



COMPARATIVE ASSESSMENT OF CLINICAL-PARACLINICAL MANIFESTATIONS OF ROTAVIRUS INFECTION VERSUS GENOTYPICAL VARIETY IN INFANTS

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Introduction. RVI (rotavirus infection) is the most common cause of dehydration in infants and young children. The implementation of the sentinel surveillance of RVI in infants from 2008 in the Republic of Moldova demonstrated the high rate of this infection (40.0%), being an argument in recommending the antirotaviral immunization in children within the National Immunization Program.

Material and methods. The study enrolled 193 children with acute diarrheal disease, included in the sentinel supervision (2012-2016) and treated in the Unit for Acute Diarrheal Diseases at Children's Clinical Hospital no. 1. The biological material was examined by serological enzyme-linked immunosorbent assay (ELISA) and genotyping, revealed by polymerase chain reaction (PCR).

Results. The rotavirus infection was confirmed in 193 infants, of which 121 children were not vaccinated against RVI, and 72 were immunized. Depending on the genotypes encountered before and after vaccination, it was found that G9P [8], G3P[8], G4P[8] were detected before vaccination, although postvaccine prevailed G2P[4], G4P[8]. In addition, the incidence of RVI is decreasing and the disease evolution is much better.

Conclusions. This article reflects the evolution of the genotypic properties of rotaviruses and the clinical-paraclinical particularities of RVI in infants, highlighting the importance of the implementation of antiretroviral immunization in children within the National Immunization Program in the Republic of Moldova.

Cuvinte cheie: infecție rotavirală, sugar, genotip, vaccin.

EVALUAREA COMPARATIVĂ A MANIFESTĂRILOR CLINICO-PARACLINICE ALE INFECȚIEI ROTAVIRALE VERSUS VARIETATEA GENOTIPICĂ LA SUGARI

Introducere. IRV (infecție rotavirală) este cauza cea mai frecventă de deshidratare la sugar și la copilul mic. Implementarea în Republica Moldova în anul 2008 a supravegherii santinelă a IRV la sugari a relevat o rată înaltă a infecției (40,0%), acest fapt servind ca argument în recomandarea imunizării antirotavirale a copiilor în cadrul Programului Național de Imunizări.

Material și metode. În studiu au fost incluși 193 de copii sugari cu boală diareică acută, aflați sub supravegherea santinelă în perioada 2012-2016 și tratați în Secția de boli diareice acute a Spitalului Clinic Municipal de Copii nr. 1. Materialul biologic s-a examinat prin metoda reacției serologice ELISA, iar evidențierea genotipurilor a fost efectuată prin reacția de amplificare genică PCR.

Rezultate. Dintre cei 193 de sugari cu infecție rotavirală, 121 nu au fost vaccinați contra IRV, iar 72 – au fost imunizați. În funcție de genotipurile întâlnite până și după vaccinare, s-a constatat că până la vaccinare au predominat genotipurile G9P[8], G3P[8], G4P[8]. În schimb, postvaccinal au prevalat genotipurile G2P[4], G4P[8], incidența IRV fiind în scădere, iar evoluția bolii – mult mai ușoară.

Concluzii. Acest articol reflectă evoluția proprietăților genotipice ale rotavirusurilor și a particularităților clinico-paraclinice ale IRV la sugari, cu o importanță majoră în contextual procesului de implementare a imunizării antirotavirale a copiilor în cadrul Programului Național de Imunizări din Republica Moldova.

INTRODUCTION

Rotaviruses (RV) are one of the most common causes of acute gastroenteritis (AGE) worldwide, affecting 95.0% of children up to the age of five. Globally, it is estimated that RV infection causes 3.6 million episodes of AGE per year (1, 2). By the time antirotaviral immunization was implemented, more than 2 million children with GEA of rotavirus etiology were hospitalized annually worldwide (2, 3).

By the age of 5, almost all children have suffered from rotavirus infection (RVI), which is the first cause of severe diarrhea with dehydration in infants worldwide. In low-income countries, the average age of primary infection with rotaviruses occurs between 6 and 9 months (80.0% of cases occur in infants under 1 year), whereas in high-income countries the first episode sometimes occurs at the age of 2-5 years, children being the most affected (65.0% of cases being found in infants) (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 younger than 5 years. Rotaviruses are associated with approximately one third of all severe diarrheal diseases in young children, with recent estimates of annual mortality associated with rotaviruses ranging from 453,000 (2008), 197,000 (2010) and 173,000 (2011) (1, 3).

