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RESEARCH ARTICLE

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Analysis of genetic and serology of hepatitis A virus infection during and after outbreak in two junior high schools in Surabaya, Indonesia

Juniastuti[1,2,3] | Dedy Wahyuddin[2] | Nihayatussa'adah[3] | Mochamad Amin[3] | Laura Navika Yamani[3,4] | Takako Utsumi[3,5] | Florentina Sustini[6] | Maria Inge Lusida[1,3]

[1]Department of Microbiology, School of Medicine, Universitas Airlangga, Surabaya, East Java, Indonesia

[2]Master Program of Tropical Medicine, School of Medicine, Universitas Airlangga, Surabaya, East Java, Indonesia

[3]Institute of Tropical Disease, Universitas Airlangga, Campus C Universitas Airlangga Jalan Mulyorejo, Surabaya, East Java, Indonesia

[4]Department of Epidemiology, Faculty of Public Health, Universitas Airlangga, Campus C Universitas Airlangga Jalan Mulyorejo, Surabaya, East Java, Indonesia

[5]Center for Infectious Diseases, Kobe University Graduate School of Medicine, Kobe, Hyogo, Japan

[6]Department of Public Health, School of Medicine, Universitas Airlangga, Surabaya, East Java, Indonesia

Correspondence
Juniastuti, Department of Microbiology, School of Medicine, Universitas Airlangga, Jalan Mayjen Prof Dr Moestopo 47, Surabaya 60131, East Java, Indonesia.
Email: junsf@fk.unair.ac.id

Abstract

Outbreaks of hepatitis A have occurred in some cities in Indonesia. In Surabaya, the capital city of East Java province, Indonesia, hepatitis A outbreaks have been reported since2013, with a marked increase in the number of cases in 2015. The aim of the present study was to analyze the genetic and serology of acute symptomatic cases (early infection) during a hepatitis A outbreak and asymptomatic cases after the outbreak in two junior high schools in Surabaya in 2015 to 2016. Students with acute symptomatic hepatitis A during the outbreak and other students who were asymptomatic 3 to 4 months after the outbreak were enrolled. Asymptomatic students had no symptoms from the outbreak until they were enrolled. Sera were collected to identify anti-hepatitis A virus (HAV) IgM (by enzyme-linked immunosorbent assay) and HAV genetic variations/genotypes (using polymerase chain reaction [PCR]-sequencing and phylogenetic analysis). A total of 33 (97.1%) out of 34 sera of students with acute symptoms were positive for anti-HAV IgM and 18% of them were positive by PCR, identified as HAV subgenotype IA. No prominent amino acid variations were observed from reported HAV sequences from Indonesia. Among 38 sera of asymptomatic students, most (55.3%) were positive for anti-HAV IgM, while none were positive by PCR. In conclusion, HAV-IA was the only subgenotype identified in acute symptomatic cases during the outbreak. The percentage of HAV-specific IgM-positive cases was very high among acute symptomatic students, but that was also high among asymptomatic students, which might contribute as the important source of infection during the outbreak.

KEYWORDS

genetic variation, hepatitis A virus, immunoglobulin

1 | INTRODUCTION

Hepatitis A virus (HAV) infection has long been and remains a major public health issue in many countries.[1] In developing countries, HAV infection is endemic, with most individuals in these countries being exposed to HAV during early childhood.[2] Due to improvements in sanitation conditions, the pattern of HAV endemicity in several developing countries has changed from high to intermediary in recent decades.[3-6] HAV infection is closely associated with unsafe water or food, inadequate sanitation, and poor personal hygiene. The

Juniastuti and Dedy Wahyuddin contributed equally to this work.

virus is primarily spread via the fecal-oral route, either by person-to-person contact or through the ingestion of food or water that has been contaminated by the fecal matter of infected individuals.[7,8]

