Preliminary Final Report:

IIS - Non-interventional immunological and epidemiological study

"The long-term immunogenicity of single-dose vaccination against hepatitis A in endemic region (Tyva Republic, Russian Federation)"

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1. Introduction

Hepatitis A is an acute liver disease caused by hepatitis A virus (HAV), a member of *Picornaviridae* family. HAV infection is preventable by vaccination. A universal mass vaccination (UMV) strategy in toddlers is shown to be beneficial in regions with HAV endemicity transition from high to intermediate [1]. The standard immunization schedule for inactivated HAV vaccine consists of two doses given with six months interval. This vaccination schedule proved to be highly immunogenic and provides protective antibody response which is expected to last for decades [2]. For economic reasons and to improve coverage rates, the universal single-dose HAV vaccination has been implemented in toddlers first in Argentina in 2005 [3], and then in Brazil in 2014 [4]. This single-dose schedule has been proved effective in terms of both long-lasting immunological response and decrease of symptomatic disease burden [5]. However, more data from different regions are needed to understand the effectiveness of single-dose immunization at the population level.

Universal single-dose HAV vaccination in children aged 3 years and older has been implemented since 2012 in Tyva Republic which was the most affected by hepatitis A region of the Russian Federation, with incidence levels 10-15-fold higher compared to national average [6]. Tyva lies at in southern Siberia and borders Mongolia to the south. According to Human Development Index (HDI), the Tyva is the least developed region in Russia [7]. The vaccination campaign in Tyva has started in August 2012 with monovalent pediatric inactivated vaccine (HAVRIX® 720 EU) given to children of 3-8 years. By the end of 2012, total 65,097 children have received single-dose immunization, resulting in 87.4% coverage among children aged 3-8 years [6]. Since then, single-dose vaccination against hepatitis A for children aged 3 years and older has been introduced into the Tyva regional immunization schedule.

Currently, UMV strategy is not implemented in the Russian Federation on a country level. With the exception of Tyva and three other regions where regional standard two-dose pediatric HAV immunization has been introduced, HAV vaccination is recommended only for professional risk groups and travelers, and for the purpose of outbreak control. This approach was shown to have a little impact on the herd immunity to HAV in Russia [8].

The previous study of five-year immunological effectiveness of single-dose HAV immunization in Tyva has demonstrated the presence of anti-HAV antibody concentrations ≥10 mIU/mL in 98.0% of children tested one month after single-dose immunization, and in 93.5% and 91.1% of children one year and five years after single-dose immunization, respectively [6]. This study has also shown the rapid decline in registered annual incidence rates in children under 18 years of age

from 450–860 per 100,000 in pre-vaccination years to 7.5 per 100,000 in this age group and to 3.2 per 100,000 in the total population one year after the start of vaccination and to zero incidence level in the region by 2016. These data have confirmed that single-dose vaccination was an effective method of bringing hepatitis A under control in a short period of time in a highly endemic region. However, further monitoring of incidence rates and antibody levels is needed to determine the sustainability of the observed effect, and to establish the need for booster immunization. Existing data on the long-term immunological and epidemiological effectiveness of pediatric hepatitis A single-dose vaccination were generated so far only in Argentina [9] and Brazil [10], and no data are available currently from Russia or Commonwealth of Independent States (CIS) countries.

The single-dose UMV strategy is expected to provide a long-lasting protecting immunity against HAV that leads to the rapid and sustainable drop of disease incidence in both vaccinated and general population, and a significant decrease or even cessation of HAV circulation. The latter is usually reflected by decrease or even absence of detectable HAV RNA in sewages. To evaluate a long-term immunological and epidemiological effectiveness of the single-dose vaccination in Tyva Republic, three key parameters were assessed in this study: i) the prevalence of protective levels of anti-HAV antibodies in vaccinated population nine and eleven years after the single-dose immunization; ii) the effect of the vaccination on the reported hepatitis A annual incidence rates in study region, both in vaccinated and non-vaccinated populations; iii) the circulation of HAV in this region after the implementation of universal child single-dose vaccination based on results of the monitoring of HAV RNA in sewage and open bodies of water. In current study, we used two seroprotection thresholds to assess prevalence of HAV protecting immunity – 20 mIU/mL, as this concentration is indicated as a seropositivity threshold in instructions to anti-HAV diagnostic assays, and 10 mIU/mL, the currently recognized minimal seroprotection cutoff level [5].

Study objectives

Primary Objective

To estimate the prevalence of protective anti-HAV IgG levels (≥20 mIU/mL) and to assess mean concentration of antiHAV IgG following the single-dose hepatitis A vaccination in children nine and eleven years post vaccination in the birth cohort 2004-2009.

Secondary objectives

1. To estimate the proportion of vaccinated individuals who contacted HAV (based on abnormally high levels of anti-HAV IgG > 6000 mIU/mL).
2. To assess the proportion of children without protective anti-HAV antibodies who need the booster immunization 9-11 years after single-dose vaccination against hepatitis A.

Exploratory objectives

1. To estimate the reported annual incidence rates of hepatitis A in children under 15 years and in total population in study region after single-dose child vaccination program (2013-2022) compared to pre-vaccination period (2004–2012).
2. To investigate all hepatitis A cases notified in Republic Tyva after vaccination program implementation (2013-2022).
3. To assess the circulation of HAV after the implementation of universal child single-dose vaccination by the monitoring of HAV RNA in city sewage.