Since 2009, the World Health Organization (WHO) has recommended that rotavirus vaccines be included in national immunization programs in each country and that this measure be considered a public health priority (5, 6).

Globally, rotavirus is the most common cause of severe gastroenteritis in children <5 years of age, accounting for an estimated 2.4 million hospital admissions and 527,000 deaths each year (1,7). Because of the tremendous global burden of rotavirus, vaccine development and introduction has been a high priority for several international agencies, including the World Health Organization (WHO) and the Global Alliance for Vaccines and Immunization (GAVI) (8).

The assessment of morbidity in children under one year of age marks an increased incidence of the most commonly encountered infections, which determines the formation of a healthy soci-

ety and of future generations. In acute gastroenteritis, primary rotaviruses are acquired through contact with the diseased being (1, 4, 9). Rotaviruses are also detected in the co-filters of children with gastroenterocolitis of indeterminate etiology, having the primary role in identifying serious gastrointestinal infections, with etiology difficult to establish (5, 8, 10).

The problem of rotavirus infection remains current during the last decades, since the discovery of this virus, the rotavirus infection being present with an increased incidence, especially among children under 5 years (11, 12). Each child can withstand from one disease episode to several episodes, most commonly in the first 5 years of life, characterized by a high incidence of serious cases, with complications, in the absence of therapy. The clinical impact in rotavirus infection is with intestinal and non-intestinal disorders, involving not only the lining of the gastrointestinal tract, but also of other systems. In infants, the severity of rotavirus infection is determined in particular by the genotype and phenotype of this condition, which determines the severity of dehydration and toxic syndrome (12, 13).

MATERIAL AND METHODS

This present prospective, descriptive study included 193 children with acute diarrheal disease, involved in the sentinel surveillance (2012-2016) from the Acute diarrheal diseases unit, at the Municipal Children's Clinical Hospital no. 1.

The research protocol was approved by the Research Ethics Committee of the *Nicolae Testemitanu* SUMPh from the Republic of Moldova (report no. 54 of 13.02.2017).

All patients were selected according to the standard case scenario. The hospitalization rules and the completion of a standardized questionnaire for this study were respected. The parents of the children gave written informed consent for their enrollment in the research.

The criteria for inclusion in the study were as following:

1. Children aged between 1 - 12 months (according to WHO recommendations);
2. diarrhea with at least 3 defecations over the last 24 hours, but not more than 7 days;

3. patients examined by serological reaction ELISA with genotypes detected in PCR for rotavirus infection within the first 24 hours after admission;

The criteria for exclusion of patients from research:

1. patients with rotavirus infection or severe comorbidities (heart defects, digestive tract development abnormalities, nervous system development abnormalities etc.).
2. patients with diarrhea of less than 3 fluid defecations over the last 24 hours.
3. children aged over 12 months.

The assessment regarding signs of dehydration was performed in children included in the study at the time of clinical examination. The biological material of all the children included in the study was virologically examined for rotavirus infection, using the ELISA serological reaction and genotyping in the chain polymerization reaction (*ProSpecT ROTAVIRUS Kit*, manufacturer – *Zhejiang Orient Gene Biotech Co. LTD*, China).

This is a qualitative immunoenzymatic test for the detection of rotavirus (group A) in human faecal samples, which help in diagnosing acute gastroenteritis. The assay uses a polyclonal antibody to detect group-specific proteins, including the main internal capsid protein (VP6), present in A rotaviruses (13).

About 1.5-2 ml of liquid faeces or 1 g of fresh semi-formed faeces, spontaneously excreted were collected in a sterile recipient from each patient on the first day of hospitalization. The container was hermetically sealed, labelled with patient data and stored at 2-8°C until being transported. Also, each patient positive for rotavirus infection, was established on the vaccine status, by questioning the caregiver with whom the child was admitted to the hospital, checking the child's development booklet and checking the vaccination register at the residence place.

