

CHARACTERIZATION OF OUTBREAK HEPATITIS A ISOLATES IN FIVE TUNISIAN CHILDCARE CENTERS

Hakima Gharbi-Khelifi^{1*}, Nabil Ben Salem Abid^{1*}, Khira Sdiri¹, Rafik Harrath¹, Abir Beji¹, Leila Bhiri¹, Sylviane Billaudel², Virginie Ferre², Mahjoub Aouni¹

¹Laboratoire des Maladies Transmissibles et Substances Biologiquement Actives LR99ES27, Faculté de Pharmacie de Monastir, Avenue Avicenne, 5000 Monastir, Tunisia ; ²Département de Microbiologie, Faculté de Pharmacie, 1 Rue gaston veil, 44035 Nantes, France.

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ABSTRACT

In the present study, epidemiological survey and molecular characterization of hepatitis A virus during an outbreak in five Tunisian childcare centers in El-Mahres during October and November 2006 were carried out. Five well-water and five drinking water samples were included in the present study. Serological investigation and molecular characterization were carried out. All patients were IgM seropositive and the viral genome was detected in all clinical and well-water samples whereas it was not detected in drinking water from the five childcare centers. Sequence analysis showed that all Tunisian strains belong to sub-genotype IA. The genetic profile of the VP1/2A junction showed that the outbreak isolates underwent an amino acid substitution which was absent in virus's strains detected previously in Tunisia. Further studies need to be conducted to evaluate the emergence of the virus's strains in clinical and water samples and more epidemiological data need to be collected about the risk factors which may contribute to acute hepatitis.

Key words: Hepatitis A Virus, VP1 gene, VP1/2A junction.

INTRODUCTION

Hepatitis A virus (HAV) constitutes the major cause of acute hepatitis throughout the world and causes substantial morbidity in both developed and developing countries (10). Although the role of the HAV in the acute hepatitis outbreaks in industrialized countries was established (3-4, 18-19, 23), little is known about its contribution to the occurring outbreaks in the developing countries.

Our studies conducted previously in Tunisia during the period from 2000 to 2004 (8) showed the prevalence of HAV in waste water and shellfish samples with clear predominance of sub-genotype IA in blood samples from seropositive patients (7). As far as we know, no clear evidence of well-water-associated HAV outbreaks has been reported in Tunisia. The severity of HAV infection is multi-factorial with age, gender, and drug toxicity. Many socio-economical factors play an important role in reducing hepatitis incidence in Tunisia (11,

*Corresponding Author. Mailing address: Laboratoire des Maladies Transmissibles et Substances Biologiquement Actives R99ES27, Faculté de Pharmacie de Monastir, Avenue Avicenne, 5000 Monastir, Tunisia. ; Tel: + 216 73 466 950 / +216 95 939 776 / + 216 95 979 283 Fax: + 216 73 465 754 / + 216 73 461 830.; E-mail: khelifi_hakima@yahoo.fr / nabilabidbensalem2008@gmail.com

These authors contributed equally to this work.

16) and some Tunisian urban regions are now considered as areas of moderate endemicity pattern of HAV seroprevalence (11). Nevertheless, in some regions with low socio-economic conditions, some people still use well-water as a drinking water supply and the consumption of such water may contribute to several infections.

HAV, member of the genus Hepatovirus of the *Picornaviridae* family, is transmitted primarily through the fecal-oral route (14). The nucleotide analysis of the VP1/2A junction classified HAV in six different genotypes (I-VI) and six sub-genotypes (IA, IB, IIA, IIB, IIIA, and IIIB) (13, 17). Sequence diversity among genotypes was estimated to 15-25% (17).

Costa-Mattioli *et al.* (5) investigated the genetic variability of HAV strains using the complete sequences of VP1 gene. The obtained results revealed the existence of five groups or genotypes (I, III, IV, V, and VII). Based upon the VP1/2A junction and the complete genome sequences, Lu *et al.* (13) proposed to re-classify genotype VII (SLF88) and genotype II (CF53/Berne) to sub-genotypes IIB and IIA, respectively. Molecular characterization and nucleotide sequence comparison during HAV outbreaks may provide a deep insight into the molecular epidemiology of the prevalent HAV strains.

