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Molecular Epidemiological Characterisation of Hepatitis A Virus in Latvia

Summary of the Doctoral Thesis for obtaining the scientific degree "Doctor of Science (*PhD*)"

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The Doctoral Thesis was developed at Riga East University Hospital, Laboratory Service, laboratory "Latvian Centre of Infectious Diseases", National Microbiology Reference Laboratory

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Abbreviations used in the Thesis

bp	base pair
CDC	Centres for Disease Control and Prevention
CDC of Latvia	Centre for Disease Prevention and Control of Latvia
DTT	dithiothreitol
ECDC	European Centre for Disease Prevention and Control
EU/EEA	European Union / European Economic Area
HAV	hepatitis A virus
HEV	hepatitis E virus
HIV	human immunodeficiency virus
M	molar
mL	millilitres
μL	microlitres
MSM	men who have sex with men
PCR	polymerase chain reaction
PWID	people who inject drugs
REUH LIC NMRL	Riga East University Hospital, Laboratory Service, laboratory "Latvian Centre of Infectious Diseases", National Microbiology Reference Laboratory
RCF	relative centrifugal force
RNA	ribonucleic acid
RT-PCR	reverse transcription polymerase chain reaction
TBE buffer	Tris-borate-EDTA buffer
USA	the United States of America
U	U unit
VHA	viral hepatitis A
WHO	World Health Organisation

Introduction

Viral hepatitis, formerly known as jaundice epidemics, has plagued humanity since ancient Greek, Roman and Chinese times (Beard & Lemon, 1999; Nainan et al., 2006; Pinto & Saiz, 2007). At the time, the pathogens causing this epidemic were elusive. It was not until the beginning of the 20th century that a form of hepatitis was associated with certain epidemic forms of infectious jaundice (Dotzauer, 2008; Pinto et al., 2010). Later, two separate entities of hepatitis were identified based on the route of transmission, "infectious" and "serum" hepatitis (Pinto et al., 2010). In the second half of the 20th century, a group of viruses were identified as the etiological agents responsible for both entities of hepatitis (Beard & Lemon, 1999; Nainan et al., 2006; Pinto et al., 2010). The viruses were shown to primarily infect hepatocytes, causing acute or chronic inflammation of the liver, and as a result were given the name "hepatitis viruses" (Nainan et al., 2006; Tang, Shetty, Andrews, 2009).

There are currently five major hepatitis viruses that cause similar clinical manifestations but differ in their morphology, genome organisation, taxonomy, and mode of replication (Tang, Shetty, Andrews, 2009; Kumar, Das, Jameel, 2010; Pinto et al., 2010). These viruses can be grouped by their predominant mode of transmission, namely enteral ("infectious" hepatitis) or parenteral ("serum" hepatitis) (Collier & Oxford, 2006). Parenterally transmitted hepatitis viruses include hepatitis B virus, hepatitis C virus, and hepatitis D virus. They cause acute hepatitis with a high probability of developing chronic infection (Kumar, Das, Jameel, 2010) and can be spread through blood and blood products, sexually or vertically (from mother to child) (Collier & Oxford, 2006; Tang, Shetty, Andrews, 2009). Enterically transmitted hepatitis viruses include hepatitis A virus (HAV) and hepatitis E virus (HEV), which have a predominantly faecal-oral mode of transmission (Collier & Oxford, 2006; Pinto & Saiz, 2007; Tang, Shetty, Andrews, 2009; Pinto et al. al., 2010), either directly

from person to person or indirectly from contaminated food and water sources (Nainan et al., 2006; Pinto et al., 2010). Infections with HAV and HEV are reported as epidemic or sporadic cases (Kumar, Das, Jameel, 2010).

Viral hepatitis A (VHA) is a viral liver disease that can cause acute hepatitis with a mild or severe course of the disease, as well as the development of acute liver failure, which can become the cause of death. HAV is spread mainly through contaminated food or water and through household contact, as well as a possible transmissible route during viremia. There is evidence of transmission among men who have sex with men (MSM) (ECDC, 2017). HAV can live in water for a long time (up to 92 days), is quite stable in the environment, remaining at room temperature for a few weeks, but at a temperature of +4 °C for a few months or even years.

The risk of spreading HAV infection is related to lack of water, poor sanitation or hygiene conditions. Once in a favourable environment, the virus can cause widespread epidemics, which in turn can cause significant economic losses. The only effective form of protection against HAV infection is vaccination.

The period from the moment of infection to the first symptoms of the disease is on average 15 to 50 days (4 weeks on average). The pre-jaundice period lasts from 3 to 7 days, when the patient has nausea, fatigue, loss of appetite, fever, pain in the right side. VHA often resembles a flu-like illness or stomach-intestinal disorders. Suspicion of hepatitis arises when the patient has dark urine and light-coloured faeces. After some days, the eyeballs and skin remain yellow. The jaundice period lasts up to 2 weeks. Young children may not develop jaundice. The infection is often asymptomatic in adults as well. Importantly, two weeks before symptoms of the disease, HAV is excreted in the faeces of patients. This means that a person, feeling healthy, can spread the infection, and personal hygiene is not observed.

The geographical distribution of HAV can be divided into three levels – high, medium and low. Places with a high prevalence rate – in developing countries with poor sanitary conditions, with an average prevalence rate – in developing countries, with transitional economies where sanitary conditions are variable, with a low prevalence rate – in developed countries with good sanitary and hygienic conditions (WHO, 2017). Latvia belongs to the countries where the prevalence rate is low. However, other factors also influence the intensity of HAV spread. As the geopolitical situation changes, the issue of refugees becomes more and more relevant, and as a result, HAV infection can be imported from countries with a high prevalence of infection. People who inject drugs (PWID), many of whom are infected with human immunodeficiency virus (HIV) and hepatitis C virus, MSM, and people traveling to HAV-endemic areas are at highest risk of HAV infection. Therefore, the mentioned factors can affect the spread of HAV in Latvia as well.

Until now, there were no accurate researched data on the distribution of HAV subgenotypes in Latvia, it was not investigated whether several genotypes can circulate at the same time, which creates additional risks for the spread of outbreaks. Therefore, the topic of the research work can be considered actual.

Aim of the Thesis

To determine the distribution of HAV subgenotypes in the territory of Latvia and to analyse the homology of HAV sequences and their belonging to different isolated HAV clusters from other countries.

Tasks of the Thesis

1. To determine HAV subgenotypes by sequencing method from 2008 to 2021, using frozen and archived blood samples.

2. Construct a phylogenetic tree and analyse HAV sequences with each other using the National Institute for Public Health and the Environment (RIVM, Netherlands) HAVNET database.
3. To study the circulation of HAV in different years (a time interval of more than two years) of samples from the same cluster.
4. To evaluate possible sources of infection with HAV.
5. To analyse the laboratory-confirmed diagnosis time and determine the number of days of hospitalisation in patients with different HAV subgenotypes.

Hypotheses of the Thesis

Clusters of HAV subgenotypes circulating in Latvia, which caused local outbreaks, including in the MSM group (or other population groups), are associated with outbreaks in EU/EEA countries.

Novelty of the Thesis

For the first time in Latvia, a study on the molecular epidemiology of HAV was carried out with a phylogenetic analysis of the virus sequence, which will give the opportunity to identify circulating subgenotypes of HAV, understand the distribution of virus and investigate outbreaks.

Personal contribution

At the beginning of 2017, the author of the work introduced HAV subgenotyping by the Sanger sequencing method, which is used to determine HAV subgenotypes in sporadic cases and to decipher outbreaks, and has also carried out scientific planning, collected, analysed and processed the obtained data, including using statistical data analysis methods. Has prepared scientific publications. The author of the paper has written this paper.

1 Materials and methods

1.1 Patients involved in the study

The study "Molecular Epidemiological Characterisation of Hepatitis A Virus in Latvia" was started in 2018 in Riga East university hospital, Laboratory Service, laboratory "Latvian Centre of Infectious Diseases", National Microbiology Reference laboratory (REUH LIC NMRL). 295 patients' samples of positive IgM class antibodies against HAV from 2008 to 2021 were analysed.

In the study, molecular biological investigation of HAV was performed from 295 blood samples that were previously positive for IgM class antibodies against HAV using immunochemical methods. Blood samples were taken from patients with suspected VHA for diagnosis. The positive blood samples were tested and stored at a temperature of −20 °C at REUH LIC NMRL. Nucleotide sequences of HAV were determined in the extracted nucleic acids of 259 blood samples for further subgenotyping of the virus and comparison of nucleotide sequences with each other.

The study carried out a molecular epidemiological investigation of the sequences of 259 HAV samples. After obtained sequences and phylogenetic tree construction, homology and affiliation with different isolated HAV clusters from other countries and/or from local cases were analysed (Figure 1.1).

1.2 Study design

The study "Molecular Epidemiological Characteristics of the Hepatitis A Virus in Latvia" is a qualitative study. It was carried out by REUH LIC NMRL in cooperation with the Centre for Disease Prevention and Control of Latvia (CDC of Latvia) in the period from 2018 to 2021. The study was approved by the Ethical Committee of Rīga Stradiņš University (No 4/08.09.2018), REUH (No ZD/08-06/01-19/210 17.07.2019) and CDC of Latvia (No 6.1.-3/13 20.12.2018).

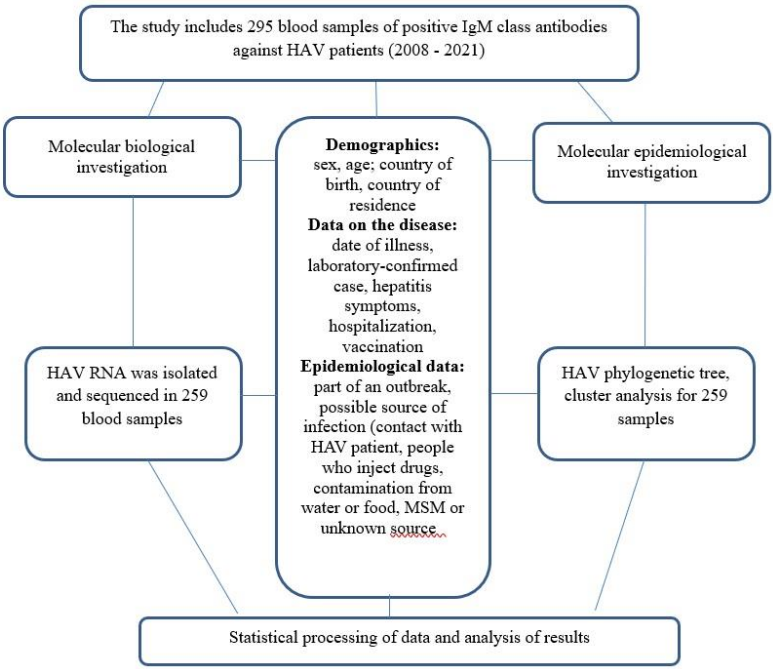

Figure 1.1 **Study design**

1.3 Inclusion and exclusion criteria

Inclusion criterion – the study included 295 patients' samples of positive IgM class antibodies against HAV patients. For molecular biological and molecular epidemiological analysis, 259 samples for which HAV RNA was isolated were recognised as valid, which served as an inclusion criterion.

Exclusion criterion – 36 samples for which no HAV RNA was isolated served as an exclusion criterion, since further subgenotyping and analysis by phylogenetic tree construction was not possible.

1.4 A hepatitis cases registered in Latvia

In the period from 1990 to 2021, 34967 cases of VHA were notified in Latvia. Evaluating the distribution of diagnosed cases by year, it can be found that the number of new cases is fluctuating in general and has decreased significantly since 2000, however, there is a tendency to increase the number of cases during outbreaks from 2008 to 2009 and from 2017 to 2018. (Figure 1.2) (Epidemiological bulletins, CDC of Latvia).

