CHAPTER ONE
INTRODUCTION AND OBJECTIVES

1.1. Introduction

Hepatitis E represents a significant public health and economic burden particularly in countries where the absence of sanitation infrastructures, or their breakdown as a consequence of wars or natural disasters, brings the hygienic conditions below a safe level (Boccia *et al*., 2006; Panda *et al*., 2007).

Hepatitis E Virus (HEV) causes large outbreaks and sporadic cases of acute hepatitis. Scientists estimated that one-third of the world's population has been infected with HEV (Jong-hoon *et al*., 2014). It is the most or second most common cause of acute viral hepatitis among adults in Asia, Middle East, and Africa (Purcell and Emerson, 2008; Kamar *et al*., 2012).

HEV is a spherical, non-enveloped, single-stranded RNA virus belonging to the *Hepeviridae* family and the *Hepevirus* genus (Aggarwal, 2012). The existing evidence suggests all human HEV strains belong to a single serotype (Emerson and Purcel, 2007). Although there are at least four genotypes (1–4) (Schlauder and Mushahwar, 2001 ; Lu *et al*., 2006) with 24 subtypes (Lu *et al*., 2006; Teshale and Hu, 2011; Purdy and khudyakov, 2011). Genotype 1 and 2, restricted to humans, are associated with epidemics in developing countries with poor hygiene conditions (mainly genotype 1 in Asia and Africa and genotype 2 in Central America and Central Africa) (Purcell and Emerson, 2010). Genotypes 3 and 4 infect swine and humans, the latter

being infected by the consumption of contaminated food, resulting in sporadic cases of hepatitis E in both developing and industrialized countries (Pavio *et al*., 2010; Zhang *et al*., 2011; Cossaboom *et al*., 2011; Colson *et al*., 2010).

HEV is mainly an enterically transmitted virus that causes waterborne epidemics in developing countries and sporadic cases in developed countries. There are four reported routes of transmission, namely waterborne, zoonotic (foodborne), bloodborne and perinatal (Mushawar, 2008). Person-to-person transmission of HEV is rare compared with Hepatitis A Virus, which might be related to the low amount of intact HEV particles present in a patient's stool (Previsani and Lavanchy, 2001).

The incubation period of HEV infection ranges from 15 to 60 days (mean 40 days). Research among non-human primates showed a direct association between infective dose and severity of disease with an inverse relation to the incubation period. HEV causes a range of clinical manifestations including asymptomatic infection, unapparent infection, and icteric hepatitis. The clinical presentation of acute hepatitis E is indistinguishable from other acute viral hepatitis. Hepatitis E is an acute disease with abrupt onset of non specific symptoms followed by right upper quadrant pain, jaundice, anorexia, malaise, nausea and vomiting. Asymptomatic infections occur more often among children than adults (Eyasu and Dale, 2011).

Diagnosis of hepatitis E was made by observance of typical symptoms with elevated aminotransferases, presence of IgM anti-HEV, and rising titer of IgG anti-HEV in exclusion of other etiology of acute hepatitis. Enzyme immunoassays (EIA) for HEV antibodies are based on detection of antibodies against the highly conserved capsid

protein. Immunoglobulin M (IgM) anti-HEV appears during the early stage of infection and detectable at 1 to 3 weeks after acute infection of immune-competent patients while seroconversion may be delayed up to 6 to 10 months in immunocompromised patients (Sook-Hyang, 2011). Detection of HEV RNA in serum or stool using nested or realtime PCR is the most sensitive and definitive diagnostic test; however, the viremic period is short (10 to 30 days after onset of symptoms) and detection of HEV RNA within the proper time for diagnosis in the clinical setting is not easy, while fecal shedding of virus may last longer with high viral titer compared to viremia in the blood (Inoue *et al*., 2006). Although there is no commercially available HEV RNA PCR assay, diagnosis of hepatitis E should be made with repeated use of anti-HEV (both of IgM and IgG) and preferably detection of HEV RNA in stool or blood (Sook-Hyang, 2011).