Hepatitis A is the most common form of acute viral hepatitis worldwide. A typical symptomatic presentation includes nonspecific prodromal symptoms with various combinations of fever, malaise, weakness, anorexia, nausea, vomiting, arthralgia, and myalgias. Prodromal symptoms tend to decrease with the onset of jaundice, which lasts for several weeks and is followed by a convalescent period. Peak infectivity occurs during the 2 weeks before the onset of jaundice. When jaundice appears, stool viral concentrations decrease and most patients are noninfectious after 1 week.[1,9] Viremia occurs within 1 to 2 weeks after HAV exposure and presents for approximately 3 to 4 weeks before the onset of jaundice,[10]while IgM antibodies to HAV are detectable at or before the onset of clinical illness and decrease within 3 to 6 months.[11] Infected children under the age of 6 years do not generally exhibit noticeable symptoms, and only 10% develop jaundice. Starting from 6 years of age to adulthood, infection typically causes more severe symptoms, with jaundice occurring in more than 70% of cases.[8] It is important to note that in most cases, asymptomatic cases are not recognized as hepatitis A, whereas they can shed HAV in feces. The asymptomatic and nonjaundiced HAV-infected individuals, particularly children, are frequently the source of infection for others.[12] Other previous findings have also suggested that HAV is mainly transmitted by children with either asymptomatic or subclinical infection.[13,14]

HAV is a nonenveloped RNA virus of 27 to 32 nm diameter in size, with an icosahedral symmetry, which belongs to the genus *Hepatovirus* of the *Picornaviridae* family, and has a positive-polarity, single-stranded 7.5-kb genome.[15] The amplification and sequencing of variable regions within capsid proteins, that is, the VP3/VP1 and VP1/P2A junctions of wild-type HAV isolates from different regions of the world, revealed significant nucleotide sequence heterogeneity, but limited amino acid heterogeneity.[10,16,17] Based on nucleotide sequence heterogeneity, HAV isolates may be differentiated into seven unique genotypes.[18] Viruses from four of the genotypes (I, II, III, and VII) were detected in human cases of hepatitis A, whereas viruses from the other three genotypes (IV, V, and VI) were isolated from animal species only.[19-22]

Outbreaks of hepatitis A in Indonesia have been consecutively reported in the following cities: Bogor (West Java province) in 1998; Jember and Bondowoso (East Java province) in 2006; Tangerang (West Java province) in 2007; Yogyakarta (Special Region of Yogyakarta province) in 2008; and Ngawi (East Java province) in 2009, with icteric cases in each area accounting for 74, 50, 17, 1160, and 146 individuals, respectively (Sub-directorate of Surveillance and Outbreak Response, Directorate General of Disease Control and Environmental Health, Ministry of Health, personal communication). Hepatitis A outbreaks occurred in 6 provinces with 495 cases in 2013, with East Java province being the most prevalent, and in 3 provinces with 460 cases in 2014.[23,24] The subgenotype Indonesian IA sublineage HAV was identified in 34 sera during outbreaks of hepatitis A in some regions between 2003 and 2007 in Indonesia.[25]

In Surabaya (the capital of East Java province), the number of hepatitis A cases increased from 20 in 2013 to 40 in 2014 (Public Health Office of Surabaya, 2013 and 2014, unpublished). In 2015, the number of hepatitis A cases markedly increased to 59, and occurred in 2 junior high schools in Surabaya (Primary Health Care of PacarKeling, 2015, unpublished). This hepatitis A outbreak was defined by the initial detection of three students who tested positive for anti-HAV IgM. The present study reported the results of genetic and serological examinations of hepatitis A in symptomatic cases (early infection) during the outbreak and in asymptomatic cases a few months after the outbreak.

2 | MATERIALS AND METHODS

2.1 | Samples

The outbreak of hepatitis A in two junior high schools (a private school and a public school) in Surabaya occurred between October 2015 and January 2016. Students with acute symptoms including those with acute symptoms during the sample collection and those with a history of acute symptoms for the past 1 month were enrolled in January 2016. An acute symptomatic case was defined by the onset of acute illness with a minimum of two of the following clinical symptoms: fever, malaise, flatulence, anorexia, nausea, vomiting, dark urine, and jaundice, which are consistent with hepatitis A during the outbreak and serological/genetic confirmation of acute hepatitis A (serum IgM antibody to HAV and/or HAV RNA being positive). A few months after the outbreak (between March and April 2016), students without acute hepatitis A symptoms (from the outbreak until the sampling period) were enrolled. Sera were collected from symptomatic and asymptomatic students.

Ethical clearance for this study was obtained from the Ethics Committee of the School of Medicine, Universitas Airlangga, Surabaya. Informed consent for participation in this study was obtained from the parents of each individual.

2.2 | Detection of anti-HAV IgM

An anti-HAV IgM enzyme immunoassay was performed using the BIOS HAV-IgM Enzyme Immunoassay Test Kit (Chemux BioScience, Inc, San Francisco, CA) according to the manufacturer's instructions.

