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ORIGINAL ARTICLE

Molecular epidemiology and case-control approaches for management of an outbreak of hepatitis A in Liguria, Italy

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Key words

Hepatitis A • Molecular epidemiology • Outbreak

Summary

Introduction and methods. *Hepatitis A remains an important public health problem in low endemicity areas, because of the social and economic high burden of cyclical outbreaks. In this study we described an outbreak of HAV infection occurred in the city of Genoa and in its proximity and the viral circulation in the post-epidemic period. In order to identify risk factors associated to the illness and to determine the source of infection and the dynamics of virus evolution, we conducted an epidemiological and molecular investigation by a case-control study and by sequence analysis of high variable regions of the genome.*

Results. *From May to October 2005, 58 HAV hepatitis cases were notified. The case-control study showed that beach establishment attending is strongly associated with HAV hepatitis (OR = 24.5, p-value < 0.01), at multivariate analysis. The profile of epidemic curve, the clinical onset of primary cases who occurred in few weeks and the geographic distribution of cases clearly indicated a common exposure to a point source: the outbreak can be probably associated with a contaminated food product dispensed in the affected area.*

The outbreak has been mainly caused by a single variant, confirming the common exposure to a point source; this variant previously circulated within homosexual man (MSM) network in Northern Europe. During the outbreak and in the following months, different variants originating from Southeast Asia, Southern America and Northern Africa, have co-circulated: all these cases were related to international travel and none of these had determined secondary cases.

Discussion. *The epidemiological picture of hepatitis A in Liguria is characterized by a wide heterogeneity of circulating HAV strains. This pattern could be associated with the increase of imported cases and transmission within network of persons with similar risk factors. Molecular approach coupled to descriptive and analytical epidemiological studies appeared un-replaceable tools for management and control of HAV outbreaks, because of their capacity to recognize infection origin, transmission patterns and dynamics of virus evolution.*

Introduction

Although the incidence of hepatitis A virus (HAV) infection in developed countries has declined over the past decade, as a consequence of both improvements in social conditions, sanitation and hygiene, and immunization of risk groups [1, 2], it remains an important public health problem, because of the social and economic high burden of cyclical outbreaks [3, 4].

Italy is considered to be an area with low/intermediate endemicity for hepatitis A. Data from the Integrated Epidemiological System of Acute Viral Hepatitis (SEIEVA) showed that the incidence has declined from 10/100,000 in 1985 to 3.5/1,000,000 in 2004, with isolate increases corresponding to outbreaks occurred in some regions in the Southern Italy, such as Puglia and Campania during 1996/1997 season [5]. Moreover, serological analysis conducted on army recruits during the last two decades recorded an anti-HAV prevalence of 66% in 1981, 30% in 1990 and 5% in 2003 (from 2% in the north to 8% in the south) [6]. These data suggest an important change in the epidemiological pattern of hepatitis A: improved health and sanitary conditions, together with better controls on food production and handling and on treatment of waste waters, caused a shift in the average age of first contact with HAV virus towards the highest incidence in young adult [7]. This age shift has heavy consequences on the clinical presentation of the disease: while in children < 6 years of age most infections (70%) are asymptomatic or usually anicteric, among older age groups infection is usually symptomatic, with jaundice at the onset in > 70% of patients, and with an increased risk of acute liver failure [1].

The decline in the rate of hepatitis A infection among children generated an increasing proportion of susceptible adolescents and young adults and explained the cyclicity of HAV infection outbreaks: they occur when a critical number of susceptible individuals is reached, while in the years between outbreaks circulation of the virus is limited to infants and young children, who remain asymptomatic [2]. After the ceasing of the transmission, reintroduction of the virus, especially in