Treatment for acute hepatitis E is generally supportive. Chronic hepatitis E in solid organ transplant (SOT) recipients on immunosuppressive treatment has been successfully treated by withdrawal or reduction of immunosuppressive drugs, administration of ribavirin, administration of interferon or a combination of these measures (Pischke *et al*., 2013; Kamar *et al*., 2012).

HEV infection can be prevented by providing clean drinking water and improving the sanitary infrastructure in developing countries. HEV3 infection may be prevented by avoiding eating undercooked meat, especially pork products. The virus is completely inactivated when heated above 70°C (Nassim *et al*., 2014).

1.2. Rationale

Hepatitis E Virus (HEV) infection is a newly recognized serious threat to global public health. Africa is suspected to be among the most severely affected regions in the world (Jong-hoon *et al*., 2014). However, there are a few studies available to determine the prevalence of HEV infection among Sudanese people, especially in food handlers.

Therefore, the detection of HEV among food handlers help to minimize spread of infection and make guideline to public health decision –making.

1.3. Objectives

1.3.1. General objective

To detect Hepatitis E Virus antibodies among food handlers in Khartoum Locality.

1.3.1. Specific objectives

1. To determine the infected food handler workers in Khartoum with HEV.

2. To estimate prevalence of HEV among food handlers.

3. To determine risk factors associated with HEV among food handlers.

CHAPTER TWO
LITERATURE REVIEW

Hepatitis E Virus (HEV) is a major public health problem, especially in resource limited countries. In an annual estimate in 2005, there had been 20.1 million HEV infections, resulting in 70 000 deaths and 3000 intrauterine fetal deaths, and a possibility of 0.019 and 0.198 mortality in symptomatic illness for non-pregnant and pregnant patients, respectively. HEV infections can occur either in the form of epidemics or sporadic cases. Epidemics of HEV have been reported in African countries, including Sudan, Ethiopia, Somalia, Chad, the Democratic Republic of the Congo, and Uganda (Duria *et al*., 2013).

In June 2004, a large hepatitis E outbreak occurred in western Darfur, Sudan. A total of 2621 cases were reported between 26 June and 31 December 2004 in Mornay Internally Displaced Persons Camp (78,800 inhabitants). The medical nongovernmental organization Médecins Sans Frontières was the main health care provider in the camp, with a hospital and 2 outpatient departments. The epidemiological investigation suggested an increased risk of HEV infection with drinking water (Guthmann *et al*., 2006)

Local study was conducted to verify the frequency of Hepatitis E Virus among pregnant women attending Khartoum hospitals. Enzyme linked immunoassay (ELISA) was done to determine the presence of anti- HEV IgG among 90 pregnant women during the period from July to September 2013. HEV IgG antibodies were

detected in 41.1% (37/90). The highest percentages were recorded in the second and third trimesters of pregnancies (37.8% and 48.7%) respectively (Zuhal *et al*., 2014).

A study in Darfur was carried out to determine the seroprevalence of HEV infection among displaced persons. The samples were tested for immunoglobulin G (IgG) and immunoglobulin M (IgM) antibody to HEV (serum) and for amplification of the HEV genome (serum and stool) over 6 months, 253 HEV cases were recorded at the hospital, of which 61 (24.1%) were in pregnant women. A total of 72 cases (39.1% of those for whom clinical records were available) had a diagnosis of hepatic encephalopathy. Of the 45 who died (case-fatality ratio, 17.8%), 19 were pregnant women (specific case-fatality ratio, 31.1%). Acute hepatitis E was confirmed in 95% (19/20) of cases sampled; 18 case-patients were positive for IgG (optical density ratio > or = 3), for IgM (optical density ratio >2), or for both, whereas one was negative for IgG and IgM but positive for HEV RNA in serum. The survey identified 220 jaundiced women among the 1133 pregnant women recorded over 3 months (attack rate, 19.4%). A total of 18 deaths were recorded among these jaundiced pregnant women (specific case-fatality ratio, 8.2%) (Boccia *et al*., 2006).