Depending on the vaccine status, the study sample (n=193) was divided into group "Unvaccinated children with rotavirus infection" (n=121) and group "Vaccinated children with rotavirus infection" (n=72).

Sample size was estimated by using the following formula:

$$n = \frac{1}{(1-f)} \times 2 \times (Z_{\alpha} + Z_{\beta})^2 \times P(1-P) / (P_0 - P_1)^2$$

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

P_0 – according to the bibliographic data (11), the success of the treatment in the unvaccinated patients constitutes on average 50.0% ($P_0=0.50$);

P_1 – expected success of treatment in the vaccinated children group will be 75.0% ($P_1=0.75$); $P=(P_0+P_1)/2=0.625$;

Z_{α} – table value, when the statistical significance is 95.0%, then the coefficient $Z_{\alpha}=1.96$;

Z_{β} – table value, when the statistical power of the comparison is 80.0%, then the coefficient $Z_{\beta}=0.84$;

f – Proportion of subjects expected to abandon the study for reasons other than the investigated effect $q=1/(1-f)$, $f=10.0\%$ (0.1).

Therefore, the L_1 research group included no less than 65 patients vaccinated against rotavirus infection and the L_0 control group included no less than 65 unvaccinated patients.

The data collected in the study were introduced into the electronic table via the Microsoft Office Excel 2007 program. The results were processed using the SPSS version 22 software. For comparing the differences between groups, the 95% confidence interval (95CI), the criterion (chi-square) χ^2 was calculated. $P < 0.05$ was considered as a significant threshold.

RESULTS

In both groups, male sex prevailed. Depending on age, children aged 6-12 months from the unvaccinated group predominated in 66.9% cases, compared to the vaccinated group, where this age group constituted only 47.2%.

The patients included in the study were admitted to the hospital during the first 3 days of illness. In all patients, the disease started with acute intoxication syndrome in 100% of cases in unvaccinated children compared with 82% in vaccinated children, characterized by alteration of the overall health condition, decreased appetite and malaise.

Table 1 shows a much more severe evolution of the unvaccinated children who suffered from rotavirus infection, compared with the vaccinated ones, thus moderate and severe dehydration accounted for 53.7% vs. 30.6% in the vaccinated group. The clinical form in which rotavirus infection occurred was manifested by gastroenterocolitis in 81% of unvaccinated and 75% of those vaccinated (tab. 1).

Table 1. Manifestations of acute diarrheal disease.

Clinical diagnosis	Unvaccinated (n=121)	Vaccinated (n=72)	X ²	p
	Abs, %	Abs. %		
Acute gastroenterocolitis, without dehydration	37(30.6)	36(50.0)	7.186	0.0073
Acute gastroenterocolitis, moderate dehydration	56(46.4)	17(23.6)	9.919	0.0016
Acute gastroenterocolitis, severe dehydration	5(4.1)	1(1.4)	1.092	0.2960
Acute enterocolitis, without dehydration	19(1.7)	14(19.4)	0.434	0.5100
Acute enterocolitis, moderate dehydration	4(3.3)	4(5.6)	0.596	0.4401

Note: statistical test applied: χ^2 .

Unvaccinated children presented vomiting in 80% of cases, fever – in 76.9%, faces with

pathological inclusions – in 97.5%, whereas vaccinated children showed less frequent symptoms (tab. 2).

Table 2. Clinical symptoms of rotavirus infection.

Symptom	Unvaccinated (n=121)	Vaccinated (n=72)	X ²	P
Vomiting	98 (80%)	50 (69.4%)	1.821	0.0686
Fever	93 (76.9%)	54 (75%)	0.293	0.7694
Liquid feces	121 (100%)	72 (100%)	1.245	0.2133
Feces with mucus	115 (95%)	60 (83.3%)	2.561	0.0104
Feces with foam	3 (2.5%)	0	0.957	0.3383

Note: statistical test applied: χ^2 .

Table 3 shows the frequency of comorbidities that occurred concurrently with rotavirus infection. The incidence of the respiratory system diseases

was higher, accounting for 61.1% of cases, followed by GI disorders - 57.8%, whereas the NS impairment ranked third among these.

Table 3. Structure of comorbidities in study groups in children with acute diarrheal disease of rotavirus etiology.