high-risk groups such as intravenous drug users (IDU), homosexual men (MSM), day-care centres and primary school personnel and travellers to areas of high endemicity, may lead to large outbreaks that could involve large cohorts of non-immunized subjects [2, 8, 9].
Liguria is situated in the North-West of Italy and is a region with low HAV endemicity, with an average incidence of 2.1 notified cases/month during the period 2000-04 [10].
Since May 2005, a virological surveillance system was established in order to define the molecular characterization of HAV virus isolated in Liguria, with the aims of (i) describing the epidemiological pattern of the virus, (ii) identifying the major determinants of the disease, such as potential risk factors and sources of infection, (iii) detecting HAV evolution over time in the community and, more importantly, (iv) contributing to management and control of outbreaks.
In this study we described an outbreak of HAV infection occurred in the city of Genoa and in its proximity from May to October 2005 and the viral circulation in the post-epidemic period. In order to identify spatial, temporal, environmental and behavioural factors associated to the illness and to determine the source of infection and the dynamics of virus evolution, we conducted an epidemiological and molecular investigation by a case-control study and by sequence analysis of high variable regions of the genome of HAV.

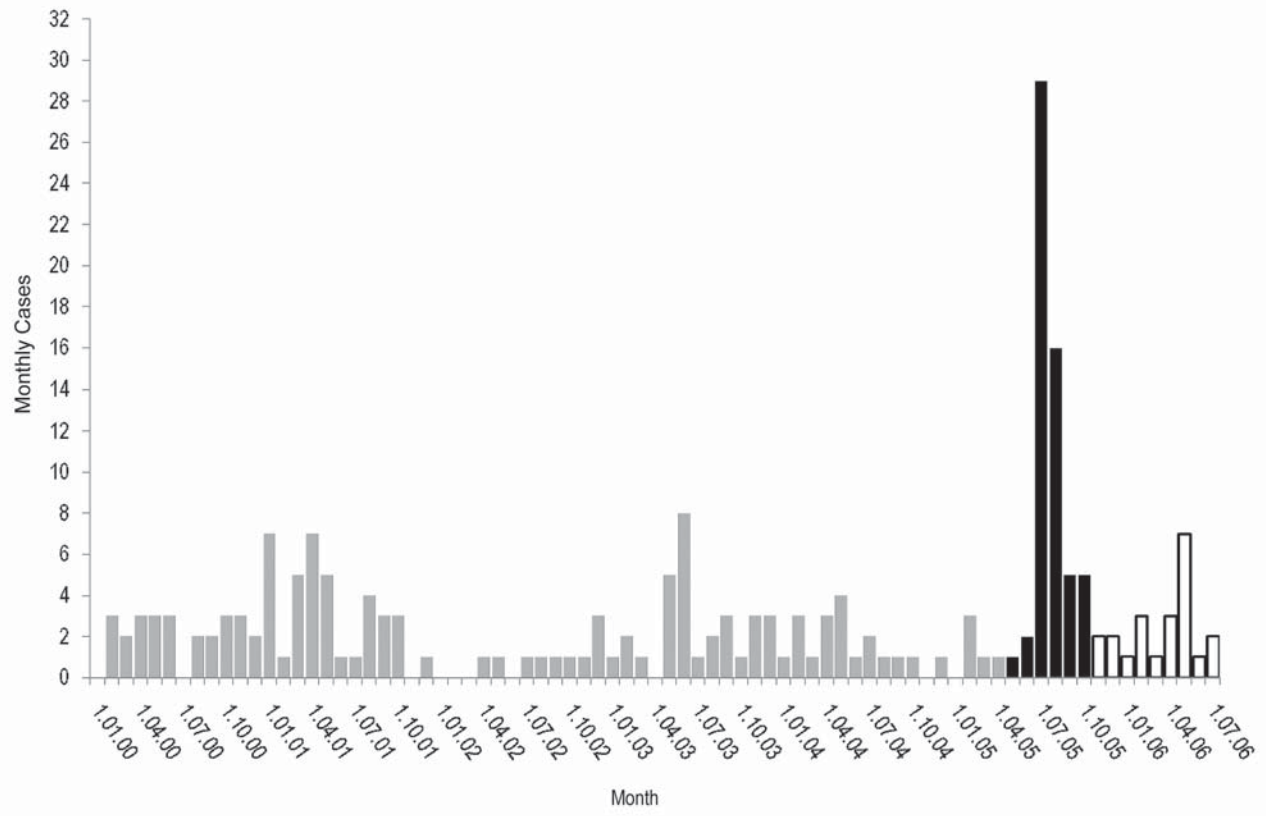
Materials and methods

Incidence data sources. Incidence data observed during the period January 2000-July 2006 were collected by regional surveillance system and reported in SIMI web site [10].
Case-control study. In order to identify spatial, temporal, environmental and behavioural factors associated to the illness and to determine the source of infection a case-control study was performed. A case patient was defined as a subject with a laboratory confirmed hepatitis A infection (IgM positivity) and with clinical onset of liver damage between 15 June and 30 July 2005: this time interval has been selected in order to analyze the only primary cases, presumptively infected by the potential source of infection looked for in the study. Cases were matched 1:2 with control subjects, who were recruited among patients to the Emergency Department of Azienda Ospedaliera Universitaria San Martino, Genoa, from 1 to 10 August, and did not present symptoms related to hepatitis A. They were matched according to cases age (± 5 years) and gender. To both patients and control subjects has been administered a standardized questionnaire approved by Department of Health Sciences, University of Genoa, and Nutrition and Public Health Units, Department of Prevention, Local Health Agency (LHA) 3 "Genovese", Genoa. Information collected from cases and controls included individual demographic characteristics such as age, sex, occupation and exposures to potential risk factors (history of travel to high-medium endemic areas, contact with a jaundiced person, sexual behaviour, history of drug use, type of consumed food, purchase and consumption place) for the period ranging 15-50 days before the date of symptom onset as regard as cases, for the period 15 June-15 July 2005 as regard as controls. Written informed consent was obtained from all participants.