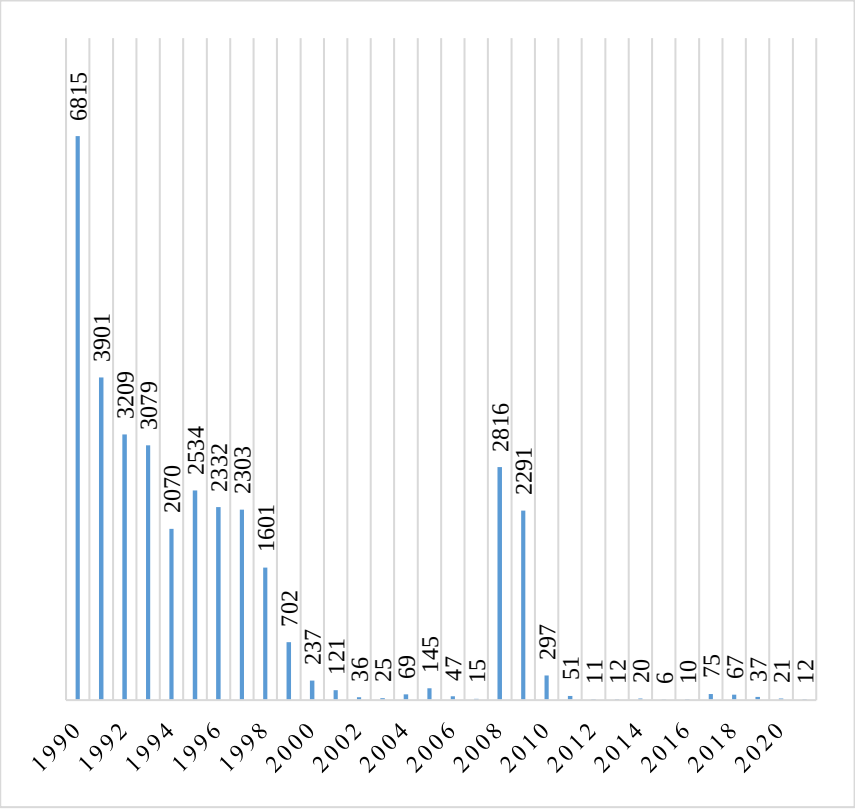

Figure 1.2 **Hepatitis A notified cases, 1990–2021**

1.5 Hepatitis A virus diagnostic

1.5.1 Serological diagnostic

Detection of IgM class antibodies by immunochemical method was used to confirm HAV infection. The assay is for the qualitative determination of anti-HAV IgM in human serum or plasma. Stages of the immunochemical reaction: covering the surface of the plate with the sample; incubation with primary antibody; washing to remove unbound antibodies; incubating with a secondary antibody conjugated to a detection enzyme; washing to remove unbound antibodies; addition of the substrate required for the enzyme reaction; stopping the reaction; determination of optical densities.

The presence of anti-HAV IgM in the sample is determined by comparing the read optical density with the calculated cut-off value. The interpretation of the results is in accordance with the manufacturer's validation.

Blood samples were collected in anticoagulant-free tubes with a coagulation activator. After receiving blood samples in the laboratory, centrifugation was performed for 10 min at 10000 RCF. After the centrifugation step, the separated serum is transferred to a sterile eppendorf and stored at –20 °C.

Serum samples were tested with immunochemical diagnostic kits: AxSYM HAVA-M 2.0 (Abbott Diagnostics, Germany) (n = 100, 2008), ETI-HA-IgMK-PLUS (DiaSorin, Italy) (n = 1, 2012) , Architect HAVAb-IgM, (Abbott Diagnostics, Germany) (n = 99; n = 2, 2013; n = 5, 2014; n = 2, 2015; n = 5, 2016; n = 27, 2017; n = 45, 2018; n = 5, 2019; n = 8, 2020), Cobas Anti-HAV IgM (Roche Diagnostic, Germany) (n = 59; n = 2, 2015; n = 1, 2016; n = 32, 2017; n = 7, 2018; n = 13, 2019; n = 3, 2020; n = 1, 2021).

1.5.2 Molecular biological detection

Isolation of HAV RNA from biological material

HAV RNA was isolated in 259 samples (n = 259) from blood serum. Nucleic acid isolation was performed with the automatic isolation system NucliSens easyMAG (Biomerieux USA) based on the manufacturer's protocol. 200 µl of serum was taken for HAV RNA isolation. The obtained RNA was eluted in 60 µl of buffer developed by the manufacturer and stored at −70 °C.

Molecular detection and typing of the HAV VP1 region

The "Molecular detection and typing of VP1 region of Hepatitis A virus (HAV)" protocol, National Institute for Public Health and Environment, was used to determine HAV genotypes. The protocol provides information on HAV molecular typing based on protocols developed by the HAV Reference Laboratory of the Netherlands National Institute for Public Health and the Environment.

HAV subgenotyping was performed using RT-PCR and Sanger sequencing. RT-PCR was performed on a Veriti cycler (Applied Biosystems, USA), product lengths were checked on a gel electrophoresis bath PS250 (Hybaid. USA) and sequencing on a 3130 xl Genetic Analyzer (Applied Biosystems, USA). The HAV VP1/2A region is between 2873 and 3376 nucleotides in length. Amplification was performed on 503 nucleotides of the VP1/2A region and the resulting nucleotide sequence is 460 bp. The following reagents were used for RT-PCR: primers, 5 x RT buffer Superscript III, Invitrogen; PCR Nucleotide Mix 10mM, Roche; Superscript III RT 10000 U (200 U/µl), Invitrogen; RNaseOUT 5000 U (40 U/µl), Invitrogen; 0.1 M DTT, Invitrogen; Taq polymerase buffer, Roche; 10 x Faststart Taq polymerase buffer, Roche; Taq Polymerase 5 Uml; Faststart Taq Polymerase 5 u/µl. For gel electrophoresis, the following were used: agarose, GelRed dye, 5 x TBE buffer. The following were used for sequencing: BigDye Terminator v3.1, Applied

Biosystems; Sequence buffer (Big Dye buffer), Applied Biosystems; 70 % ethanol, 3.0 M sodium acetate, HiDi Formamide, Applied Biosystems.

1.5.3 Molecular epidemiological and phylogenetic analysis

The phylogenetic tree was constructed using the maximum likelihood method based on the Tamura-Nei model with bootstrap analysis (1000 replicates). All items with gaps and missing data were eliminated. The phylogenetic tree was generated with MEGA (6.0) software (Tamura et al., 2013).

Demographic data (sex, age; country of birth, country of residence), disease data (date of illness, laboratory-confirmed case, hepatitis symptoms, hospitalisation, vaccination), epidemiologic data (part of the outbreak, possible source of infection (contact with HAV patients, people who inject drugs, contamination from water or food, MSM or unknown source) were collected from CDC of Latvia.

1.6 Statistical methods

Descriptive statistical methods were used to characterise the patients. For quantitative data, the arithmetic mean (Mean, M) with the standard deviation (Standard Deviation, SD) of the dispersion indicators was evaluated, and if the data did not correspond to the normal distribution – the median (Median, Me).

The results were evaluated with a 5 % α-error, thus, if the p value obtained in the results was less than 0.05, the null hypothesis was rejected and the test result was recognised as statistically significant.

If statistical data did not correspond to normal distribution, non-parametric tests such as Mann-Whitney U Test and Kruskal-Wallis Test were applied.

Spearman's rank correlation coefficient (rs) was used to analyse the relationships.

Statistical data processing was performed using Statistical Package for Social Sciences IBM (SPSS) version 27 (IBM Corporations, USA).

2 Results

2.1 Molecular epidemiological data

Molecular epidemiological data were analysed for 259 sequenced HAV samples between 2008 and 2021. From the large HAV outbreak of 2008–2009, 100 samples were sequenced in collaboration with the Netherland National Institute for Public Health and the Environment and 159 samples at the RAKUS LIC NMRL (Figure 2.1).

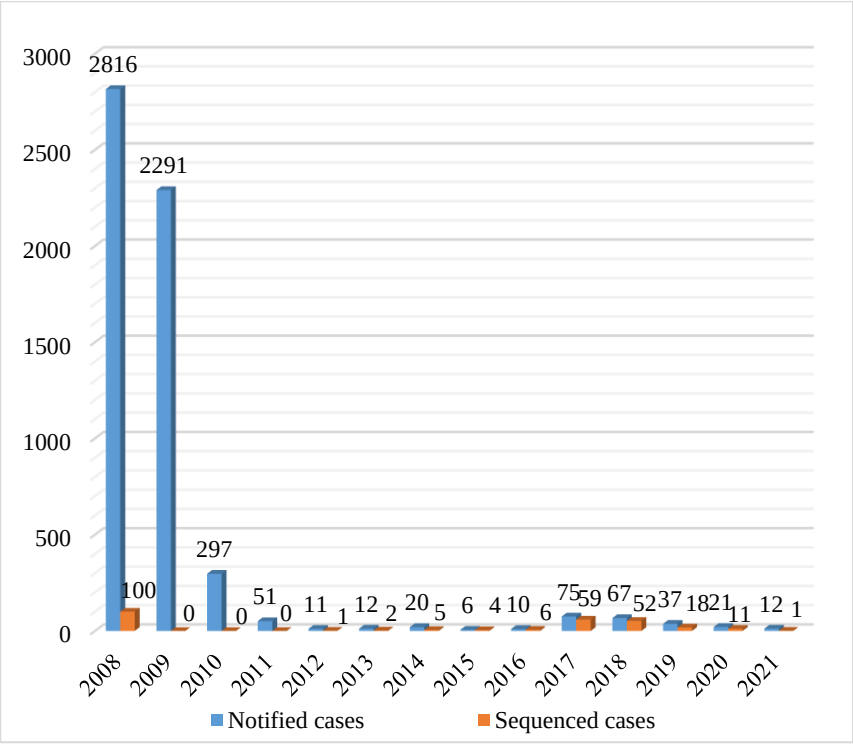

Figure 2.1 **Number of HAV samples sequenced from 2008 to 2021**

Of the 259 HAV sequenced samples, the majority were HAV subgenotype IA – 72.0 % (n = 187), subgenotype IB – 23.0 % (n = 59), subgenotype IIIA – 5.0 % (n = 13) (Figure 2.2, Table 2.1).

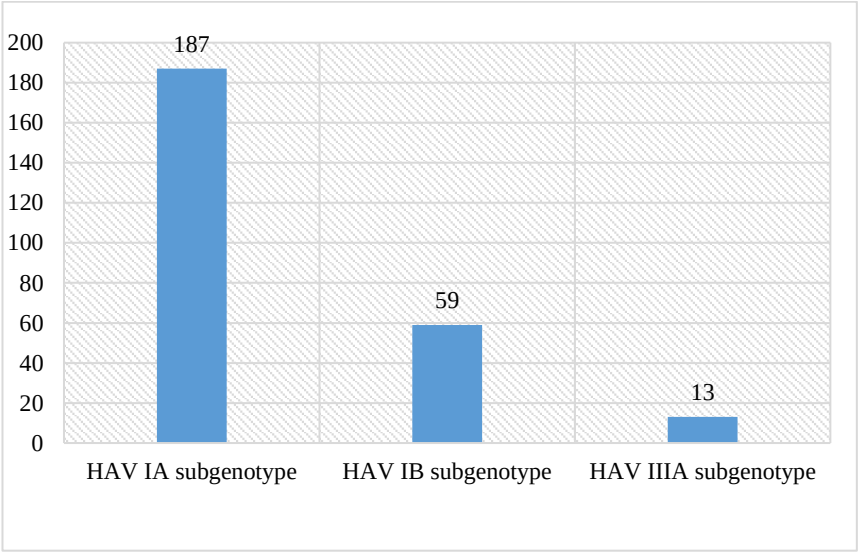

Figure 2.2 **HAV subgenotypes, 2008–2021**

Table 2.1

Distribution of HAV subgenotypes by years

	Years											**Total**	
	2008	2012	2013	2014	2015	2016	2017	2018	2019	2020	2021	number	%
Samples	100	1	2	5	4	6	59	52	18	11	1	259	100
IA	95	1	2	4	2	2	55	20	4	2	0	187	72
IB	1	0	0	1	0	1	3	29	14	9	1	59	23
IIIA	4	0	0	0	2	3	1	3	0	0	0	13	5

Among Latvian HAV patients' samples, HAV subgenotype IA strains were found, associated with the VHA outbreak in the EU/EEA countries among MSM persons belonging to one of the three isolated clusters in 2016–2017: VRD_521_2016, n = 30; RIVM-HAV16-090, n = 7; V16-25801, n = 2 (Table 2.2).

