



THESIS OF DOCTORAL DISSERTATION

Molecular investigation of hepatitis viruses

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1. INTRODUCTION

The one of the most emerging health problems in the world is the inflammation of the liver caused by several viruses. Hepatitis viruses differ in morphology, genome structure and also in the route of transmission. The diseases caused by these viruses are different also in the outcome of the infection. The development of new molecular techniques and methods provide new possibilities for virologists in the discovery of new viral agents and in deeper investigation of known pathogens.

In the past 2-3 decades more new viruses associated with hepatitis have been discovered than in the past 100 years. In spite of this there are many patients suffering from hepatitis with unknown origin. The newly discovered viral pathogens, which are presumably associated with hepatitis of unknown etiology are usually mentioned as „*nonA-nonG hepatitis viruses*” instead of the collective noun of „*nonA-nonB hepatitis viruses*” used until the late 80-90’s.

The opening of the Hungarian borders 20 years ago resulted the easy migration of population. As a result of migration of people from a country or continent to another, the different viruses carried by the hosts have also started to migrate.

2. AIMS

The aims of the study were

- 2.1. to determine the genotypes of hepatitis B and Torque Teno (TT) viruses in Hungary. The genotype of HBV or TTV may have impact on therapy. The practical aspect of HBV genotyping was the molecular investigation of a presumably nosocomial outbreak (which occurred among patients of an onco-hematology unit) to verify or exclude the common source of their infections. Our further aim was to survey risk groups for vaccine-escape hepatitis B virus variants in Hungary, which viruses can infect vaccinated individuals.
- 2.2. to determine the prevalence of GBV-C/HGV and TT viruses in different risk groups (patients of an onco-hematology unit and patients with hepatitis of unknown origin) who underwent invasive procedures during their treatment several times. We compared our results with the prevalence of GBV-C/HGV and TT viruses in the healthy population.
- 2.3. to determine the prevalence of TTV in swine in Hungarian piggeries.

Table 1. Characteristics of the viruses revealed in the thesis.

VIRUS denomination	HBV	HGV or GBV-C	TTV/ SENV
Family	<i>Hepadnaviridae</i>	<i>Flaviviridae</i>	suggested: <i>Anelloviridae</i>
Genus	<i>Orthohepadna virus</i>	not ordered to any genus yet	<i>Anellovirus</i>
VIRION			
size (diameter in nm)	42	?	30-50
genome	circular, 3,2 kilobases, partially double stranded DNA	linear, not segmented, 9,6 kilobases, single stranded (positive sense) RNA	circular, 3,6-3,8 kilobases, single stranded DNA
lipid membrane	yes	yes	no
route of transmission	parenteral	parenteral	parenteral and/or enteral
DISEASE			
fulminant hepatitis	rare	?	?
chronic hepatitis	~5 %	?	?
hepatocellular carcinoma	yes	?	?
IMMUNIZATION			
passive	yes	no	no
active	recombinant HBsAg	no	no

3. MATERIALS AND METHODS

3.1. Materials

The serum samples were aliquoted after receiving and stored at -20°C until testing.

3.1.1. Serum samples for HBV genotyping

Sera of 24 HBsAg positive persons from different regions of the country were used for genotyping. The criteria for selection of samples were the place of residence and HBsAg positivity of the individuals.

3.1.2. Molecular investigation of nosocomial HBV outbreak

Sera of 30 HBsAg/HBV DNA positive persons (29 patients treated at the onco-hematology unit, 1 person from the hospital staff) of a presumably nosocomial outbreak were collected. The 24 samples used for genotyping also served as controls for the molecular epidemiological survey. Collection of samples occurred between 2001-2003.

3.1.3. Samples for detecting vaccine induced „escape” virus variants in Hungary

Two different risk groups were tested:

- 40 pregnant women (presumably carrying HBV of genotype B or C) with different serological results. Samples were collected between 2004 and 2006.

- 28 children, who received active and/or passive immunization at birth but proved to be HBsAg, and/or HBe antibody positive at control date. Samples were collected between 2004 and 2006.

We used sera of 9 symptomless virus carriers from different parts of the country, as controls.