Statistical analysis. Contingency table tests (Chi-square or Fisher exact tests) were used for the assessment of the association between HAV infection and risk factors. Multivariate logistic regression was performed to evaluate the relationship between HAV infection and the variables statistically significant in univariate analysis and associations were expressed using Odds ratio (OR) and 95% confidence intervals (95% CI).
Sample collection and molecular procedures. Fifty-five serum and/or stool samples were obtained from patients with specific clinical symptoms in association with the presence of IgM antibody to HAV, notified to Genoa LHA 3 and Chiavari LHA 4 during the period May 2005-July 2006.
Viral nucleic acid was extracted from 250 μl of serum or from 200 μl of a 10% fecal suspension in NaCl 0.9% solution using QIAamp MinElute Virus Spin kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. RNA elution was performed using 40 μl of Buffer AVE.
HAV RNA amplification was performed by reverse transcription-nested-PCR of VP1-P2A and VP3-VP1 junctions as previously described [11].
PCR products were purified using the QIAquick purification kit (Qiagen, Valencia, CA, USA). Each purified PCR product underwent cycle sequencing (forward and reverse) using the Big-dye Terminator v3.1 Kit (Applied Biosystems, Foster City, CA, USA) and analyzed on ABI Prism 3100-Avant (Applied Biosystems).
Virus characterization was performed by sequence analysis after alignment of the isolate sequences with reference strains. Construction of phylogenetic trees was carried out by the Neighbour-Joining method using MEGA package, version 3.1, of the Pennsylvania State University (PA, USA).

Results

Descriptive epidemiology. Notified cases of hepatitis A during the period January 2000-July 2006 in Liguria are reported in Figure 1. During the pre-outbreak period (January 2000-April 2005) the average monthly cases were 2 ± 1.8, ranging between 0 and 8 cases. From May to October 2005, 58 HAV hepatitis cases were notified, with a monthly average of 9.8 ± 10.9 cases, reaching the higher incidence on July with 29 registered cases. The epidemic curve of hepatitis A cases showed a characteristic bell-shaped profile (Kolmogorov-Smirnov test p-value = 0.52), indicating a common or a point source of infection, with the epidemic peak in correspondence to the 27th-28th week. The median age of the patients was 40.3 ± 14.6 years (range 21-85 years), and more

Fig. 1. Notified cases of hepatitis A during the period January 2000-July 2006 in Liguria. Grey, white and black histograms represent pre-, post- and outbreak cases, respectively.



than 75% of the cases occurred in subjects 25 to 64 years old. After October 2005, the incidence decreased, showing incidence data similar to that observed in the pre-outbreak period (monthly average 2.5 ± 1.9 cases, range 1-7).