Table 2.2

HAV strains associated with outbreaks among MSM

HAV subgenotype strains	Sequenced casas, years		Total	
	2017	2018	Number	%
IA (VRD_521_2016)	27	3	30	17,1
IA (RIVM-HAV16-090)	5	2	7	3,7
IA (V16-25801)	2	0	2	1,2

The country of birth and residence of the majority of patients was Latvia (97.7 %, n = 253). The remaining cases (2.3 %, n = 6): in two cases, the country of birth was India and the place of residence was Latvia, HAV subgenotype IIIA was determined from the isolated HAV RNA, and the possible country of origin of the virus was India; in two cases, the country of birth was Uzbekistan and the place of residence – Latvia, HAV subgenotype IA was determined and the possible country of origin of the virus was Uzbekistan; in one case, the country of birth was Latvia, but the place of residence was Germany, HAV subgenotype IA was determined and the possible country of origin of the virus was Germany; in one case, the country of birth was Bulgaria and the country of residence was Latvia, the HAV subgenotype IA was determined and the possible country of origin of the virus was Bulgaria, and this case and the HAV sequence found are similar and related to 31 registered cases of HAV in EU/EEA countries (Denmark, Germany , the Netherlands, Sweden, Norway and Latvia), as well as with two registered cases in New Zealand. Cases have been linked to travel to

Bulgaria and consumption of fresh blueberries and other fresh berries. Frozen berries were suspected as the source of infection.

Data on possible source of infection showed that only 27.4 % (n = 71) had a known source of infection and 72.6 % (n = 188) had an unknown source of infection. Among identified sources of infection, one case was associated with fruit from Azerbaijan, one case was associated with fruit from Uzbekistan, four cases were among individuals who self-identified as MSM, isolated HAV RNA nucleotide sequences belong to cluster RIVM-016-90, seven cases were related to each other from the outbreak in 2008, 23 cases were INL, 35 cases were determined to be in contact with a HAV patient.

Data on travel history during HAV incubation and possible country of virus origin showed that 81.5 % (n = 211) were local cases and 18.5 % (n = 48) were linked to other countries: Austria (n = 1), Bulgaria (n = 1), Philippines (n = 1), France (n = 1), Greece (n = 1), Estonia (n = 1), Italy (n = 1), Nepal (n = 1), Netherlands (n = 1), Nigeria (n = 1), Sri Lanka (n = 1), Turkmenistan (n = 1), Ukraine (n = 1), Great Britain (n = 2), Morocco (n = 2), Tajikistan (n = 2), Spain (n = 3), Egypt (n = 4), India (n = 4), Kazakhstan (n = 4), Russia (n = 4), Uzbekistan (n = 4), Germany (n = 6) (Figure 2.3).

2.2 HAV subgenotypes phylogenetic analysis

259 Latvian HAV sequences with HAV subgenotypes IA, IB, IIIA were included in the phylogenetic tree. The tree contains three clusters that were associated with an outbreak of VHA among MSM in the EU/EEA in 2016/2017 (VRD_521_2016, RIVM-HAV16-090, V16-25801 clusters), and other clusters and sporadic cases with and with no identified epidemiological link.

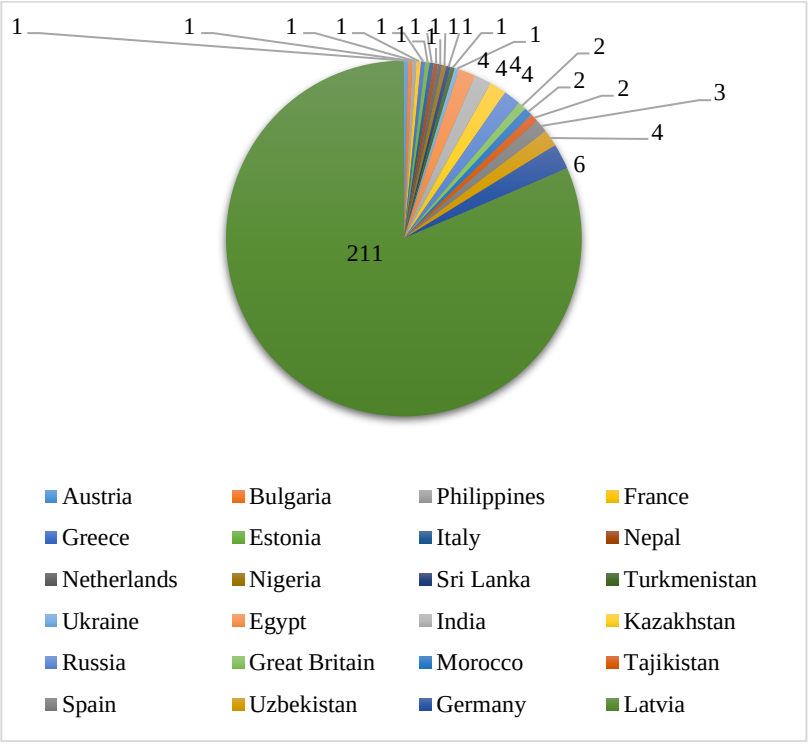

Figure 2.3 **Possible country of origin of the virus**

2.2.1 HAV subgenotype IA results

Subgenotype HAV IA results showed that 187 HAV sequences fall into 13 clusters and 12 sporadic cases with no identified epidemiological association. The average age of patients with HAV subgenotype IA was 35.5 years, men were 103, women – 84, adults – 165, children – 22.

The first cluster consists of 96/187 (51.3 %) identical sequences, 95 cases were notified in 2008 and one in 2012. 23/96 cases were associated with PWID from the 2008 HAV outbreak, 6/96 were associated with the 2008 outbreak, 67/96 were of unknown source. The case from 2012 was linked to travel in Great Britain, the other cases were local.

The cluster named VRD_521_2016, which was associated with the HAV outbreak in EU/EEA countries, consists of 30/187 (16 %) identical sequences, 27 cases were notified in 2017, 3 – in 2018. In 11/30 cases, the source of infection was determined – contact with HAV patients, in 19/30 – the source of infection is unknown, but two cases were associated with travel to Germany, one to Spain and Austria, the remaining cases were local.

The cluster named RIVM-HAV16-090, which was associated with the HAV outbreak in the EU/EEA countries, consists of 7/187 (3.7 %) identical sequences, five cases were notified in 2017, two cases in 2018. In 1/7 cases, the source of infection was determined – contact with an HAV patient, 4/7 had identified themselves as MSM persons, 2/7 – the source of infection is unknown and two cases were associated with travel to Germany, one to Spain, France and Russia, the other cases were local.

A cluster named V16-25801, which was associated with an outbreak of HAV in EU/EEA countries, consists of 2/187 (1.1 %) identical sequences, cases were notified in 2017. The source of infection is unknown, but the cases were linked to travel to Germany and Estonia.

The second cluster consists of 16/187 (8.5 %) identical sequences, six cases were notified in 2017 and ten cases in 2018. In 3/16 cases, the source of infection was determined – contact with an HAV patient, for the rest the source of infection is unknown. One case was linked to travel to Ukraine and one to the Netherlands, the other cases were local.

The third cluster consists of 3/187 (1.6 %) identical sequences, one case was notified in 2015, one in 2016 and one in 2018. The source of infection is unknown, but one case was linked to travel to Bulgaria and one to Greece.

The fourth cluster consists of 4/187 (2.1 %) identical sequences, one case was notified in 2014, two in 2017, one in 2019. The source of infection is unknown, but two cases were linked to travel to Russia and Uzbekistan.

The fifth cluster consists of 5/187 (2.7 %) identical sequences, one case was notified in 2013, one in 2015, three in 2017. The source of infection is unknown, but one case was linked to travel to Russia, one to Italy, the rest were local.

The sixth cluster consists of 3/187 (1.6 %) identical sequences, two cases were notified in 2017, one in 2020. The source of infection is unknown, but one case was linked to travel to Spain, the others were local.

Recurrent HAV subgenotype IA virus circulation has been observed in different (more than two) years in the first, third, fourth, fifth and sixth clusters (Table 2.3).

Table 2.3

Circulation of recurrent HAV subgenotype IA viruses (a time interval of more than two years)

HAV subgenotype IA cluster	Circulation of the virus over the years	Number
The first	2008	95
	2012	1
The third	2015	1
	2016	1
	2018	1
The fourth	2014	1
	2017	2
	2019	1
The fifth	2013	1
	2015	1
	2017	3
The sixth	2017	2
	2020	1

The seventh cluster consists of 2/187 (1.1 %) identical sequences, two cases were notified in 2014. In one case, the source of infection was fruit brought from Uzbekistan, in the second case, the source of infection is unknown. The cases were local.

The eighth cluster consists of 2/187 (1.1 %) identical sequences, two cases were notified in 2017. The source of infection is unknown, the cases were linked to travel to Uzbekistan and Kazakhstan.

The ninth cluster consists of 3/187 (1.6 %) identical sequences, two cases were notified in 2018, one in 2019. The source of infection is unknown, but one case was linked to travel to Morocco, the others were local.

The tenth cluster consists of 2/187 (1.1 %) identical sequences, the cases were notified in 2017, the source of infection is unknown and the cases were local.

Sporadic cases accounted for 12/187 (6.4 %) sequences. Cases were notified: one in 2013, one in 2014, one in 2016, four in 2017, two in 2018, two in 2019, one in 2020. In 11 cases, the source of infection is unknown and one had contact with an HAV patient. Two cases were associated with travel to Kazakhstan, one to Germany, Uzbekistan, Morocco, the Philippines. The rest were local (Table 2.4 and Figure 2.4).

Table 2.4

Data of HAV subgenotype IA

HAV subgenotype IA clusters		**1 cluster**	**VRD_521_2016**	**RIVM-HAV16-090**	**V16-25801**	**2 cluster**	**3 cluster**	**4 cluster**	**5 cluster**	**6 cluster**	**7 cluster**	**8 cluster**	**9 cluster**	**10 cluster**	**Sporadic cases**
n =		96	30	7	2	16	3	4	5	3	2	2	3	2	12
Sex	male	56	16	5	2	8	1	1	2	1	1	0	1	1	8
	female	40	14	2	0	8	2	3	3	2	1	2	2	1	4
Age	0–9	0	1	0	0	0	0	0	1	0	0	0	0	0	1
	10–19	8	3	0	0	4	0	1	0	1	0	0	0	1	1
	20– 9	40	6	0	1	0	2	0	0	1	0	0	0	0	4
	30–39	22	8	5	1	0	0	1	1	0	0	1	2	1	3
	40–49	12	4	1	0	8	0	2	1	1	2	0	1	0	2
	50–59	9	7	1	0	3	1	0	0	0	0	1	0	0	1
	60–69	1	0	0	0	0	0	0	1	0	0	0	0	0	0
	70–79	3	1	0	0	1	0	0	1	0	0	0	0	0	0
Country of birth	Latvia	96	30	7	2	16	2	3	5	3	2	2	2	2	11
	other	0	0	0	0	0	1 Bulgaria	1 Uzbekistan	0	0	0	0	0	0	1 Uzbekistan
Country of residence	Latvia	96	30	7	2	16	3	4	5	3	2	2	2	2	11
	other	0	0	0	0	0	0	0	0	0	0	0	0	0	1 Germany
Possible source of infection	unknown	67	19	2	2	13	3	4	5	3	1	2	3	2	11
	PWID	23	0	0	0	0	0	0	0	0	0	0	0	0	0
	outbreak	6	0	0	0	0	0	0	0	0	0	0	0	0	0
	Contact with HAV patient	0	11	1	0	3	0	0	0	0	0	0	0	0	1
	MSM	0	0	4	0	0	0	0	0	0	0	0	0	0	0
	Fruits from Uzbekistan	0	0	0	0	0	0	0	0	0	1	0	0	0	