2.3 | RNA extraction and reverse transcription polymerase chain reaction

Hepatitis A virus RNA was extracted from 140 μl plasma using a commercially available kit (QIAamp Viral RNA kit; Qiagen, Tokyo, Japan).

Extracted RNA was reverse-transcribed and amplified using SuperScript One-Step RT-PCR (Invitrogen, Tokyo, Japan) and a set of primers. Polymerase chain reaction (PCR) amplification was subsequently performed to detect the regions of the VP1-2A and VP3-VP1 junctions. First- and second-round PCRs using primers

representing the VP1-P2A junction were performed with 35 cycles at 95°C for 2 minutes, 55°C for 1 minute, 72°C for 1 minute, and a final extension step at 72°C for 10 minutes. The first round of PCR using primers representing the VP3-VP1 junction was performed at 94°C for 5 minutes, 40 cycles at 94°C for 45 seconds, 57°C for 1 minute, 72°C for 1 minute, and a final extension step at 72°C for 10 minutes, whereas the second round was performed at 94°C for 5 minutes, 40 cycles at 94°C for 45 seconds, 55°C for 1 minute, 72°C for 1 minute, and a final extension step at 72°C for 10 minutes (Table 1).[10,16,17,26]

PCR products were electrophoresed on a 2% agarose gel containing ethidium bromide and visualized under UV illumination. Amplified cDNA fragments were sequenced by a direct sequencing method with the BigDye Terminator v1.1 Cycle Sequencing Kit and an ABI Prism 310 sequencer (Applied Biosystems, Foster City, CA).

2.4 | Sequence analysis

Nucleotide sequences were aligned with reference strains by the program Molecular Evolutionary Genetic Analysis (MEGA) X (http://www.megasoftware.net). Genotypes have more than 15% nucleotide variations and subgenotypes have 7% to 7.5% nucleotide variations.[22] Phylogenetic trees were constructed by the Neighbor-Joining method and bootstrap resampling was performed 1000 times.

3 | RESULTS

The outbreak occurred in two junior high schools between October 2015 and January 2016. The location of the two schools was very close. Among 1219 students during the outbreak, 59 (4.8%) hepatitis A cases were reported in the 2 schools. The definition of a case of hepatitis A was an individual exhibiting acute hepatitis A symptoms. A total of 34 students with acute symptoms from the 2 schools in January 2016 and 38 asymptomatic students between March and April 2016 (3-4 months after the outbreak) were enrolled. All 34 students with acute symptoms had jaundice accompanied by other symptoms. All students (with and without acute symptoms) were aged between 12 and 14 years old and were not vaccinated. The workflow and results obtained are shown in Figure 1.

In consideration of possible sources of HAV transmission, investigations focused on canteens, water supply systems, toilets, and the locations of septic tanks. Washbasins were not adequate in canteens (2-3 canteens in the 2 schools), with only a bucket/sink filled with water being available for dish washing, but not for hand washing. Water supplies in both schools were from three to seven wells, respectively, and also from tap water. Although the isolation of HAV was not performed from the water supplies, a bacterial analysis revealed that water from the seven wells and tap water were contaminated with *Escherichia coli* (unpublished data from the Center for Health Laboratory, Surabaya). Many toilets were available in the 2 schools: 6 toilets in the private school and 19 in the public school. Although toilets were separated from other rooms, the majority of the septic tanks were located close to the wells (less than 10 m).

3.1 | Detection of anti-HAV IgM

Positivity for anti-HAV IgM was 97.1% among 34 students with acute symptoms (33/34), and 55.3% among 38 asymptomatic students (21/38).

3.2 | Detection of the HAV RNA genome in serum

Among 33 samples from students with acute symptoms and testing positive for anti-HAV IgM, 6 (18%) were positive for HAV RNA, whereas 1 sample from a student with acute symptoms, but negative for anti-HAV IgM was negative for HAV RNA. Among 38 samples from asymptomatic students who tested positive for anti-HAV IgM ($n = 21$) and negative for anti-HAV IgM ($n = 17$), none were positive for HAV RNA. The VP1/P2A junction region was detected in 4 sera, while the VP3/VP1 junction region was detected in 2.

