POINT OF CARE TEST FOR THE DETECTION OF EXPOSURE TO HEV IMMUNOGLOBULIN A (IGA)

FIELD OF THE INVENTION

The present invention relates to a point of care test (POCT) of detecting recent exposure of a human to HEV or equivalent thereof. More particularly, the present invention relates to a rapid and easy analysis of a biological sample taken from a subject (human) in order to determine if the subject had been exposed specifically to a HEV or equivalent thereof. The present invention further provides point of care (POC) diagnostic kits and sero-evaluation by detecting HEV specific antibody (IgA) on a rapid platform using reverse flow technique due to HEV infection or an equivalent thereof.

BACKGROUND

Introduction

Hepatitis E virus ("HEV") is an enterically transmitted virus causing acute liver disease (hepatitis). Along with the Norwalk gastroenteritis virus and the hepatitis A virus (HAV), HEV is easily acquired through consumption of contaminated food and water. HEV is the most common cause of clinical hepatitis in residents of many developing countries[1,2]. Among travellers to regions with poor sanitation, HEV infection remains a problem despite the effective use of specific vaccines and immune serum globulin against HAV as there is no cross-protection.HEV has been reported to infect rats and some domestic/farm animals like pigs, cows, sheep etc in both developing and developed countries and cases of HEV has been identified in patients with no history of travel outside the USA[3]. The risk factors for HEV infection are related poor sanitation in large areas of the world, and HEV shedding in faeces. Person-to-person transmission is uncommon. There is no evidence for sexual transmission or for transmission by transfusion.

Hepatitis E ("HEV") was not recognized as a distinct human disease until 1980. Hepatitis E is caused by infection with the hepatitis E virus, a non-enveloped, positive-sense, single-stranded RNA virus known as Calcivirus.

The highest rates of infection occur in regions where low standards of sanitation promote the transmission of the virus. Epidemics of hepatitis E have been reported in Central and South-East Asia, North and West Africa, and in Mexico, especially where faecal contamination of drinking water is common. However, sporadic cases of hepatitis E have also been reported elsewhere and serological surveys suggest a global distribution of strains of hepatitis E of low pathogenicity.

The Disease

The incubation period following exposure to HEV ranges from 3 to 8 weeks, with a mean of 40 days. The period of communicability is unknown. There are no chronic infections reported.

Hepatitis E virus causes acute sporadic and epidemic viral hepatitis. Symptomatic HEV infection is most common in young adults aged 15-40 years. Although HEV infection is frequent in children, it is mostly asymptomatic or causes a very mild illness without jaundice (anicteric) that goes undiagnosed.

Typical signs and symptoms of hepatitis include jaundice (yellow discoloration of the skin and sclera of the eyes, dark urine and pale stools), anorexia (loss of appetite), an enlarged, tender liver (hepatomegaly), abdominal pain and tenderness, nausea and vomiting, and fever, although the disease may range in severity from subclinical to fulminant.

In general, hepatitis E is a self-limiting viral infection followed by recovery. Prolonged viraemia or faecal shedding are unusual and chronic infection does not occur.

Occasionally, a fulminant form of hepatitis develops, with overall patient population mortality rates ranging between 0.5% - 4.0%. Fulminate hepatitis occurs more frequently in pregnancy and regularly induces a mortality rate of 30% among pregnant women in the 3rd trimester. As such the early diagnosis of HEV infection may allow the use of appropriate clinical management to reduce this mortalty[4]. Death usually occurs as a result of liver damage or secondary symptoms such as dehydration or malnutrition. Due to a lowered immune status, pregnant women might be at higher risk for acquiring Hepatitis E. These patients develop an unusual form of DIC (diffuse intravascular coagulation) besides fulminant hepatitis. If the mother

survives the acute stage of the disease, the foetus is not usually adversely affected. No chronic carrier state develops in either.

Diagnostic

Since cases of hepatitis E are not clinically distinguishable from other types of acute viral hepatitis and antigens cannot be routinely detected in clinical specimens, diagnosis is made by blood tests which detect elevated antibody levels of specific antibodies to hepatitis E in the body or by reverse transcriptase polymerase chain reaction (RT-PCR). The detection of anti-HEV IgM antibodies provides the most suitable marker of acute infection, especially in population where prior infection with HEV and thus IgG reactivity to HEV, may be common.

Immunoglobulin M (IgM) against HEV is used as a reliable and sensitive marker for recent HEV infection[5]. However, the specificity of solid-phase assay for anti-HEV IgM has been questioned in patients with IgM rheumatoid factors in serum. It was reported that anti-HEV IgA can be utilized as an additional complementary antibody for detecting recent HEV infection[6]. But the clinical and epidemiological implication of HEV IgA in HEV infection has been reported by several authors [7-11]. They concluded that HEV-specific IgA in serum has better potential to be a diagnostic target compared to IgM due to its higher sensitivity and specificity.

The present invention provides an effective and sensitive point of care test (POCT) for the detection of IgA reactive to HEV that alleviates some of the problems of the prior art.

SUMMARY OF THE INVENTION

The important aspect of the present invention provides a method called point of care test ("POCT") for detecting IgA in a subject that is specific for a HEV or equivalent thereof said method comprising: contacting a biological sample/s from the subject with a mixture of HEV specific immunogenic components; and determining the presence of a complex that forms between a binding partner in the biological sample and a HEV specific immunogenic component; and characterizing the binding partner in the complex with an anti-IgA antibody conjugated with gold colloid using reverse flow technology.

The method identifies only the IgA in the biological sample.

The present invention results from a need to develop POCT with low cost, rapid and straightforward assay to determine present or prior recent exposure to HEV. The invention preferably utilizes nitrocellulose membrane to hold HEV immunogen, which subsequently capture binding partners identified as anti-HEV IgA from a biological sample such as whole blood, serum/plasma or saliva/urine of HEV infected patients and finally visualized by anti-IgA antibody conjugated with gold particle.

Accordingly, the present invention shows greater specificity and sensitivity compared to other conventional and most currently used HEV capture IgM ELISA by providing a reverse flow rapid platform that can specifically identify antibody (IgA) produced against HEV or immunological relatives thereof at an early stage of HEV infection. Most preferably, the method identifies HEV infection.