In the present study, an HAV outbreak in five Tunisian childcare centers in a sub-urban area of El-Mahres (governorate of Sfax, south of Tunisia) between October and December 2006 was reported. Molecular studies were conducted to characterize the detected HAV isolates.

MATERIALS AND METHODS

Survey design and case definition

El-Mahres is a suburban region in the south of Tunisia with moderate to low socio-economic conditions. An HAV outbreak in five childcare centers was registered in this region during the period from October to December 2006. An interview was performed to examine the available medical and

epidemiological information. A questionnaire was carried out according to the Tunisian Ministry of Health Regulations. Stool and serum samples from children (n=50) under 6 years old with acute hepatitis were collected. Five well-water samples from different wells in the region and five drinking water samples from the five childcare centers were included for analysis. Actual case (AC) was identified by the presence of clinical signs and anti-HAV IgM seropositive. A total of 50 stool, 50 serum, 5 well-water, and 5 drinking-water samples were collected. Informed consent was obtained from all patients.

Biochemical and serological analysis

The alanine aminotransferase (ALT) blood test is typically used to detect liver injury. It is often ordered in conjunction with aspartate aminotransferase (AST) or as part of a liver panel to help diagnose liver disease. The normal level of ALT is assumed to be 4-36 U/L. Very high ALT levels (more than 10 times the highest normal level) are usually due to acute hepatitis, often due to a virus infection.

In acute hepatitis, ALT levels usually keep high for about 1-2 months but can take as long as 3-6 months to return to its normal level. The serum samples were screened for the presence of ALT by biochemical tests according to the manufacturer's instructions. Anti-HAV IgM enzyme immunoassay was carried out using a diagnosis kit for HAV IgM antibody according to the manufacturer's instructions (Abbott Laboratories, Rungies, France).

Virus concentration and RNA extraction

HAV was concentrated from the water samples using the method described by the US Environmental Protection Agency (6). Samples were diluted with 1% (v/v) of 0.05 M aluminium chloride and the pH was then adjusted to 3.5. The mixture was homogenized and centrifuged at 2 500 x g for 15 min. The pellet was suspended in 100 mL of 10% beef extract (w/v) (Oxoid, Basingstoke, UK) and adjusted to pH 7.0. The mixture

was again homogenized and centrifuged at 10 000 x g for 30 min. The extraction of viral RNA from stool and water samples was carried out using QIAamp viral RNA kit according to the manufacturer's instructions (Qiagen, Paris, France). Viral RNA was stored at -80°C until use or used immediately.

RT-PCR and gel electrophoresis

Viral RNA was amplified using specific primers for entire VP1 and VP1/2A junction (1070 bp) as described previously (5). Reverse-transcription polymerase chain reaction (RT-PCR) was performed using Qiagen OneStep RT-PCR kit according to the manufacturer's instructions (Qiagen, Paris, France).

RT-PCR products were resolved by gel electrophoresis on 2% agarose gel in TBE (Tris–boric acid–EDTA) buffer and stained by ethidium bromide (Sigma, NY, USA). The product of the RT-PCR was visualized using UV transilluminator and gel images were recorded digitally with the Gel Doc 2000 system (Bio-Rad Laboratories, NY, USA).

Cloning and sequencing of the amplified fragment

RT-PCR products from stool and water samples collected in the present work were purified by phenol–chloroform extraction (22). DNA fragments were ligated directly into the pMOSBlue-T vector (Amersham, Les Ullis, France) using a blunt-ended cloning kit (Amersham). These plasmids were then used to transform *Escherichia coli* pMOSBlue-competent cells, prepared as described by Tounsi *et al.* (22) using a method based on the conventional calcium chloride method. Plasmid DNA was extracted using the rapid alkaline extraction procedure described by Birnboim and Doly (1). DNA sequencing was carried out using ABI Prism BigDye Terminator cycle sequencing ready reaction kit and 373A DNA sequencing system (Applied Biosystems, Foster City, CA).