3.1.4. Samples for detecting Torque Teno virus in risk groups and for molecular analysis of TTV

Two risk groups were tested for TTV DNA:

- sera of 228 patients suffering from hepatitis of unknown origin
- sera of 30 HBsAg/HBV DNA positive persons (29 patients treated at the onco-hematology unit, 1 person from the hospital staff) of a presumably nosocomial outbreak.

For genotyping TTV in patients with hepatitis of unknown origin, virus DNA positive samples were used. Samples from hepatitis patients were collected from 1999 to 2001.

3.1.5. Samples for detecting TTV in swine in Hungarian piggeries

Sera of 82 adult swine from 13 piggeries from different parts of Hungary were tested for the presence of TTV DNA. Samples were collected during a period of approximately 12 months.

3.1.6. Samples for detecting GBV-C/HGV virus in risk groups and for GBV-C/HGV molecular analysis

Two risk groups were tested for viral RNA:

- sera of 247 patients suffering from hepatitis of unknown origin
- sera of 30 HBsAg/HBV DNA positive persons (29 patients treated at the onco-hematology unit, 1 person from the hospital staff) of a presumably nosocomial outbreak.

PCR products from 9 of GBV-C/HGV RNA positive samples were sequenced for further analysis.

Parallel to the RT-PCR, samples of 51 patients for anti-E2 IgG antibody (against membrane protein of the virus) also were determined.

3.2. Methods

3.2.1. Serology kits

For detection of virus specific antigens and/or antibodies, serology kits were used as follows: **HBsAg** — Hepanostika HBsAg UniForm II (Biomerieux), **Anti HBe IgM** — HBe IgM ELISA (DiaPro), **Anti HBe Ab** — Hepanostika anti-HBe UniForm (Biomerieux), **HBe Ag/Ab** — HBe Ag/Ab ELISA (DiaPro), **Anti-HAV Ab** — Bioelisa HAV (Biokit), **Anti HDV Ab** — HDV Ab

ELISA (DiaPro), **Anti HCV IgG** — Bioelisa HCV (Biokit), **GBV-C/HGV E2 Ab** — Anti GBV-C Immunoassay (R and D Systems).

3.2.2. Molecular investigations

Nucleic acid extraction/purification: Viral RNA or DNA were extracted from serum samples with phenol-chloroform purification and ethanol precipitation, or silica based column purification. **Reverse transcription:** The viral RNA of GBV-C/HGV was transcribed to cDNA by reverse transcription, using „*random hexamer*” as a primer. The extracted and resuspended nucleic acid was added to reaction mix (2-2 µl/tube). **Polymerase chain reaction (PCR):** for detecting TT virus in swine samples a single PCR, while for detecting HBV, human TTV, and GBV-C/HGV in human samples nested PCRs with virus specific primer sets were used. To check the molecular weight of each amplified DNA fragment an **agarose gel electrophoresis** was performed using a molecular weight control („DNA-ladder”). **Cloning:** Because of the known heterogeneity of TT viruses, the sequence analysis was carried out after cloning of the PCR amplicons into a pCR2.1 plasmid of TOPO TA Cloning (Invitrogen) kit. **DNA – sequencing:** The TTV clones were sequenced using the AutoRead Sequencing Kit (Amersham Pharmacia Biotech) and using „*M13 Reverse primer*”. The electrophoresis was carried out on A.L.F. DNA Sequencer. The PCR amplicons of **GBV-C/HGV** were directly sequenced by the same method. For analysis of **HBV**, the purification of PCR products was carried out using Viogene PCR-M Clean up System (Viogene) kit. For sequencing of samples for HBV genotyping ABI PRISM 3.1 BigDye Terminator — Perkin Elmer kit was used, the electrophoresis was carried out on an ABI PRISM 3100 Genetic Analyzer. For the sequencing of samples collected for investigation of „vaccine-escape” virus variants DYEnamic ET — *Dye Terminator Cycle Sequencing Kit for MegaBACE DNA Analysis System* kit was used, electrophoresis was carried out on MegaBACE 1000 capillary sequencer. **Sequence analysis:** Our results were compared to representative sequences from the GenBank (*EMBL/GenBank – Nucleotide Sequence Database*) using the BLAST [*Basic Local Alignment Search Tool*] software. The **alignment** of the sequences was performed by *Multiple alignment - ClustalW DNA sequences* software at the website of „*Pôle Bioinformatique Lyonnais*”. DNA sequences were translated to amino acid sequences using the *BCM - 6 Frame Translation* software at the website of „*Baylor College of Medicine HGSC*”. The alignment of amino acid sequences were performed at the website of „*Pôle Bioinformatique Lyonnais*” using *Multiple alignment - ClustalW Protein sequences* software. **Phylogenetic analysis:** Representative sequences of the GenBank were used for the construction of the phylogenetic trees. In case of TTV the analysis was performed by v3.573c PHYLIP software, using *dnadist* method, with Kimura two-parameter substitution model. Bootstrap analysis with 1000 replications was performed to confirm the topology of the tree. For