Table 2.4 continued

HAV subgenotype IA clusters		1 cluster	VRD_521_2016	RIVM-HAV16-090	V16-25801	2 cluster	3 cluster	4 cluster	5 cluster	6 cluster	7 cluster	8 cluster	9 cluster	10 cluster	Sporadic cases
Possible country of origin of the virus	Latvia (local cases)	95	26	2	0	14	1	0	3	2	2	0	2	2	6
	Germany	0	2	2	1	0	0	0	0	0	0	0	0	0	1
	Spain	0	1	1	0	0	0	0	0	1	0	0	0	0	0
	Great Britain	1	0	0	0	0	0	0	0	0	0	0	0	0	0
	Austria	0	1	0	0	0	0	0	0	0	0	0	0	0	0
	France	0	0	1	0	0	0	0	0	0	0	0	0	0	0
	Estonia	0	0	0	1	0	0	0	0	0	0	0	0	0	0
	Ukraine	0	0	0	0	1	0	0	0	0	0	0	0	0	0
	Nether-lands	0	0	0	0	1	0	0	0	0	0	0	0	0	0
	Russia	0	0	1	0	0	0	2	1	0	0	0	0	0	0
	Uzbekistan	0	0	0	0	0	0	2	0	0	0	1	0	0	1
	Morocco	0	0	0	0	0	0	0	0	0	0	0	1	0	1
	Bulgaria	0	0	0	0	0	1	0	0	0	0	0	0	0	0
	Kazakhstan	0	0	0	0	0	0	0	0	0	0	1	0	0	2
	Philippines	0	0	0	0	0	0	0	0	0	0	0	0	0	1
	Italy	0	0	0	0	0	0	0	1	0	0	0	0	0	0
	Greece	0	0	0	0	0	1	0	0	0	0	0	0	0	0

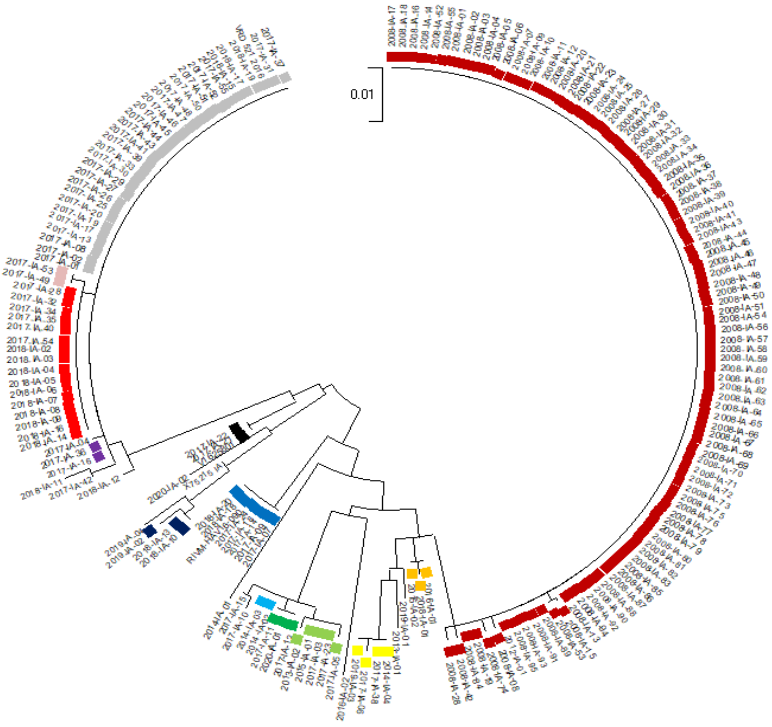

Figure 2.4 **Maximum likelihood phylogenetic tree of the hepatitis A virus VP1/2A genomic region sequences from hepatitis A cases subgenotype IA (n = 187)**

Reference sequence IA X75215 for subgenotype HAV IA from the GeneBank database and VRD_521_2016, RIVM-HAV16-090, V16-25801 from HAVNET database have been included for comparison. VRD_521_2016 (grey bars), RIVM-HAV16-090 (blue bars), V16-25801 (black bars), HAV IA 1 cluster (dark red bars), HAV IA 2 cluster (red bars), HAV IA 3 cluster (orange bars), HAV IA 4 cluster (yellow bars), HAV IA 5 cluster (bright green bars), HAV IA 6 cluster (green bars), HAV IA 7 cluster (bright blue bars), HAV IA 8 cluster (purple bars), HAV IA 9 cluster (dark blue bars), HAV IA 10 cluster (rose bars).

2.2.2 HAV subgenotype IB results

Results for HAV subgenotype IB showed that 59 HAV sequences fall into eight clusters and eleven sporadic cases with no identified epidemiological association. The average age of patients with HAV subgenotype IB was 27.8 years, men were 30, women – 29, adults – 38, children – 21.

The first cluster consists of 4/59 (6.8 %) identical sequences, two cases were notified in 2018 and two in 2019, the source of infection is unknown and the cases were local.

The second cluster consists of 2/59 (3.4 %) identical sequences, two cases were notified in 2020, the source of infection is unknown and the cases were local.

The third cluster consists of 4/59 (6.8 %) identical sequences, three cases were notified in 2019, one case in 2020, the source of infection is unknown and the cases were local.

The fourth cluster consists of 3/59 (5.1 %) identical sequences, one case was notified in 2018, one in 2019, one in 2020, the source of infection is unknown, but two cases are local and one case was associated with traveling to Egypt.

The fifth cluster consists of 20/59 (33.9 %) identical sequences, 18 cases were notified in 2018 and two in 2020. 16/18 cases in 2018 were related to HAV infection among relatives in one family, where the first recorded case was in a two-year-old child and the suspected source of infection was food from Egypt. Of the two registered cases in 2020, one was linked to travel to Egypt. In 16/20 cases, the source of infection was determined – contact with an HAV patient, 4/16 – the source of infection is unknown.

The sixth cluster consists of 4/59 (6.8 %) identical sequences, one case was notified in 2018, three in 2019, the source of infection is unknown and the cases were local.

The seventh cluster consists of 6/59 (10.2 %) identical sequences, two cases were notified in 2017, one in 2018, one in 2019, two in 2020.

Recurrent HAV subgenotype IB virus circulation has been observed in different (more than two) years in the fourth, fifth and seventh clusters (Table 2.5).

Table 2.5

Circulation of recurrent HAV subgenotype IB viruses (a time interval of more than two years)

HAV subgenotype IB cluster	Circulation of the virus over the years	Number
The fourth	2018	1
	2019	1
	2020	1
The fifth	2018	18
	2020	2
The seventh	2017	2
	2018	1
	2019	1
	2020	2

The eighth cluster consists of 5/59 (8.5 %) identical sequences, all notified in 2018. The possible source of infection in four cases was linked to contact with an HAV patient, all cases were local.

Sporadic cases accounted for 11/59 (18.6 %) sequences. One case was notified in 2008, 2014, 2016, 2017, 2018, 2020, 2021, four in 2019. Possible sources of infection are unknown. Two cases were associated with travel to Egypt, one to Nigeria, one to Sri Lanka (Table 2.6 and Figure 2.5).

Eight cases of subgenotype HAV IB that were associated with travel had a direct link to the geographic spread of HAV subgenotype IB.

Table 2.6

Data of HAV subgenotype IB

HAV subgenotype IB clusters		**1 cluster**	**2 cluster**	**3 cluster**	**4 cluster**	**5 cluster**	**6 cluster**	**7 cluster**	**8 cluster**	**Sporadic cases**
n =		4	2	4	3	20	4	6	5	11
Sex	male	4	1	2	1	10	1	5	1	5
	female	0	1	2	2	10	3	1	4	6
Age	0–9	0	0	0	0	6	0	0	3	2
	10–19	1	0	0	0	6	1	1	0	0
	20–29	0	1	0	2	1	2	0	1	3
	30–39	1	1	2	0	2	1	1	0	2
	40–49	1	0	1	1	2	0	2	1	2
	50–59	1	0	1	0	2	0	2	0	2
	60–69	0	0	0	0	1	0	0	0	0
	70–79	0	0	0	0	0	0	0	0	0
Country of birth	Latvia	4	2	4	3	20	4	6	5	11
	other	0	0	0	0	0	0	0	0	0
Country of residence	Latvia	4	2	4	3	20	4	6	5	11
	other	0	0	0	0	0	0	0	0	0
Possible source of infection	unknown	4	2	4	3	4	4	6	1	11
	Contact with HAV patient	0	0	0	0	16	0	0	4	0
Possible country of origin of the virus	Latvia (local cases)	4	2	4	2	19	4	4	5	7
	Egypt	0	0	0	1	1	0	0	0	2
	Nepal	0	0	0	0	0	0	1	0	0
	Great Britain	0	0	0	0	0	0	1	0	0
	Nigeria	0	0	0	0	0	0	0	0	1
	Sri Lanka	0	0	0	0	0	0	0	0	1

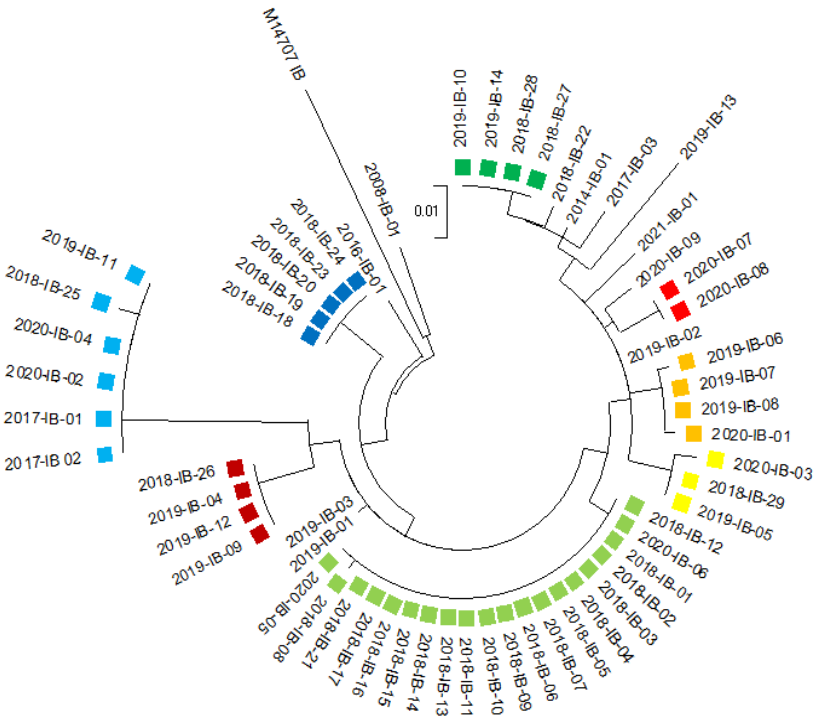

Figure 2.5 **Maximum likelihood phylogenetic tree of the hepatitis A virus VP1/2A genomic region sequences from hepatitis A cases subgenotype IB (n = 59)**

Reference sequence IB M14707 for subgenotype HAV IB from the GeneBank database has been included for comparison. HAV IB 1 cluster (dark red bars), HAV IB 2 cluster (red bars), HAV IB 3 cluster (orange bars), HAV IB 4 cluster (yellow bars), HAV IB 5 cluster (bright green bars), HAV IB 6 cluster (green bars), HAV IB 7 cluster (bright blue bars), HAV IB 8 cluster (blue bars).

2.2.3 HAV subgenotype IIIA results

Results for HAV subgenotype IIIA showed that 13 HAV sequences fall into one cluster and nine sporadic cases with no identified epidemiological association. The average age of patients with HAV subgenotype IIIA was 34.5 years, men were 7, women – 6, adults – 12, child – 1.