In South Korea a study was conducted to determine hepatitis E seroprevalence in 1848 cases: 1434 slaughter workers and 414 residual products handlers. Anti-HEV IgG and IgM were measured using HEV IgG and IgM enzyme-linked immunospecific uassay kits and HEV antigen was measured by reverse transcription polymerase chain reaction (RT-PCR). The seropositivity of anti-HEV IgG was 33.5% (slaughter

workers 32.8% and residual products handlers 36.2%) and among the seropositive individuals the seroprevalence of anti-HEV IgM was 0.5% (slaughter workers 0.5%, residual products handlers 0.7%). The response rate of HEV-antigen as measured by RT-PCR was 0.2% (Byung-Seok *et al*., 2015).

A cross-sectional study in Nigeria was undertaken to determine the epidemiology, seroprevalence and associated risk factors of HEV. A total of 462 subjects were used for the study, categorized into four groups: apparently healthy persons, pregnant women, HIV positive subjects and animal handlers. Blood samples were collected and analyzed for HEV antibodies (IgG and IgM) using enzyme-linked immunosorbent assay (ELISA) technique. The overall seroprevalence of IgG and IgM was 42.7 and 0.9%, respectively. Animal handlers had the highest seroprevalence (66.7%) (Surajudeen *et al*., 2014).

Study in Iran investigated 324 chronic hemodialysis patients attending three different units in the city of Tabriz, for anti-HEV antibody. A specific solid- phase enzyme-linked immunoassay was used. The overall seroprevalence of hepatitis E was 7.4 % (Mahnaz *et al*., 2005).

Another study in Iran was carried out to determine the seroprevalence of HEV infection among volunteer blood donors. A total of 530 consecutive blood donor samples collected from Blood Transfusion Organization, Central Province of Iran. All samples were tested for the presence of IgG Hepatitis E antibody (anti-HEV) using enzyme-linked immunosorbent assay (ELISA). From 530 blood donors, 91.9% were

males and 8.1% were females. Overall, anti-HEV was found in 76 of 530 samples (14.3%). There was no significant difference in HEV seropositivity between the subjects regarding gender and area of residence (urban vs. rural). Anti-HEV was distributed among all age groups. Although people aged 31-50 years had the highest prevalence, but there was no statistical difference between the age groups (Hassan *et al.*, 2013).

A study in France was carried out to determine HEV seroprevalence in 593 forestry workers and 421 wild boars. Anti-HEV was detected in 31% of the forestry workers and 14% of the wild boars (Audrey *et al*., 2012).

In Zambia a study was conducted to determine prevalence of hepatitis E. Blood samples from 194 children and 106 adults were examined for immunoglobulin G and immunoglobulin M antibodies for HEV. HEV data were correlated with HIV status and morphometric analysis of small intestinal biopsies. Seroprevalence rose throughout childhood, from 8% in children aged 1–4 years, to 36% in children aged 10–14 years. In adults, the overall prevalence was 42%, with 28% in HIV-seronegative adults and 71% in HIV-seropositive adults (odds ratio, 6.2; 95% confidence interval, 2.2 – 18; $P = .0001$) (Choolwe *et al*., 2014).

A cross-sectional study to determine the frequency of anti-HEV IgG antibodies in 273 adults living in rural Durango, Mexico, was carried out using an enzyme-linked immunoassay. One hundred (36.6%) of the 273 rural adults (mean age: 39.85 ± 17.15 years) had anti-HEV IgG antibodies (Cosme *et al*., 2014).

Study in China investigated seroprevalence and molecular characteristics of hepatitis E virus (HEV) in the illegal blood donors (IBDs). A total of 546 blood samples were collected from the IBDs in Maanshan city, a questionnaire was completed by each subject, detailing the age, sex, and periods of blood or plasma donation. The seropositive samples were subjected to nested reverse transcription-polymerase chain reaction and sequencing to analyze HEV partial genome. The prevalence of IgG and IgM HEV antibody in IBDs was 22.7% and 1.8%, respectively (Xian-Feng *et al.*, 2012).

