COMPARATIVE STUDY BETWEEN MOLECULAR HYBRIDIZATION AND ELECTRON MICROSCOPY FOR THE DETECTION OF HEPATITIS E VIRUS

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Abstract. The study describes a comparison between molecular hybridization using a non-radiolabeled, thymine-thymine (TT) dimerized synthetic oligonucleotide complementary DNA probe and electron microscopy for the detection of hepatitis E virus genome in bile. Spot hybridization with the TT dimerized probe was found to be more sensitive and specific compared to electron microscopy.

INTRODUCTION

Spot hybridization using synthetic oligonucleotide probe is a uniquely powerful and sensitive assay in which a minute amount of genomic material in a sample can be detected. Recently the technology has been successfully applied to various samples for detection of a large number of viruses including hepatitis B virus (Blum *et al,* 1984) and human immunodeficiency viruses (Balachandran *et al,* 1991). The thymine-thymine (TT) dimerized cDNA probes are advantageous in terms of probe stability, personal safety and simple technique (Koji and Nakane, 1990). This report demonstrates sensitive and specific detection of hepatitis E virus (HEV) genome in bile by spot hybridization using a TT dimerized cDNA probe.

MATERIALS AND METHODS

Animal transmission and sample collection

Animal transmission of HEV in monkeys has been described (Soe Soe *et al,* 1989). Briefly the animals were serially passaged with HEV by intravenous injection of HEV containing either stool suspension or bile. Bile was collected through a minilaparotomy weekly after inoculation for four weeks. Preinoculation bile was also collected as control. In this experiment bile obtained from the 5th passage monkey was used.

Electron microscopic examination of bile

20 µl of bile was diluted 1:10 with phosphate buffered saline (PBS) pH 7.4 and deposited onto colloidin and carbon coated 400 mesh cupper grids by means of an Airpax machine (Beckman). Two grids were used for each sample. The grids were dried and negatively stained with 2% phosphotungstic acid and examined under JEOL 100S electron microscope at 50,000 magnification.

Synthesis of cDNA probe

An antisense probe, which has a DNA (cDNA) sequence complementary to a portion of the HEV genome (Reyes *et al,* 1990) was synthesized and used as a test probe. A sense probe which has identical DNA sequence to the HEV genome was also synthesized and used as a control. Both sense and antisense probes were irradiated with UV light to produce TT dimerized probes.

Preparation of bile for hybridization

50 µl of bile was diluted 1:40 with PBS, mixed and ultracentrifuged in an ultracentrifuge (Hitachi Koki, rotor RP 50-2-423) at 40,000 rpm at 4°C

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for 2 hours. The virus pellet was dissolved in 50 µl Tris-EDTA solution and spotted on the nitrocellulose paper (4 µl/spot). Post 1st and 2nd weeks bile preparation were spotted at position A and B of both papers respectively. At positions C, 1:10 dilution of post 2nd week was spotted. Preinoculation bile was spotted at positions D.

Spot hybridization

Hybridization was performed according to the method described by Koji and Nakane (1990). Briefly the nitrocellulose papers were digested with proteinase K solution (1 µg/ml in PBS) for 10 minutes at 37°C. They were then washed three times with PBS to remove remaining proteinase K. After washing the membranes were prehybridized at 42°C for 2 hours. Hybridization was performed separately with TT dimerized antisense and sense probes with papers 1 and 2 respectively at 42°C overnight. After hybridization the papers were reacted for 3 hours at room temperature with rabbit serum against TT dimers of the probes (kindly supplied by Dr Koji). Washed several times in PBS and reacted again with commercial horseradish peroxidase labeled goat antirabbit immunoglobulins (MBL, Japan). Color production was performed in the presence of diaminobenzidine, hydrogen peroxide, cobalt and nickel.

RESULTS

Electron microscopic findings

As shown in Fig 1, numerous rounded virus-like particles (VLPs) measuring about 27nm in diameter with both electron lucent (empty) and dense (full) centers were detected in the post 3rd week bile. The amount of VLPs in bile was semiquantitatively graded into +1 to +3. If only a single VLP was detected in whole 400 mesh grid, it was graded as +1 (Fig 2). If small groups of VLPs were seen as in Fig 3, it was graded as +2. VLPs were not demonstrated in preinoculation, post 1st and 4th week bile. The +1 +3 VLPs were visualized in post 2nd and 3rd weeks, bile respectively.