In another aspect of the present invention there is provided a solid support for use in a method for detecting exposure of a subject to a HEV or equivalent thereof, said method comprising: contacting a biological sample from the subject with a mixture of HEV specific immunogenic components or an equivalent thereof; determining the presence of a complex that forms between a binding partner present in the biological sample and a HEV specific immunogenic component; and optionally characterizing the binding partner in the complex to correlate the binding partner to exposure to the HEV; said support comprising HEV specific immunogenic components immobilized on the support.

In a preferred embodiment, the biological sample may be applied to a nitrocellulose membrane previously loaded with HEV. Preferably the antigen or immunogenic component is derived from a HEV infected cells. The complex formed by an immunogenic component of the HEV antigen and the binding partner may then be detected using a detection agent that contains a reporter group and which specifically binds to the component/binding partner complex more specifically the IgA binding partners.

Yet another aspect of the present invention provides a kit in a cassette form for detecting IgA in a subject that is specific for a HEV or equivalent thereof or for detecting HEV exposure comprising: a solid support including a HEV specific immunogenic component or equivalent thereof; or a solid support including a HEV

specific immunogenic component or equivalent thereof attached to a second support; at least one detection agent conjugated to a reporter group for detecting a binding partner in a biological sample that forms a complex with the HEV specific immunogenic component; and optionally instructions for using said kit to further identify the binding partner of the complex. The reporter group is anti-IgA antibodies conjugated with gold particles and the platform is reverse flow technology.

The present invention also provides individual components of the POCT kit for use in the method of the present invention. The present invention also serves as a method of assessing the relative risk of one or more subjects being exposed to HEV or an equivalent thereof within a defined location (e.g. geographical area, housing estate, means of transport or center for medical treatment or assessment), comprising; obtaining samples from a representative population within a defined location; and assessing evidence of exposure of individual members of a sample population to a HEV or equivalent thereof by the method comprising the steps of - contacting a biological sample from the subject with a mixture of HEV specific immunogenic components; and determining the presence of a complex that forms between a binding partner in the biological sample and a HEV specific immunogenic component and Wherein the presence of the complex is indicative of exposure of the subject to a HEV or equivalent thereof; and assessing the relative risk of exposure within the defined location by characterizing the binding partner in the complex.

Risk analysis may be conducted using software in a computer readable form. Consequently, the present invention further relates to a computer readable program and computer comprising suitable for analyzing exposure of subjects or group of subjects or a risk of exposure of subject or group of subjects to a HEV or equivalent thereof.

BRIEF DESCRIPTION OF DRAWINGS

Figure-1 shows the comparative effectiveness of HEV IgA POCT with HEV IgM Rapid Test.

Figure-2 shows a Reverse Flow Rapid Test.

DETAILED DESCRIPTION OF THE INVENTION

Development of point of care test (POCT) for the detection of anti-HEV IgA using reverse flow technique from biological samples of a individual who is exposed to HEV infection/immunization.

A point of care test (POCT) was developed for the detection of anti-hepatitis E virus (HEV) IgA from the patient's biological samples (blood, serum and saliva). The test utilizes reverse flow technique where HEV recombinant antigen was coated onto the nitrocellulose membrane which captured anti-HEV IgA from the biological samples of HEV infected patient and later detected by goat anti-human IgA antibody conjugated with gold colloid. The total assay time of the test was 15 minutes and can be used at all level of health care system. The sensitivity and specificity of the POCT HEV-IgA was found better than HEV IgM ELISA and comparable with IgM rapid test. One of the major advantages of the POCT is the utilization of a wide spectrum of biological samples like whole blood, serum/plasma and saliva. HEV-IgA POCT tests can be able to make accessible in areas with limited laboratory facilities and can provide immediate result without the need for sophisticated laboratory equipment and highly trained manpower. (See Figure 1 where HEV IgA POCT showed high level of test line intensities compared to IgM rapid test which indicates IgA as a better signature molecule for the detection of acute HEV infection).

The major important aspect of the present invention provides a POCT for detecting IgA in a subject that is specific for a HEV or equivalent thereof said method comprising: contacting a biological sample from the subject with a mixture of HEV specific immunogenic components; and determining the presence of a complex that forms between a binding partner in the biological sample and a HEV specific immunogenic component; and characterizing the binding partner in the complex with an anti-IgA antibody conjugated with gold colloid.

This method is specific for the identification of those binding partners in the biological sample that is IgA. This method may be further enhanced by the use of immunogenic components that have been isolated using a HEV specific IgA. Accordingly, the immunogenic components may be HEV IgA specific immunogenic components. The introduction of the IgA reactive immunogenic components will attract the IgA in the biological sample that is specific for HEV.

In another aspect of the present invention there is provided a POCT using reverse flow technique for detecting exposure of a subject to a HEV or equivalent thereof said method comprising: contacting a biological sample from the subject with a mixture of HEV specific immunogenic components; determining the presence of a complex that forms between a binding partner in the biological sample and a HEV specific immunogenic component; characterizing the binding partner in the complex; and correlating the binding partner to exposure to the HEV.

The present invention results from a need to develop a POCT which is rapid (15 minutes), low cost and straightforward without laboratory facility and expert technical hands to determine present or prior recent exposure to HEV. In accordance with the present invention, the subjects including animals such as mammals and in particular humans are screened for the presence of binding partners preferably IgA to HEV or an equivalent thereof using reverse flow technology. The preferred binding partners are subject-derived binding partners such as, but not limited to immunointeractive molecules. The most immuno-interactive molecules are antibodies particularly immunoglobulin A (IgA). The identification of such binding partners is then used as evidence of present or prior recent exposure of the subject to HEV or an equivalent thereof.

The invention specifically utilizes reverse flow technique, where HEV antigen is sprayed or loaded onto the nitrocellulose for the capturing HEV reactive IgA from the biological samples of the subject exposed to HEV infection. The resultant complex is later detected by anti-IgA antibody conjugated with gold particle and the technique can be useful at any level of health care system which is called point of care (POC) testing.

Accordingly, the present invention shows greater sensitivity and specificity compared to other conventional and most currently used IgM ELISA by providing a platform that can identify antibody produced against HEV or an equivalent thereof at an early stage of the infection by reverse flow technique.