Phylogenetic analysis

Nucleotide sequences of HAV strains and isolates were included for phylogenetic analysis (Table 1). Sequence alignment and analysis were performed using FASTA software (version 3), ClustalW software (21), and MEGA 4.0 program (20).

Table 1. Nucleotide sequences of HAV reference strains and Tunisian isolates included for phylogenetic analysis

Strains	Accession numbers	Year	Sequence	Sub-genotype	References
HM-175	M14707		VP1 and VP1/2A	IB	
GBM	X75215		VP1 and VP1/2A	IA	
Tun105-01	AY875657	2001	VP1 and VP1/2A	IA	Hakima Gharbi-Khelifi <i>et al.</i> (2006)
TunS3-01	AY875663		VP1 and VP1/2A	IA	
TunS4-01	AY875664		VP1 and VP1/2A	IA	
Tun102-01	AY875672		VP1 and VP1/2A	IA	
Tun113-02	AY875652	2002	VP1 and VP1/2A	IA	Hakima Gharbi-Khelifi <i>et al.</i> (2006)
Tun159-02	AY875656		VP1 and VP1/2A	IB, IA	
Tun116-02	AY875661		VP1 and VP1/2A	IA	
Tun40-03 ^s	AY875649	2003	VP1 and VP1/2A	IA	Hakima Gharbi-Khelifi <i>et al.</i> (2006)
Tun148-03	AY875650		VP1 and VP1/2A	IA	
Tun165-03	AY875651		VP1 and VP1/2A	IA	
Tun153-03	AY875653		VP1 and VP1/2A	IA	
Tun29-03 ^s	AY875654		VP1 and VP1/2A	IA	
Tun97-03	AY875655		VP1 and VP1/2A	IA	
Tun92-03	AY875658		VP1 and VP1/2A	IB	
Tun143-03	AY875659		VP1 and VP1/2A	IA	
Tun152-03	AY875660		VP1 and VP1/2A	IA	
Tun147-03	AY875662		VP1 and VP1/2A	IA	
Tun25-03 ^s	AY875665		VP1 and VP1/2A	IA	
Tun18-03 ^s	AY875666		VP1 and VP1/2A	IA	
Tun27-03 ^s	AY875667		VP1 and VP1/2A	IA	

Tun77-03	DQ380523		VP1 and VP1/2A	IA	Hakima Gharbi-Khelifi et al. (2006)
Tun49-03	DQ380510		VP1 and VP1/2A	IA	Hakima Gharbi-Khelifi et al. (2007b)
Tun1-03	DQ380511		VP1 and VP1/2A	IA	
Tun17-03	DQ380512		VP1 and VP1/2A	IA	
Tun19-03	DQ380513		VP1 and VP1/2A	IA	
Tun42-03	DQ380514		VP1 and VP1/2A	IA	
Tun62-03	DQ380515		VP1 and VP1/2A	IA	
Tun75-03 ^s	DQ380516		VP1 and VP1/2A	IA	
Tun66-03	DQ380517		VP1 and VP1/2A	IA	
Tun28-03	DQ380518		VP1 and VP1/2A	IA	
Tun36-03	DQ380519		VP1 and VP1/2A	IA	
Tun70-03	DQ380520		VP1 and VP1/2A	IA	
Tun80-03	DQ380521		VP1 and VP1/2A	IA	
Tun79-03	DQ380522		VP1 and VP1/2A	IA	
Tun216-04 ^s	AY875668	2004	VP1 and VP1/2A	IA	Hakima Gharbi-Khelifi et al. (2006)
Tun225-04 ^s	AY875669		VP1 and VP1/2A	IA	
Tun228-04 ^s	AY875670		VP1 and VP1/2A	IA	
Tun295-04	AY875671		VP1 and VP1/2A	IA	

^s HAV strains detected in the same region (Sfax) as the outbreak isolates.

Plot similarity was carried out using Simplot, version 3.5.1 (12).

The evolutionary history of the sequences was inferred using the UPGMA method. The tree was drawn to scale, with the branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. All the positions containing gaps and missing data were eliminated from the dataset (complete deletion option).