CHAPTER THREE
MATERIALS AND METHODS

3.1. Study design

3.1.1. Type of study

This is a cross-sectional study.

3.1.2. Study area

The study was conducted in Khartoum Locality.

3.1.3. Study duration

This study was conducted during the period from January to May, 2015.

3.2. Study population

Food handlers, both males and females with different ages were the source of samples.

3.3. Ethical consideration

This study was approved by the College Ethical Committee Board, Sudan University of Science and Technology. An informed consent was obtained from each food handlers before collecting the demographic and clinical data (Appendix 1).

3.4. Sample size and sampling technique

3.4.1. Sample size

A total of ninety (n=90) food handlers working in Khartoum Locality were included.

3.4.2. Sampling technique

This study was based on non-probability convenience sampling technique.

3.5. Data collection

Data were collected by interviewing questionnaire (Appendix 2).

3.6. Laboratory methods

3.6.1. Collection of blood samples

A volume of 5 ml of whole blood was obtained from each food handler by venipuncture and collected into a plain container.

3.6.2. Sample processing

Blood samples were then centrifuged at 3000 *g* for 5 min. Sera were gently separated into cryotubes (Nalgene®) and stored at −20°C until the serological analysis.

3.6.3. Detection of the virus

The serum samples of the participants were analyzed for anti-HEV IgG antibodies by a commercially available enzyme immunoassay “HEV-IgG ELISA” kit (EUROIMMUN medizinische labordiagnostika AG, Germany). The assays were performed following the instructions of the manufacturer. Positive and negative

controls were included in each assay. According to the information included in the kit's insert (Appendix 3), the immunoassay used has a sensitivity of 100% and a specificity of 100%.

3.6.4. Principle

The ELISA test kit provides a quantitative or semiquantitative *in vitro* assay for human antibodies of the IgG class against hepatitis E antigens. The test kit contains microtiter strips each with 8 break-off reagent wells coated with recombinant antigens of hepatitis E virus. In the first reaction step, diluted patient samples are incubated in wells. In case of positive samples, specific IgG antibodies (also IgA and IgM) will bind to the antigens. To detect the bound antibodies, a second incubation is carried out using an enzyme-labeled anti-human IgG (enzyme conjugate) catalyzing reaction.

3.6.5. Procedure

All reagents and specimens were settled to the reach room temperature. 100µl of the calibrators, positive and negative controls or diluted patient samples were added into the individual microplate wells according to the pipetting protocol. Incubated for 30 minutes at room temperature (+18c° to +25c°). The wells were emptied and subsequently washed 3 time using 300µl of working strength wash buffer (Tween 20) for each wash. Then 100µl of enzyme conjugate (peroxidase-labelled anti human IgG) was added into each of the microplate wells. Incubated for 30 minutes at room temperature (+18c°to +25c°). The wells were emptied and washed as described. 100µl of chromogen/substrate solution (TMB/H_2O_2) was added into each of the microplate

wells. Incubated for 15 minutes at room temperature (+18c° to +25c°). Finally 100µl of stop solution (0.5M Sulphuric acid) was added into each of the microplate wells in the same order and at the same speed as the chromogen /substrate solution was introduced. Photometeric measurement of colour intensity should be made at a wavelength of 450 nm and a reference wavelength between 620 nm and 650 nm within 30 minutes of adding the stop solution.

3.6.6. Quality control

Reagent, standard and control were checked for storage, stability and preparation before starting work.

3.7. Date analysis

The data that collected from questionnaire and laboratory results were analyzed by SPSS version 15 computerized program.

CHAPTER FOUR
RESULTS

The study was carried out during the period from January to May 2015 to detect Hepatitis E Virus among food handlers working in Khartoum.