analysis of HBV, the phylogenetic trees were constructed with the Neighbor-Joining method using Kimura two-parameter substitution model; to estimate the reliability of the tree topology, a bootstrap analysis of 1000 replicates was performed using MEGA 3.1 (*Molecular Evolutionary Genetics Analysis*) software.

To confirm the HBV genotype data obtained by sequencing, hybridization with Inno-Lipa HBV (Innogenetics) kit was performed.

4. RESULTS

4.1. Results of molecular investigations of HBV

4.1.1. Genotyping

Sera of 24 HBsAg positive carriers from different parts of the country were collected to identify the genotypes of hepatitis B virus prevalent in Hungary. The viruses of Hungarian carriers could be clustered into two main groups: the **genotypes A** (5/24 — 21 %) and **D** (19/24 — 79 %) [Szomor, 2007]. These results are similar to published international genotype distribution data in Europe.

4.1.2. Results of molecular investigations of a nosocomial HBV outbreak

Twenty-eight of the 30 sequences (29 from patients, 1 from the hospital staff) obtained from the nosocomial outbreak were clustered into **genotype D**. The viruses in this group were genetically very close to each other. This suggests that the source of their infection was common. Two samples (belonging to one of the patients and the member of the staff) proved to be **genotype A**. Based on the phylogenetic distance of their sequences, the common origin of infections could be excluded.

The possible route of nosocomial infection could not be determined. Epidemiological investigations were carried out after the fact of the infections was acknowledged. The possible source of nosocomial infections might have been a HBV positive patient previously or continually treated at the unit. The spread of the infections was likely due to inappropriate infection control measures, and the impact of overcrowding of the hospital unit. As a result of the epidemiological investigations, strict infection control measures were introduced to prevent new infections [Szomor, 2007].

4.1.3. „Vaccine-escape” virus variants in Hungary

Nineteen of 40 samples of pregnant women were HBsAg/HBV DNA positive. The HBV-DNA positive samples were sequenced, the viruses could be clustered into genotypes B and C. Ten of 19

samples had different polymorphisms on the surface antigen coding region of the virus genome, but only one of them occurred on the hydrophilic loop (“loop 2”) of the protein.

A total of 12 samples of 28 actively/passively immunized children proved to be HBV-DNA positive. Five of the samples (42 %) had mutations on the surface protein coding region, but only one of them occurred on the „loop 2”.

The most sites of amino acid substitutions could be referred to known, previously described mutation sites, although a few substitutions leading to amino acid changes in our isolates could be found and determined as a newly described polymorphism [Szomor, 2008].

4.2. Results of study on the Torque Teno virus

4.2.1. Prevalence of TTV in risk groups; TTV molecular analysis

The DNA of TT virus could be detected in the samples of 115 patients (50.4 %) with hepatitis of unknown origin. Seventeen PCR products have been cloned giving 26 clones. All of the sequences were found to be different from each other. Twenty-four of 26 sequences belonged to the genogroup 1, 2 proved to be members of genogroup 2. Only the genotype 1 TTV (member of genogroup 1) is associated with clinical symptoms. Two samples from the hepatitis patients (3 clones) could be clustered into genotype 1. Mixed infections with different genotypes also occurred. In spite of the genetic heterogeneity observed in the nucleotide sequences, the amino acid sequences were identical in several cases [Takács, 2003].

The patients of the onco-hematology unit were also tested for TTV. Nineteen of 29 (65.5 %) were found to be positive.

The prevalence of TTV was higher in both risk groups compared to the healthy Hungarian population (18.5 %). The higher rate of virus carriers can be explained with invasive interventions (such as transfusion, transplantations etc.) during their treatment.