2. Methods

2.1. Study design

A graphic representation of this prospective non-interventional observational single-center surveillance study is shown in Figure 1. The anti-HAV IgG antibodies were determined at two time points in two independent cohorts of children who were vaccinated with single dose of monovalent pediatric inactivated vaccine (HAVRIX® 720 EU) in Tyva in 2012 and had available HAV vaccination records. The sample collection and anti-HAV IgG antibody testing was performed in two cohorts, in 2021, i.e. nine years after immunization (Year 9 Cohort), and in 2023, i.e. eleven years after immunization (Year 11 Cohort). The study involved only one study visit per subject. However, since the study comprised of two independent time points (Year 9 and Year 11), the same subject who participated in the Year 9 Cohort could be enrolled for the Year 11 Cohort. In that case, the subject was enrolled as a new subject and was not associated to his/her participation in the Year 9 survey. To supplement the data on immunogenicity of single-dose HAV vaccination with epidemiology data, the annual hepatitis A incidence rates reported in Tyva Republic in 2013-2023 were analyzed in comparison to pre-vaccination period (2001–2012). During the study period, in 2021-2023, samples of sewage, sources of drinking water (springs, wells) and open bodies of water were collected for HAV RNA monitoring. Sampling was performed twice a year, in June and October.

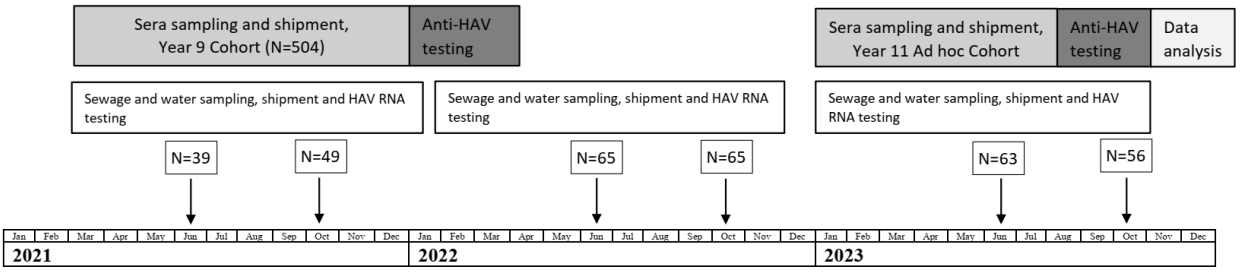

Figure 1. Study design and the timeline. N in boxes indicates the number of samples.

2.2. Study cohorts and blood sampling

The population sample size was calculated using the effect size calculation [11] with significance level 5% and power 99%. The minimal sample size was calculated to be 470 for both cohorts and then rounded to 500. In 2021, total 504 children (aged 11–18 years, median age 14 years) who were vaccinated with one dose of HAV vaccine were recruited for the study. This group of participants is referred thereafter as Year 9 Cohort. In 2023, the independent cohort of 500 children (aged 13–18 years, median age 15 years) vaccinated with one dose of HAV vaccine in 2012 was recruited. This group of participants is referred thereafter as Year 11 Cohort. However, due to high demand from parents of vaccinated children for voluntary sampling and anti-HAV antibody testing, additional 835 children were recruited in 2023 and tested within the study budget. These additional samples were included as "ad hoc" analysis, resulting in the total number of 1335 participants in 2023 (referred thereafter as Year 11 Ad hoc Cohort), with median age 15 years.

In Year 9 Cohort the male/female ratio was 1:1.2, and the urban/rural population ratio was 1:1.2. Similarly, in Year 11 Cohort the male/female ratio was 1:0.97, and the urban/rural population

ratio was 1:1.4. In Year 11 Ad hoc Cohort, the male/female ratio was similar - 1:0.94, but the proportion of rural population was slightly higher, with urban/rural population ratio - 1:1.4.

The study was conducted in accordance with the principles expressed in the World Medical Association Declaration of Helsinki regarding ethical medical research involving human subjects. The signed informed consent of each participant's parents was obtained before the recruitment. Additionally, the signed informed consents of subjects of age 15 to 18 years were obtained in addition to the signed informed consent from their parents.

The vaccination status of participants retrieved from medical records (individual vaccination cards, Form No.63) stored at children's polyclinics.

In compliance with study protocol, all enrolled participants met the following inclusion criteria:

- Subjects whose parents comply with the requirements of the protocol.
- Written informed consent obtained from the subject and/or subject's parents.
- Children who have received one dose of HAV monovalent pediatric inactivated vaccine (HAVRIX® 720 EU) that has been implemented since August 2012 in Tyva Republic with available HAV vaccination records.
- Not more than 6 months deviation of the time point: time between the date of vaccination and the date of blood sampling should be between 8.5 and 9.5 years in Year 9 Cohort, and between 10.5 and 11.5 years in Year 11 Cohort.

In compliance with study protocol, from the enrollment were excluded: children in care, children who received 2 doses of vaccine or received hepatitis A vaccines other than Havrix, children with known past history of hepatitis A infection before vaccination.

Serum samples with volume ca. 3 ml were obtained from each enrolled participant and shipped using cold chain to Moscow, to Mechnikov Research Institute for Vaccines and Sera. All sera were coded and aliquoted, and 0.5 ml aliquots were stored at -70°C until anti-HAV testing. Following the anti-HAV antibody testing, children with antibody concentrations below 20 mIU/mL were offered to receive a booster dose of vaccine. No additional measurement of antibodies was performed thereafter for these children.

2.3. Anti-HAV testing

Total anti-HAV antibodies were tested in sera of vaccinated children using two commercially available quantitative immunoassays: Elecsys® Anti-HAV (Roche, Mannheim, Germany) on cobas e 411 analyzer and ELISA Vectohep A-IgG kit (Vector-Best, Novosibirsk, Russia). Both assays had similar performance characteristics. Testing was performed according to the instructions provided by the manufacturers of the respective kits. Seropositivity was defined as antibody levels of ≥20 mIU/ml according to kit manufacturer's instruction. The alternative, or surrogate, seropositivity cut-off level was defined as anti-HAV antibody concentration of ≥10 mIU/ml, as this concentration was previously reported as a minimal protective in humans [12]. All samples with anti-HAV antibody concentrations above the upper limit of quantification range of the test were diluted and repeatedly tested. The final concentrations were obtained by multiplying the result on the dilution factor.