A total of ninety food handlers, 87 (96.7%) males and 3 (3.3%) females were enrolled in this study (Table1). Among these 67 (74.4%) were Sudanese and 23 (25.6%) Ethiopian. Their ages ranged from 19 to 57 years, with a mean age 29.5 years; 17 (18.9%) were less than 25 years old, 72 (80%) were 26 to 41 years old and one (1.1%) was above to 42 years old (Table 1). Almost all food handlers 85 (94.4%) had medical check-up previously.

The serodetection revealed that anti-HEV IgG among food handlers was 10 (11.1%) positive, 1 (1.1%) borderline while 79 (87.8%) negative (Table 2). The seroprevalence was high (60%) among food handlers aged 26 - 41 years, followed by 10 - 25 years (40%), and 42 - 57 years (0%) . However, there was no statistically significant difference in prevalence of anti-HEV IgG by age groups (P = o.47). The seroprevalence was also higher in Sudanese food handlers (90%) than Ethiopian (10%) (Table3). There was no significant differences between the prevalence of anti-HEV antibodies in Sudanese and Ethiopian food handlers (P = 0.4). P. values ≤ 0.05.

Table 1. Distribution of food handlers according to age groups and gender

Age group (year)	Gender		Total
	Male No. (%)	Female No. (%)	
10 – 25	16 (17.8%)	1 (1.1%)	17 (18.9%)
26 – 41	70 (77.8%)	2 (2.2%)	72 (80%)
42 – 57	1 (1.1%)	0	1 (1.1%)
Total	**87 (96.7%)**	**3 (3.3%)**	**90 (100%)**

Table 2. HEV-IgG detection among food handlers relation to age

Age group (year)	Result		
	Positive NO. (%)	Borderline NO. (%)	Negative NO. (%)
10 – 25	4 (4.4%)		13 (14.5%)
26 – 41	6 (6.7%)	1 (1.1%)	65 (72.2%)
42 – 57	0		1 (1.1%)
Total	**10 (11.1%)**	**1 (1.1%)**	**79 (87.8%)**

Table 3. Distribution of sero-positivity of food handlers according to nationality

Nationality	No.	%
Sudanese	9	90
Ethiopian	1	10
Total	10	100

CHAPTER FIVE
DISSCUSSION

5.1. Discussion

Hepatitis E Virus (HEV) infection represents an important public health concern in many developing countries, where it is primarily transmitted through the orofecal route due to inadequate sanitary conditions and contaminated water supplies (Worm *et al*., 2002; Meng, 2010).

This study investigated HEV among food handlers working in Khartoum Locality. Ninety food handlers, (67 (74.4%) Sudanese and 23 (25.6%) Ethiopian) were included in this study. Of them 87 (96.7%) were males, and 3 (3.3%) females, with mean age of 29.5 years. The sero-positive HEV IgG antibody among food handlers was 10 (11.1%). This result was less than that reported among pregnant women in Khartoum (41.1%) and Darfur (31.1%) (Zuhal *et al*., 2014; Boccia *et al*., 2006). The differences between these two groups could be due to the fact that pregnant women considered to be potential risk factor for the virus (Mushahwar, 2008). The result was also less than that reported in South Korea (33.5%), Nigeria (42.7%), Zambia (42%), France (31%), China (22.7%) and Mexico (36.6%) but higher than that reported in Iran (7.4%) (Byung-Seok *et al*., 2015; Surajudeen *et al*., 2014; Choolwe *et al*., 2014; Audrey *et al*., 2012; Xian-Feng *et al*., 2012; Cosme *et al*., 2014; Mahnaz *et al*., 2005). These differences in prevalence rate could be due to differences in the population or the size of the sample studied. Other reasons might be lie in the differences in socioeconomic,

cultural and hygienic and climatic factors across geographic. These factors need to be further evaluated.