RESULTS

Serological and biochemical analysis

A total of 100 stool and serum samples were collected. The patients showed acute hepatitis including jaundice, nausea, fever, vomiting, and abdominal pain. The results of ALT test in children's blood with acute hepatitis showed high levels of ALT. Anti-HAV IgM was detected in all children.

DNA sequencing and phylogenetic analysis

The HAV genome fragment with a size of 1070

nucleotides of the entire VP1 and VP1/2A junction region was amplified by RT-PCR (5). Although HAV RNA was detected in all the stool and well-water samples, it was not detected in the five drinking water samples.

Fifteen HAV isolates were selected and their nucleotide sequences were deposited in GenBank under accession numbers HM011106, HM011107, HM011108, HM011109, HM011110, HM011111, HM011112, HM011113, HM011114, HM011115, HM011116, HM011117, HM011118, HM011119, and HM011120. Sequence analysis showed high similarity (99%) among HAV strains isolated in stool and well-water samples.

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The phylogenetic analysis classified the detected isolates into sub-genotype IA (Figure 1, C). The detected HAV isolate

in well-water (Tun15-2006W) was clustered with the clinical strains (Figure 1, A).

For plot similarity analysis, HAV isolate Tun1-2006 was used as a sequence query. Plot of similarity showed a high similarity among all Tunisian HAV isolates and the reference strain GBM (sub-genotype IA, accession number X75215)

(Figure 2, A). The exception was for HAV isolates Tun159-02 (sub-genotype IB, IA, accession number AY875656), and Tun92-03 (sub-genotype IB, accession number AY875658). These strains are classified with the reference strain HM-175 (sub-genotype IB, accession number M14707) (Figure 2).