Therefore, the present invention provides a new specific, rapid and economical POC detection method preferably using HEV antigen, preferably HEV envelope protein immunogenic components of the virus described above, which permits the specific detection of HEV binding partners, preferably IgA that may be present in the test samples (whole blood, serum/plasma or in saliva). The test is rapid, preferably

providing results within 15 minutes at room temperature (RT). Apart from it being a simple and convenient technique for specific antibody detection. The major advantages of the present invention is the preferred usage of reverse flow platform in a cassette format and anti-IgA antibodies conjugated with gold for the detection of anti-HEV IgA from saliva, whole blood, serum or plasma. Furthermore, the present invention makes test useful for testing a single sample with high sensitivity due to the use of much volume of sample (15µl) with greater exposure of anti-HEV IgA present in human biological samples.

Throughout the description and claims of this specification, use of the word "comprise" and variations of the word, such as "comprising" and "comprises", is not intended to exclude other additives, components, integers or steps.

HEV

The term "HEV" is used herein the claims and description refers to all HEV serotypes (serotype-1, serotype-2, serotype-3 and serotype-4) associated with a HEV infection. Preferably, the present invention is applicable to detecting HEV infection or exposure in any subjects including human. Human subjects, however, are preferred in accordance with the present invention. However, the invention includes any subject that can respond to an infection or immunization by the HEV or an equivalent thereof.

HEV is a non-enveloped single stranded positive-sense RNA virus. Its genome is approximately 7.2 kb long, and consists of a short 5' untranslated region (UTR) followed by three partially overlapping open reading frames (ORFs: ORF1, ORF2 and ORF3), and then a short 3'UTR terminated by poly (A) tract[12-14]. HEV was recently classified as the sole member of the genus *Hepevirus in the family Hepeviridae*[15] *(http://www.ncbi.nlm.nih.gov/ICTVbd/ictv/fs_hepev.htm).*

HEV is transmitted via the faecal-oral route. Hepatitis E is a waterborne disease, and contaminated water or food supplies have been implicated in major outbreaks. Consumption of faecally contaminated drinking water has given rise to epidemics, and the ingestion of raw or uncooked shellfish has been the source of sporadic cases in endemic areas. There is a possibility of zoonotic spread of the virus, since several non-human primates, pigs, cows, sheep, goats and rodents are susceptible to infection. The risk factors for HEV infection are related poor sanitation in large areas of the world, and HEV shedding in faeces. Person-to-person transmission is

uncommon. There is no evidence for sexual transmission or for transmission by transfusion.

The incubation period following exposure to HEV ranges from 3 to 8 weeks, with a mean of 40 days. The period of communicability is unknown. There are no chronic infections reported.

Hepatitis E virus causes acute sporadic and epidemic viral hepatitis. Symptomatic HEV infection is most common in young adults aged 15-40 years. Although HEV infection is frequent in children, it is mostly asymptomatic or causes a very mild illness without jaundice (anicteric) that goes undiagnosed.

Most patients are not seen until the onset of an icteric state. The patient will experience jaundice, anorexia, hepatomegaly, abdominal pain, nausea and vomiting. At this stage, the clinical features resemble other acute hepatitis. Here the use of sensitive serological tests is necessary to pinpoint the specific virus causing the infection.

Most current methods for the diagnosis of HEV are determined by the presence of IgM anti-HEV antibodies or an increase in the amount of IgG anti-HEV. It is important to detect subclinical, acute and convalescent cases of HEV. It is especially important in cases of children and pregnant women who are at high risk of fulminate cases. The severity of the hepatitis increases with each trimester and can reach up to 20%.

Death usually occurs as a result of liver damage or secondary symptoms such as dehydration or malnutrition. Due to a lowered immune status, pregnant women might be at higher risk for acquiring Hepatitis E. These patients develop an unusual form of DIC (diffuse intravascular coagulation) besides fulminant hepatitis. If the mother survives the acute stage of the disease, the foetus is not usually adversely affected. No chronic carrier state develops in either.

Protective antibodies after the infection are apparently effective to prevent reinfection even with strains from distant regions but the duration of this protection in humans is still questioned. Immune serum globulin will considerably reduce mortality in the 3rd trimester of pregnancy but will not block incidence of infection on contact.

Equivalents

The term "equivalent" as used herein and applied to the HEV is intended to include similar molecules that can elicit the same or similar response that the HEV or a structural or non-structural protein of the HEV could elicit. For instance, various antigens expressed by the HEV at various stages of infection or various virus particles or fragments may cause similar effects that the whole virus causes. The response may be an immunological response (non-clinical response) or it may be an infectious response (clinical response) or due to vaccination.

Exposure

The present invention is applicable to detecting exposure to the HEV or an equivalent thereof. Exposure may be present or prior exposure to the HEV or an equivalent thereof. Preferably, the exposure is sufficient to elicit an immune reaction or response in the body so as to induce a binding partner in response to the HEV or equivalent thereof. Once the subject is exposed, the method of the present invention may be applied at any stage of exposure as described above. Preferably, the method is used to detect exposure where there are no signs and symptoms that are obvious of a HEV infection. Preferably, the method detects exposure of the subject at a phase of HEV infection at an early acute phase for secondary infection or late convalescence stage of exposure to HEV or equivalent thereof for the primary infection or vaccination. The exposure may not always manifest in a HEV infection or notable signs or symptoms but it will cause a response so as to induce a binding partner. Preferably, the response is an immunological response.

The subject may have been exposed to HEV but need not show visual symptoms of the infection. The present method detects exposure that may lead to infection or may indicate prior exposure with no symptoms manifested.

Immune response or Immunological response

An "immune response" or "immunological response" is understood to be a selective response mounted by the immune system of vertebrates in which specific antibodies or fragments of antibodies and/or cytotoxic cells are produced against invading pathogens and antigens which are recognized as foreign in the body.

Binding partner

The binding partner is any molecule or cell that is produced against the foreign HEV or equivalent thereof. Preferably, the binding partner is an antibody or immunologically active fragment thereof, or a cytotoxic cell. The binding partner includes an immuno-interactive molecule that can interact with HEV antigen or equivalent and is preferably an IgA molecule.

As indicated herein, the preferred binding partner is an immuno-interactive molecule, which preferably refers to any molecule comprising an antigen binding portion or a derivative thereof. Preferably, the immuno-interactive molecule is an antibody against any portion of HEV proteins produced during a humoral response in the subject of a HEV infection or exposure.