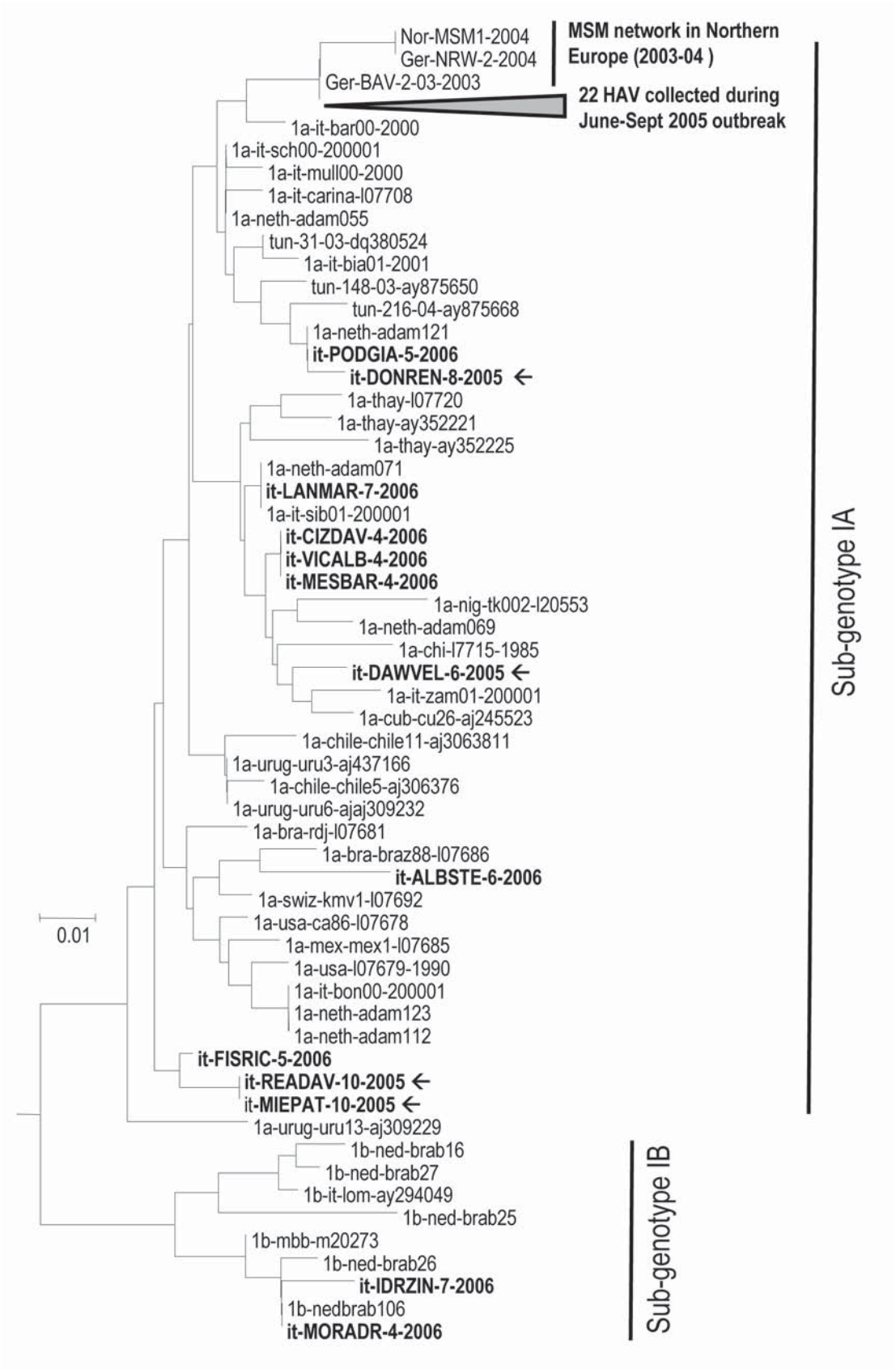
Geographic distribution of cases observed between May and October 2005 was characterized by higher incidence in Genoa (77.8% of total cases) and in its proximity with a cluster in east districts of the city (63.9% of total cases).