2.4. Incidence analysis

Data on the reported annual hepatitis A incidence rates were retrieved from the database of the Russian Federal Service for Surveillance on Consumer Rights Protection and Human Wellbeing (Rospotrebnadzor). Hepatitis A incidence rates in Tyva in 2001–2023 were analyzed in comparison to the national average rates for the following population groups: i) children aged 0-14 years; ii) children aged 0-17 years (data from 2006 to 2023 were only available for analysis for this age group); iii) total population. Additionally, hepatitis A incidence rates in 2013-2023 in regions neighboring Tyva (Republic of Buryatia, Republic of Khakassia, Republic of Altai, Irkutsk Region, Krasnoyarsk Territory) were analyzed. To assess the possible changes in incidence of other enteric infections during the HAV vaccination period (2013-2023), the incidence rates of enterovirus infections and shigellosis in total population of Tyva were analyzed.

2.5. HAV RNA testing in environment samples

Total 337 samples of sewage and water from different sources were collected, including 120 sewage samples from Kyzyl city, the capital of Tyva; 82 samples from sources of drinking water such as springs and wells; and 135 samples from open basins near settlements, including lakes and rivers at places popular for swimming and sunbathing. Samples were collected in six time points, twice a year, in July and in October throughout 2021-2023. The sampling was performed at the same locations in each time point.

The volume of each sample was 2 liters. The concentration of water samples was carried out immediately after the sampling using commercially available kit "Virosorb-M" (Bioservice, Russia) according to manufacturer's protocol. The method of processing is based on the concentration of negatively charged viral particles on magnetic particles coated with polymer silicon dioxide modified with amino groups. The volume of resulting concentrated sample was 2 ml. The isolation of total nucleic acids was performed from 1 ml concentrated samples using the MagNA Pure Compact Nucleic Acid Isolation Kit I - Large Volume (Roche Applied Science, Mannheim, Germany). HAV RNA was detected by polymerase chain reaction combined with reverse transcription (RT-PCR) with primers to VP1/2A region of virus genome using the previously described protocol [13]. All HAV positive samples were sequenced to obtain genetic information on strains circulating in Tyva. For this purpose, amplified fragments of HAV genome were sequenced in 3130 Genetic Analyzer (ABI, Foster City, USA) automatic sequencer using the BigDye Terminator v3.1 Cycle Sequencing Kit according to the manufacturer's protocol. HAV sequences were subjected to phylogenetic analysis using the maximum likelihood (ML) method in the IQ-TREE software together with HAV sequences obtained in different part of Russia including Tyva in different years.

2.6. Statistical analysis

Data analysis was performed using graphpad.com. Statistical analysis included calculation of geometric mean concentration (GMC) for anti-HAV antibody concentrations, the calculation of a 95% confidence interval (95% CI) and assessing the significance of differences of mean values between study cohorts using Fisher's exact test (for categorical data) and unpaired t test (for continuous data) with significance threshold $p < 0.05$.

3. Results

3.*1. Prevalence of protective anti-HAV IgG levels and mean concentration of anti HAV IgG following the single-dose hepatitis A vaccination in children nine and eleven years post vaccination (primary objective)*

Results of anti-HAV antibody testing in Year 9 and Year 11 cohorts are summarized in Table 1.

In Year 9 Cohort, protective anti-HAV antibody concentrations (≥20 mIU/mL) were detected in 99.4% (95% CI: 98.2-99.9% [501/504]) of children tested nine years after single-dose immunization. Among 501 seropositive samples, 440 samples contained anti-HAV antibodies in concentrations in the range 20 to 6,000 mIU/mL (minimum – 30 mIU/mL, maximum – 1,632 mIU/mL).

Table 1. Distribution of serum anti-HAV antibody concentrations in samples collected 9 and 11 years following single-dose vaccination

Anti-HAV concentrations	Year 9 Cohort, n=504		Year 11 Cohort, n=500		Year 11 Ad hoc Cohort, n=1335	
	Number of samples	Detection rate, % (95% CI)	Number of samples	Detection rate, % (95% CI)	Number of samples	Detection rate, % (95% CI)
0-9 mIU/mL	3	0.6% (0.1-1.8%)	102	20.4% (17.1-24.2%)	329	24.6% (22.4-27.0%)
*p**			< 0.0001		< 0.0001	
10-19 mIU/mL	0	0%	22	4.4% (2.9-6.6%)	48	3.6% (2.7-4.7%)
*p**			< 0.0001		< 0.0001	
20-6,000 mIU/mL	440	87.3% (84.1 -89.9%)	330	66.0% (61.7-70.0%)	839	62.9% (60.2-65.4%)
*p**			< 0.0001		< 0.0001	
>6,000 mIU/mL	61	12.1% (9.5 – 15.3%)	46	9.2% (6.9-12.1%)	119	8.9% (7.5-10.6%)
*p**			0.1523		0.0432	

* When compared data from Year 11 Cohort and Year 11 Ad hoc Cohort with data from Year 9 Cohort (Fisher's exact test)

In Year 11 and Year 11 Ad hoc Cohorts, the proportions of serum samples with anti-HAV antibody concentrations ≥20 mIU/mL were significantly lower compared to Year 9 Cohort, 75.2% (95% CI: 71.2-78.8% [376/500]) and 71.8% (95% CI: 69.3-74.1% [958/1335]), respectively (p <0.0001). The proportions of samples with low anti-HAV concentrations, between 10 and 20 mME/mL, were 0%, 4.4%, and 3.6% in Year 9, Year 11 and Year 11 Ad hoc cohorts. Given that the possible protective threshold level could be as low as 10 mIU/mL, we additionally calculated the proportion of samples with anti-HAV antibody concentrations ≥ 10 mIU/mL in each study cohort (Table 2).