Nosological entity	Unvaccinated (n=121)	Vaccinated (n=72)	X ²	P
Respiratory diseases (bronchitis, pneumonia)	74 (61.1%)	38 (52.7%)	1.300	0.2541
GI disorders	70 (57.8%)	27 (37.5%)	7.402	0.0065
Nervous system diseases (HIPE, TIE)	48 (40%)	6 (8.3%)	22.299	0.0001
The reno-urinary system pathologies (UTI)	27 (22.3%)	10 (13.8%)	2.097	0.1476
Hematopoietic system disorders (anemia)	27 (22.3%)	12 (16.6%)	0.906	0.3412
Allergic dermatitis	9 (7.4%)	6 (8.3%)	0.051	0.8214
Malnutrition	7 (5.7%)	2 (2.8%)	0.857	0.3545
ENT diseases	11 (9%)	1 (1.4%)	4.484	0.0342

Note: statistical test applied: χ^2 . HIPE – hypoxiischemic perinatal encephalopathy; TIE – toxi-infectious encephalopathy; UTI – urinary tract infections.

The hospital stay of most unvaccinated children was doubled compared to the vaccinated ones. Thus, the mean length of hospitalization of unvaccinated children was 6.7 days, whereas vaccinated children had a mean hospital stay of 6 days (fig. 1).

The rotavirus etiology of acute diarrheal disease was confirmed in all patients included in the study. 73.6% of cases of acute diarrheal disease of viral etiology was associated with bacterial flora, more significantly in unvaccinated children. Thus, in the unvaccinated group, *Klebsiella pneumoniae*

and *Proteus mirabilis* predominated in 5.0% of patients, double compared with the vaccinated group, followed by *Staphylococcus aureus* and *Citrobacter freundii*, accounting for 4%. Atypical

h+ Escherichia coli and *Klebsiella oxytoca* were detected in 2.5% of children. The etiological structure of rotavirus infection is shown in Table 4.

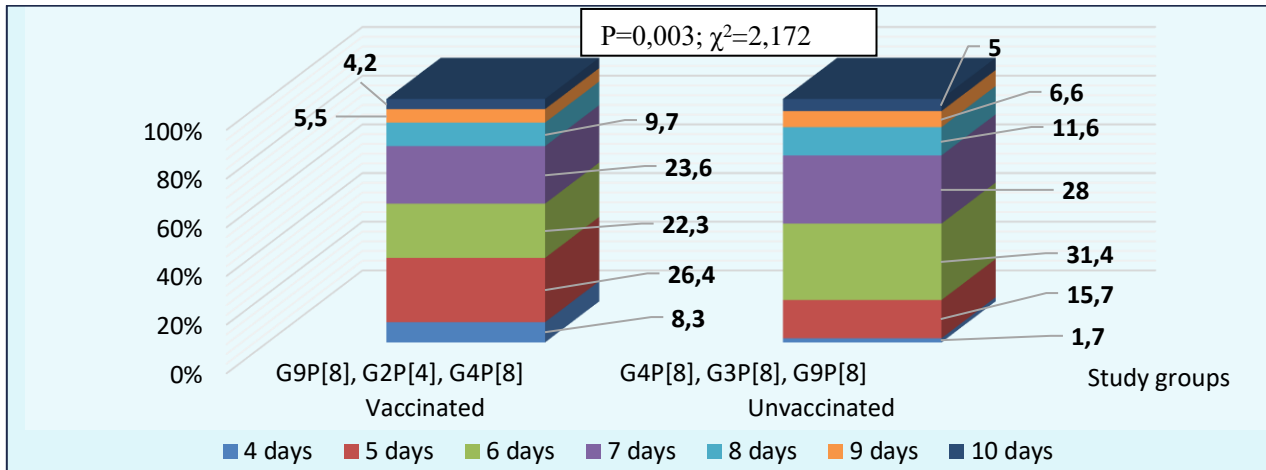


Figure 1. The RVI severity depending on the hospital stay length within both study groups.

Table 4. Bacterial over infection of children affected by rotavirus infection.