The first cluster consists of 4/13 (30.7 %) identical sequences, the cases were notified in 2008, the source of infection is unknown and the cases were local. It is possible that this HAV subgenotype was imported to Latvia, as homology with HAV sequences with the country of origin Pakistan was found.

The first branch consists of 2/13 (15.4 %) similar sequences, cases were notified in 2017–2018 and were associated with travel in India. One case was associated with an Indian citizen whose country of residence was Latvia, in both cases the source of infection is unknown.

The second branch consists of 2/13 (15.4 %) similar sequences, cases were notified in 2016 and were associated in one case with travel in Turkmenistan and the source of infection is unknown, and in the second case with the consumption of unwashed fruits brought from Uzbekistan.

The third branch consists of 2/13 (15.4 %) similar sequences, the cases were notified in 2018 and were associated with travel in India, and one case was associated with an Indian citizen whose country of residence was Latvia, in both cases the source of infection is not known.

The fourth branch consists of 2/13 (15.4 %) similar sequences, cases were notified in 2015–2016 and were associated with travel in Tajikistan, both cases with an unknown source of infection.

The fifth branch consists of 1/13 (7.7 %) sequences, the case was notified in 2015 and was linked to travel in Kazakhstan, the source of infection is unknown (Table 2.6 and Figure 2.6).

Table 2.7

Data of HAV subgenotype IIIA

HAV subgenotype IIIA clusters and similar sequences		Cluster	Sporadic cases, HAV sequences from one phylogenetic tree branch				
		1 cluster	1st branch	2nd branch	3rd branch	4th branch	5th branch
n =		4	2	2	2	2	1
Sex	male	2	1	1	1	2	0
	female	2	1	1	1	0	1
Age	0 – 9	0	0	0	0	0	0
	10 – 19	1	0	0	0	0	0
	20 – 29	1	0	1	1	0	0
	30 – 39	0	2	0	1	1	1
	40 – 49	2	0	0	0	1	0
	50 – 59	0	0	1	0	0	0
	60 – 69	0	0	0	0	0	0
	70 – 79	0	0	0	0	0	0
Country of birth	Latvia	4	1	2	1	2	1
	other	0	1 India	0	1 India	0	0
Country of residence	Latvia	0	2	2	2	2	1
	other	0	0	0	0	0	0
Possible source of infection	unknown	4	2	1	2	2	1
	fruits	0	0	1 Uzbeki-stan	0	0	0
Possible country of origin of virus	Latvia (local cases)	4	0	0	0	0	0
	India	0	2	0	2	0	0
	Tajikistan	0	0	0	0	2	0
	Uzbekistan	0	0	1	0	0	0
	Turkmenistan	0	0	1	0	0	0
	Kazakhstan	0	0	0	0	0	1

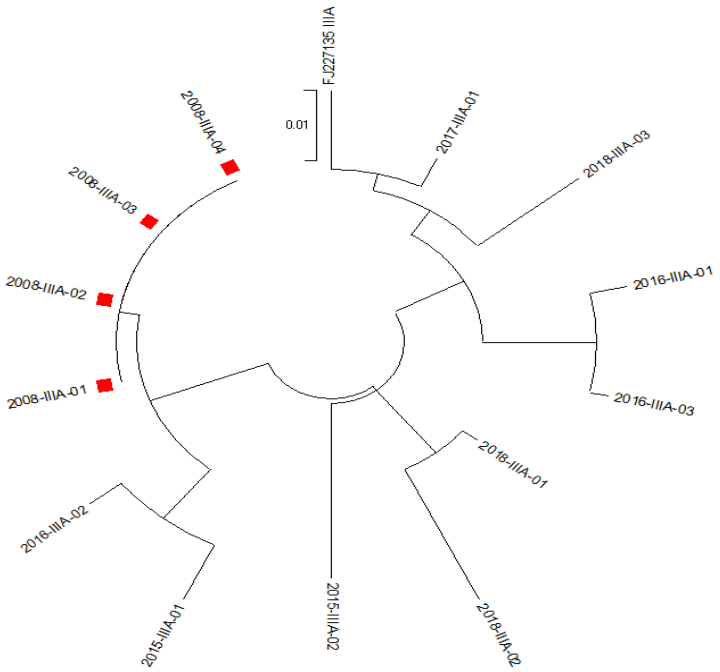

Figure 2.6 **Maximum likelihood phylogenetic tree of the hepatitis A virus VP1/2A genomic region sequences from hepatitis A cases subgenotype IIIA (n = 13)**

Reference sequence IIIA FJ227135 for subgenotype HAV IIIA from the GeneBank database has been included for comparison. HAV IIIA 1 cluster (red bars).

All 13 cases have a direct link to the distribution of subgenotype HAV IIIA in the Asian region.

2.3 Demographic data

The study group, which analysed demographic data in relation to the HAV molecular epidemiology, included data from 259 patients, of whom more – 140 (54 %) were male and 119 (46 %) were female (Figure 2.7).

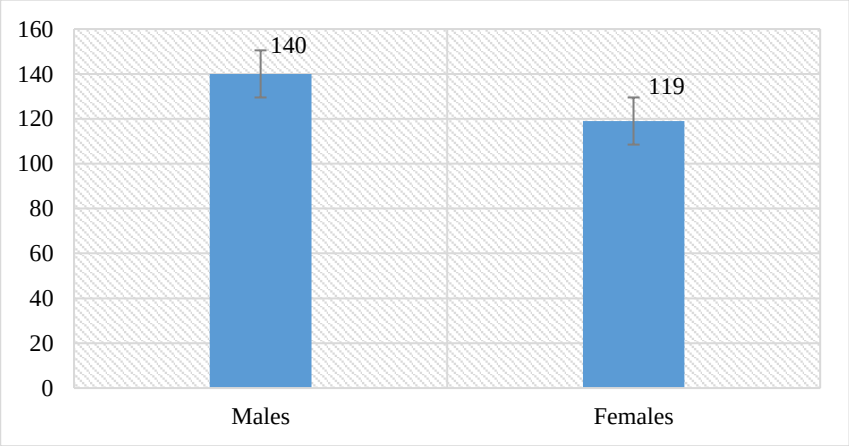

Figure 2.7 **Distribution of hepatitis A patients by sex**

The mean age of patients at diagnosis was 33.7 years (SD ± 14.9 years). The youngest patient was 3 years old, while the oldest patient was 77 years old. The median age was 32 years. 41 (16 %) of this group of patients were children, and overwhelmingly more – 218 (84 %) were adults (Figure 2.8).

Comparing the age of patients at the time of diagnosis, the age of men and women differs statistically significantly (p = 0.049) (Figure 2.9).

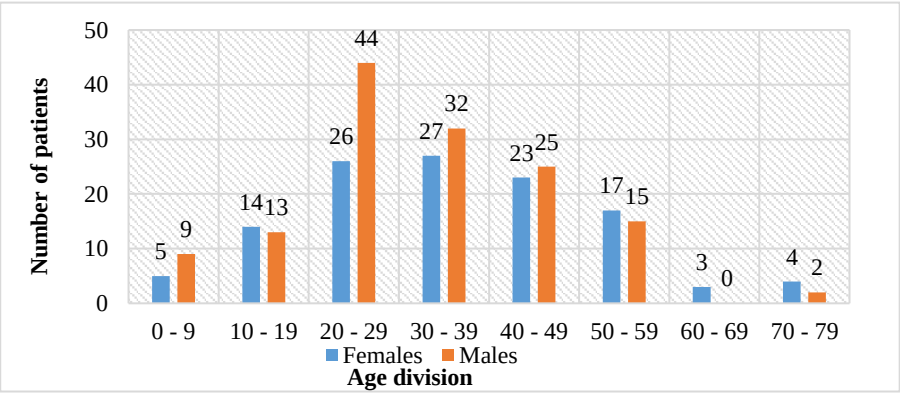

Figure 2.8 **Classification of study patients into sex and age groups**

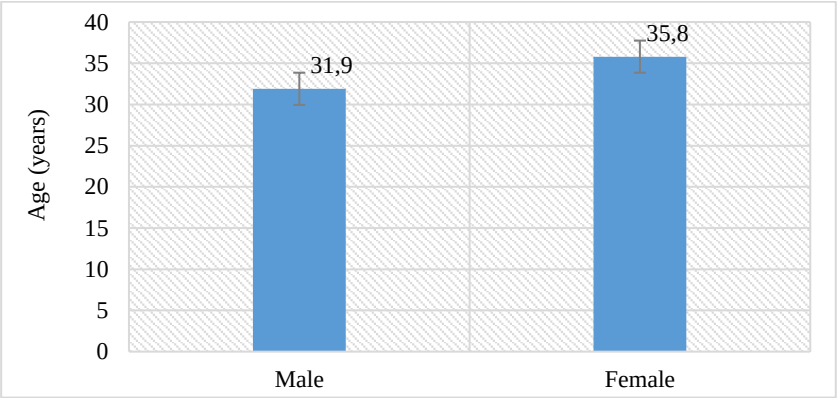

Figure 2.9 **Distribution of patients by age and sex**

2.4 Data of laboratory confirmed cases

In an analysis of 259 patients with case definition for acute hepatitis A, the mean time from the date of illness to a laboratory-confirmed case was 7.9 days (SD ± 5.3 days), with a median of 7.0 days. The minimum number of days was one day when a laboratory-confirmed diagnosis was established after hospitalisation of patients, while the maximum number of days was 32 days (Figure 2.10).

When comparing the length of days from the date of illness to the laboratory-confirmed case between HAV subgenotypes, the length of days is statistically identical among HAV subgenotypes ($p = 0.584$).

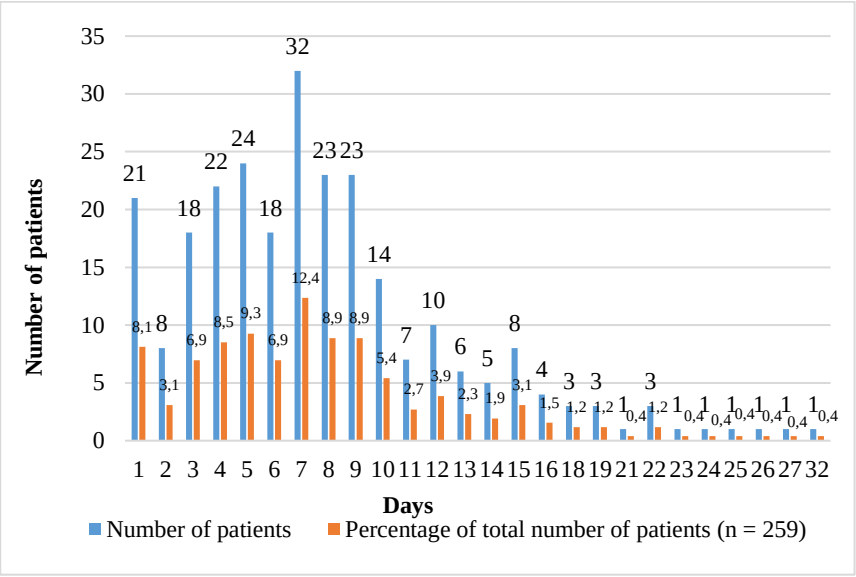

Figure 2.10 **Laboratory confirmed hepatitis A diagnosis after the date of illness**

2.5 Data of hepatitis symptoms, hospitalisation and vaccination

All 259 patients had symptoms of hepatitis. The total number of hospitalised patients was 242/259 (93.4 %), the number of non-hospitalised patients was 17/259 (6.6 %) (Figure 2.11).

Among hospitalised patients, adults were 205/242 (84.7 %) and children were 37/242 (15.3 %). The average age of hospitalised patients was 33.7 years (SD ± 14.9 years), with a median of 32 years, the sex distribution was uneven: men – 54.1 % (n = 131), women – 45.9 % (n = 111). Among hospitalised patients, HAV subgenotype IA was 178, subgenotype IB-52, subgenotype IIIA-12.