As indicated herein the preferred binding partner is an antibody produced in the subject to a HEV or related virus components. However, a binding partner of the targeted antibody may also be used. An example of such a binding partner is an anti-idiotypic antibody or an antibody specific for and discriminatory of a subject antibody specific for a HEV or related virus components.

As used herein, an "anti-idiotypic antibody" is an antibody which binds to the specific antigen binding site of another antibody generated in response to exposure to a component derived from HEV or immunological relative thereof.

As used herein, the terms "antibody" or "antibodies" include the entire antibody and antibody fragments containing functional portions thereof. The term "antibody" includes any monospecific or bispecific compound comprised of a sufficient portion of the light chain variable region and/or the heavy chain variable region to effect binding to the epitope to which the whole antibody has binding specificity. The fragments can include the variable region of at least one heavy or light chain immunoglobulin polypeptide, and include, but are not limited to, Fab fragments, F(ab') 2 fragments, and FC fragments.

Preferably the binding partner is an antibody. More preferably it is a HEV IgA molecule or a HEV IgA molecule.

Biological sample

The method of the present invention detects exposure to the HEV or equivalent thereof via the use of a biological sample obtained from a subject having been potentially exposed to the virus. The biological sample may be any sample from the body that may contain a binding partner. Such biological samples may be selected from the group including blood, saliva, cord fluid, B cells, T-cells, plasma, serum, urine and amniotic fluid. Preferably, the biological sample is serum or plasma. Most preferably, the biological sample is serum or saliva.

It should be noted that a biological sample might also be devoid of a binding partner that can interact with HEV or an equivalent thereof. This occurs when the subject has not been exposed to HEV or an equivalent thereof. Hence "determining the presence of a complex that forms between a binding partner in the biological sample and a HEV specific immunogenic component" may yield a zero result, as a complex cannot form in the absence of binding partners. A control may be performed with HEV specific immunological agent such as a monoclonal antibody designed to compete with binding partners in the biological sample.

Reference to a biological sample being placed in contact with a component, preferably an immunogenic component or its immunological relative thereof should be understood as a reference to any method to facilitating the interaction of one or more immuno-interactive molecules of the biological sample with a component preferably of HEV antigen derived from infection of cells with HEV or an equivalent thereof. The interaction should be such that coupling or binding or otherwise association between the immuno-interactive molecule and a specific immunogenic component of HEV antigen derived from cells infected with the HEV or an equivalent thereof can occur.

The biological sample is contacted with a mixture of HEV specific immunogenic components preferably derived from cells infected with HEV or equivalent thereof. The HEV provides viral immunogenic components that may be provided by the HEV at any stage of its development. In the early convalescent stages of HEV infection, antibody preferably IgA developed or derived from previous HEV infection is one of the indications of either primary or secondary HEV infection and this may be detected by the formation of a complex between it and a HEV specific immunogenic component, preferably an immunological component of the virus.

HEV antigen

HEV antigen used in this development recombinant capsid protein known as PET2.1 which is constructed as a fusion protein containing the ORF2.1 fragment (amino acids 394 to 660) from the capsid protein of HEV (Chinese isolates) and a six-histidine tag[15]. This fusion protein was expressed in *E. coli* subsequently stabilized in 5M urea, and purified in the presence of 5M urea using TALON resin (Clontech Laboratories, Palo Alto Calif). The purified protein was refolded by serial dilution in carbonate buffer (pH 9.6) at 4^0C prior to application.

Formation of a complex

A complex will form between an antibody, preferably an IgA reactive with HEV or equivalent thereof.

The methods and kits of the present invention seek to detect components and binding partners, which form complexes and are indicative of a HEV infection. These components and binding partners are generated in the course of a HEV infection/immunization.

The complex may comprise one or more binding partners bound to one or more components derived from HEV or an equivalent thereof. However, not all will be HEV specific IgA. Other immunological molecules such as IgG and IgM may also bind.

A second binding molecule is also loaded on the nitrocellulose membrane which binds all immunoglobulins (IgA, IgG, and IgM) called Protein-L and this protein is used as a control line to indicate the validity of the assay.

The biological sample is left in contact with the component derived from HEV or an equivalent thereof for a period of time sufficient and conditions, which allow the stable formation of the complex.

The HEV specific immunogenic components and the biological sample are contacted so that a complex may form between the components and a binding partner present within the biological sample. Preferably, immunogens of the HEV antigen, including but not limited to those of the structural and non- structural proteins preferably

captured by anti-HEV IgA having a binding site specific to HEV antigen, will form complexes with either binding partners or a competing HEV specific immunological agent such as a specific IgA.

Preferably, the specific binding partners are antibodies or fragments thereof present in the biological sample. These will only be present when the subject has been exposed/immunized to the HEV.

Preferably, the complex will form between an antibody, preferably an IgA specific for the member of the HEV antigen or equivalent thereof and a anti-HEV IgA captured HEV viral component. This is then indicative of HEV specific IgA in the sample and hence recent or prior exposure.

The preferred method of the present invention relies upon the detection of HEV specific binding partners preferably IgA present in the biological sample that are specific for a component of the HEV antigen or an equivalent thereof which has been captured using anti-HEV IgA. The complex may comprise one or more binding partners bound to one or more components derived from HEV or an equivalent thereof. However, it is the identification of an IgA bound to the complex that is indicative of prior exposure in this present invention.

A non-immunological complex is formed with biological samples (saliva, serum) along the line loaded with protein-L containing all three types of immunoglobulins (IgA, IgG and IgM). This complex does not indicate the exposure of HEV to the subject but does provides the validity of the assay.

Supports for the detection of HEV specific IgA

Accordingly in another aspect of the present invention there is provided a solid support for use in a method for detecting exposure of a subject to a HEV or equivalent thereof, said method comprising: contacting a biological sample from the subject with a mixture of HEV specific immunogenic components or an equivalent thereof; determining the presence of a complex that forms between a binding partner present in the biological sample and a HEV specific immunogenic component; and optionally characterizing the binding partner in the complex to correlate the binding partner to exposure to the HEV; said support comprising HEV specific immunogenic components immobilized on the support.