TABLE 1 Primers used for HAV RNA amplification

Region	Name of primers	Sequence	Nucleotide number	Product size (bp)
VP1/P2A				
1st PCR	BR-5[a]	TTGTCTGTCACAGAACAATCAG	2950-2972	361
	BR-9 [a]	AGTCACACCTCTCCAGGAAAACTT	3310-3286	
2nd PCR	RJ-3 [b]	TCCCAGAGCTCCATTGAA	2984-3002	234
	BR-6 [a]	AGGAGGTGGAAGCACTTCATTTGA	3217-3193	
VP3/VP1				
1st PCR	HAV-1 [c]	GCTCCTCTTTATCATGCTATGGAT	2172-2196	244
	HAV-2 [c]	CAGGAAATGTCTCAGGTACTTTCT	2415-2391	
2nd PCR	HAV-3 [c]	ATGTTACTACACAAGTTGGAGAT	2195-2218	186
	HAV-4 [c]	GATCCTCAATTGTTGTGATAGCT	2380-2357	

Abbreviation: PCR, polymerase chain reaction.
[a] (17).
[b](16).
[c] (10).

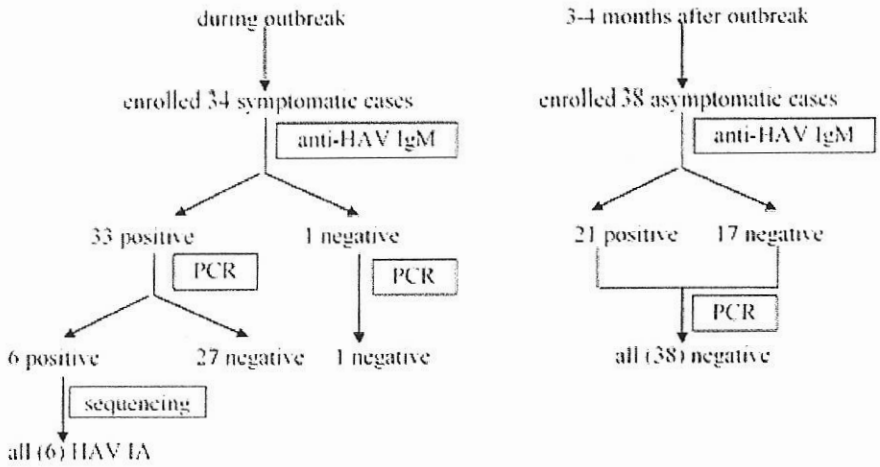

FIGURE 1 Workflow with the obtained results. HAV, hepatitis A virus; PCR, polymerase chain reaction

3.3 | Genetic analysis of HAV isolates

The nucleotide sequence identities of the VP1/P2A junction region with the HAV subgenotype IA strain (AB917146) from Surabaya, Indonesia ranged between 99% and 100%. The nucleotide sequence identities of the VP3/VP1 junction region with HAV subgenotype IA strain (AB839697) from Tangerang, Indonesia were 98%. No prominent amino acid variations were observed from those of other reported sequences from Indonesia (Figures 2 and 3).

Phylogenetic analyses of sequences revealed that all strains ($n = 6$) belonged to subgenotype IA (Figures 4 and 5).

4 | DISCUSSION

Anti-HAV IgM has been used diagnostically as the primary marker of acute infection,[27] and mainly comprises antibodies against capsid proteins. IgM anti-HAV enzyme immunoassays are available commercially.[28] The sensitivity, specificity, and positive predictive value of IgM anti-HAV measurements for acute hepatitis were previously reported to be 100%, 99%, and 88%, respectively.[29] IgM antibodies to HAV are detectable at or before the onset of clinical illness, and decrease within approximately 3 to 6 months.[11] A previous study reported that IgM levels were often elevated in acute hepatitis A infection (85%).[30] Anti-HAV IgM was shown to be negative 4 months after the onset of infection in 70% of patients and by 7 months in 86%.[11] Furthermore, lower concentrations were 4 to 6 months after the onset of infection.[31]