Infection	Unvaccinated	Vaccinated	X ²	P
Rotaviral mono-infection	84 (69.4%)	53 (73.6%)	0.620	0.5354
Bacterial association (total)	37 (30.6%)	19 (26.4%)	0.620	0.5354
<i>Staphylococcus aureus</i>	13.5%	21%	0.515	0.4730
<i>St. aureus</i> associated with other gram (+) bacteria	13.5%	21%	0.515	0.4730
<i>Klebsiella pneumoniae</i> și <i>oxytoca</i>	24.3%	16%	0.504	0.4780
<i>Klebsiella</i> associated with other gram (+) bacteria	2.7%	0	0.467	0.4944
<i>Escherichia coli</i>	8.1%	21%	1.879	0.1705
<i>Proteus vulgaris</i> și <i>mirabilis</i>	19%	10.5%	0.659	0.4169
Other pathogens (<i>Citr. freundii</i>, <i>Ps. aeruginosa</i>)	19%	10.5%	0.659	0.4169

Note: The assessment was based on Fisher's exact test.

The present study identified acetonuria in 47 unvaccinated children vs. 20 vaccinated children. Thus, a high amount of ketone bodies in the urine (≥ 150 mg/dl) was found in 21.3% of the cases among unvaccinated children, compared with 5% in children from the vaccinated group.

Of the total number of genotypes samples, the incidence of genotypes identified in patients with rotavirus infection during the prevaccine period, the most commonly encountered genotypes were G4P[8], G3P[8] and G9P[8]. In the postvaccine period, their frequency decreased first, being the genotypes G2P[4] and G4P[8] (fig. 2).

DISCUSSIONS

Rotavirus is one of the most important causative agents of acute dehydrating diarrheal disease, be-

ing involved in 12.0-71.0% of acute hospitalized gastroenteritis cases. In developed countries rotaviruses cause from 1/3 to 1/2 of all serious diarrhea. Thus, about 3 million cases of rotavirus infection (RVI) are registered annually in the USA, which causes 67 thousand hospitalizations (250 thousand day/bed) and more than 100 cases of deaths (14).

In 2006, two live attenuated vaccines were developed and authorized: *Rotarix*[®] and *RotaTeq*[®]. *Rotarix* is a monovalent vaccine derived from a human G1P isolate [8]. *RotaTeq*[®] is a pentavalent, consisting of a mixture of monoreassortants human bovine rotavirus, which transports genes encoding human G1, G2, G3, G4 and P[8] proteins into a genetic background of Wi79 bovine rotavirus (G6P[5]).

Both vaccines have proven to be very effective in clinical trials and have been included in the mandatory national vaccination scheme for children

in over 100 countries since 2006. Post-marketing studies have shown that both vaccines are highly effective at the population level (7, 15, 16).