The solid support may be any material known to those of ordinary skill in the art to which a binding partner or HEV specific immunogenic component may be attached. For example, the solid support may be a test well in a microtitre plate or a nitrocellulose or other suitable membrane. Alternatively, the support may be a bead or disc, such as glass, fiberglass, latex or a plastic material such as polystyrene or polyvinylchloride.

The binding partner or the HEV specific immunogenic component may be immobilized on the solid support using a variety of techniques known to those of skill in the art, which are amply described in the patent and scientific literature. In the context of the present invention, the term "immobilization" refers adsorption of antigen on the nitrocellulose membrane following spraying by BioDot machine. Immobilization by adsorption of antigen is onto the membrane (Prima-85). In such cases, adsorption may be achieved by spraying of antigen at 7" from the bottom of membrane using BioDot machine in a suitable buffer. Two more lines are sprayed on the same membrane at 14" and 12" using crystal violet and Protein-L. Following spraying membrane is allowed dry in a dryer and finally is blocked by blocking buffer.

Detecting, characterizing and identifying the binding partner of the complex

The HEV specific immunogenic component derived from a HEV or an equivalent thereof loaded/sprayed onto the membrane is allowed to contact with biological samples which is passed over the membrane once added onto the sample pad, which allow the stable formation of a complex. Once the biological sample meets the indicator line (crystal violet) a detection system which is anti-IgA antibodies conjugated with gold p[article is allowed to flow over the membrane from other side of the cassette which then detect the specific immunocomplex through the specific binding with IgA molecule.

Detecting the complex between the components derived from HEV or an equivalent thereof and a subject-derived binding partner such as immunoreactive molecules, may be based on any convenient method, which will be known to those of the skill of the art.

It is contemplated that procedures useful for detecting components and binding partners which form complexes and are indicative of a HEV infection in a biological

sample include, but are not limited to, immunological assays, such as immunoblotting, immunocytochemistry, immunohistochemistry or antibody-affinity chromatography, Western blot analysis, lateral flow or variations or combinations of these or other techniques such as are known in the art.

In general, components and binding partners, which form complexes and are indicative of a HEV infection, may be detected in a biological sample obtained from a subject by any means available to the skilled addressee. In a preferred embodiment, the method of detection employs a further detection agent such as specific anti-body conjugated with enzyme, gold particle/colloid which permits detection of said complexes and the binding partners.

In a preferred embodiment, the methods as herein described involve the use of a HEV antigen onto nitrocellulose membrane to which a binding partner of a biological sample may absorb/bind. The complex formed by a component of the HEV antigen and the binding partner may then be detected using a detection agent that contains a reporter group and specifically binds to the component/binding partner complex. Such detection agent may comprise, for example, an anti-antibody or other agent that specifically binds to the binding partner, such as an antiimmunoglobulin (i.e. antibody), protein G, protein A, protein-L or a lectin.

In a preferred embodiment, the detection reagent is an antibody or secondary antibody or an antigen-binding fragment thereof, capable of binding to the binding partner of the biological sample. Antibodies may be prepared by any of a variety of techniques known to those of ordinary skill in the art (See, for example, Harlow and Lane, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, 1988).

The secondary antibody which may be conjugated to a label, can be added to the complex to facilitate detection. A range of labels providing a detectable signal may be employed. The label may be selected from a group including chromogen, an enzyme, a catalyst, a fluorophore, gold colloid and a direct visual label. In the case of a direct visual label, use may be made of a colloidal metallic or non-metalic particle, a dye particle, an enzyme or a substrate, an organic polymer, or a latex particle. A large number of enzymes suitable for use as labels are disclosed in United States Patent Nos. 4366241, 4843000 and 4849338. Suitable enzyme labels in the present invention include alkaline phosphates, horseradish peroxidase, preferably horseradish peroxidase. The enzyme label may be used alone or in combination with

a second enzyme, which is in solution. In the present invention a secondary antibody attached with horseradish peroxidase, which then reacts with its substrate DAB and produces a visually detectable colour change, preferably achievesjhe detection of the complex.

Preferably, the antibody is an anti IgA antibody conjugated with gold colloid and therefore detects IgA binding partners that have bound to the HEV specific immunogenic components.

General description of the process

This assay may be performed by first contacting a binding partner of a biological sample that has been immobilized on a solid support, the nitorcellulose membrane, with the HEV specific immunogenic components as herein described, such that a component is allowed to bind to the immobilized binding partner such as an antibody. Alternatively, the HEV specific immunogenic components may be bound to the solid support such that binding partners are allowed to bind to the immobilized component and then detection reagent is added which may be specific reporter group. Unbound sample is then removed from the immobilized complex through reverse flow technique.

More specifically, once the binding partner or a HEV specific immunogenic component is immobilized on the support as described above, the remaining binding sites on the support are typically blocked. Any suitable blocking agent known to those of ordinary skill in the art, such as bovine serum albumin or milk casein with either Triton X 100 or Tween 20 ™ (Sigma Chemical Co., St. Louis, Mo.). The component or binding partner is used undilued form during contact with HEV immunogenic component. In general, an appropriate contact time is 5-10 minutes that is sufficient to allow a HEV specific immunological component presence in the biological samples to bind to the immobilized binding partner, or vice versa. Preferably, the contact time is sufficient to achieve a level of binding to the target epitope on the attached HEV specific immunogenic component that is at least about 95% of that achieved at equilibrium between the bound and unbound binding partner or HEV specific immunogenic component. Those of ordinary skill in the art will recognize that the time necessary to achieve equilibrium may be readily determined by assaying the level of binding that occurs over a period of time.

The detection agent is generally an anti-IgA antibody. Preferred reporter groups include those groups recited herein. The detection agent is then released from the gold-pad over the membrane where immunocomplex is formed with HEV antigen and anti-HEV IgA from biological samples. An appropriate amount of time may generally be determined by assaying the level of binding that occurs over a period of time. Unbound detection agent is then removed and bound detection agent is detected using the reporter group. The method employed for detecting the reporter group depends upon the nature of the reporter group. For radioactive groups, scintillation counting or autoradiographic methods are generally appropriate. Spectroscopic methods may be used to detect dyes, luminescent groups, chromogenic enzymes and fluorescent groups. Chromogenic enzymes include, but are not limited to, peroxidase and alkaline phosphatase. Fluorescent groups include, but are not limited to, fluorescein isothiocyanate (FITC), tetramethylrhodamine isothiocyanate (TRITC) 1 rhodamine, Texas Red, Gold colloid and phycoerythrin. Biotin may be detected using avidin, coupled to a different reporter group (commonly a radioactive or fluorescent group or an enzyme).