Comparing the sex of hospitalised patients and HAV subgenotype, there is no statistically significant association (p = 0.790), but comparing the age

distribution of hospitalised patients with HAV subgenotypes is statistically significantly different (p = 0.08).

Among non-hospitalised patients, adults were 14/17 (82.4 %) and children were 3/17 (17.6 %). The mean age of non-hospitalised patients was 33.4 years (SD ± 16.0), with a median of 34 years, the gender distribution was even: men – 52.9 % (n = 9), women – 47.1 % (n = 8). Among non-hospitalised patients, HAV subgenotype IA was 9, subgenotype IB-7, subgenotype IIIA-1.

When comparing non-hospitalised patients' sex and HAV subgenotype, there is no statistically significant difference (p = 0.758), as well as the age distribution by HAV subgenotype is not statistically significantly different (p = 0.837).

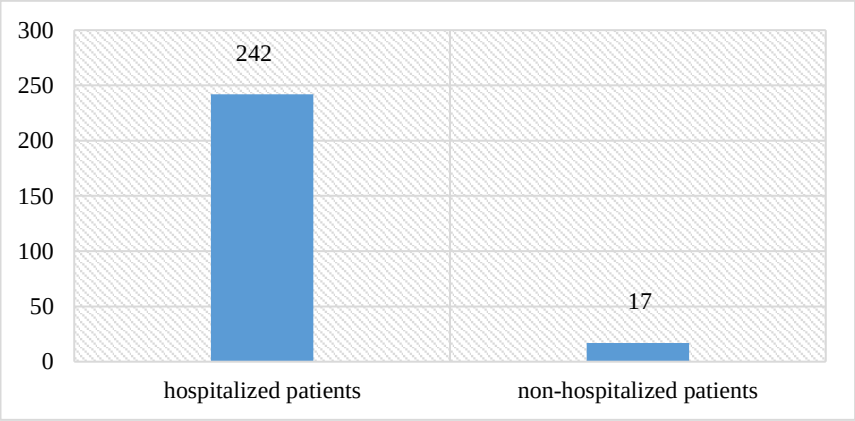

Figure 2.11 **Hospitalised and non-hospitalised patient's number**

The number of analysed patients who were hospitalised in REUH inpatient LIC from 2012 to 2021 amounted to 97/259 (37.5 %).

In 65 cases, a laboratory-confirmed VHA diagnosis was established on the day of hospitalisation, for the remaining 32 cases, the average time from the date of hospitalisation to a laboratory-confirmed case was 2.5 days (SD ± 1.7 days), with a median of 2 days (Figure 2.12).

Patients were hospitalised for an average of 6.9 days (SD ± 4.5 days) from the date of illness, with a median of 6 days. The minimum number of days was one day, when the diagnosis of acute hepatitis A was also laboratory confirmed and established, while the maximum number of days was 27 days after the illness (Figure 2.13).

The number of days of hospitalisation for the examined group was on average 8.7 days (SD ± 7.8 days), with a median of 7.0. The minimum number of days was one day, the maximum number of hospitalised days was 73 days (Figure 2.14).

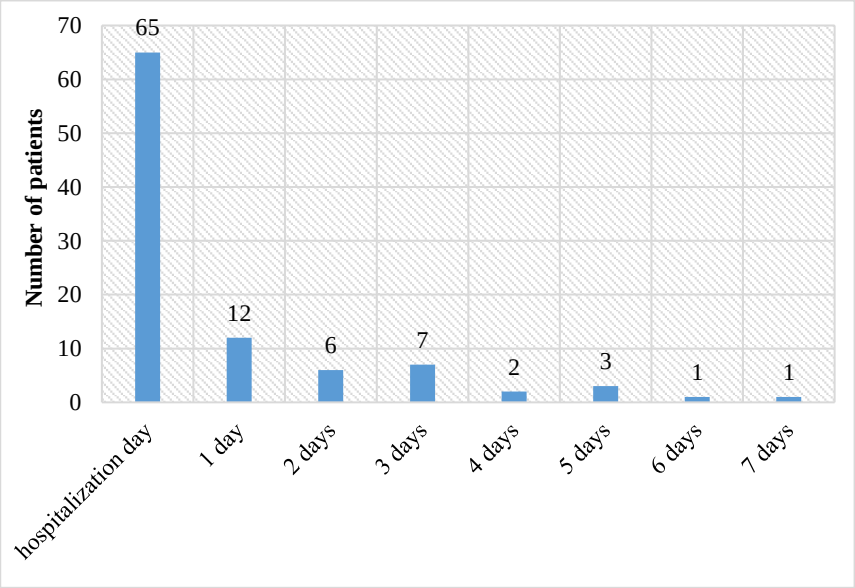

Figure 2.12 **Laboratory-confirmed hepatitis A after hospitalisation day**

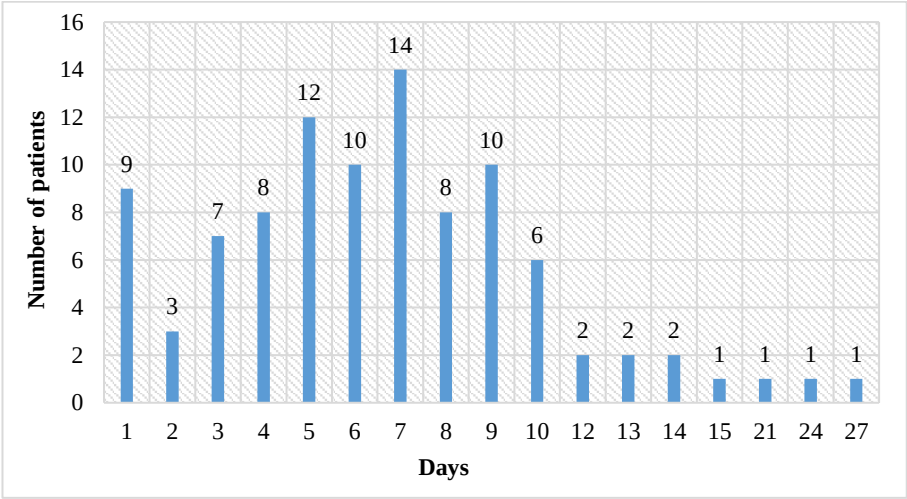

Figure 2.13 **Sick Day during hospitalisation**

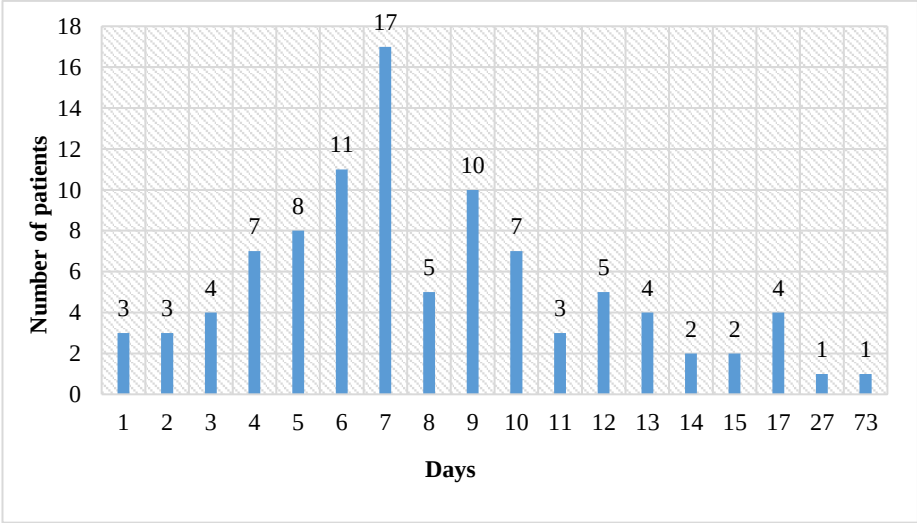

Figure 2.14 **Number of days of hospitalisation**

Of the 97 hospitalised patients, the sex distribution was even: men – 51.5 % (n = 50), women – 48.5 % (n = 47). The mean age was 38.8 years

(SD ± 12.6 years), with a median of 38. The youngest patient was 19 years old and the oldest patient was 74 years old. There is no statistically significant correlation between age and days of hospitalisation (k = 0.037; p = 0.717).

The number of patients with subgenotype HAV IA was 69.1 % (n = 67), with subgenotype HAV IB 24.7 % (n = 24), with subgenotype HAV IIIA 6.2 % (n = 6). The average number of hospitalised patients with HAV subgenotype IA was 9.3 days, (minimum day was one day, maximum days – 73 days), with subgenotype IB was 7.3 days (minimum day was two days, maximum days – 15 days), with subgenotype IIIA 7.7 days (minimum day was one day, maximum days – 12 days) (Figure 2.15).

Data on vaccination of the study group showed that the majority of patients were not vaccinated against HAV – 89.6 % (n = 232), 10.0 % (n = 26) did not know their vaccination status and in one case – 0.4 % (n = 1), when the patient was in contact with an HAV patient and was vaccinated with a one dose of HAV vaccine, but still got sick (Figure 2.16).

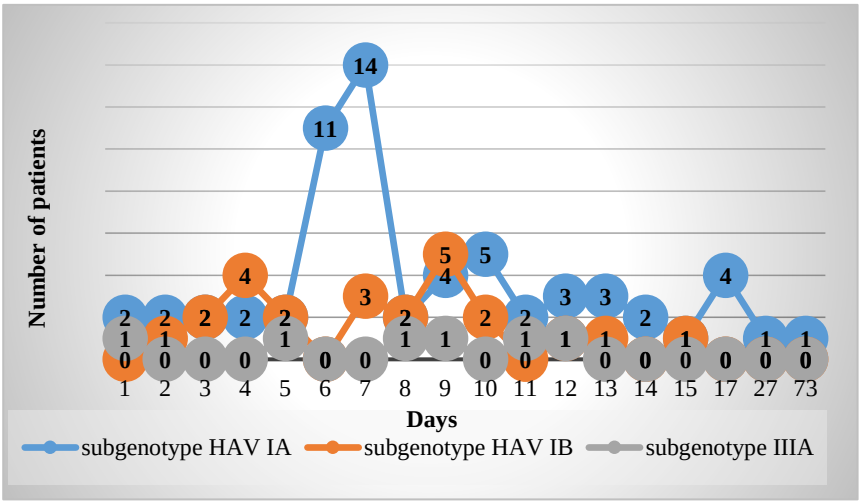

Figure 2.15 **Number of hospitalisation days of patients with HAV subgenotypes IA, IB, IIIA**

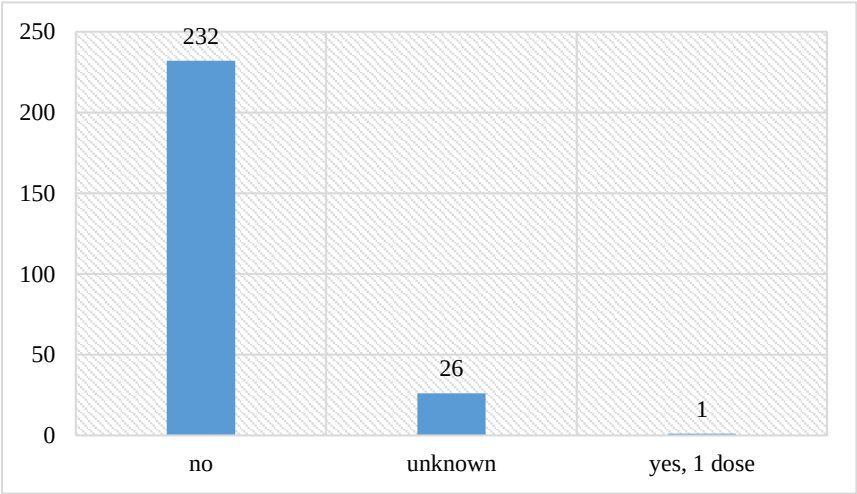

Figure 2.16 **Vaccination against HAV**

3 Discussion

Viral hepatitis A remains a major problem for the health sector in all countries. According to official statistics, HAV affects 1.5 million people worldwide each year (WHO, 2009). According to a WHO expert report, the true incidence of this infection could be ten times higher. The WHO reported that in 2016 hepatitis A caused an estimated 7134 deaths (representing 0.5 % of viral hepatitis mortality) (WHO, 2020).