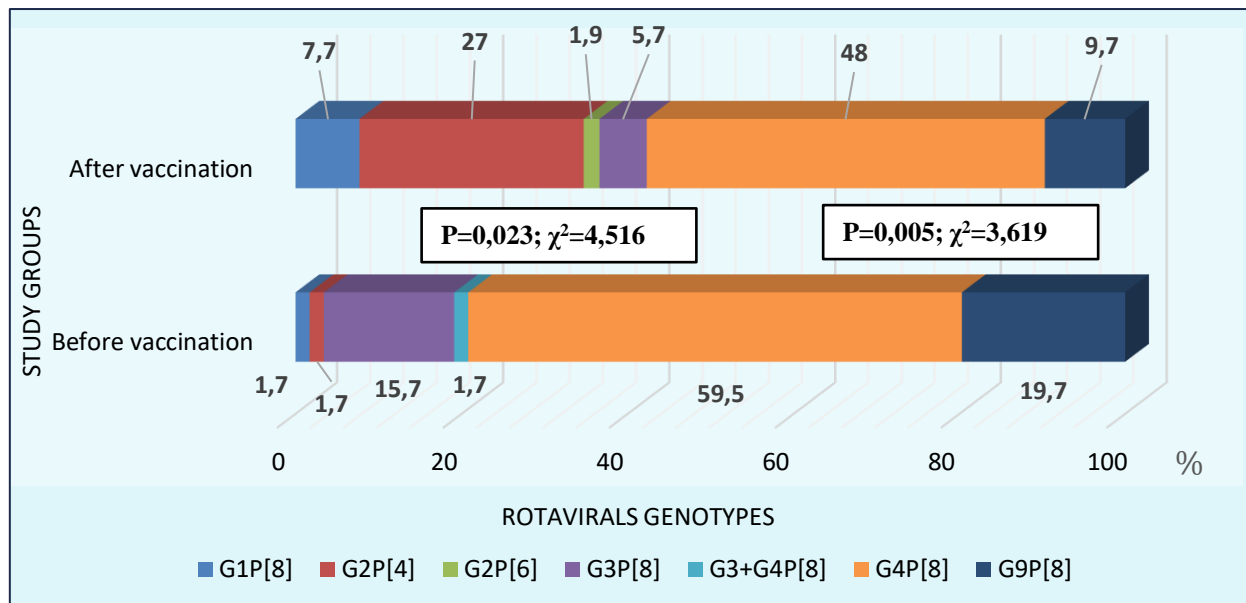


Figure 2. The evolution of the frequency of genotype incidence among RVI patients before and after vaccination (%).

Moldova was the first country in the WHO European Region to introduce rotavirus vaccination into the routine immunization program for children. The vaccine used in Moldova includes G4, G2, G9 genotypes that cover most strains of circulating rotaviruses in the population of the country according to the monitoring and sentinel surveillance data in children up to the age of 5 years.

In their study, Codruța Iliescu Halițchi et al. (2013), also noted that most cases (85.0%) occurred between January and July, with 2 peaks in February (25.7%) and June (21.4%) (15).

The study performed by Stela Gheorghita highlighted the favorable impact of the vaccination program on rotavirus disease among children from Chisinau, Moldova. Two-dose rotavirus vaccination reduced hospitalization by 79%, and severe disease progression was reduced by 82%. Generally, hospitalizations with rotavirus decreased by two-thirds until the second year of the program, in a model compatible with the impact of the vaccine. The major decrease was among vaccinated cohorts children <1 year in the first year and <2 years in the second year after vaccine implementation. In addition, the number of children <5 years old hospitalized with rotavirus decreased significantly,

including unvaccinated cohorts, suggesting indirect protection resulting from children’s immunization (17).

According to our data, this is the first research study of patients with rotavirus infection, following the vaccination program in the Republic of Moldova, which analyzed the prophylactic potential of the antirotaviral vaccine in our country. Given that vaccines have been very effective in high-income countries, they have proven to be considerably less powerful in low- and middle-income countries. The disease associated with rotavirus was the cause of death in more than 200,000 children aged <5 years worldwide in 2013.

A long-term study was carried out by Joshua Gikonyo et al., lasting from January 2015 until December 2017. Patients with rotavirus gastroenteritis were supervised within in several hospitals in Kenya. The subjects of the study were infants and young children under 5 years of age, who had an episode with three liquid or watery stools for 24 hours for up to 7 days, with or without episodes of vomiting. In this study, the distribution of cases of rotavirus infection during the year was more frequently recorded in August-September, with a reduction in the number of cases in November-January (18).

According to Ulrich Desselberger, vaccine effectiveness was higher in high-income countries, with severe rotavirus disease protection rates at 80-90%, whereas in low- and middle-income countries it was 30-50% lower. Different factors were assessed to identify or suggest the differences in efficacy of the rotavirus vaccine, including malnutrition, intestinal microbiota status, vitamin D3 administration, co-infections, immunity of the infant immune system and genetic factors (19).

Raúl F. Velázquez performed a systematic review and metaanalysis to describe, compare and synthesize the effectiveness of the vaccine, from randomized clinical trials prior to authorization, finding a decrease in hospitalizations and addresses to the children's emergency department with rotavirus infections (20).

Alkali B. R. et al, in 2015 conducted a pediatric study on a sample of 200 children with diarrhea. Of these, 51 (25.5%) children were positive for rotavirus. Among children with rotavirus infection, 79.1% of cases had watery stools and 75.0% - semi-liquid stools. Short-term diarrhea lasted 2 days in most cases (43.1%), the liquid stool lasted 7 days in 27.5%, and only in 2.0% it lasted 10 days. 40 children out of 51 had vomiting that occurred in the first two days of illness, accounting for 90.0%, on the 7th day it was found in only 7.5% of children. Chi-square analysis indicated a significant association between rotavirus diarrhea and vomiting ($P < 0.05$) (16, 21).