Table 2. Proportion of samples with anti-HAV antibody concentrations ≥ 10 mIU/mL collected 9 and 11 years following single-dose vaccination

Anti-HAV concentration	Year 9 Cohort, n=504		Year 11 Cohort, n=500		Year 11 Ad hoc Cohort, n=1335	
	Number of samples	Detection rate, % (95% CI)	Number of samples	Detection rate, % (95% CI)	Number of samples	Detection rate, % (95% CI)
≥ 10 mIU/mL	501	99.4% (98.2–99.9%)	398	79.6% (75.8-82.9%)	1006	75.4% (73.0-77.6%)
*p**			< 0.0001		< 0.0001	

* When compared data from Year 11 Cohort and Year 11 Ad hoc Cohort with data from Year 9 Cohort (Fisher's exact test)

The proportions of such samples in Year 11 and Year 11 Ad hoc cohorts were similar between these two cohorts (79.6% and 75.4%, respectively, p =0.0634), but were significantly lower compared to Year 9 Cohort (99.4%, p <0.0001).

Samples with anti-HAV concentrations <10 mIU/mL and >6,000 mIU/mL were excluded from the calculation of GMC. Anti-HAV antibody GMC values for study cohorts are shown in Table 3.

Table 3. Anti-HAV antibody geomantic mean concentrations in samples collected 9 and 11 years following single-dose vaccination

Cohort	Anti-HAV GMC, mIU/mL (95% CI)	*p**
Year 9 Cohort	1446.3 (1347.1-1545.4)	
Year 11 Cohort	271.2 (219.1-323.1)	<0.0001
Year 11 Ad hoc Cohort	282.6 (203.8-360.8)	<0.0001

* When compared data from Year 11 Cohort and Year 11 Ad hoc Cohort with data from Year 9 Cohort (unpaired t-test)

Anti-HAV antibody GMC value nine years following single-dose vaccination was 1446.3 mIU/mL. In Year 11 and Year 11 Ad hoc Cohorts, anti-HAV GMC values were significantly lower, 271.2 mIU/mL and 282.6 mIU/mL, respectively (p <0.0001, unpaired t test).

3.2. The proportion of vaccinated individuals with abnormally high levels of anti-HAV IgG antibodies (secondary objective)

In Year 9 Cohort, 61 out of 504 samples (12.1%) contained anti-HAV antibodies in concentration above 6,000 mIU/mL, indicating the possible virus exposure resulted in the boosted antibody response (Table 1). When compared between Year 11 Cohort and Year 11 Ad hoc Cohort, the proportion of samples with anti-HAV antibody concentrations above 6,000 mIU/ml was similar, 9.2% and 8.9%, respectively (p = 0.8548). However, when compared to Year 9 Cohort, the proportion of samples with abnormally high levels of anti-HAV IgG antibodies was significantly lower in Year 11 Ad hoc Cohort (12.1% vs. 8.9%, p=0.0432).

3.3. The proportion of children without protective anti-HAV antibodies (secondary objective)

In Year 9 Cohort, the proportion of samples with anti-HAV concentrations below 20 mIU/mL was 0.6% (95% CI: 0.1-1.8%, [3/504]). All these samples contained anti-HAV in concentrations below

10 mIU/mL (Table 1). The proportions of samples with anti-HAV concentrations below 20 mIU/mL were significantly higher in Year 11 and Year 11 Ad hoc cohorts, 24.8% (95% CI: 21.2-28.8% [124/500] and 28.2% (95% CI: 25.9-30.7% [377/1335], respectively (p <0.0001). If consider the anti-HAV concentration ≥ 10 mIU/mL as a protective threshold level, the proportions of samples In Year 11 and Year 11 Ad hoc cohorts, the proportions of children without protective antibody levels were also significantly higher in Year 11 and Year 11 Ad hoc cohorts compared Year 9 Cohort, 20.4% and 24.6%, respectively, vs. 0.6% (p <0.0001) (Table 1).

3.4. Hepatitis A incidence analysis (exploratory objective)

Registered incidence of any infectious diseases in Russia is reported by Rospotrebnadzor for three categories of people: the total population, children aged 0-14 years, and children aged 0-17 years. The hepatitis A annual incidence rates in Tyva are shown in Figure 2 for these three categories in comparison to respective national average rates.