4.2.2. Prevalence of TTV in swine in Hungarian piggeries

Thirteen piggeries from different parts of Hungary were selected for the survey. Virus carrier animals were found in 10 of 13 of the farms (77 %). Twenty five sera of 82 adult swine (30 %) proved to be positive for TTV DNA [Takács, 2008].

4.3. Results of study on GBV-C/HGV

4.3.1. Prevalence of GBV-C/HGV in risk groups; GBV-C/HGV molecular analysis

The RNA of GBV-C/HGV virus could be detected in 36 of 247 patients (14.6 %) with hepatitis of unknown origin. PCR products of 9 virus carrier patients were sequenced, one of them was followed up for years. There were no nucleotide changes at all in this patient’s viral sequences during the examined period [Takács, 2002].

The patients of the onco-hematology unit were also tested for GBV-C/HGV. Nine of 29 (31 %) proved to be positive.

The prevalence of GBV-C/HGV was higher in both risk groups compared to the healthy Hungarian population (8 %). The higher rate of virus carriers can be explained with invasive interventions (such as transfusion, transplantations etc.) during their treatment.

4.3.2. Seroprevalence of GBV-C/HGV

The anti-E2 membrane protein of GBV-C/HGV could be detected in the samples of 20 of selected 51 patients with hepatitis of unknown origin. The ratio of seropositive samples was 39 %. The viral RNA could not be detected in seropositive samples. Sera of 27 patients contained neither RNA nor antibodies against the virus [Takács, 2002].

5. DISCUSSION, CONSEQUENCES OF THE RESULTS

5.1. The majority of Hungarian hepatitis B isolates belonged to either group A or D. These two types seem to be predominant in Hungary. These results are similar to international findings.

5.2. It was proven, that the outbreak was nosocomial among patients treated at the onco-hematology unit. The infections of the member of the staff and one of the patients was not related to this outbreak, the common source of their infection could be excluded. As earlier epidemiological data were not available, it remains a question, which patient's virus was the origin of the epidemic.

5.3. The presence of hepatitis B virus variants were verified in Hungary. Some of these variants, such as „vaccine-escape” mutants can infect the actively or passively immunized persons. In both of the risk groups (individuals carrying presumably the genotype B or C HBV; and the newborns of HBsAg carrier mothers, who are vaccinated at birth) examined, several virus variants could be detected. The first described amino acid substitution associated with „vaccine-escape” variants was the glycine to arginine substitution at 145th amino acid position of the surface protein (G145R). We could not detect G145R, but found other point mutations on the significant „loop2” (the hydrophilic region of the surface protein).

In conclusion: there are challenges in the surveillance of HBV virus variants. First of all, discrepant serological results and infections of previously vaccinated persons have to be verified or investigated by molecular methods. The results should be published in journals for professionals. Based on the results the sensitivity of serology kits should be evaluated and their specificity should be increased. If the ratio of vaccine-escape virus variants reaches

a critical level in the population, epidemiological authorities (such as CDC) should order the modification of the vaccine using also recombinant proteins of emerging new variants. The evaluated vaccine can provide a wider immunity against these variants. According to the presentations at the symposium „*Drug-resistant and Vaccine-escape Hepatitis B Virus Mutants: Emergence and Surveillance*” held by CDC (Atlanta, USA) at 4-5. June, 2009, this is not yet necessary.