In the two junior high schools, high percentages of anti-HAV IgM were observed in symptomatic (97.1%, 33/34) and asymptomatic students (55.3%, 21/38). Of note, the percentage of anti-HAV IgM in asymptomatic students was higher than expected. These asymptomatic students had no symptoms from the time of outbreak until the time of this study (3-4 months after the outbreak), but the presence of antibodies (anti-HAV IgM) after the outbreak can confirm that they were in fact related to the outbreak. The result indicates that hepatitis A represented a great threat in the two schools, with asymptomatic students potentially facilitating the transmission of HAV during the outbreak. CDC surveillance data[32] showed that children aged 5 to 14 years historically have the highest incidence of hepatitis A, although the incidence of HAV infection may be the highest among those <5 years old.[33] Among older children (older than 6 years old) and adults, HAV infection is generally symptomatic with jaundice occurring in >70% of patients.[8] The ratio of symptomatic to asymptomatic students in an outbreak appears to reflect the age and immunity of individuals. In a previous outbreak of hepatitis A in a religious community, a household serosurvey detected anti-HAV IgM in 15 individuals younger than 20 years, 2 (13%) of whom developed jaundice.[34] In this present study, a higher percentage of students with jaundice (33/54, 61%) was noted among those who tested positive for anti-HAV IgM, while the remainder (39%) did not have jaundice, whereas the age of all students ranged between 12 and 14 years. The unidentified source shedding HAV may be important for the spread of HAV to other individuals. Students with less scrupulous hygiene may serve as a major source of transmission. The lack of proper hygiene facilitates HAV transmission from these children to their relatives and friends.[13,14]

Previous hepatitis A outbreaks in schools were reported in 2001 in Malaysian boarding schools and in a large private school in Manila.[35] Environmental issues, such as the chlorination of water and improvements in the personal hygiene standards of food handlers working in the schools were addressed. A number of recommendations made as a result of the investigation into this outbreak included continued surveillance for the disease, the provision of health education, and better sanitation practices in the canteen, as well as encouraging students to bring their own food and drink to school. Investigations on the two schools in Surabaya found that poor hygiene and sanitation in canteens (no available washbasins), water supplies (contaminated with *E. coli*, a known marker of feces contamination), and the close proximity of septic tanks to wells may have contributed to the spread of HAV. In the present study, the water supply was the likely transmission source because it may have been contaminated by the septic tanks. Some recommendations for these two schools may be similar to those for previous outbreaks of hepatitis A in other schools.