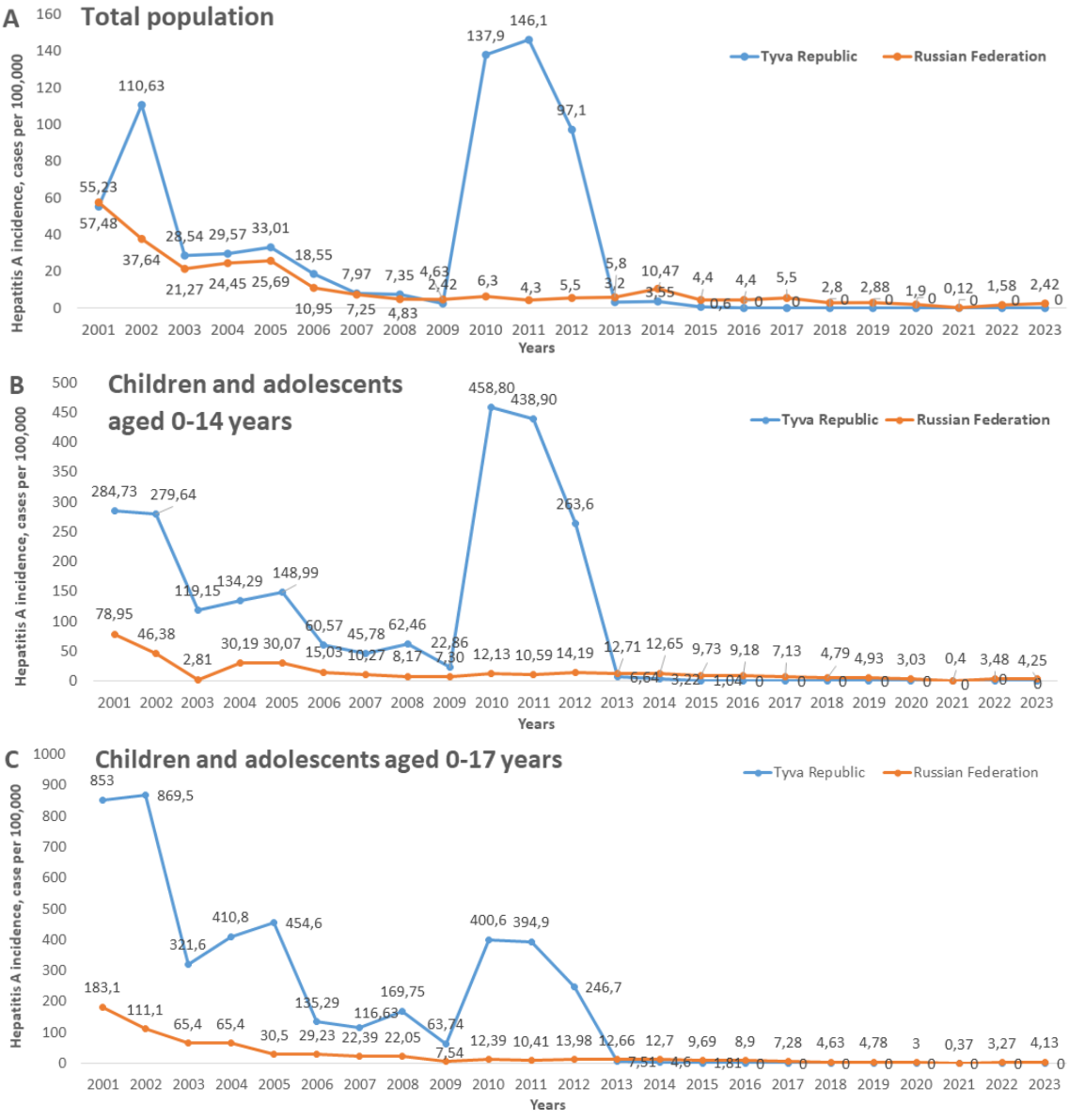

Figure 2. Hepatitis A annual incidence rates in 2001-2023 in Tyva and in the Russian Federation on average, among total population (A), children and adolescents aged 0-14 years (B), children and adolescents aged 0-17 years (C).

Hepatitis A incidence rates in Tyva in the pre-vaccination period (2001-2012) were the highest in the country, with the majority of cases registered in children and adolescents under 18 years. In 2020–2023, during the study period, no cases of hepatitis A were registered in Tyva, as well as in previous years, starting from 2016. Interestingly, the incidence dropped after the start of vaccination campaign not only in children aged 0-14 years (Fig. 2B), i.e. the age group that includes vaccinated children, but also in older children (Fig. 2C) and in total population as well (Fig. 2A).

A detailed description of all cases reported in Tyva in 2013–2015 was published previously [6]. We provide these data here for illustrative purposes in Table 4. All pediatric cases were reported in unvaccinated children. Three of these pediatric cases, along with one adult case, evidently were imported from neighboring Kyrgyzstan, as all these patients confirmed the contact with an HAV-positive individual in this country. In 2015, only two cases of hepatitis A were registered, including one case in unvaccinated child aged 13 years.

Table 4. Cases of hepatitis A reported in Tuva, 2013–2015. Adapted from [6].

Case #	Age, years	Sex	Place of permanent residence	History of hepatitis A vaccination	Infection risk factors	Year	Severity of disease
1	3	Male	Tuva	No	Intrafamilial cluster case (together with cases #2 and #3)	2013	Mild, anicteric
2	16	Female	Tuva	No	Intrafamilial cluster case (together with cases #1 and #3)	2013	Moderate severity, anicteric
3	16	Female	Tuva	No	Intrafamilial cluster case (together with cases #1 and #2)	2013	Moderate severity, anicteric
4	9	Female	Tuva	No	Contact with an infected child in another region in Russia	2013	Moderate severity, icteric
5	10	Female	Tuva	No	No risk factors identified	2013	Moderate severity, icteric
6	12	Male	Tuva	No	No risk factors identified	2013	Moderate severity, icteric
7	12	Female	Tuva	No	No risk factors identified	2013	Mild, anicteric
8	16	Female	Tuva	No	No risk factors identified	2013	Moderate severity, icteric
9	19	Male	Tuva	No	No risk factors identified	2013	Moderate severity, icteric
10	1.5	Male	Tuva	No	Contact with an infected individual in another country	2014	Moderate severity, icteric
11	3	Male	Tuva	No	Contact with an infected individual in another country	2014	Moderate severity, icteric

12	23	Female	Tuva	No	Contact with an infected individual in another country	2014	Moderate severity, icteric
13	35	Female	Tuva	No	No risk factors identified	2014	Subclinical
14	56	Female	Tuva	No	Contact with an infected individual in another country	2014	Subclinical
15	14	Male	Tuva	No	No risk factors identified	2014	Mild, anicteric
16	36	Female	Tuva	No	No risk factors identified	2014	Moderate severity, icteric
17	15	Female	Tuva	No	No risk factors identified	2014	Mild, anicteric
18	34	Male	Tuva	No	No risk factors identified	2015	Moderate severity, icteric
19	13	Male	Tuva	No	No risk factors identified	2015	Moderate severity, icteric

Next, we analyzed the hepatitis A incidence rates during the vaccination period (2013-2023) in regions neighboring Tyva, to confirm that the drop of incidence in this particular territory did not result from the decrease in HAV circulation in the whole region. Almost every year, with the exception of the COVID-19 pandemic, in all regions neighboring Tyva there was a registered incidence of hepatitis A, which in some years had pronounced increases (Table 5). Thus, against the background of zero or almost zero incidence of hepatitis A in Tyva during the vaccination period, a substantial incidence rates were registered continuously in neighboring regions.