- 5.4.** More than the half (115/228) of the samples of patients with hepatitis of unknown origin (50.4 %), and more than the half (19/29) of the samples collected from the patients of the onco-hematology unit (65.5 %) proved to be positive for TT virus. Comparing to the healthy Hungarian population, the prevalence of the virus was higher in both risk groups (18.5 %). The higher rate of virus carriers can be explained by invasive interventions during their treatment.
- 5.5.** The TTV sequences found in the samples of patients suffering from hepatitis predominantly proved to be members of genogroup 1, while some of them belonged to the genogroup 2. Only genotype 1 TTV (member of genogroup 1) is associated with clinical symptoms. Two samples from the hepatitis patients (3 clones) could be clustered into genotype 1. Mixed infections with different genotypes also occurred. The heterogeneity of the TT virus sequences observed rarely lead to changes in amino acid sequences.
- 5.6.** We found TTV carrier animals in the majority (10/13) of Hungarian piggeries examined, although only 30 % of swine proved to be positive. It was the first detection of swine TTV in pigs in Hungary.
- 5.7.** Thirty-six of the 247 samples of patients with hepatitis of unknown origin (14.6 %), and about one third (19/29) of the samples of an onco-hematology patients (31 %) proved to be GBV-C/HGV RNA positive. The prevalence of GBV-C/HGV virus was higher in both risk groups compared to the healthy Hungarian population (18.5 %). The higher rate of virus carriers can be explained by invasive interventions during their treatment.
- 5.8.** Nine of the GBV-C/HGV RNS positive samples were sequenced, one of the patients was followed up for years. The viral sequences obtained during the examined period were identical.
- 5.9.** Parallel to molecular investigations of hepatitis patients, some of the samples (51/247) were examined also for seroprevalence. Twenty of 51 (39 %) were positive for anti-E2 antibodies. The viral RNA and the antibodies were not detectable at the same time, as the appearance of antibodies leads to the elimination of the virus from the patient’s blood.

6. THE THESIS IS BASED ON THE FOLLOWING PUBLICATIONS

Publication in peer-reviewed journals:

1. Takács M., Rusvai E., Brojnás J., Tóth G., **N. Szomor K.**, Tóth E., Szendrői A., Mezey I., Berencsi Gy. (2001): A hepatitis G és TT vírus diagnosztikájának bevezetése Magyarországon. *Lege Artis Medicine* 11, 274-282.
2. Takács M, **Szomor KN**, Szendroi A, Dencs A, Brojnás J, Rusvai E, Berencsi G (2002): Prevalence of GB virus C/hepatitis G virus in Hungary. *FEMS Immunol Med Microbiol* 34:283-7.
3. Takács M, Balog K, Tóth G, Balogh Z, **Szomor KN**, Brojnás J, Rusvai E, Minárovits J, Berencsi G (2003): TT virus in Hungary: sequence heterogeneity and mixed infections. *FEMS Immunol Med Microbiol* 35:153-7.
4. **Szomor KN**, Dencs A, Tóth G, Kovács GM, Saleh Ali Y, Berencsi G, Takács M (2007): Variability of the PreS1/PreS2/S regions of hepatitis B virus in Hungary. *Arch Virol* 152:697-704.
5. **Szomor KN**, Dencs A, Garai E, Rusvai E, Berencsi G, Takács M (2008): Mutation spectra of the surface-protein-coding region of the HBV genome in HBV-vaccinated and non-vaccinated individuals in Hungary. *Arch Virol* 153:1885-92.
6. Takács M, Dencs A, Csiszár C, Hettmann A, Rusvai E, **Szomor KN**, Pálfi V, Nagy B (2008): First description of swine Torque teno virus (TTV) and detection of a new genogroup in Hungary: short communication. *Acta Vet Hung* 56:547-53.
7. Dencs A, Hettmann A, **Szomor KN**, Kis Z, Takács M (2009): Prevalence and genotyping of group 3 torque teno viruses detected in health care workers in Hungary. *Virus genes* 39:39-45.

Other publications:

- **N. Szomor Katalin**, Dencs Ágnes, Kis Zoltán, Takács Mária: *Vírusgenom vizsgálatok a diagnosztikában*. Könyvfejezet az *Orvosi molekuláris virológia* (szerkesztette: Berencsi György, 2005.) c. könyvben.

Participation on Hungarian and international conferences:

1. 14th International Congress of the Hungarian Society for Microbiology, Balatonfüred, Hungary, 9-11 October, 2003.
 - **Katalin N. Szomor**, Ágnes Dencs, Gábor Tóth, Younes Saleh-Ali, György Berencsi, Mária Takács: Variability in Hepatitis B Virus DNA in Different Regions of Hungary. (angol nyelven)
2. 3rd European Meeting on Molecular Diagnostics, Scheveningen, The Netherlands, 16-17. October 2003. (poster)
 - **Katalin N. Szomor**, Ágnes Dencs, Gábor Tóth, Younes Saleh-Ali, György Berencsi, Mária Takács: Variability in Hepatitis B Virus DNA in Different Regions of Hungary. (*Journal of Microbiological Methods* 55:507. 2003)
3. Magyar Molekuláris és Prediktív Epidemiológiai Társaság kongresszusa, Pécs, 2003. november 28-29.
 - **N. Szomor Katalin**, Dencs Ágnes, Tóth Gábor, Berencsi György, Takács Mária: A hepatitis B vírus DNS-ének variabilitása Magyarországon.
4. Third Croatian Congress of Microbiology with International Participation. Poreč, Croatia October 4-7. 2004.