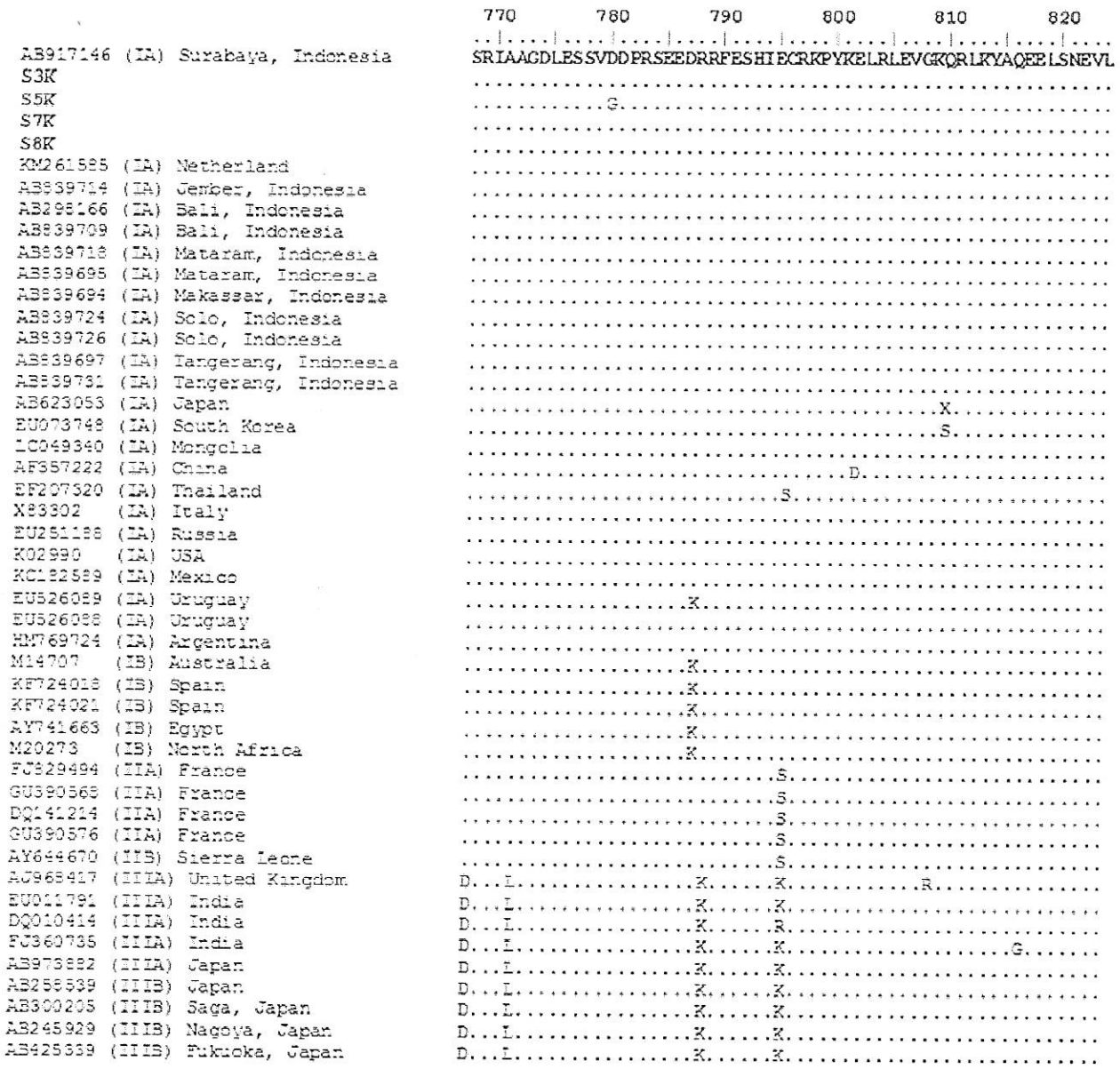

FIGURE 2 Alignments of amino acid sequences in the VP1/P2A junction region of 4 HAV isolates from Surabaya (code: S, shown in bold) and 43 reported sequences of HAV genotypes/subgenotypes. HAV, hepatitis A virus

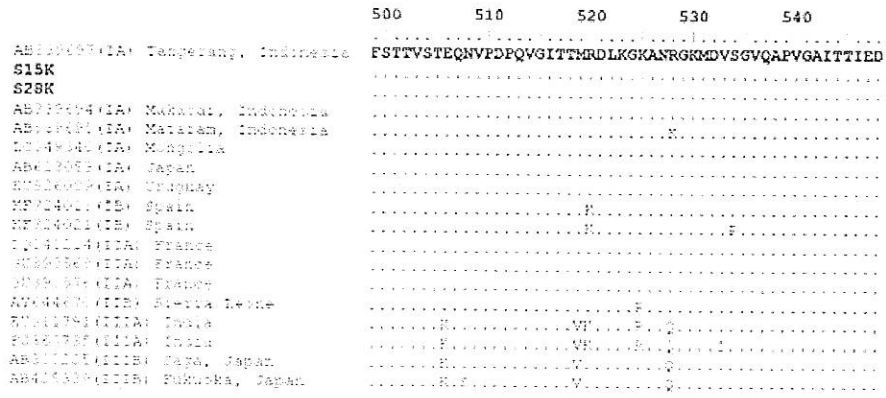

FIGURE 3 Alignments of amino acid sequences in the VP3/VP1 junction region of 2 HAV isolates from Surabaya (code: S, shown in bold) and 16 reported sequences of HAV genotypes/subgenotypes. HAV, hepatitis A virus