Reporting of HAV is mandatory, and the surveillance system is on a national level in all EU Member States, Iceland and Norway, except the United Kingdom, which has organised surveillance differently (ECDC, 2020).

Based on the data published by the ECDC on the HAV situation in the EU/EEA countries from 2008 to 2020, it can be concluded that the occurrence of HAV in Latvia is rare, except for outbreaks that occurred in 2008–2009 and 2017. From 2008 to 2020, 183191 laboratory-confirmed cases of HAV were registered in the EU/EEA (ECDC, Surveillance Atlas of Infectious Diseases, 2008 – 2020):

- in 2008, 16791 cases were detected in the EU/EEA countries – the number of cases registered in Latvia was 2816 (16.8 %), and the highest number of HAV cases was registered in Romania (3161), Spain (1877), and the Czech Republic (1649). The smallest number was registered in Iceland – 1. In the Baltic States – Estonia (13), Lithuania (20);
- in 2009, 17453 cases were detected in EU/EEA countries – the number of cases registered in Latvia was 2291 (13.1 %), and the highest number of HAV cases was registered in Romania (3734), Spain (1808), and Italy (1580). The smallest number was registered in Austria – 1. In the Baltic States – Estonia (19), Lithuania (16);

- in 2010, 13471 cases were detected in the EU/EEA countries – the number of cases registered in Latvia was 297 (2.2 %), and the highest number of HAV cases was registered in Romania (3493), Bulgaria (2350), Slovakia (1449). The smallest number was registered in Cyprus – 2. In the Baltic States – Estonia (6), Lithuania (10);
- in 2011, 12785 cases were detected in EU/EEA countries – the number of cases registered in Latvia was 51 (0.4 %), and the highest number of HAV cases was registered in Bulgaria (5587), Romania (2581), France (1115). The smallest number was registered in Iceland – 1. In the Baltic States – Estonia (153), Lithuania (17);
- in 2012, 13369 cases were detected in the EU/EEA countries – the number of cases registered in Latvia was 11 (0.08 %), and the highest number of HAV cases was registered in Bulgaria (4896), Romania (3603), France (1096). The smallest number was registered in Luxembourg – 2. In the Baltic States – Estonia (63), Lithuania (113);
- in 2013, 12659 cases were detected in EU/EEA countries – the number of cases registered in Latvia was 12 (0.09 %), and the highest number of HAV cases was registered in Romania (4173), Bulgaria (1819), Italy (1388). The smallest number was registered in Cyprus – 2. In the Baltic States – Estonia (6), Lithuania (64);
- in 2014, 14113 cases were detected in EU/EEA countries – the number of cases registered in Latvia was 20 (0.14 %), and the highest number of HAV cases was registered in Romania (6646), Hungary (1548), France (933). The smallest number was registered in Malta – 2. In the Baltic States – Estonia (12), Lithuania (17);
- in 2015, 12528 cases were detected in EU/EEA countries – the number of cases registered in Latvia was 6 (0.05 %), and the highest number of HAV cases was registered in Romania (5176), Bulgaria (1061),

Hungary (963). The smallest number was registered in Croatia – 4. In the Baltic States – Estonia (6), Lithuania (7);

- in 2016, 12430 cases were detected in EU/EEA countries – the number of cases registered in Latvia was 10 (0.08 %), and the highest number of HAV cases was registered in Romania (3190), Bulgaria (1625), Slovakia (1358). The smallest number was registered in Cyprus – 3. In the Baltic States – Estonia (7), Lithuania (17);
- in 2017, 26145 cases were detected in EU/EEA countries – the number of cases registered in Latvia was 75 (0.3 %), and the highest number of HAV cases was registered in Spain (4528), Italy (3766), France (3387). The smallest number was registered in Iceland – 5. In the Baltic States – Estonia (45), Lithuania (38);
- in 2018, 15680 cases were detected in EU/EEA countries – the number of cases registered in Latvia was 67 (0.4 %), and the highest number of HAV cases was registered in Romania (4527), Spain (2294), France (1525). The smallest number was registered in Iceland – 1. In the Baltic States – Estonia (15), Lithuania (13);
- in 2019, 11370 cases were detected in the EU/EEA countries – the number of cases registered in Latvia was 37 (0.3 %), as well as the highest number of HAV cases was registered in Romania (3351), Bulgaria (1512), France (1375). The smallest number was registered in Iceland – 2. In the Baltic States – Estonia (20), Lithuania (8);
- in 2020, 4397 cases were detected in EU/EEA countries – the number of cases registered in Latvia was 21 (0.5 %), and the highest number of HAV cases was registered in Bulgaria (1297), Romania (1010), Germany (550). The smallest number was registered in Iceland – 1. In the Baltic States – Estonia (30), Lithuania (9).

From 1990 to 1999, hepatitis A was a very common infectious disease in Latvia and 28546 cases were notified (Epidemiological bulletins, CDC of Latvia). The spread of HAV occurred mainly through the faecal-oral route or through contact with an HAV patient. Between 2000 and 2007, the number of HAV cases (695) decreased significantly and mainly sporadic cases were recorded. Although the incidence of VHA was the lowest in 2007, the risk of introducing and spreading the infection still exists, as the virus spreads easily when personal hygiene is not followed. Disease outbreaks can occur in cases where water or food products are contaminated.

From 1990 to 2008, the laboratory diagnosis of HAV in Latvia was based on the detection of Anti-HAV IgM in blood serum and the detection of HAV Ag in faeces by immunochemical methods. As technology developed, the Sanger sequencing method was applied since 2008, which started molecular epidemiology and also made it possible to determine not only the circulating subgenotypes of HAV, but also to build a phylogenetic tree based on nucleotide sequences to trace HAV clusters (Nainan et al., 2006).

In 2008–2009, an outbreak of HAV was registered in Latvia (5107 cases) and initially the spread of infection was associated with PWID and then spread widely to the rest of the population, continuing to be registered in 2009 (Perevoscikovs et al., 2009). The 2008 outbreak can be attributed to the large number of susceptible individuals caused by rapidly declining population immunity to VHA. Control measures taken included: case contact tracing; vaccination recommendations for contacts of cases; quarantine and medical observation of cases; public health education through mass media and specific prevention recommendations for food handlers, schools and the general public. Since September 2008, uptake of HAV vaccination in the population has been observed (ECDC, 2008). As a result, sporadic cases of acute hepatitis A were

reported in Latvia every year until 2017, and most cases were associated with travel to endemic regions.

In late 2016 and early 2017, outbreaks of HAV were reported in EU/EEA countries with a common source of infection, mostly among MSM (ECDC, 2018; Ndumbi et al., 2018), and a trend of increasing HAV cases in 2017 was observed in Latvia with similar HAV sequences from EU/EEA outbreaks, as well as sporadic cases that were associated with travel or contact with an HAV patient (Savicka, Zeltmatis, Storozenko, 2021).

Analysing the molecular epidemiological data on the spread of HAV in Latvia from 2008 to 2021, it can be established that three subgenotypes of HAV have been found in Latvia – IA, IB, IIIA. The majority of HAV cases were of HAV subgenotype IA (72 %), which is also the most common genotype worldwide (Holmberg, 2012). The remaining cases were with HAV subgenotype IB (23 %) and HAV subgenotype IIIA (5 %). The prevalence of HAV subgenotypes is not different compared to other EU/EEA countries (Foster et al., 2019). Our study identified 13 clusters and 12 sporadic cases of HAV subgenotype IA, eight clusters and 11 sporadic cases of HAV subgenotype IB, one cluster and nine sporadic cases of HAV subgenotype IIIA.

Analysing the gender of the patients, the majority of patients – 54 % (140) were men and 46 % (119) were women, similar data are also from the Eurosurveillance of Infectious Diseases selection, where the gender-specific rate (N/100000) by year is as follows: 2008 year M = 3.45, F = 2.26; 2009 M = 4.22, F = 2.77; 2010 M = 2.75, F = 2.07; 2011 M = 1.62, F = 1.77; 2012 M = 1.86, F = 1.54; 2013 M = 2.40, F = 1.96; 2014 M = 3.11, F = 2.49; 2015 M = 2.63, F = 2.25; 2016 M = 2.85, F = 1.98; 2017 M = 7.43, F = 2.75; 2018 M = 3.54, F = 2.53; 2019 M = 2.39, F = 2.0; 2020 M = 1.08, F = 0.99. As can be seen from the data, there is mostly no gender difference, but it was observed in 2008–2009, 2016–2018, when most patients were men. These gender differences could be

related to HAV outbreaks in certain risk groups, as, for example, our data indicate that in 2008 there was a predominance of men who were PWID. Between 2016 and 2018, a gender gap was associated with HAV outbreaks among MSM in EU/EEA countries (ECDC, 2018).

At the time of diagnosis, the median age of the patients included in the study was 32 years, the majority of patients were in the age group from 20 to 29 years, which could be explained by active spending of time, including traveling. When analysing whether the age groups at diagnosis differed for any of the sexes, a statistically significant difference was found. Comparing age data from the Eurosurveillance of Infectious Diseases selection with EU/EEA countries, where age is reflected in an age-standardised rate (N/100000), it was found that from 2008 to 2021, the majority of patients are in the age group from 5 to 14 years and 0–4 years, but the age group 65+ years is the same in all countries: 0–4 age group = 4.67, 5–4 age group = 6.95, 15–24 age group = 3.52, 25–44 age group = 2.65, 45–64 age group = 1.31, 65+ age group = 0.59. Comparing the age groups between the Baltic States, the data are similar to our data. Also, our data coincides with the data of the Scandinavian countries and with Germany, Spain, France. However, reported laboratory-confirmed cases of HAV from countries such as Romania and Bulgaria, the countries where the highest number of HAV were recorded, contradict our results but are similar to the EU/EEA data combined, where HAV cases prevail among children in the age groups 0–4 years and 5–14 years. Taking into account the analysed data, it can be concluded that in many European countries, there are no significant differences in the age of patients. Patients are mostly middle-aged, of course, in regions where the disease is very common, patients are younger (ECDC, Surveillance Atlas of Infectious Diseases, 2008–2020).

The likelihood, which was associated with the clinical course of HAV infection, increases with age. In children under 6 years of age, the majority of

HAV infection (70 %) is asymptomatic (Morais et al., 2006; Quiros-Tejera, 2022) and if the disease does develop, it is usually non-icteric. In older children and adults, infection is usually symptomatic, with jaundice occurring in 70 % of patients (Shin & Jeong, 2018). The data included in our study indicate that all patients had symptoms of hepatitis according to the clinical criteria from the case definition for hepatitis A: any person with a discrete onset of symptoms (for example, fatigue, abdominal pain, loss of appetite, intermittent nausea and vomiting) and at least one of the following three: fever, jaundice, Elevated serum aminotransferase levels (European Commission, 2018).

It is important to note the differential diagnosis of laboratory – specific acute viral hepatitis. Analysis of our data concluded that the median time from the date of illness to a laboratory-confirmed case of VHA was 7.9 days after the date of illness. A study from South Korea reported 6.3 days (Hyun et al., 2012). Timely diagnosis of VHA makes it possible to identify contact persons and take preventive measures, since the contagious period begins one to two weeks before the appearance of symptoms and is minimal about a week after the appearance of jaundice. For example, food workers must be excluded from work for at least two weeks after the onset of clinical symptoms of VHA. In cases where food workers are jaundiced, they should not return to work for at least one week after the onset of jaundice (Department of health, 2019).

Patients included in our study were hospitalised for an average of 6.9 sick days. A study from Sri Lanka reported an average of 7.8 days of hospitalisation (Niroshama et al., 2016), while a study from South Korea reported an average of 5.3 days of hospitalisation (Hyun et al., 2012).