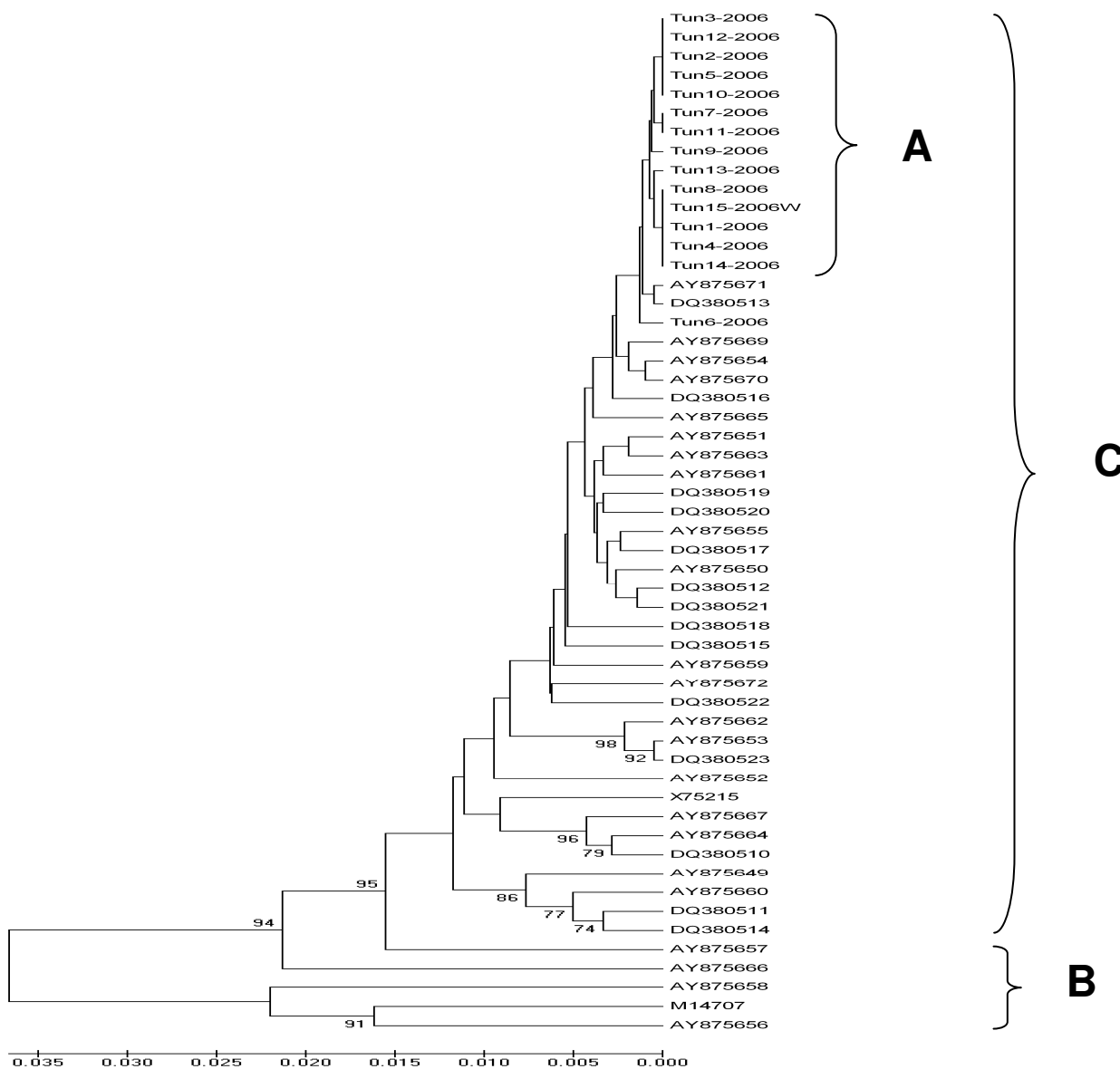


Figure 1. Phylogenetic analysis. Phylogenetic tree of HAV strains and Tunisian HAV strains isolated from 2001 to 2006. The entire VP1 and VP1/2A nucleotide sequences were analyzed using UPGMA bootstrap test of phylogeny. The number at nodes indicates bootstrap percentages after 1,000 replicate sampling. The bar indicates genetic distance and values under 70% were hidden. Cut-off value for condensed tree is 50%. A: The Tunisian HAV strains detected in the present work; B: HAV strains genotype IB; and C: HAV strains genotype IA.

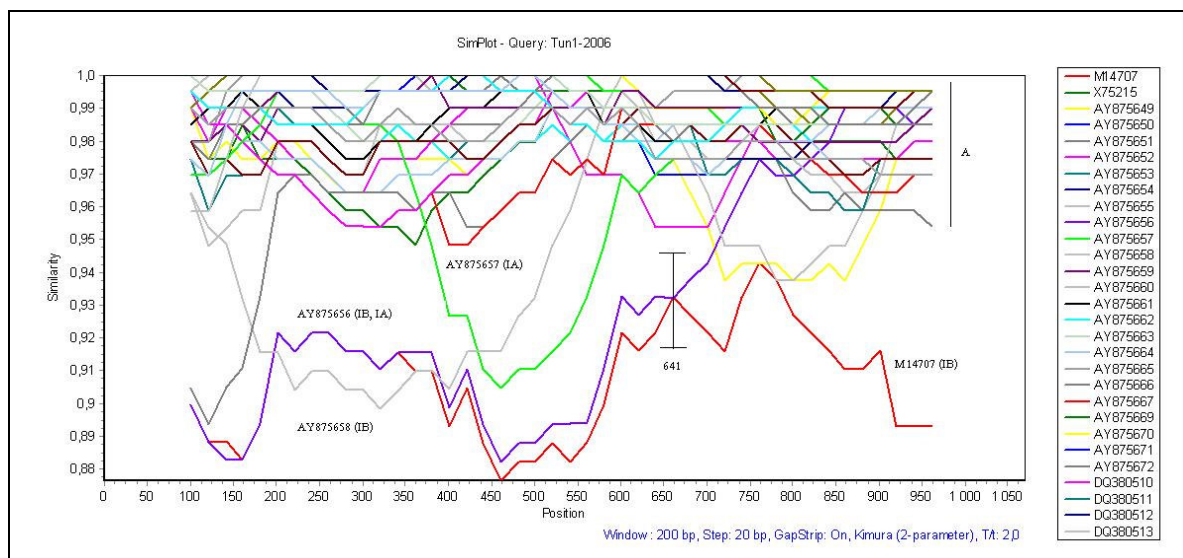


Figure 2. Plot Analysis.