Spot hybridization

As depicted in Fig 4, positive staining was seen in positions A, B and C of paper 1 which was hybridized with test or antisense probe. No staining in position D of paper 1 and A, B, C and D of paper 2 which was hybridized with control or sense probe. Thus HEV genome was detected in post 1st, 2nd and 1:10 dilution of post 2nd week bile.

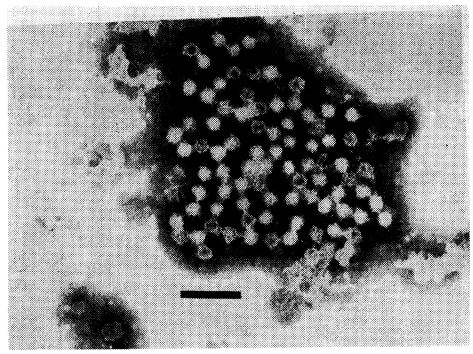

Fig 1—Electron micrograph of bile showing large aggregate of VLPs (+3) containing both full (arrow head) and empty (arrow) particles. Bar = 100nm.

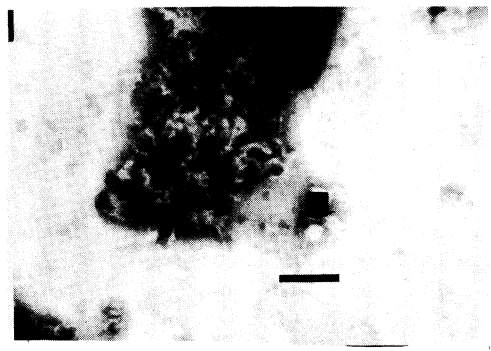

Fig 2—Electron micrograph of bile showing a group of 5 VLPs (+2). Bar = 100nm.

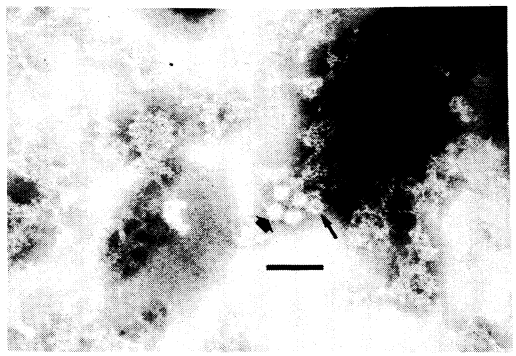

Fig 3—Electron micrograph of bile showing a single full VLP (+1). Bar = 100nm.

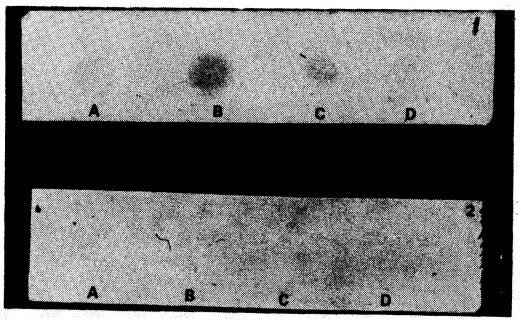

Fig 4—Spot hybridization results of bile and TT dimerized probes. Post 1st and 2nd week bile were spotted at A and B positions of both papers. At positions C and D 1:10 dilution of post 2nd week and preinoculation bile were spotted. Papers 1 and 2 were separately hybridized with test (antisense) and control (sense) probes respectively. Positive stainings were seen in A, B and C of paper 1 and no reaction in D of paper 1 and A, B, C and D of paper 2.

DISCUSSION

With the electron microscope VLPs were identified only at post 2nd week and VLPs were not visualized in post 1st week bile. However, with spot hybridization HEV genome could be identified as early as post 1st week at 1:10 dilution of the +1 graded VLPs. Thus spot hybridization is more sensitive than the electron microscopy. This is in agreement with others working on hepatitis A virus. They have reported that molecular hybridization was more sensitive than the electron microscopy (Tassopoulos *et al,* 1986). Under the electron microscope only the size and shape of the particles are visualized, so that we can only say that they could be viruses, thus the term VLPs was used. In this case since the particles were roughly rounded without envelope and measured 27nm that it could be either HEV or HAV or even a small round virus. By contrast, because of the specific sequence interaction with the HEV genome, a positive reaction in spot hybridization can definitively identify the HEV genome. Therefore, spot hybridization with TT dimerized cDNA probe is superior to electron microscope in terms of sensitivity and specificity.

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