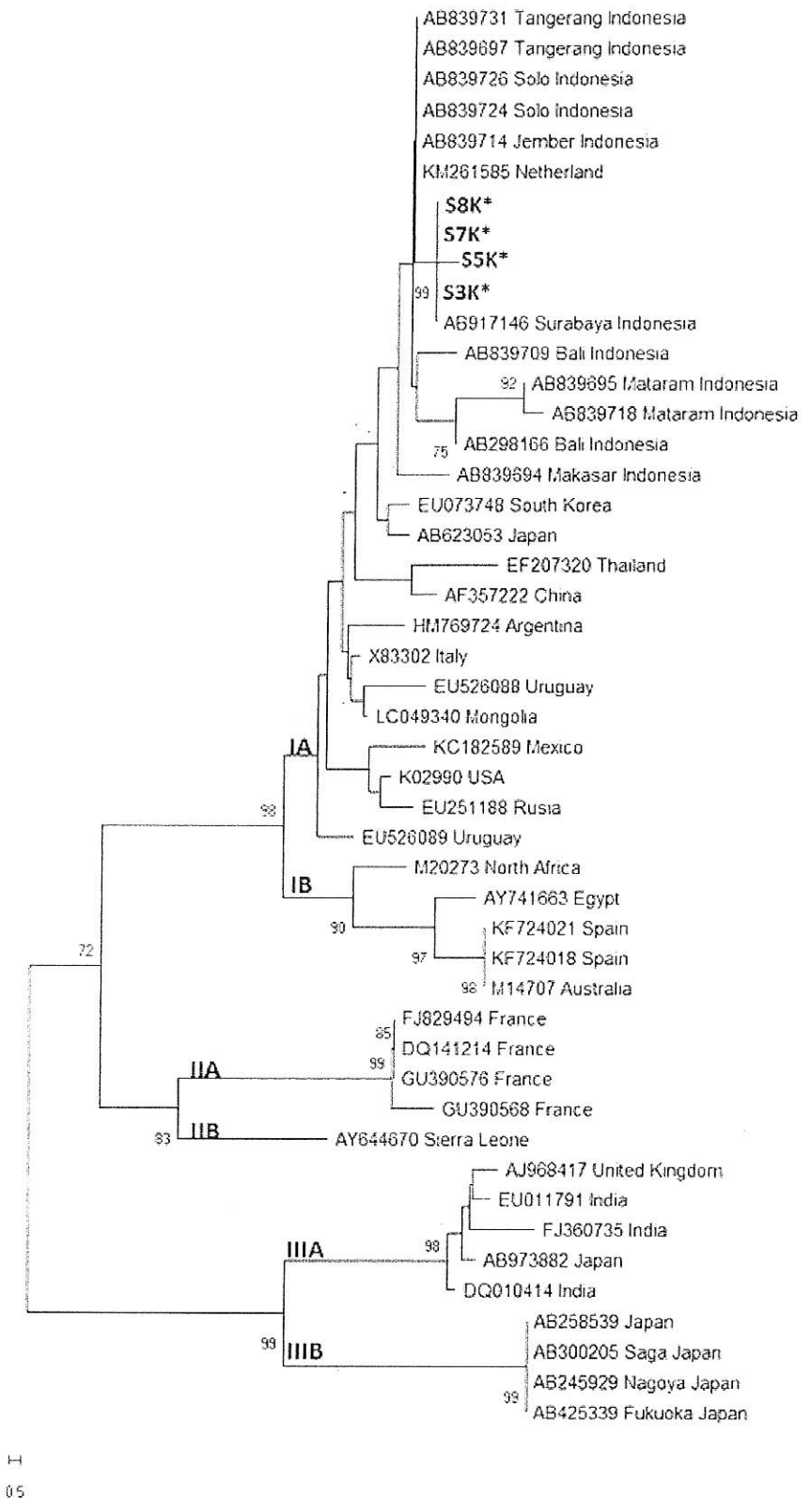

FIGURE 4 Neighbor-joining phylogenetic tree of nucleotide sequences in the VP1/P2A junction region of 4 HAV isolates from Surabaya (code: S*, shown in bold) and 43 reference strains. The genotypes/subgenotypes are indicated on the branches. Bootstrap values are indicated for each branch. The nucleotide sequences reported in this study were deposited in the DDBJ database under accession numbers LC432511-LC432514. HAV, hepatitis A virus

A study in China also found that an increased risk of HAV was associated with no hand washing before food preparation and cooking, before eating, and after defecation,[36] and a study in Brazil reported that a higher number of water taps in the home, which may promote the use of water for washing, was associated with a decreased risk of HAV.[37] The prevalence of hepatitis A in Indonesia is generally very high, and mass hepatitis A vaccinations have not been performed for the prevention and control of HAV infection.[38] The hepatitis A