As used herein, "binding" refers to a non-covalent association between two separate molecules such that a complex is formed. The ability to bind may be evaluated by, for example, determining a binding constant for the formation of the complex. The binding constant is the value obtained when the concentration of the complex is divided by the product of the component concentrations. In general, two compounds are said to "bind," in the context of the present invention, when the binding constant for complex formation exceeds about 10 3 L/mol. The binding constant may be determined using methods well known in the art.

Membranes contemplated by the method and kits of the present invention include any membrane to which either the binding partner or components derived from the flavivirus or equivalent thereof can bind. Examples of membranes include without being limited to, nitrocellulose membranes, polytetrafluorethylene membrane filters, cellulose acetate membrane filters and cellulose nitrate membrane filters with filter paper carriers. Most preferably, the membrane is a nitrocellulose membrane.

Alternatively, the diagnostic methods of the present invention may adopt an automated analytic method using a biological microchip. For instance, a diagnostic kit can be structured to perform immuno-blotting using a glass slide coated with the component of the cell lysate. This diagnostic kit may comprise a biological microchip

onto the surface of which a flavivirus specific immunogenic component is immobilized, an appropriate buffer, a standardised sample comprising a detectable level of binding agent, and a secondary detection reagent, as herein described.

The method and kits of the present invention can detect specific exposure of human or animals to a HEV or any specific member of the family or equivalent thereof either during acute infection or in convalescent phase. As used therein "acute infection" refers to the period of time when a virus has infected a host and is actively replicating and/or causing symptoms associated with infection like fever, rush, joint pain and or abdominal pain. The "convalescent phase" refers to the stage of HEV infection cycle when HEV is no longer multiplying or remains in the host blood and has developed binding partners such as, but not limited to antibodies. Using the method and kit of the present invention, exposure can be detected at any time after generation of a binding partner in the infected patient or patient derived it from his/her previous infection/infections.

Rapid Kit

In another aspect of the present invention there is provided a POCT kit for detecting IgA in a subject that is specific for a HEV or equivalent thereof or for detecting HEV exposure comprising: a solid membrane support including a HEV specific immunogenic component or equivalent thereof; or a solid support including a HEV specific immunogenic component or equivalent thereof attached to a second support; at least one detection agent conjugated to a reporter group for detecting a binding partner in a biological sample that forms a complex with the HEV specific immunogenic component; and optionally instructions for using said kit to further identify the binding partner of the complex.

Optionally, the kit will also include additional parts such as plastic dropper, chase buffers, cassette and instructions as are necessary for conducting the method.

Accordingly the present invention provides a kit for detecting exposure of a subject to HEV or any member of the family or an equivalent thereof. The kit may be any convenient form of POC testing which allows for a binding partner in a biological sample to interact with an anti-HEV IgA captured HEV viral component. The result is an indication, by the presence of HEV specific binding partners such as IgA in the biological sample, of prior exposure to HEV. Preferably the kit comprises a solid

support such as described herein adapted to receive or comprise anti-HEV IgA captured components of HEV or an equivalent thereof. The kit may also comprise reagents, reporter molecules capable of providing detectable signals and optionally instructions for use.

The kit may be a modular kit comprising one or more members wherein at least one member is a solid support comprising loaded/sprayed HEV component of virus particle or equivalent or cell lysate comprising an immunogenic component derived from a HEV or equivalent thereof.

The present invention also provides individual components of the kit for use in the method of the present invention. The invention provides solid supports that include anti-HEV IgA reactive components of the HEV for use in the detection of exposure to the HEV. In one embodiment, a nitrocellulose membrane is used to attach viral antigen, either for use as an immobilized anti-HEV IgA reactive HEV viral components or as a line blot. Preferably the membranes include components selected from the group of HEV viral antigens structural and non- structural proteins, flavivirus particles and fragments thereof, glycoproteins, lipids and carbohydrates derived from the flavivirus or any mixture thereof.

The solid support may also be a microtitre plate, glass slide or biological microchip wherein the components of the cell lysate are immobilised. These solid supports can then be subjected to the biological sample to detect flavivirus exposure. Preferably nitrocellulose membrane is used to attach HEV antigen by spraying onto the membrane from HEV infected cells.

Assessing relative risk of infection

In yet another aspect, the present invention also provides a method of assessing the relative risk of one or more subjects being exposed to HEV or an equivalent thereof within a defined location (e.g. geographical area, housing estate, means of transport or center for medical treatment or assessment), comprising; obtaining samples from a representative population within a defined location; and assessing evidence of exposure of individual members of a sample population to a HEV or equivalent thereof by the method comprising the steps of - contacting a biological sample from the subject with a mixture of HEV specific immunogenic components; and determining the presence of a complex that forms between a binding partner in the

biological sample and a HEV specific immunogenic component and wherein the presence of the complex is indicative of exposure of the subject to a HEV or equivalent thereof; and assessing the relative risk of exposure within the defined location by characterizing the binding partner in the complex.

Risk analysis may be conducted using software in a computer readable form. Consequently, the present invention further relates to a computer readable program and computer comprising suitable for analyzing exposure of subjects or group of subjects or a risk of exposure of subject or group of subjects to a HEV or equivalent thereof.

The method or technique of the present invention allows for the epidemiological study or sero-surveillance of outbreaks of infection caused by HEV or any member of the family or equivalent thereof. Such studies provide valuable information, which advance multiple facets of research in the area of HEV disease. For example, epidemiological studies aid in the identification of the index of an infection. Such information enables the identification of a defined location from which the source of virus responsible for a viral outbreak originated.

Additionally the technique/method of the present invention permits for the rapid identification or isolation of subjects who are infected with a HEV or equivalent thereof without major laboratory equipment or even in field conditions. Such information aids in identifying subjects, who require medical treatment as well as defining locations that require further investigation or disease control approaches such as identification of breeding places and its control. Further, the technique of the present invention allows for the monitoring of an infected patient to determine the presence of - anti-HEV specific IgA. Alleviation of IgA titre or its presence in an early phase of infection may be the indication acute phase of infection.

