Hepatitis E virus infection in two different regions of Indonesia with identification of swine HEV genotype 3

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学位論文の内容要旨

Hepatitis E virus infection in two different regions of Indonesia with identification of swine HEV genotype 3

インドネシアの異なる地域におけるE型肝炎ウイルス感染とブタ由来遺伝子型3の同定

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INTRODUCTION

Hepatitis E is an emerging disease with globally high incidence, caused by the Hepatitis E Virus that is classified in the genus *Hepevirus* and is the only member of the *Hepeviridae* family. Hepatitis E virus is a polyadenylated, single-stranded RNA genome, approximately 7.2 kb long with short 5' and 3' untranslated regions, and three open reading frames (ORFs). Genomic sequence analysis has classified HEV isolates from humans and other mammals into four genotypes with at least 24 subgenotypes (1a-1e, 2a-2b, 3a-3j, and 4a-4g). Regions with low standards of sanitation, which promotes viral transmission, have the highest rates of HEV infection. HEV epidemics predominantly occur in regions where faecal contamination of drinking water is common. Generally, these outbreaks and sporadic water-borne infections are caused by genotypes 1 and 2. However, sporadic cases of hepatitis E in patients without history of traveling to endemic countries have also been reported and particularly to be genotype 3 and 4. These autochthonous infections are believed to be caused by zoonotic HEV or foodborne infections related to swine, and that swine handlers are at higher risk of infection.

This study represents an investigation on the prevalence of HEV infection in swine and humans from multiple areas in Java and Bali, Indonesia, which are two geologically distinct communities with different customs and swine breeding condition and focus on the molecular aspects of the partially conserved nucleotide sequences of HEV strains among Indonesian isolates.

MATERIAL AND METHOD

Sample Collection

In the Java region, swine farms are located far from housing with relatively better hygiene system and the local residents do not have close relation or contact with swine, whereas in Bali swine farms are located near houses and usually bred as domestic animals in the back yard and local residents have close relation and contact with swine. Serum samples from 137 swine farm workers, 100 blood donors and 100 swine (including 27 faecal) in Yogyakarta (Central of Java), along with 12 and 64 swine farm workers, 42 and 135 local residents also 89 and 119 swine serum samples in Tulungagung (East Java) and Mengwi (Bali), respectively from our previous study, were collected.

Serological Marker Testing

Serological test of anti-HEV antibodies were done by using a species-independent, double-antigen, sandwich enzyme-linked immunosorbent assay (ELISA) (MPD HEV ELISA 4.0v; MP Biomedicals Asia Pacific Pte., Ltd., Singapore).

Detection of Viral RNA

HEV-RNA was detected by reverse transcriptase (RT)–polymerase chain reaction (PCR). Viral RNA was extracted from 140 µl of serum using a QIAamp Viral RNA Kit (QIAGEN GmbH, Hilden, Germany). The extracted RNA was reverse-transcribed to cDNA using SuperScript™ III RT (Invitrogen, Carlsbad, CA) with a specific primer for the HEV ORF2 region. The transcribed cDNA was used to amplify HEV-RNA by nested PCR.

Determination of Genotyping by Phylogenetic Analysis

The amplified PCR products from the second round were sequenced directly by dideoxy sequencing using the Taq Dye Deoxy Terminator Cycle Sequencing Kit with a 3100-Avant genetic analyser (Applied Biosystems, Foster City, CA, USA). The designations and accession numbers of the full-length reference sequences representing the different genotypes for analysis of HEV ORF1 and ORF2 were retrieved from GenBank. Sequences were aligned using ClustalX software. The Neighbour-Joining method was used to construct the phylogenetic trees. The analyses were carried out using Molecular Evolutionary Genetics Analysis software.

Statistical Analysis

Categorical variables were compared using χ^2 test or Fisher's exact test. Results with P values of <0.05 were considered statistically significant. SPSS/PASW Statistics Software version 18.0 (SPSS Inc., Chicago, IL) was used for all analyses.