The prevalence of HEV IgG in the different age groups of food handler revealed a high prevalence in the group 26 - 41year olds. These results were similar to those obtained by Hassan *et al* (2013) in Iran which revealed a high prevalence in aged group 31 –50 years. The increase prevalence in these aged groups might be attributed to the high exposure of virus due to activates of these group.

Although no statistically significant difference was observed with regard to nationality, the current study recorded proportionately higher prevalence among Sudanese food handlers than Ethiopian. Logical reasoning could be the members of Sudanese in this study were higher than Ethiopian.

Therefore, a population-based study to confirm or exclude this speculation is urgently required. In addition, the sanitary conditions under which food handlers working need to be improved.

5.2. Conclusion

The study concluded that Hepatitis E Virus (HEV) circulates at low but considerable levels especially among food handlers; that may be a source of outbreak. The seroprevalence of HEV was higher in Sudanese food handlers than Ethiopian and among food handlers aged 26 – 41 years. However, there was no statistically significant difference in HEV seropositivity between the subjects regarding to

nationality ($P = 0.40$) (Sudanese vs. Ethiopian) and age groups ($P = 0.47$), *P*. values $\leq$ 0.05.

5.2. Recommendations

1. Further studies with large sample size are needed to evaluate the risk factors associated with HEV among food handlers.

2. Reinforce the importance of food safety and provide training for food handlers to reduce the risk of HEV and other enteric infections.

3. Raise the awareness of general public about the risks of HEV and other enteric infections through various channels and advise the importance of good personal and food hygiene.

4. Food handlers should be considered for prophylaxis (HEV vaccine).

5. Finally, further nationwide study is required to validate the results of the present study.

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APPENDICES

Appendix 1

INFORMED CONSENT

Serodetection of Hepatitis E Virus among Food Handlers in Khartoum Locality

By

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Purpose of study

The purpose of this study will be to detect Hepatitis E virus antibodies among food handlers working in Khartoum Locality.

Procedures

5ml blood samples will be collected by venipuncture in dry tube. Blood samples will then centrifuged at 3000 *g* for 5 min. Sera were gently separated into cryotubes (Nalgene®) and stored at −20°C until the serological analysis.

Benefits of study

The detection of HEV among food handlers that helps to minimize spread of infection and make guideline to guide public health decision –making.

The choice whether or not to donate your specimens and information is your to make. Your decision will not affect your medical care. It also will not affect wether you can take part in other research projects. Please read this form carefully, and ask any questions you have before signing

Participant's signature ……

Appendix 2

QUESTIONNAIRE

1. Name…….

2. Gender male □ female □

3. Age ……. …….

4. Nationality ……..

5. Type of food handler……..

6. Medical checkup yes □ No □

7. Case details

Symptom	Onset date	resolution date
• Jauntice	…….	…….
• Fever	…….	…….
• Abdominal pain	……..	……..
• Vomiting	……...	…….
• Nausea	……..	…….
• Diarrhea	……	…….

8. Did you travel abroad in the two months before falling ill?

YES □ *NO* □

If you answered YES to Question 8:

Which country/countries did you travel to? ……

9.Result…….……

……

Appendix 3

EUROIMMUN Medizinische Laboratoriumsdiagnostika AG

Anti-Hepatitis E Virus (HEV) ELISA (IgG)
Test instruction

ORDER NO.	ANTIBODIES AGAINST	IG-CLASS	SUBSTRATE	FORMAT
EI 2525-9601 G	Hepatitis E antigens	IgG	Ag-coated microplate wells	96 x 01 (96)

Indication: Infections with hepatitis E virus and associated diseases such as acute hepatitis or icterus.

Application: The Anti-Hepatitis E Virus ELISA (IgG) is based on recombinant target antigens of hepatitis E virus genotypes 1 and 3. The careful selection of antigens provides highly specific detection of anti-HEV antibodies of class IgG. Since the standard curve of the Anti-Hepatitis E Virus ELISA (IgG) is largely based on the WHO standard (NIBSC 95/584), the assay allows quantitative result analysis in international units (IU/ml).