Further, the technique of the present invention provides a means for identifying subjects who are infected with any specific member of the genus of HEV and serotypes involved, allowing for the rapid detection, risk of further infection, pointing to the location of an infection and disease control strategy.

A reference herein to a patent document or other matter which is given as prior art is not to be taken as an admission that the document or matter was known or that the

information it contains was part of the common general knowledge as at the priority date of any of the claims.

Examples of the procedures used in the present invention will now be more fully described. It should be understood, however, that the following description is illustrative only and should not be taken in any way as a restriction on the generality of the invention described above.

EXAMPLES

Example 1:

a) HEV antigen: HEV recombinant protein pte2.1 (Burnet Institute, Australia and MP Biomedicals, Singapore) was used to develop POCT using reverse flow technology. HEV recombinant antigen was expressed in E. Coli system and purified using MP Biomedicals existing protocol and certified by QC depertment.

b) Protein-L: Protein L (Pierce Biotechnology, USA) is an immunoglobulins-binding recombinant protein that was originally derived from the bacteria ***Peptostreptococcus magnus.*** Protein L has the ability to bind with kappa light chain of all classes and subclasses of immunoglobulins without interfering antigen-antibody reactions and was used to bind IgA molecule from the biological samples used in this development.

c) Anti-human IgA-Gold conjugate (40nm): Goat anti-human IgA antibodies conjugated with gold colloid (Code-HIA10, Millenia Diagnostics, Inc, USA) was used in this experiment as secondary antibodies and was used at a dilution of 7 OD (520 nm).

d) Serum samples: A total 200serum samples positive either by HEV IgM or IgG ELISAs were used as HEV positive samples. 216 serum samples collected from healthy donors were used as negative sample experiment. Another 150 samples obtained from non-HEV illness including hepatitis B, hepatitis C was used as cross-reactive sera.

e) Serological assays: IgM capture ELISA, a commercial kit (Pan-bio, Australia) was used for the measurement of anti-HEV specific IgM antibodies in the samples

used in this study. The test was performed according to the procedures described by the manufacturer.

f) HEV rapid diagnostics: One type of HEV rapid diagnostic kit available in the market (MP Biomedicals) in the form of cassette was used as competitor kits to validate and evaluate HEV-IgA POCT.

g) HEV IgM and IgG ELISAs: HEV IgM ELISA (2.0, 3.0 MP Biomedicals and Adultis, Italy) were used for the detection of HEV specific IgM and IgG from the serum samples used in this study. The test was carried out according to the procedures described by the manufacturer.

h) HBV and HCV diagnostics: HBsAg rapid antigen detection kit (MP Biomedicals) and Biokit HBsAg ELISA were used to screen all samples used in this study to detect the presence of HBV infection. HCV antibody (IgG) rapid test (MP Biomedicals) was also used to screen all sera samples used in this study. Positive serum against HCV was detected and confirmed by PCR technique.

i) Optimization of HEV Antigen

The optimization of HEV antigen (MP Biomedicals) is carried out 1x PBS according to the table. A minimum volume of 500µl was made for each concentration before starting the optimisation.

Name	Pte2.1- Protein (mg/ml)
1	0.3
2	0.2
3	0.1

HEV r-protein antigen was sprayed at position set for HEV-IgA Rapid Test striping on 3 pieces of membrane (15 cm each). Use Protein L as control line and crystal violet as indicator line. Following spraying, the membrane is dried, blocked using blocking buffers and dried again and then laminated, cut and assembly for test.

j) Optimization of Protein L

Protein L (Pierce Biotechnology, USA) was diluted in 1 x PBS according to the table mentioned below. A minimum volume of 500µl was made for each concentration for the process of optimisation.

Name	Protein A (mg/ml)
New lot (concentration 1)	0.1
New lot (concentration 2)	0.05
New lot (concentration 3)	0.025

Following assembly cassette were used for the optimization of antigen and protein-L sprayed/load onto the membrane-using panel of strong, weak and negative anti-HEV IgA positive serum samples. For protein-L the C-line with 2.0 to 3.0 intensity with serum was considered as optimum level of reaction. For the optimization of viral protein the T-line with strong positive serum 3.0, weak positive 1.5 while for negative no line or line with +/- - intensity were considered as optimum level of reactions.

Following optimization of antigen and protein-L, 500 cassette were prepared for the study of sensitivity, specificity, positive predictive value and negative predictive value.

Assay procedure

Serum / Plasma/saliva Samples

Step 1- Added 15µl of whole blood sample to the samples square well followed by 1 drop of chase buffer to the same well.

Step 2 – Sample absorbed by sample pad and then move over the membrane and once reached the blue control line (A), three (3) drops Chase Buffer was added to the conjugate pad.

Step 3 -Pulled the "plastic tab (labeled with HEV-IgA) until resistance is felt and added another drop of chase buffer onto the sample pad square and started timing. Read the result after 15 minutes.

See Figure 2 where HEV IgA POCT device contains three lines; one visible blue line known as indicator line while rest two lines (C and T) only visible following test with positive sample. The details description and interpretations are given below.

Interpretation of HEV-IgA POCT

The result of POCT was interpreted using interpretation card which has a range of color intensities varied from 0.25 to 3.0. For test line (T-line) any intensity between 0.5 to 3.0 was considered as den-IgA positive sample while 0.0 to 0.25 is considered as negative. For control line (C-Line) intensity between 1.0 to 3.0 was considered as validate test.

k) Antigen captures anti-HEV IgA ELISA (in-house): The 96 well plate (Max-absorb-NUNC) was coated with 100 µl per well of HEV antigen (3µg) using 1x PBS and incubated either over-night at 4^0C or 1 hour at 37^0C. After blocking the plate with blocking buffer for 1 hour at 37^0C, plate was washed 4 washes with wash and used for HEV IgA ELISA. 100µl of test serum at 1:20 dilution was added to each well and 2 positive and 3 negative serum controls were kept in each plate. After 30 minutes of incubation at 37^0C plate was washed again 6 times using wash buffer and 100µl of mouse anti- human IgA monoclonal antibody conjugated with HRP (1:3000) was added to each well and further incubated at 37^0C for 30 minutes. Following incubation, the plate was washed again 6 times and 100µl of TMB (Sigma, USA) was added to each well and incubated for 10 minutes at room temperature. Further colour development was stopped using sulphuric acid (2.75%) and the plate was read in an ELISA reader at 450 nm. The cut-off value was calculated by an average OD of negative samples plus 0.2.

l) All other HEV ELISAs (IgM 2.0, 3.0 and IgG 3.0) were conducted according to manufacturer's instruction.

m) Comparative analytical sensitivity of HEV-IgA rapid test (POCT):

The analytical sensitivity of HEV-IgA POCT was studied by using strong, medium and weak HEV IgA positive serum samples. Serum samples were diluted at 1:5 to 1:160 in whole blood, serum or plasma from a healthy person negative to HEV antibodies (IgG, IgM and IgA).Twenty-microlite (25µl) of each diluted sample was

then added onto the cassette sample pad and test was performed as described above.