RESULTS

Prevalence of Anti-HEV Antibody Seropositivity among Swine farm workers and local residents in Java and Bali

A total of 5.1% of participants were anti-HEV antibody positive, consisting 10 of 149 (6.7%) swine farm workers, and 5 of 142 (3.5%) local residents. The total prevalence of anti-HEV in

Bali was as high as 11.6%. In 64 workers and 135 local residents from Bali, 12 (18.8%) and 11 (8.1%), respectively, were anti-HEV antibody positive. The overall prevalence of anti-HEV antibody seropositivity in Bali was significantly higher than that in Java (P = 0.015). Significantly more swine farm workers from Bali were anti-HEV antibody seropositive compared with workers from Java (P = 0.013). Although the prevalence of HEV antibody seropositivity in local residents from Bali was also higher than that in Java, this difference was not statistically significant. The rate of anti-HEV antibody seropositivity was also not significantly different between local residents and swine farm workers in Java.

Prevalence of Anti-HEV Antibody Seropositivity among Swine in Java and Bali

A total of 281 swine sera were obtained from different regions in Java and Bali. The age of swine ranged from 1 to 6 months. The unification of the Yogyakarta and Tulungagung samples as from Java showed the prevalence of anti-HEV antibody seropositivity in 1 and 2 month old swine were 5.2% (1 of 19) and 43.3% (13 of 30), respectively. Eleven of 16 (68.8%) 1 month old swine with 18 of 20 (90%) 2 month old swine in Bali were positive for anti-HEV antibodies. There were significant differences in the rates of anti-HEV antibody seropositivity between Java and Bali, where seropositivity in the 1 and 2 month old swine was higher in Bali than in Java (P < 0.001 and P = 0.01). The prevalence rates in the 3–6 month old swine among Java and Bali were not significantly different, nor were the overall prevalence rates of anti-HEV antibody seropositivity in swine from both regions.

Identification of HEV-RNA in Swine Sera

In this study, 2 new HEV RNA isolates were identified from 3 month old swine and 1 isolate was identified from a 5 month old pig from Yogyakarta. The Java (Yogyakarta) strains were reported to GenBank, and were given the accession numbers AB714131 for YKSB26, AB714132 for YKSB51 and AB714133 for YKSB52. Nucleotide sequences of the two previously detected isolates from Tulungagung and Mengwi (Bali) strains had been reported under the accession numbers AB541111 and AB541112.

Identification and Confirmation of Swine HEV-RNA in Swine Faeces

Twenty-seven faecal samples were collected in Java for this study. HEV-RNA was detected in 4 (14.8%) of these samples. The Java strains from swine faeces YKSF2, YKSF23, YKSF24, and YKSF25, were reported to GenBank and were given accession numbers AB714127, AB714128, AB714129, and AB714130, respectively. The YKSF23, YKSF24, and YKSF25 strains were isolated in 2 month old pigs, whereas the YKSF2 isolate was from a 4 month old pig. All of the faecal strains were identified as HEV genotype 3 based on ORF2, and the genetic similarity with serum samples was 96.2%–99.7%. This genotype was also confirmed based on ORF1, and the genetic similarity to the closest nucleotide sequences was 88.5%–92.5%. Amino acid comparison with other genotype 3 strains showed similarity of 97.8% - 100%.

DISCUSSION

From our study, regions with local customs involving close contact to swine showed to have higher prevalence of anti-HEV seropositivity among the people. However, significant differences in the prevalence of anti-HEV antibodies between swine farm workers and local residents in a population that did not develop close relationships with swine could not be

revealed. A previous study has identified HEV infection from rats in Indonesia, but further studies are needed to proof the zoonosis risk as a way of transmission.