Plot of nucleotide similarity (generated by SimPlot) of a set of HAV reference sequences and Tunisian strains isolated previously to the Tun1-2006. Each point plotted is the percent identity within a sliding window of 200 bp, with a step size between points of 20 bp. Positions containing gaps were excluded from the comparison. A: outbreak isolates and previously detected Tunisian HAV isolates grouped in a cluster. The accession numbers of HAV type strain HM-175 (genotype IB), Tunisian HAV isolates Tun159-02 (accession number AY875656, genotype IB/IA), Tunisian HAV isolate Tun92-03 (accession number AY875658, genotype IB), and Tunisian HAV isolate Tun105-01 (accession number AY875657, genotype IA) are shown. The cross position between Tun159-02 and reference type HM-175 is indicated by a vertical line in position 641.

Simplot analysis showed further characteristics of HAV isolate Tun159-02 (Blue line) detected and characterized previously as genotype IA and IB (7). A cross point at position 641 showed a recombination event between co-circulating HAV strains of different genotypes. Furthermore, HAV strains Tun92-03 (genotype IB, gray line) and Tun105-01 (genotype IA, green line) showed cross events between genotypes in positions 470-700 and 400-600, respectively.

One nucleotide substitution in the VP1/2A junction of the HAV outbreak isolates has modified the amino acid sequence at the position 291 (Arg > Lys) (Figure 3). At this amino acid position, the HAV isolates detected in the present work seem to be more related to genotype IB. This substitution was absent in the HAV isolates detected previously in Tunisia. These findings arise an important question about the source of such

circulating strains.

Epidemiologic studies conducted later by investigators in the region of Sfax did not support the emergence of new HAV variant with the same profile as for the strains detected in the present study (unpublished data). Further studies need to be conducted to further characterize the emergent HAV strains in the country from clinic and environment samples.

The analysis of the deduced amino acid sequences showed that HAV Tun11-2006 and Tun8-2006 strains are distinguished from the other outbreak isolates by two amino acids in positions 270 and 281, respectively. HAV strains Tun11-2006 and Tun8-2006 may be more related to Tunisian HAV strains Tun62-03 and Tun143-03 under accession numbers DQ380515 and AY875659, respectively (Figure 3).