28 studies from 12 countries included data on the proportion of GERV among hospitalized children under the age of five in the Middle East and North Africa. These studies included 17,233 cases of diarrhea that were tested for rotavirus infection. Of these, 7,366 (42.7%) were RV positive. Depending on the country, the average share of cases with rotaviral infection ranged from 316 (22.5%) to 1,885 cases (63%). Egypt, Tunisia and the Islamic Republic of Iran reported the lowest proportion with 316 (22.5%), 65 (23.3%) and 537 cases (27.4%). The highest proportion was observed in Turkey 1,885 (63%), the United Arab Emirates 381 (50.3%), and Saudi Arabia 1,226

cases (48.7%). The other countries reported a percentage between 93 (35.8%) and 358 cases (45.2%) (22).

Another study carried out in the neighboring country Romania (2014) by Victoria Birlutiu and Rares Mircea Birlutiu between January 1, 2011 - December 31, 2012, aimed at tracking the seasonality of the disease, the clinical aspects, the severity of the disease, the laboratory examinations, the need for parenteral rebalancing, the costs of hospitalization. The study group consisted of 236 children (2011 - 114 cases, 2012 - 122 cases) between 0 and 16 years old with rotavirus infection in the infectious diseases services in children in Sibiu. Thus, 114 cases were diagnosed in 2011, respectively 122 in 2012, commonly in the cold months, more frequently in the male gender, sex ratio M/F 1.42:1 in 2011, 1.18:1 in 2012, among children aged 1-3 years - 58.90%. 91 cases in 2011 - 79.82%, and 112 cases in 2012 - 91.80% respectively, with an average / severe score. 15 cases showed neurological disorders and 15 cases acute renal failure. Severe onset cases led to hospitalization in the first 24 hours: 41.23% of cases in 2011 and 51.64% in 2012, prolonged with diarrhea over 6 days, established in 62 cases (54.39%) in 2011 and 75 cases (61.48%) in 2012. Cases with severe dehydration were found in children aged 1 to 12 months, being associated with thrombocytopenia, leukopenia, PCR increase (probability 0.42), hydroelectrolyte imbalances associated with signs of encephalopathy. This study highlighted the frequent association of hyponatremia (< 130 mEq/l) from electrolyte imbalance with rotavirus gastroenteritis, 83 cases in 2011 and 51 cases in 2012. Therefore, it is worth mentioning that in Romania the rotavirus vaccine was not included in National Immunization Program (7, 21).

Estimation of 2-dose vaccine effectiveness in Moldova, especially against severe cases at 84% (95CI: 65% to 93%), is largely compatible with that in other countries with low mortality (in the mortality layers A and B of WHO), namely VE is 85% (95CI: 80% to 88%) based on the overall analysis of 8 studies, including $> 32,000$ participants (17, 22).

CONCLUSIONS

1. The clinical and etiological evolution of rotavirus infection was presented by the following genotypes: G4P[8], G9P[8], G2P[4] and G3P[8]. The peak incidence of rotavirus infection being in February, more commonly found in boys over 6 months.
2. The polymorphism of the clinical manifestations in the rotavirus infection identified in the study groups, showed a severe and extremely severe evolution of the entity among unvaccinated children, which was 2.85 times higher than in the group of vaccinated ones ($p < 0.005$).
3. Stool test indices in unvaccinated children exhibited a massive inflammatory process due to statistically significant values ($p < 0.005$, $\chi^2 = 0.397$). The increase in the level of transaminases (TGO and TGP) in the unvaccinated group proved a poor prognosis due to a more serious and lasting evolution of rotavirus infection ($p > 0.026$).
4. Determination of circulating genotypes (G4, G9, G3) by molecular biology techniques in the infant population proved and confirmed the usefulness of the Rotarix vaccine included in the National Immunization Calendar, manifested by a considerably lower morbidity of gastroenteritis of rotavirus etiology in the group of vaccinated children.

CONFLICT OF INTERESTS

Nothing to declare.

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