Principles of the test: The ELISA test kit provides a quantitative or semiquantitative in vitro assay for human antibodies of the IgG class against hepatitis E antigens in serum or plasma. The test kit contains microtiter strips each with 8 break-off reagent wells coated with recombinant antigens of hepatitis E virus. In the first reaction step, diluted patient samples are incubated in the wells. In the case of positive samples, specific IgG antibodies (also IgA and IgM) will bind to the antigens. To detect the bound antibodies, a second incubation is carried out using an enzyme-labelled anti-human IgG (enzyme conjugate) catalysing a colour reaction.

Contents of the test kit:

Component	Colour	Format	Symbol
1. **Microplate wells** coated with antigens: 12 microplate strips each containing 8 individual break-off wells in a frame, ready for use	–	12 x 8	STRIPS
2. **Calibrator 1** 25 IU/ml (IgG, human), ready for use	red coloured in decreasing intensity	1 x 2.0 ml	CAL 1
3. **Calibrator 2** 10 IU/ml (IgG, human), ready for use		1 x 2.0 ml	CAL 2
4. **Calibrator 3** 2 IU/ml (IgG, human), ready for use		1 x 2.0 ml	CAL 3
5. **Calibrator 4** 0.2 IU/ml (IgG, human), ready for use		1 x 2.0 ml	CAL 4
6. **Positive control** (IgG, human), ready for use	blue	1 x 2.0 ml	POS CONTROL
7. **Negative control** (IgG, human), ready for use	green	1 x 2.0 ml	NEG CONTROL
8. **Enzyme conjugate** peroxidase-labelled anti-human IgG (rabbit), ready for use	green	1 x 12 ml	CONJUGATE
9. **Sample buffer** ready for use	light blue	1 x 100 ml	SAMPLE BUFFER
10. **Wash buffer** 10x concentrate	colourless	1 x 100 ml	WASH BUFFER 10x
11. **Chromogen/substrate solution** TMB/H_2O_2, ready for use	colourless	1 x 12 ml	SUBSTRATE
12. **Stop solution** 0.5 M sulphuric acid, ready for use	colourless	1 x 12 ml	STOP SOLUTION
13. **Test instruction**	–	1 booklet	
14. **Quality control certificate**	–	1 protocol	
LOT Lot	CE	Storage temperature	
IVD In vitro determination		Unopened usable until	

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Preparation and stability of the reagents

Note: All reagents must be brought to room temperature (+18°C to +25°C) approx. 30 minutes before use. After first use, the reagents are stable until the indicated expiry date if stored at +2°C to +8°C and protected from contamination, unless stated otherwise below.

- **Coated wells:** Ready for use. Tear open the resealable protective wrapping of the microplate at the recesses above the grip seam. Do not open until the microplate has reached room temperature to prevent the individual strips from moistening. Immediately replace the remaining wells of a partly used microplate in the protective wrapping and tightly seal with the integrated grip seam (Do not remove the desiccant bag).
  Once the protective wrapping has been opened for the first time, the wells coated with antigens can be stored in a dry place and at a temperature between +2°C and +8°C for 4 months.
- **Calibrators and controls:** Ready for use. The reagents must be mixed thoroughly before use.
- **Enzyme conjugate:** Ready for use. The enzyme conjugate must be mixed thoroughly before use.
- **Sample buffer:** Ready for use.
- **Wash buffer:** The wash buffer is a 10x concentrate. If crystallization occurs in the concentrated buffer, warm it to 37°C and mix well before diluting. The quantity required should be removed from the bottle using a clean pipette and diluted with deionized or distilled water (1 part reagent plus 9 parts distilled water).
  For example: For 1 microplate strip, 5 ml concentrate plus 45 ml water.
  The working strength wash buffer is stable for 4 weeks when stored at +2°C to +8°C and handled properly.
- **Chromogen/substrate solution:** Ready for use. Close the bottle immediately after use, as the contents are sensitive to light. The chromogen/substrate solution must be clear on use. Do not use the solution if it is blue coloured.
- **Stop solution:** Ready for use.