Table 5. Hepatitis A incidence rates in Tyva and in neighboring regions in total population in 2013-2023

Year	Hepatitis A incidence, cases per 100,000					
	Tyva Republic	Republic of Buryatia	Republic of Khakassia	Republic of Altai	Irkutsk Region	Krasnoyarsk Territory
2013	3.20	5.35	4.13	3.37	5.61	11.29
2014	3.55	6.48	34.55	2.39	9.37	34.93
2015	0.60	1.44	23.99	2.37	9.83	13.86
2016	0.00	2.25	6.54	0.00	5.46	7.91
2017	0.00	2.96	2.80	1.87	8.91	5.17
2018	0.00	1.02	3.91	1.85	4.94	2.44
2019	0.00	1.63	1.68	32.50	5.46	2.78
2020	0.00	0.1	1.31	5.47	3.93	2.02
2021	0.00	0.00	0.19	0.00	0.04	0.00
2022	0.00	0.71	0.75	0.00	1.73	1.89
2023	0.00	0.82	0.38	0.00	1.57	1.93

To assess, whether the observed drop in hepatitis A incidence in Tyva could be possibly associated with the general improvement in sanitation and consequent decrease in enteric

infections burden, we analyzed the incidence rates of enterovirus infections and shigellosis in 2013-2023 in Tyva (Table 6). The high annual rates of these enteric infections indicate the maintaining poor sanitation in the region, which is associated with the sustained risk of transmission of communicable diseases. Moreover, these data clearly indicate that hepatitis A incidence drop observed in 2013-2023 was not associated with the improved sanitation, but was associated entirely with HAV vaccination program.

Table 6. Enterovirus infections and shigellosis incidence rates in total population of Tyva in 2013-2023.

Year	Incidence, cases per 100,000	
	Enterovirus infections	Shigellosis
2013	0.65	197.6
2014	10.00	207.48
2015	5.14	157.82
2016	93.36	119.26
2017	22.24	101.36
2018	19.55	70.64
2019	174.56	49.83
2020	28.23	10.13
2021	1.23	0.00
2022	77.83	9.96
2023	113.73	7.42

3.5. HAV RNA monitoring in sewage and environment samples (exploratory objective)

Data on HAV RNA detection in sewage and water samples are summarized in Table 7. HAV RNA was detected in 7 out of 337 samples tested, including 2 out of 120 (1.7%%) sewage samples, 2 out of 82 (2.4%) sources of drinking water and 3 out of 135 (2.2%) samples from open bodies of water.

Table 7. Results of HAV RNA monitoring in sewage and water samples

Sampling time point	Sewage samples		Sources of drinking water (springs, wells)		Open bodies of water	
	Number of tested samples	Number of positive samples (%)	Number of tested samples	Number of positive samples	Number of tested samples	Number of positive samples
June, 2021	16	0 (0%)	9	0 (0%)	14	1 (7.1%)
October, 2021	16	0 (0%)	13	0 (0%)	20	0 (0%)
June, 2022	24	0 (0%)	15	0 (0%)	26	0 (0%)
October, 2022	24	1 (4.2%)	15	0 (0%)	26	0 (0%)
June, 2023	24	0 (0%)	15	0 (0%)	24	0 (0%)
October, 2023	16	1 (6.3%)	15	2 (13.3%)	25	2 (8.0%)

One HAV containing sample was collected in June 2021 in the lake near the beach popular among local residents. Another positive sample was obtained in October 2022 from sewage collected in Kyzyl city. Five HAV RNA positive samples were identified in October 2023, including one from sewage collected in Kyzyl city, two samples from springs in rural areas, and two samples from the river downstream the place where treated sewage from Kyzyl city are discharged.

All HAV positive samples were sequenced. The results of phylogenetic analysis are shown in Figure 1.