Example 2: In-house comparative analysis of HEV-IgA POCT against HEV IgM, ELISAs (2.0 and 3.0) and HEV IgG ELISA.

The level of sensitivity of Den- IgA POCT carried out using HEV positive sera (PCR and IgM ELISA) obtained from R & D lab and showed that HEV-IgA POCT showed 99% sensitivity and 97% specificity respectively (See Table-1 which shows diagnostic performances of HEV IgA POCT in 2x2 table).

Table-1 Diagnostic performance of HEV IgA POCT using 2x2 table

		HEV IgM ELISA (3.0) and PCR		
		POS	*NEG*	*Total*
HEV IgA POCT	***POS***	***258***	***5***	***203***
	NEG	***2***	*211*	*213*
	Total	***260***	*216*	*416*

Diagnostic Parameters	**Per cent**	**95% Confidence Level**
Sensitivity	99.23%	97.24% to 99.88%
Specificity	97.69%	94.68% to 99.24%
Positive Likelihood Ratio	42.87	18.02 to 101.95
Negative Likelihood Ratio	0.01	0.00 to 0.03
Disease prevalence	54.62%	50.03% to 59.16%
Positive Predictive Value	98.10%	95.62% to 99.37%
Negative Predictive Value	99.06%	96.64% to 99.86%

The overall sensitivity and specificity of HEV IgA POCT is very good and is one of the appropriate diagnostic tools for the acute diagnosis of HEV infection.

Example 3: The performance of HEV-IgA POCT was further evaluated using against in-house IgM and commercially available IgM rapid test (MP Biomedicals) using 84 HEV positive and 66 negative sera which showed 100% agreement in terms of sensitivity and specificity (See Table-2 which shows the comparative performance of HEV IgA POCT against HEV IgM rapid test).

Table-2 Comparative performance of HEV IgA POCT and HEV IgM Rapid test (2x2 table)

		HEV IgA POCT		
		POS	*NEG*	*Total*
HEV IgM Rapid Test	***POS***	***111***	***0***	***111***
	NEG	***1***	*80*	*81*
	Total	***112***	*80*	*192*

Diagnostic Parameters	**Per cent**	**95% Confidence Level**
Sensitivity	99.11%	95.11% to 99.85%
Specificity	100.00%	95.45% to 100.00%
Positive Likelihood Ratio		
Negative Likelihood Ratio	0.01	0.00 to 0.06
Disease prevalence	58.33%	51.02% to 65.39%
Positive Predictive Value	100.00%	96.70% to 100.00%
Negative Predictive Value	98.77%	93.29% to 99.79%

The overall diagnostic agreement of HEV IgM Rapid test and HEV IgA POCT is close and only one sample positive to PCR and HEV IgA POCT was found negative with HEV IgM Rapid test.

Example 4: The performance of HEV IgA POCT was also compared with in-house HEV IgA ELISA and was found 100% agreement in terms of sensitivity and specificity (See Table-3 which shows the comparative performance of HEV-IgA POCT with HEV IgA ELISA (in-house)).

Table-3 Comparative performance of HEV IgA POCT against HEV IgA ELISA using 2x2 table

		HEV IgA ELISA		
		POS	NEG	Total
HEV IgA POCT	**POS**	**84**	**0**	**84**
	NEG	**0**	91	91
	Total	**84**	91	175

Sensitivity	100.00%	95.45% to 100.00%
Specificity	100.00%	95.99% to 100.00%
Positive Likelihood Ratio		
Negative Likelihood Ratio	0.00	
Disease prevalence	46.78%	39.13% to 54.55%
Positive Predictive Value	100.00%	95.45% to 100.00%
Negative Predictive Value	100.00%	95.99% to 100.00%

In 2x2 table analysis of HEV IgA POCT and HEV IgA ELISA showed 100% agreement in diagnostic performance which indicates the consistency of IgA molecule produced during acute of HEV infection.

Example 5: Specificity of HEV-IgA POCT using sera collected from non-HEV febrile illness including HBV and HCV infections.

The cross-reactivity of HEV IgA POCT was carried against non-HEV viral hepatitis infections (HAV, HBV and HCV) along with other five febrile illnesses. HBV positive sera were confirmed as HBV using both HBsAg rapid test (MP Biomedicals) as well as antigen ELISA (Biokit Ag ELISA) while HCV positive samples were positive to PCR technique. (See Table-4 which shows specificity of HEV IgA POCT against non-HEV viral hepatitis and other febrile illness).

Table 4: Performance with Cross-reactive serum samples

Serum group and patient status	No. of positive & Performance %
From other hepatitis patients:	
HCV antibody positive	1/45
HBV antibody positive	3/66
HAV antibody positive	1/37
Other conditions	
Rheumatoid Arthritis	0/10
High Bilurubin	1/10
Total	6/168
Total Specificity	**96.43%**
	(95% CL: 92.38% to 98.67%)

HEV IgA POCT showed very low level of reactivity against other viral hepatitis serum samples. Serum samples positive with HEV IgA POCT need to be confirmed with other HEV antibodies serological Tests like ELISA.

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CLAIMS:

1. A method for detecting exposure of a subject to an anti-hepatitis E virus ("HEV") thereof, said method comprising: contacting a biological sample from the subject with a mixture of HEV specific immunogenic components; and determining the presence of a complex that forms between a binding partner in the biological sample and a HEV specific immunogenic component; characterizing the binding partner in the complex; and correlating the binding partner to exposure to HEV.