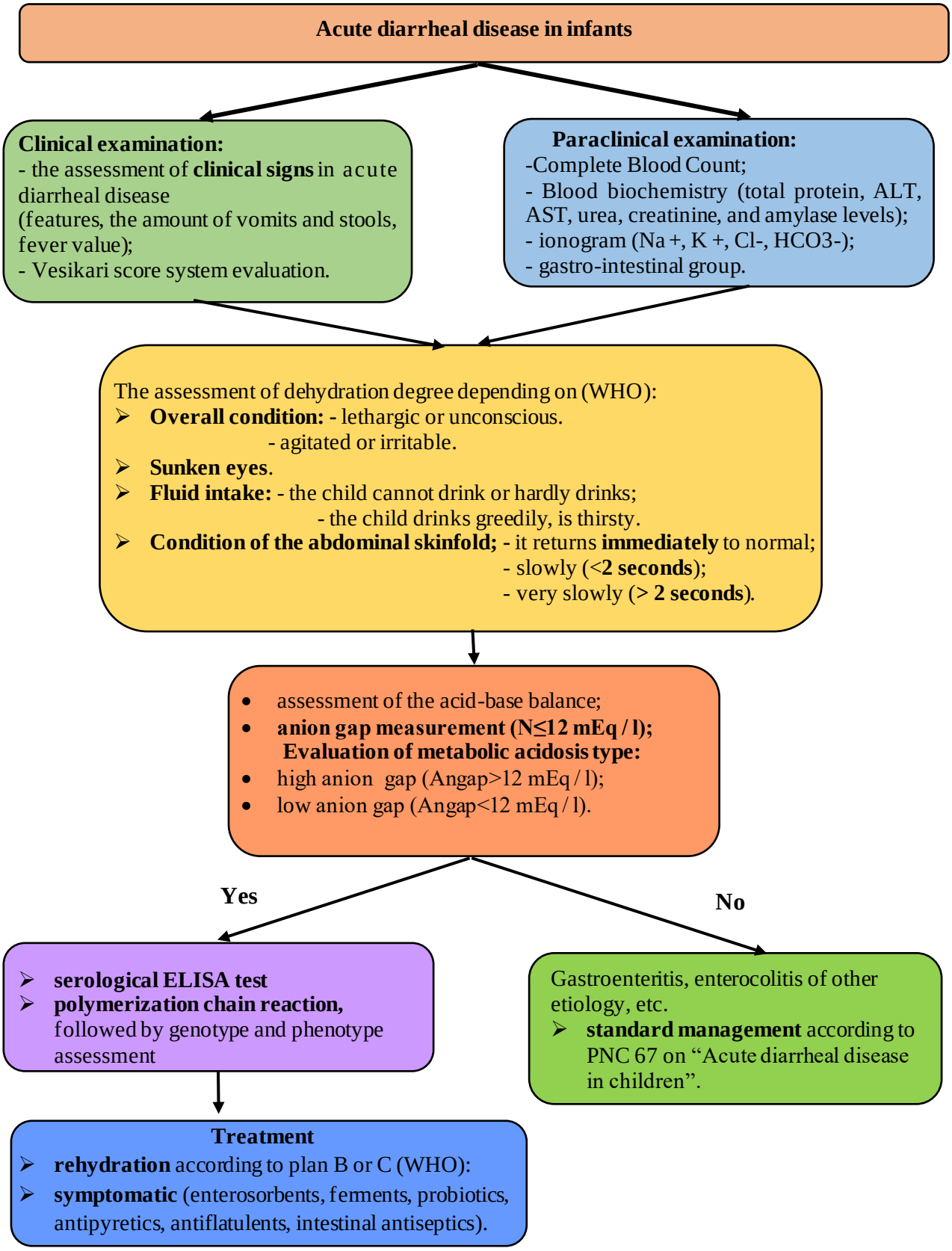


Figure 14. **Differential diagnostic algorithm for rotavirus infection in infants**

The data obtained in our study on the virus genotype and phenotype were used as scientific basis in the selection of the anti-rotavirus vaccine introduced in the National Immunization Program. The Rotarix vaccine included the following rotavirus strains: G1P[8], G2P[4], G3P[8], G4P[8] and G9P[8].