Case-control study. Participants to the case-control study included 29 primary cases of HAV infection and 48 controls. Geographic distribution of case and control residences was not significant different (Pearson Chi-square p-value = 0.82). Case and control groups were perfectly overlapped according to age and sex (p-value ≥ 0.9 for both the variables). Univariate analysis revealed that the only significant positive associations with HAV infection was found with the consumption of snacks or sandwiches not prepared at own residence (Pearson Chi-square p-value < 0.01) and with beach establishment attending (Pearson Chi-square p-value < 0.01). None of the associations with the other numerous exposures to potential risk factors showed statistical significance, including the consumption of raw seafood. Consumption of snacks or sandwiches not prepared at own residence and beach establishment attending were included in the multivariate logistic model, together with dumping variables, such as the habits to have lunch outside home: to frequent beach establishment is strongly associated with HAV hepatitis (OR = 24.5, 95% CI 5-120.6, p-value < 0.01), while the association with consumption of snacks or sandwiches not prepared at own residence was not significant (OR = 2.7 95% CI 0.5-13.9, p-value = 0.25). The case-control study lacked to identify a single business trade liable to HAV infection: the beach establishment and the delicatessen attended by cases during the incubation period are predominantly distributed in a limited area of the seafront at the east of Genoa.

Molecular characterization. Fifty-five (68.8%) clinical samples were collected from cases occurred during the period May 2005-July 2006; HAV RNA was detected in 63.6% (35 out of 55) of clinical samples collected during the outbreak (n. = 26) and post-outbreak (n. = 9) periods. Figure 2 shows the genetic relatedness among outbreak and "post-epidemic" strains to other HAV reference isolates in the VP1-P2A regions. The phylogenetic pattern of VP3-VP1 junction region was identical to that of VP1-P2A and was not shown. All strains belonged to genotype I: most of the collected strains (33/35, 94.3%) belonged to sub-genotype IA, while two isolates (5.7%) were assigned to sub-genotype IB.

Twenty-two (84.6%) out of 26 outbreak strains showed identical VP1-P2A sequences and high homology with

Fig. 2. Phylogenetic analysis of HAV VP1-P2A sequences. A Neighbor-Joining tree was built using reference strains circulating in different areas of the world and sequences of HAV detected in Liguria (in bold) during outbreak (←) and in the following months.



viruses detected among MSM network in Northern Europe in 2003 and 2004 (Nor-MSM1-2004 and Ger-BAV-2003 in Figure 2). These sequences seem phylogenically close to it-bar00-2000 (nucleotide distance 2% ± 1.2%), a IA strain previously circulating in Southern Italy and in the Mediterranean areas.
The other 4 HAV detected during the outbreak showed sequences belonging to 3 different clusters including viruses circulated in Northern Africa (it-DONREN-8-2005), in Southeast Asia (it-DAWVEL-6-2005) and South America (it-READAV-10-2005; it-MIEPAT-10-2005) (marked with arrows in Figure 2). The epidemiological investigation revealed that the 3 last viruses were detected in immigrants and travellers from the above mentioned areas and were considered imported cases.
Six HAV isolated during the spring 2006 presented a high degree of heterogeneity: in April 2006, 3 patients were infected by the same variant belonging to sub-genotype IA (it-MESBAR-4-2006; it-CIZDAV-4-2006; it-VICALB-4-2006), and 1 patient became infected with a virus (it-MORADR-4-2006) belonging to sub-genotype IB and responsible of an outbreak in Netherland in travellers from Morocco [12]; it is noteworthy that it-MORADR-4-2006 was isolated in a patient who initially declared no journey in Northern Africa. After that molecular analysis suggested the Northern Africa origin of the virus, a deeper epidemiological investigation allowed to discover a brief stay in Morocco forgotten by the patient.
In May 2005, 2 phylogenically distant IA-strains were detected in 2 travellers from North Africa and South America. Genetic analysis showed the relatedness with viruses circulating in the above mentioned areas, confirming that they were imported cases with no relationship with the other observed cases.
In the following months, 2 patients were infected by 2 phylogenically distant IA-strains and 1 by IB-virus: it-ALBSTE-6-2006, a variant previously characterized in South America, was detected in a traveller from Brazil and it-IDRZIN-7-2006, a Northern African HAV, was revealed in a Moroccan child, belonging to an immigrant community in Genoa, with unknown risk factors. No risk behaviours were known as far as patient infected with it-LANMAR-7-2006.