- **Katalin N. Szomor**, Ágnes Dencs, Gábor Tóth, Gábor Kovács, Dániel Papp, Erzsébet Rusvai, György Berencsi, Mária Takács: Sequence analysis of hepatitis B viruses derived from Hungarian virus-carriers.
- 5. Magyar Mikrobiológiai Társaság Nagygyűlése, 2004. október 07-09. Keszthely
 - **N. Szomor Katalin**, Papp Dániel, Dániel, Dencs Ágnes, Rusvai Erzsébet, Brojnás Judit, Takács Mária Aktív és passzív immunizálás ellenére HBsAg hordozóvá vált gyermekek hepatitis B vírusainak nukleotidsorrend-vizsgálata.
 - Rusvai Erzsébet, Szilágyi Emese, **N. Szomor Katalin**, Ferenczi Emőke, Brojnás Judit, Böröcz Karolina, Takács Mária, Mezey Ilona: A vérrel terjedő hepatitis vírusok előfordulási gyakorisága (prevalenciája) a magyar egészségügyi dolgozók között.
- 6. IUMS XIII. International Congress of Virology July 23-28, 2005. San Francisco, CA, USA (poster)
 - M. Takacs, **K.N. Szomor**, A. Dencs, G. Berencsi: Phylogenetic Analysis of Hepatitis B Sequences Derived from Hungarian Virus-carriers.
- 7. Magyar Orvosi Laboratóriumi Szakdolgozók Egyesületének IX. Nagygyűlése, Bük, 2005. szept. 29-30.
 - Kerékgyártóné Zalka Cecília, Nagyné Péntek Ilona, **N. Szomor Katalin**, Takács Mária: Vérrel terjedő hepatitisz vírusok diagnosztikája PCR módszerrel.
- 8. MedVetNet 2nd General Scientific Meeting, Malta, May 3-6, 2006.
 - Mária Takács, Ágnes Dencs, **Katalin N. Szomor**, György Berencsi: Prevalence and genotypes of Anelloviruses in Hungary.
- 9. 1st Central European Forum for Microbiology (CEFOM) and the Annual Meeting of the Hungarian Society for Microbiology, Keszthely, Hungary, October 26-28, 2005.
 - Mária Takács, **Katalin N. Szomor**, Ágnes Dencs, György Berencsi: Phylogenetic analysis of hepatitis B sequences derived from Hungarian virus-carriers. (*Acta Microbiologica et Immunologica Hungarica*, 2005, 52. 159. Supplement)
- 10. Annual Meeting of the Hungarian Society for Microbiology, Keszthely, Hungary, October 18-20, 2006.
 - Ágnes Dencs, **Katalin N. Szomor**, Erzsébet Rusvai, Mária Takács: Analysis of hepatitis C genomes from haemodialysed patients. (*Acta Microbiologica et Immunologica Hungarica*, 2006, 53. 3:261.)
 - Mária Takács, Ágnes Dencs, **Katalin N. Szomor**, György Berencsi: Prevalence and genotypes of anelloviruses in Hungary, (*Acta Microbiologica et Immunologica Hungarica*, 2006, 53. 3:350.)
- 11. XIV. International Congress of Virology, Istanbul, Turkey, August 10-15, 2008
 - **N. Szomor K**, Dencs Á, Garai E, Rusvai E, Berencsi G, Takács M: Mutations on Surface Protein Coding Region of HBV in Hungary.
 - Dencs Á, **N. Szomor K.**, Hettmann A Takács M: prevalence and genotypes of SEN virus in Hungarian healthcare workers.
- 12. Magyar Mikrobiológiai Társaság Nagygyűlése, Keszthely, 2008. október 15-17.
 - Dencs Á., **N. Szomor K.**, Hettmann A., Takács M.: A SEN vírus prevalenciája és genotípusai egészségügyi dolgozóknak Magyarországon. (*Acta Microbiologica et Immunologica Hungarica*, 2009, 56 Suppl.:21)