The Republic of Moldova was the first country in the WHO European Region [18, 29] to introduce rotavirus vaccination into its routine immunization program among children. These results reveal a deep impact of the immunization program on RV diseases among young children in Moldova.

During the research, we found that the acid-base balance and anion gap measurement exhibited greater values than 12 mEq/l in RVI-diseased children. This fact proves that these patients had a high anion gap metabolic acidosis, which refers to the presence of a viral infection with pronounced toxic infectious syndrome, followed by a severe general condition. Thus, following both clinical and paraclinical investigations, as well as the assessment of dehydration signs (according to the WHO), it is recommended to study the acid-base balance, to calculate the anion gap and to identify the type of metabolic disorders. Proper diagnosis of types of metabolic disorders might enable possible suspecting of virus-related gastroenteritis or enterocolitis in infants, followed by an appropriate medical approach for RVI management.

GENERAL CONCLUSIONS

1. According to the clinical-epidemiological study, the peak incidence of rotavirus infection was registered in February, in both groups during the winter-spring time period. Rotaviral infection was developed more frequently in boys over 6 months old. The clinical evolution of rotaviral infection, medium, severe, and very severe occurred depending on the genotypes of rotaviral viruses: G4P [8], G9P [8], G2P [4] and G3P [8] identified in infants.

2. The polymorphism of RVI clinical manifestations found in the study groups, according to the Vesikari score system, reported that the non-immunized group showed a severe and extremely severe disease development, which is 2,85 times higher than in immunized group ($p < 0,005$).

3. The stool analysis parameters (Leukocytes, Erythrocytes, Neutral fats) of non-immunized children revealed a massive inflammatory response, based on statistically significant values ($p < 0,005$, $\chi^2 = 0,397$). The increased transaminase level (TGO and TGP) in the non-immunized group displayed a poor prognostic value, due to a severe and lasting evolution of rotavirus infection ($p < 0,026$).

4. The acid-base disturbances were more pronounced in the control group, compared to the main group. Anion gap metabolic acidosis was significantly increased (>15 mEq/l) in non-immunized children ($p = 0,0435$, $\chi^2 = 0,7431$), whereas the highest normal value did not exceed within the immunized children. The mean value of the anion gap in non-immunized group was twice increased ($15,1 \pm 3,51$ vs $7,9 \pm 2,12$), due to genotypes G4, G9 and G3 being the main determinants in assessing the severity degree of RVI clinical evolution.

5. Molecular biology techniques used in determining the circulating genotypes (G4, G9, G3) among infants have proved and confirmed the usefulness of the Rotarix vaccine implementation, which was included within the National Immunization Calendar , subsequently it is characterized by a considerable reduction in morbidity rate by 40,0%, caused by RV-related gastroenteritis in immunized children.

6. In the Republic of Moldova, the sentinel surveillance system in rotavirus infection was implemented with the dynamic evaluation of the initially dominant strains G9 P [8] – 40,0% and G1 P [8] – 36,0%, which determined an increased morbidity with severe and very serious. Following the implementation of the rotaviral vaccine and the evaluation of the impact of sentinel surveillance measures, the genotypic variety was shown to change with the presence in infants of the dominant strains G2 P [4] –60,0% and G4 P [8] –40,0% and the evolution of clinical features by decreasing severity and morbidity. by rotavirus infection in infants.

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- **Articles submitted to scientific journals abroad:**

✓ **Articles submitted to accredited international scientific journals:**

1. **Alsaliem T.,** Donos A., Iliev A-M., Bozomitu L. Aspecte clinico-epidemiologice ale infecției determinate de rotavirus la sugari în Republica Moldova. *Revista Pediatru.ro*, 18 decembrie 2019; **DOI**: 10.26416/Pedi.56.4.2019.2792**, ISSN 1841-5164 (B+ conform Colegiului Medicilor din România).**
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✓ **Articles in category B journals**

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- **Active participations within various scientific forums:**

✓ **International attendance**

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