Discussion

An useful molecular epidemiologic analysis should include some key elements: an accurate and rigorous collection of epidemiologic information, an exhaustive analysis of the circulating genotypes and a thorough determination of the genetic relationship of HAV isolates obtained from infected subjects [13-15]. Molecular techniques and viral genetic analysis have become an essential tool in epidemiological investigation, because of their ability to link cases and probable sources of infection and to describe HAV transmission patterns [16-19]. In this study we characterized the circulating strains from hepatitis A laboratory-confirmed cases in Genoa and in its proximity during an outbreak from May to October 2005 and in the following months.
In the 6-months outbreak period, 58 subjects were infected: the case-control study conducted on primary cases indicated an association between illness and beach establishment attending at the East of the Genoa seafront, but it lacked to identify the exact source of infection.
The bell-shaped profile of epidemic curve, the clinical onset of primary cases who occurred in few weeks and the geographic distribution of cases who were clustered in a limited geographic area, clearly indicated a common exposure to a point source and the outbreak can be probably associated with a contaminated food product dispensed in the affected area. Contaminated food and water are an infrequent source of infection in low endemic areas, although they have been associated with outbreaks [16, 18, 20, 23]. Routine hepatitis A surveillance fails to identify a source of infection for 40 to 50% of reported cases [21]: several factors, such as long incubation period, focal viral contamination, viral availability in the environment for weeks and memory bias can explain this relative insensitivity, especially as regard as food-borne sources [22].
As far as molecular characterization, the outbreak occurred from May to October 2005 has been mainly caused by a single variant, confirming the common exposure to a point source, suggested by descriptive and analytical epidemiology; this variant circulated within MSM network in North Europe, but the epidemiologic link between cases observed in Liguria and MSM network is hard to recognize, mainly because homosexuality or other risk behaviours, such as illicit drug use, are unwillingly stated. The outbreak variant has circulated for 12 weeks, from July to September 2005, showing no genetic changes in the genome regions used for molecular characterization, as already observed by several Authors [16, 18, 23, 24]. In the same period, different variants originating from Southeast Asia and Northern Africa, have co-circulated, and 6 out of 11 cases observed since October 2005 were closely related to variants circulating in Southern America and Northern Africa: all these cases were related to international travel and none of these had determined secondary cases. Travels to high endemicity areas is a well stated risk factor, especially when the traveller starts from a low-intermediate endemicity country [25, 26].
Moreover, the usefulness of molecular epidemiology approach to understand the chain of transmission of the virus clearly emerged when risk factors are unknown, i.e. in the patient who initially declared no journey in Morocco and in the Moroccan child immigrated from her country of origin with no travel or case-contact history, both infected by 2 Northern African variants.
Molecular analysis showed that 3 patients with very close temporally clinical onset (11, 12 and 29 April 2006) were infected by the same variant suggesting a common exposure [16, 23], although the standard epidemiological investigation were not able to identify the link between cases.

This survey highlighted that the epidemiological picture of hepatitis A in Liguria is characterized by a wide heterogeneity of circulating HAV strains, due to the contemporary presence of strains belonging to both sub-genotype IA and IB and to different genetic clusters. This pattern could be associated with the increase of imported cases: most of recent collected isolates resulted closely related to strains isolated in distant geographical areas. Some events showed that HAV is often transmitted within network of persons with similar risk factors for infection.

In conclusion, molecular approach coupled to descriptive and analytical epidemiological studies appeared un-replaceable tools for management and control of HAV outbreaks, because of their capacity to recognize infection origin, transmission patterns and dynamics of virus evolution.

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