Data included in the study indicate that patients had a median length of hospitalisation of 7 days, which is less than in Norway and Sweden, where the median length of hospitalisation was 3 days, in the Netherlands, 4 days, in Spain, 5 days, and more than in Italy, where the median length of hospitalisation was 8

days (Severi et al., 2022). Data from our study indicate that the mean duration of hospitalisation with subgenotype HAV IA was 9.3 days, with subgenotype IB – 7.3 days, and with HAV IIIA – 7.7 days. A study from Sri Lanka reported that the duration of hospitalisation was 7.4 days with subgenotype IA and 7.8 days with subgenotype IIIA (Niroshama et al., 2016). Our data and those of the Sri Lankan study indicate that no significant difference was found between hospitalisation days with different HAV subgenotypes.

Analysing the distribution of hospitalised patients between age and duration of hospitalisation, it was found that the data do not differ statistically significantly. Similar reports are from the Eurosurveillance of Infectious Diseases during the period from 2008 to 2021 in EU/EEA countries.

The main challenge of the study was to understand the possible source of infection due to the long incubation period of HAV infection. Our data show that only 27.4 % had a source of infection and 72.6 % had an unknown source of infection. Comparing the data obtained in our study on the sources of infection, it can be concluded that similar data are also available in other studies in different parts of the world. Among our data, the most frequently reported source of infection was related to close contact with an infected person, similarly mentioned in a study from the USA (Nelson et al., 2020).

Other possible sources are among PWID, which have been studied in Scandinavia and North America (Sunthornchart et al., 2008; Luquero et al., 2009; Removille et al., 2011) and travel to countries where HAV is endemic (Beaute et al. al., 2018). Previous studies have shown that travel to countries with high or moderate HAV endemicity is a risk factor for residents of countries with low HAV endemicity. Travel remains a major risk factor for HAV infection in the EU/EEA. In participating European countries, 27.8 % of reported cases of viral hepatitis A were travel related (Beaute et al., 2018). The results of our study

showed that 18.5 % of HAV cases during the incubation period were also travel related and 81.5 % were local cases.

Also, some of our cases involved individuals who self-identified as MSM, which has also been implicated as a source of HAV infection in studies from the Netherlands and England (Bruisten et al., 2001; Regan et al., 2016). In our study, MSM cases belong to the isolated HAV RNA nucleotide sequence cluster RIVM-016-90, and our study lacks information on sexual behaviour because only four patients self-identified as MSM, but these cases were also travel-related. The ECDC epidemiological update of 12 September 2018 informed that since January 2017, 24 EU/EEA countries have reported 25032 laboratory-confirmed cases of hepatitis A. Between January 2017 and August 2018, 5537 (22 %) HAV samples from 25032 laboratory-confirmed cases of hepatitis A in 24 EU/EEA countries were sequenced. Of these 5537 sequenced cases, 4217 (76 %) were infected with one of three outbreak strains: laboratory-confirmed HAV subgenotype IA and sequence similarity of ≥ 99.3 % based on overlapping fragments in the VP1/2A genomic region: VRD_521_2016, RIVM-HAV16-090, V16-25801 (ECDC, 2018; Gozlan et al.; 2017, Ndumbi et al., 2018). Of our sequenced samples, 21.9 % of sequences belong to HAV subgenotype IA, which were associated with MSM clusters. Among the sequences we obtained belonging to one of the three MSM clusters, HAV subgenotype IA was predominant with 17.1 % identity to the VRD_521_2016 cluster, 3.7 % to the RIVM-HAV16-090 cluster, 1.2 % to the V16-25801 cluster. These HAV subgenotypes IA were probably imported from EU/EEA countries and spread through contact with a HAV patient or through travel. From the HAV sequences and the phylogenetic tree analysis, we assume that the reason for the increase in the number of cases of viral hepatitis A in Latvia in 2017 is related to the outbreak of viral hepatitis A among MSM in the EU/EEA countries. Similar data on belonging to one of the three clusters are described in other studies in England

(Bradley-Stewart et al., 2019), the Netherlands (Freidl et al., 2017), data on EU/EEA countries (Ndumbi et al., 2018), as these clusters were also identified among MSM individuals in Brazil (Mello et al., 2019).

In our study, in two cases, the source of HAV infection was linked to the consumption of unwashed fruits brought from Azerbaijan and Uzbekistan, and by analysing the sequences from the phylogenetic tree, it was found that the IA sequences of this HAV subgenotype were also found in those countries. Although mentioned in other studies, contaminated food and water are rare sources of infection, although they have been linked to outbreaks (Nainan et al., 2006).

Despite the fact that the largest number of cases was local and with an unknown source of infection, there was a trend in the distribution of the identified HAV sequences in different years, which may indicate local circulation of the virus.

The most effective means of specific prevention of hepatitis A virus is vaccination. In order to develop long-term immunity against viral hepatitis A, it is necessary to receive two doses of the vaccine with an interval of 6 to 12 months. When carrying out vaccination, it should be taken into account that immunity will develop within 2 – 4 weeks after receiving the first vaccination (CDC of Latvia website). Although the incidence of hepatitis A has decreased in recent years, mainly due to immunisation, our data show that 89.6 % of cases were unvaccinated, 10.0 % had unknown vaccination status, and 0.4 % had received one dose of vaccine. The vaccine against viral hepatitis A in Latvia is not included in the national immunisation calendar and is not paid for from the state budget. Vaccination is recommended for uninfected persons who plan to travel to moderately or highly endemic countries (including Africa, Central Asia, South America and Central America), especially if they plan to stay there for a long time or go there repeatedly; plans to travel to countries where an outbreak

of hepatitis A (increased incidence) has been registered; practice risky sexual activities, including when there is a possibility of faecal-oral infection; uses narcotic substances; after contact with an HAV patient. In order to prevent the disease, vaccination against hepatitis A is recommended for persons who were at risk of infection, if it is possible to do it within two weeks of contact. The experience of other countries shows that vaccination makes it possible to successfully control outbreaks of hepatitis A, including in institutions affected by the infection, and to stop the spread of the infection (DCD of Latvia website).

Researches of recent years confirm that vaccination against hepatitis A with the latest generation vaccines provides lifelong immunity already after the first vaccination, and re-vaccination is not necessary (Shauval, 2019; Herzog, Herck, Damme, 2021).

A similar retrospective study of molecular surveillance of hepatitis A was conducted in Sweden. HAV data from 2009 to 2018 was collected. That study was the first comprehensive evaluation of the use of sequencing-based typing data to detect hepatitis A outbreaks in Sweden. Despite the fact that hepatitis A surveillance in Sweden includes typing, the detection of HAV outbreaks together with epidemiological investigation has not been fully evaluated (Ries et al., 2021).

This is the first detailed molecular epidemiological study of the hepatitis A virus in Latvia. This study highlights the genetic diversity of HAV circulating in the country. The combination of diagnostic methods, molecular biology methods and epidemiological data allows public health to identify clusters, establish links with other outbreaks and compare Latvian strains with other strains. This approach helps to understand the epidemiological process of VHA. Overall, molecular epidemiology has provided valuable insights into the genetic diversity, transmission patterns, and global distribution of HAV.

Conclusions

1. HAV subgenotypes IA, IB and IIIA circulate in the territory of Latvia, which are also the most common in the world.
2. For the HAV subgenotype IA, 13 clusters and 12 sporadic cases were identified, including three clusters (VRD_521_2016, RIVM-HAV16-090, V16-25801) associated with HAV outbreaks in EU/EEA countries among MSM in 2016–2017. For the HAV subgenotype IB 8 clusters and 11 sporadic cases were identified and for the HAV subgenotype IIIA one cluster and nine sporadic cases. The identification of HAV clusters allows to follow the spread of infection and also all new cases of hepatitis A virus could be quickly detected by the phylogenetic tree and with the already known cluster.
3. The recurrent circulation of viruses of some clusters of HAV subgenotypes IA and IB has been observed:
   - HAV subgenotype IA: first cluster – 95/96 in 2008, 1/96 in 2012; third – 1/3 in 2015, 1/3 in 2016, 1/3 in 2018; fourth – 1/4 in 2014, 2/4 in 2017, 1/4 in 2019; fifth – 1/5 in 2013, 1/5 in 2015, 3/5 in 2017; sixth – 2/3 in 2017, 1/3 in 2020.
   - HAV subgenotype IB: fourth cluster – 1/3 in 2018, 1/3 in 2019, 1/3 in 2020; fifth – 18/20 in 2018, 2/20 in 2020; seventh – 2/6 in 2017, 1/6 in 2018, 1/6 in 2019, 2/6 in 2020.
4. It was found that the sources of infection were related to contact with a HAV patient (35 cases), travel (48 cases), injecting drug users (23 cases) and men who have sex with men (4 cases), with fruits (2 cases), for the other cases the source of infection is unknown.
5. The average time from the date of illness to a laboratory-confirmed case was 7.9 days. Patients were hospitalised for an average of 6.9 sick days with a mean number of days of hospitalisation of 8.7 days.

Patients with HAV subgenotype IA had the longest hospitalisation days and averaged 9.3 days, while patients with subgenotype IB – 7.3 days, subgenotype IIIA – 7.7 days.

Hypothesis of the Thesis – clusters of HAV subgenotypes circulating in Latvia, which caused local outbreaks, including in the MSM group (or other population groups), are associated with outbreaks in EU/EEA countries – **was confirmed**.

Proposals

Recommendations for HAV typing:

1) to perform molecular biological typing for all HAV cases and to identify the subgenotype of the HAV sequence and belonging to clusters or sporadic cases in the phylogenetic tree based on the existing HAV database.2. To perform molecular biological typing for all HAV cases and to identify the subgenotype of the HAV sequence and belonging to clusters or sporadic cases in the phylogenetic tree based on the existing HAV database;
2) further supplement the HAVNET database with HAV sequences from Latvia, entering all the requested information.

List of publications, reports and patents on topic of Doctoral Thesis

Publications:

1. **Savicka, O.,** Zeltmatis, R., Storoženko, J. Molecular epidemiology of hepatitis A outbreak and sporadic cases, Latvia, 2017 to 2019. Eurosurveillance, Volume 27, Issue 11, 17.03.2022 DOI link: https://doi.org/10.2807/1560-7917.ES.2022.27.11.2100415. PMID: 35301978 PMCID: PMC8971918

2. **Savicka, O.,** Dusacka, D., Zeltmatis, R., Nikisins, S., Azina, I., Ivancenko, L., Tolmane, I., Rozentale, B. Hepatitis A virus subgenotypes in Latvia, 2008 to 2021. Journal of Infection and Public Health, Volume 16, Issue 9, September 2023, p. 1462-1470. DOI link https://doi.org/10.1016/j.jiph.2023.07.012. PMID: 37531706

Reports and theses at international congresses and conferences:

1. Savicka, O. Molecular typing results of hepatitis A, Latvia, 2008-2018. In: International scientific conference, Minsk, Belarus, 2018. (Oral presentation).

2. Savicka, O., Zeltmatis, R., Aniscenko, A., Storozenko, J., Rozentale, B., Korotinska, R., Perevoscikovs, J. Hepatitis A virus genotypes detection by sequencing for outbreaks and sporadic cases investigations in Latvia from 2008 till 2018. In: Rīga Stradiņš University Scientific conference 2019, Riga, Latvia, 2019, abstract book: 194. (Poster presentation).

3. Savicka, O., Zeltmatis, R., Aniscenko, A., Storozenko, J., Rozentale B., Korotinska R., Perevoscikovs J. Molecular epidemiology of hepatitis A outbreak in Latvia 2017-2018. In: 29th European Society of Clinical Microbiology and Infectious Diseases congress, Amsterdam, Netherlands, 2019, abstract book: P0783. (Poster presentation).

4. Savicka, O. Molecular characterisation of hepatitis A virus in Latvia from 2017 till 2019. In: 15th Baltic Congress of Laboratory Medicine congress, Riga, Latvia, 2020. (Oral presentation).

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