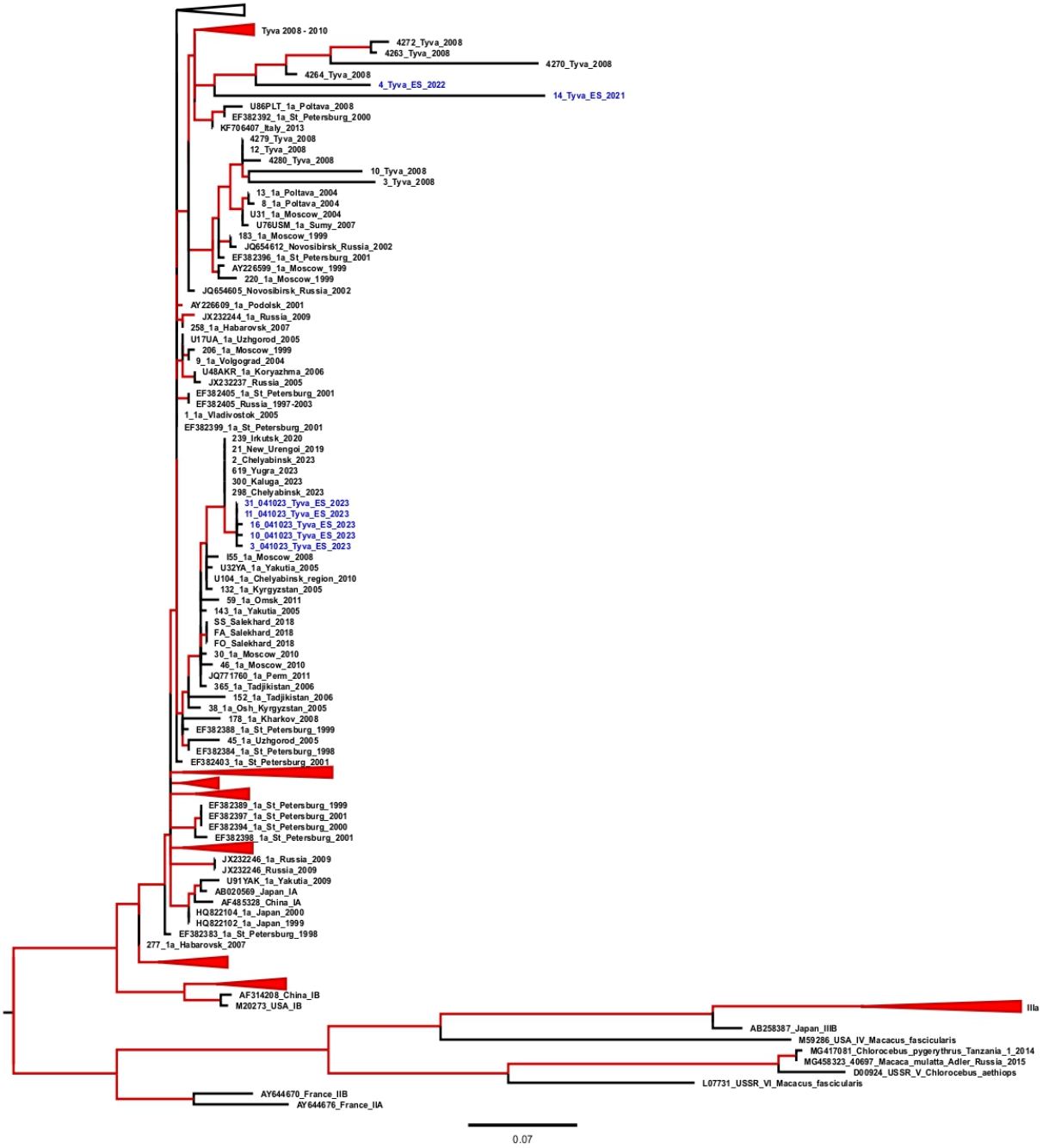

Figure 1. Maximum likelihood phylogenetic tree for HAV VP1/2A sequences. For each sequence, the number in the GenBank database, the country, the city (in case of Russian sequences), and the year of isolation are indicated. Sequences from environmental samples collected in Tyva Republic are shown in blue with indicated year of isolation. The tree branches shown in red have a posterior probability >90%.

HAV isolates detected in 2021-2022 belong to genotype IA and are grouped together with the sequences obtained in Tyva in 2008-2010, before the start of vaccination, i.e. refers to a local epidemic strain. Meanwhile, HAV sequences isolated from sewage and other environmental samples in 2023 belong to another cluster of genotype IA sequences that were isolated in 2019-2023 in different parts of the Russian

Federation. Thus, HAV sequences isolated in Tyva in 2023 are indicative of the recent importation of infection to this region from other parts of the Russian Federation.

Discussion

The primary objective of this study was to assess the immunological effectiveness of single-dose HAV vaccination, i.e. the long-term persistence of seroprotection among children who received one dose of inactivated HAV vaccine in Tyva. Although anti-HAV IgG antibody concentrations of ≥20 mIU/mL are widely used as the seroprotection cutoff in vaccine licensing and clinical studies, antibody level ≥ 10 mIU/mL is considered to be seroprotective [5]. Thus, we assessed the level of seroprotection using both cutoff values in two pediatric cohorts, nine and eleven years after the single-dose immunization. In Year 9 Cohort, the estimated effectiveness of one HAV vaccine dose was 99.4%, regardless of the seroprotection cutoff applied. This data are consistent with 97.4% seroprotection level reported in vaccinated children up to 9 years of age following single-dose vaccination at the age of one year in Argentina, where cutoff ≥10 mIU/mL was applied [14]. The level of seroprotection in Year 9 Cohort was similar to the 91% seroprotection rate observed earlier in Tyva in children five years after single-dose vaccination [6]. However, we observed the significant reduction in seroprotection rates in two Year 11 cohorts, to 71.8%-75.2% when ≥20 mIU/mL cutoff was applied, and to 75.4%-79.6% when ≥10 mIU/mL cutoff was applied. Correspondingly, GMCs in Year 11 cohorts were significantly lower compared to Year 9 Cohort, indicating the decrease of humoral immunity over time.

The level of HAV seroprotection observed in our study 11 years following the single-dose immunization is substantially lower compared to 93% rate observed in cohort of 27 children followed 12 years after single-dose immunization with inactivated HAV vaccine in Argentina [9]. However, de Brito with coauthors reported detectable anti-HAV IgG antibodies in 64% of children after 6 to 7 years of single-dose vaccination with of inactivated HAV vaccine [10]. Taken together, these data suggest that the humoral immunity to HAV following the single-dose vaccination might decrease faster compared to standard two-dose schedule which provides seroprotection in >90% vaccinated children for up to 15 years [1,15]. This observation is confirmed by the data from the study that directly compared the duration of humoral immunity to HAV 10 years following the single-dose and two-dose vaccination and demonstrated a significant differences both in seroprotection rates (71.9% versus 96.3%) and GMCs (26.0 mIU/mL versus 82.1 mIU/mL) [16]. Nevertheless, the waning humoral immunity might not necessarily indicate the lack of protection against HAV. A recent study on HAV-specific T-cell response in children up to 12 years following single-dose vaccination demonstrated the presence of memory CD4+ and CD8+ T cell responses in 53.8% and 26.9% of seronegative children, respectively [9]. Likewise, the production of interferon-gamma in peripheral blood mononuclear cells (PBMCs) stimulated with the HAV VP1 antigen was demonstrated in 32.4% of seronegative children 6 to 7 years following the single-dose vaccination with inactivated vaccine, indicating the cell-mediated immune memory [10]. Nevertheless, all children who had anti-HAV IgG antibodies in our study below 20 mIU/mL were recommended the boosted dose of vaccine.