Based on a global genotyping study of HEV isolates, nucleotide differences among isolates belonging to sub genotype 3a were to be 0.7%–6.0%. Phylogenetic tree analysis in this study showed all of the Yogyakarta isolates formed a cluster divergent from the branch of the US, Korean, and Japanese isolates with nucleotide differences as high as 8.6%–9.7%. This suggests that the Yogyakarta strain is likely to be independent from other strains in sub genotype 3a. The Bali cluster and the TP42 isolate from Tulungagung (East Java) showed a divergent branch from swDQ and swGX40 of sub genotype 4b from China, to which they showed the closest nucleotide similarity of 86.2 to 91.5%. Therefore it is a possibility of that these isolates are indigenous to Indonesia.

In conclusion we suggest the importance to identify ways of transmission from factors other than close and direct contact with swine that may contribute to HEV transmission. Swine isolates from Indonesia belonging to genotypes 3 and 4 were phylogenetically different from previously reported HEV strains. To the best of our knowledge, this is the first report of swine HEV belonging to genotype 3 in Indonesia.

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論文審査の結果の要旨

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（要旨は１，０００字～２，０００字程度）

背景と目的

E型肝炎ウイルスは、非A非B肝炎として流行したインドにおいて1983年にヒトから同定されたウイルスである。E型肝炎ウイルスはヘペウイルス科に属する直径約33ナノメートルのウイルスで、長さ約7,300塩基の1本鎖RNAを内包している。遺伝子配列の解析から少なくとも4種類の遺伝子型と24種類の遺伝子亜型に分類され、地域によりその分布が異なっている。遺伝子型1,2は水や食料の汚染により媒介され、遺伝子型3,4は散発的に発生することが多い。またE型肝炎ウイルスは、ブタやシカなどにも感染する人獣共通感染症として知られている。

インドネシアではこれまでE型肝炎ウイルスの流行が3回報告されているが、バリ島におけるE型肝炎ウイルスに関する報告はほとんどない。今回の検討では、生活習慣やブタの飼育環境の異なるインドネシアの各地域における現地住人およびブタ飼育職員のE型肝炎ウイルス抗体陽性率と、ブタにおけるE型肝炎ウイルスの保有率、またその分子遺伝学的な解析を行うこととした。

対象と方法

2011 年にジャワ島中心部のジョクジャカルタからブタ飼育職員 137 名、現地住人 100 名から血液を採取するとともに、ブタ 100 頭（血液 73 検体、便 27 検体）からも検体を採取した。これについて、以前に東ジャワのツルナグンで採取された検体（飼育職員 12 名、現地住人 42 名、ブタ 89 頭）とバリ島のメンギで採取された検体（飼育職員 64 名、現地住人 135 名、ブタ 119 頭）と比較検討を行った。ELISA 法により血清学的に抗 HEV 抗体を測定した。HEV-RNA については RT-PCR 法により検出し、系統樹解析法にて遺伝子型を決定した。

結 果

地域住民における HEV 抗体は、ジャワ島では 142 名中 5 名（3.5%）で陽性、バリ島では 135 名中 11 名（8.1%）に陽性であり、両群に有意差は見られなかった。ブタ飼育職員については、ジャワ島 149 名中 10 名（6.7%）陽性に対して、バリ島 64 名中 12 名（18.8%）で陽性であり、有意にバリの職員に高かった。ヒトにおける HEV 抗体陽性率は、バリ島 11.6%であったのに対して、ジャワ島 5.1%であり、有意にバリ島に高かった。これに対して、ブタにおける抗体陽性率は、ジャワ島 162 頭中 114 頭（70.3%）、バリ島 119 頭中 97 頭（81.5%）と有意な差は認めなかった。しかし、生後 1,2 か月のブタにおける HEV 抗体については、バリ島 68.8%, 90.0%に対してジャワ島 5.2%, 43.3%と有意にバリ島で高頻度であった。ブタ血清から HEV-RNA が陽性であったのはジャワ島 162 例中 4 例（2.4%）、バリ島 119 例中 1 例（0.8%）であった。また、遺伝子系統樹解析によると、ジャワ島ツルナグンおよびバリ島メンギより分離された HEV については、遺伝子型 4b であったのに対し、ジャワ島ジョクジャカルタより分離された検体については、すべて遺伝子型 3a であった。