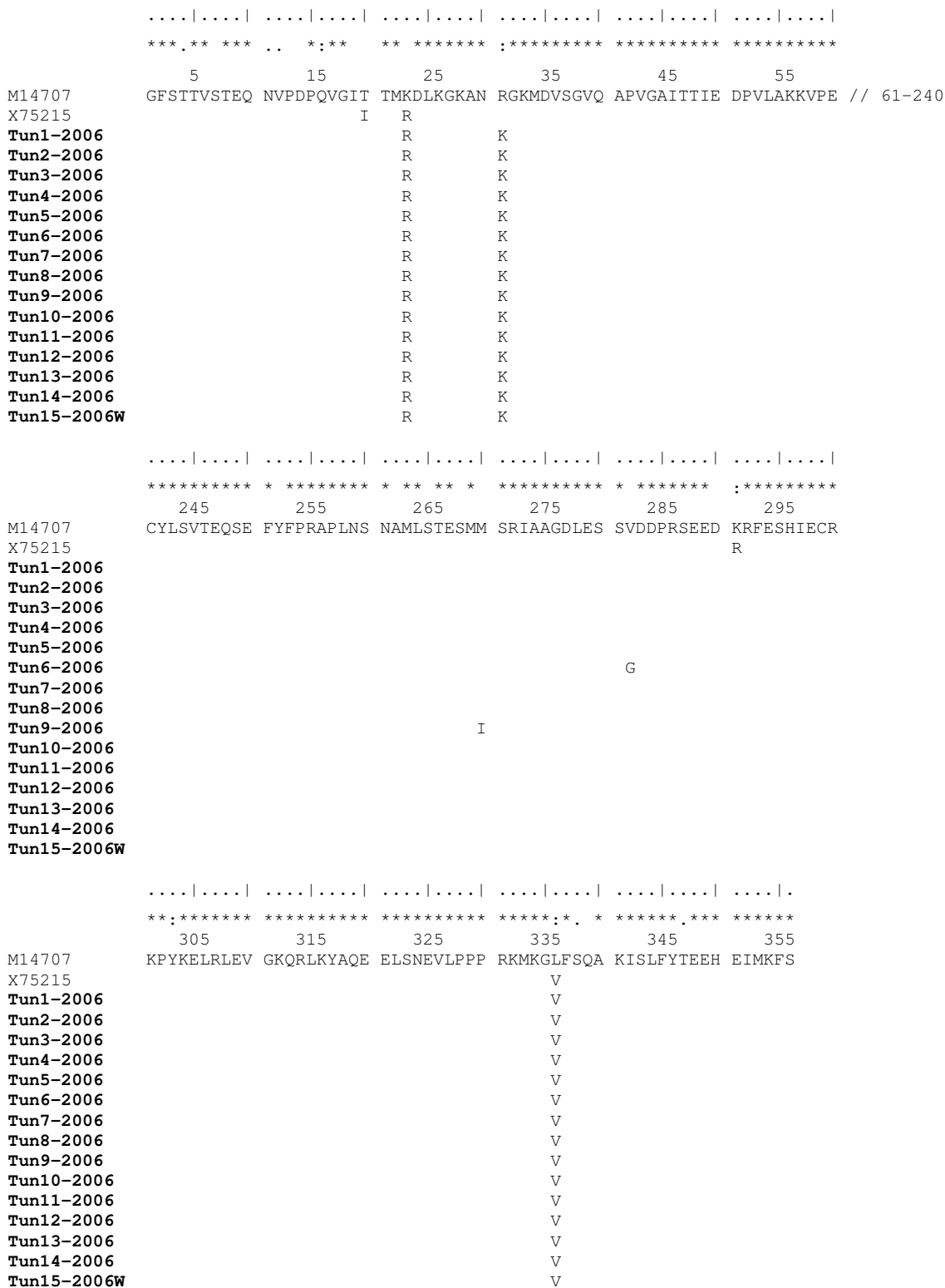


Figure 3. Alignment of the deduced amino acid sequences of the entire VP1 and VP1/2A junction of HAV strains and Tunisian isolates. Consensus sequence was generated for HAV strain and isolates. Amino acid sequences of the Tunisian HAV strains detected in the present study are shown in bold. Amino acid sequences from 61 to 240 showed high degree of similarity and were excluded from the figure.

DISCUSSION

Although many outbreaks in childcare centers associated with foods, water, and person-to-person were described previously in USA, China, and Thailand (2, 15, 17, 23), HAV transmission by water in Tunisia is probably underestimated since outbreaks appear to be infrequently reported, poorly documented, and the virus has rarely been isolated from this source.

The present study constituted an epidemiologic survey and molecular studies of an HAV outbreak among children under six years old. Data obtained in the present study showed the consumption of contaminated water by patients during the period of September 2006 which was marked by a high level of precipitation in the region.

Molecular epidemiologic studies conducted in the region after the outbreak period (in 2007) did not reveal any significant epidemiologic peculiarities and the nucleotide sequences of the positive samples were more related to Tunisian strains detected previously (2001-2004) (unpublished data).

The present work showed that although its incidence has declined over the past decades, HAV outbreaks continue to occur in Tunisia. The HAV isolates identified during this outbreak were clustered within sub-genotype IA as the most HAV isolates detected previously in Tunisia (7-9).

Our finding emphasizes the importance of the epidemiological studies and the molecular characterization of the emergent HAV strains.

Sequence analysis of the detected HAV isolates supported the evolutionary approach of HAV in Tunisia (9). Nevertheless, further studies need to be conducted to evaluate the prevalence of new HAV variants in the Tunisian population and in the environment.

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