Interestingly, significant proportions of children, 8.9% to 12.1% depending on the cohort, had anti-HAV concentrations above 6,000 IU/l. There are two possible explanations for such a large proportion of samples with abnormally high anti-HAV concentrations in vaccinated children. First, and most likely, is a vaccination of children previously exposed to HAV, as a screening for antibody to HAV was not performed prior the vaccination. The history of past hepatitis A prior the vaccination was the exclusion criterion in our study, but some participants could have subclinical infection. In this case, high levels of anti-HAV antibodies could have been associated with post-infection immune response. The second explanation is the exposure to HAV after the vaccination. However, the latter is highly debatable, as no proof of possible transient subclinical HAV infection in vaccinated individuals is reported so far, and no

evidences of “natural boosting” of HAV immunity are documented [5]. Nonetheless, such samples with abnormally high anti-HAV concentrations were excluded from the GMC calculation as being highly likely not related to single-dose immunization.

In addition to the immunological effectiveness, we assessed the epidemiological effectiveness of single-dose HAV vaccination program in Tyva. The implementation of single-dose HAV vaccination in Tyva resulted in sharp decline in hepatitis A incidence rates, both in vaccinated children and in non-vaccinated adolescents and adults. Since 2016 until now, no single hepatitis A case was reported in Tyva, making this particular region the first territory free of symptomatic hepatitis A in the Russian Federation. The absence of registered hepatitis A cases is apparently not related to underreporting, since there is mandatory reporting of hepatitis A in Russia by both laboratories and clinicians. The public health definition of confirmed hepatitis A includes any confirmed positive anti-HAV IgM antibody or positive HAV RNA. Moreover, the zero incidence rates in Tyva are not resulted solely from the global decline in HAV circulation in Russia, since hepatitis A cases were registered regularly though the last decade in regions neighboring Tyva. Furthermore, the disappearance of symptomatic HAV infections in Tyva definitely was not associated with the general sanitary improvement, since annual incidence rates of other enteric infections, both viral and bacterial, remained high in the region within last decade.

The possible explanation of the hepatitis A incidence drop in total population observed in Tyva may be the fact that the highest incidence rates in pre-vaccinated period in the region were observed in children under 14 years, who obviously served as a main source of infection. Thus, vaccine-induced HAV immunity in children together with high rates of infection-induced immunity among adults reported in this region [8] provide a sufficient level of herd immunity and significantly reduce the space for HAV circulation. Likewise, a significant and rapid decline of incidence rates in all age groups following the implementation of single-dose UMV strategy was observed in Argentina and Brazil [17,18,19].

It is expected that the significant reduction in symptomatic HAV infections is accompanied by the decrease in HAV detection rates in sewage. However, the three-year monitoring of HAV RNA in sewage and various open bodies of water in Tyva confirmed the persistence of virus shedding. Moreover, the phylogenetic analysis confirmed that HAV sequences isolated from water samples in Tyva in 2021 and 2022 belonged to the same group of strains that were detected in HAV patients in this region in 2008, in pre-vaccination period, suggesting the stable circulation of this particular variant of the virus. However, in 2023, another HAV strain was identified in sewage and environmental samples that belonged to the group of strains identified in 2019-2023 in different regions of Russia, including Irkutsk Region that borders Tyva. This finding confirms the importation of new strain into Tyva. It is impossible to conclude, whether UMV strategy resulted in decrease in the number of sewage samples positive for HAV RNA, since no such monitoring was performed in Tyva before our study. However, HAV antigen testing of sewage samples was performed in Tyva in 2002-2004 that resulted in 12.5%-63.3% positivity rates depending the study year [20]. Considering the average proportion of HAV RNA positive samples in current study was below 2.5%, one might assume that vaccination resulted in significant decrease in virus shedding in the region. Interestingly, the environmental monitoring of HAV RNA in Argentina performed following the introduction of single-dose HAV UMV strategy in 2005 demonstrated the continuous detection of HAV RNA in sewage both in 2009-2010 and in 2017-2022 studies [21,22]. However, unlike Tyva, detection of HAV RNA in wastewater samples in Argentina correlated with cases of acute hepatitis A [22]. A possible explanation for the continued shedding of the virus in Tyva and its detection in environmental samples and wastewater despite the absence of registered cases of acute hepatitis A may be associated with the age of when the vaccine is given to children. Obviously, the isolation of the virus in the absence of reported disease cases indicates most probably the presence of asymptomatic infection. Most often, HAV infection is asymptomatic in children under 5 years of age [23]. Typically, HAV vaccination is recommended for children aged 12 months or older [24]. In Tyva,

single-dose vaccination has been introduced for children aged 3 years and older, primarily in order to not exclude the only domestically produced HAV vaccine that is licensed for children of that age. Thus, children under 3 years of age are not immunized and remain susceptible to HAV. Therefore, the most likely hypothesis explaining the persistence of HAV circulation in the absence of symptomatic cases is the virus transmission among children under 3 years of age. Our data on continuing HAV circulation despite the decade of UMV program in Tyva strongly suggest that immunization should be started at the age of 12 months to diminish the vaccination gaps.

In conclusion, the proportion of participants with protective anti-HAV antibody concentrations was as high as 99.4% nine years following the single-dose vaccination in childhood, but decreased significantly in cohort tested eleven years after the immunization. The drop in seroprotection rates observed between 9 and 11 years after single-dose vaccination was accompanied by a significant decrease in anti-HAV geometric mean values. However, considering the possible seroprotection cutoff as 10 mIU/mL, 75.4% of children remain protected eleven years after the single-dose immunization. The UMV strategy based on single dose of HAV vaccine resulted in zero rates of hepatitis A incidence in Tyva Republic. However, the monitoring of HAV RNA in sewage and environmental samples demonstrated the ongoing circulation of both regional epidemic strain of the virus and the strain imported recently from other parts of the Russian Federation, probably due to subclinical infections in non-vaccinated children under 3 years. All together, these data indicate the immunological and epidemiological effectiveness of single-dose HAV vaccination strategy and suggest the need to expand the vaccination program to include children aged 12 months and older to achieve its maximum effectiveness.

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