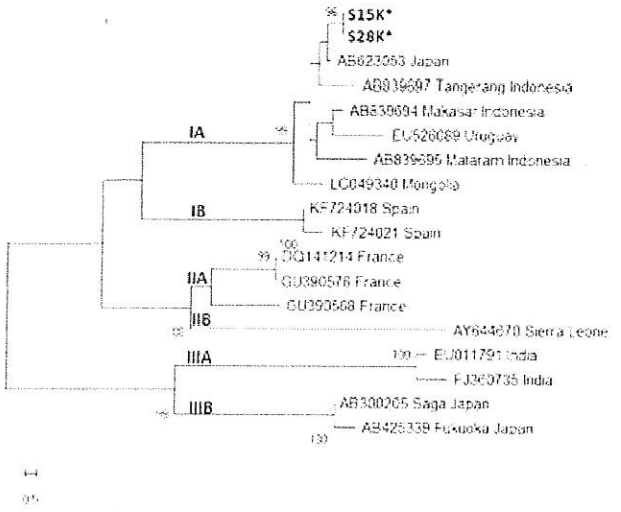

FIGURE 5 Neighbor-joining phylogenetic tree of nucleotide sequences in the VP3/VP1 junction region of 2 HAV isolates from Surabaya (code: S*, shown in bold) and 16 reference strains. The genotypes/subgenotypes are indicated on the branches. Bootstrap values are indicated for each branch. The nucleotide sequences reported in this study were deposited in the DDBJ database under accession numbers LC432515-LC432516. HAV, hepatitis A virus

vaccination currently has few indications in areas of the world in which infection is highly endemic and where most of the population is already immune[1]; however, as an outbreak response, site-specific vaccinations have been recommended to control community-wide outbreaks in small communities, such as in schools.[8]

A total of 6 (18%) out of the 33 symptomatic students who tested positive for anti-HAV IgM and none of the asymptomatic students (positive or negative for anti-HAV IgM) were positive for HAV RNA. Viremia occurs within 1 to 2 weeks of HAV exposure, presents for approximately 3 to 4 weeks before the onset of jaundice, and persists until the icteric phase in immunocompetent individuals.[39] The duration of jaundice is quite variable, the mean length was 7 days, with a range of 4 to >22 days.[27] Blood collected before the onset of symptoms appears to have the highest potential risk of transmitting HAV infection because of high viral concentrations and essentially no antibodies. Serum HAV RNA was generally detected before IgM anti-HAV. However, Bower et al[10] showed that viremia may persist long after the onset of symptoms (average, 79 days) and may present for a markedly longer period during the convalescent phase of hepatitis A. It should be noted that they used the PCR method, which detects HAV RNA at a level of~4 HAV particles/mL and appears to have been more sensitive than assays used in other studies.[10] In this present study, a low frequency of positivity for HAV RNA was obtained in the sera of symptomatic students who tested positive for IgM anti-HAV. The sampling time should be considered as a possible reason for this finding. This may have been due to a low HAV titer in blood at the time of specimen collection or the viremia period of HAV infection, which generally only occurred for 3 to 4 weeks, having passed.[39] In this study, not all blood samples were collected at the time of jaundice, some of them were collected within 1 month of jaundice onset. Time of collecting samples is critical, however, outbreaks always represent challenges in the investigation. The low frequency of positivity for HAV RNA was consistent with a previous finding. Mulyanto et al reported that only 21.52% of 158 serum samples from patients with acute hepatitis during hepatitis A outbreaks were positive for HAV RNA.[25]

Genetic analyses have played a crucial role in epidemiological investigations. To study the degree of variability among HAV isolates during the outbreak, a genetic analysis of selected genome regions (VP1/P2A and VP3/VP1 amino acids) was performed. The VP1/P2A junction region was detected in four sera, while the VP3/VP1 junction region was detected in two. Diverse primer pairs that amplify different regions of the HAV genome may affect their sensitivity. Based on phylogenetic analyses, all six isolated HAV strains were clustered with subgenotype IA strains (Figures 4 and 5), whereas there were no unique amino acid sequence changes from other reported isolates in other areas of Indonesia (Figures 2 and 3). Subgenotype IA is the most abundant type worldwide. Previous studies showed that subgenotype IA was the only genotype of isolated HAV strains in some areas of Indonesia, including an imported case from Surabaya.[25,40] The results of this outbreak in Surabaya confirmed the findings of previous studies.

In conclusion, most students with acute symptoms (97.1%) during the outbreak and even those without symptoms (55.3%) after the outbreak were positive for anti-HAV IgM. Among the 6 (18%) sera from symptomatic students with anti-HAV IgM, all were identified as HAV subgenotype IA. In addition to symptomatic cases, asymptomatic cases may have played a role as a crucial source during the outbreak. Control measures need to be adapted, according to the further epidemiology investigation.

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CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

ORCID

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