Storage and stability: The test kit has to be stored at a temperature between +2°C to +8°C. Do not freeze. Unopened, all test kit components are stable until the indicated expiry date.

Waste disposal: Patient samples, calibrators, controls and incubated microplate strips should be handled as infectious waste. All reagents must be disposed of in accordance with local disposal regulations.

Warning: The controls and calibrators used have been tested negative for HBsAg, anti-HCV, anti-HIV-1 and anti-HIV-2 using enzyme immunoassays and indirect immunofluorescence methods. Nonetheless, all materials should be treated as being a potential infection hazard and should be handled with care. Some of the reagents contain the toxic agent sodium azide. Avoid skin contact.

Preparation and stability of the patient samples

Sample material: Human serum or EDTA and heparin plasma.

Stability: Patient samples to be investigated can generally be stored at +2°C to +8°C for up to 14 days. Diluted samples should be incubated within one working day.

Sample dilution: Patient samples are diluted **1:101** in sample buffer. For example: dilute 10 µl serum in 1.0 ml sample buffer and mix well by vortexing (sample pipettes are not suitable for mixing).

NOTE: Calibrators and controls are prediluted and ready for use, do not dilute them.

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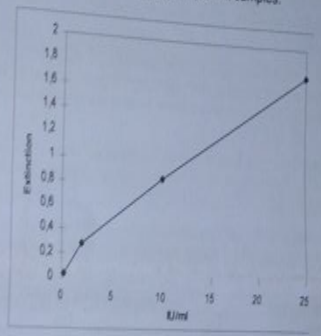
EUROIMMUN recommends interpreting results as follows:

Ratio <0.8:	**negative**
Ratio ≥0.8 to <1.1:	**borderline**
Ratio ≥1.1:	**positive**

In cases of borderline test results, an additional patient sample should be taken 7 days later and re-tested in parallel with the first patient sample. The results of both samples allow proper evaluation of titer changes.

Quantitative: The standard curve from which the concentration of antibodies in the patient samples can be taken is obtained by point-to-point plotting of the extinction values measured for the 4 calibrators against the corresponding units (linear/linear). Use "point-to-point" plotting for calculation of the standard curve by computer. The following plot is an example of a typical calibration curve. Please do not use this curve for the determination of antibody concentrations in patient samples.



If the extinction of a serum sample lies above the value of calibrator 1 (25 IU/ml), the result should be given as ">25 IU/ml". It is recommended that the sample be re-tested at a dilution of 1:400. The result in IU/l read from the calibration curve for this sample must then be multiplied by a factor of 4.

The upper limit of the reference range of non-infected persons (**cut-off value**) recommended by EUROIMMUN is **2 International Units (IU/ml)**. EUROIMMUN recommends interpreting results as follows:

<1.6 IU/ml:	**negative**
≥1.6 to <2.2 IU/ml:	**borderline**
≥2.2 IU/ml:	**positive**

For duplicate determinations the mean of the two values should be taken. If the two values deviate substantially from one another the sample should be retested.

For diagnosis, the clinical symptoms of the patient should always be taken into account along with the serological results.

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Incubation

For **semiquantative analysis** incubate **calibrator 3** along with the positive and negative controls and patient samples. For **quantitative analysis** incubate **calibrators 1 to 4** along with the positive and negative controls and patient samples.

(Partly) manual test performance

Sample incubation: (1st)	Transfer 100 µl of the calibrators, positive and negative controls or diluted patient samples into the individual microplate wells according to the pipetting protocol. Incubate for **30 minutes** at room temperature (+18°C to +25°C).
Washing:	Manual: Empty the wells and subsequently wash 3 times using 300 µl of working strength wash buffer for each wash. Automatic: Wash reagent wells 3 times with 450 µl of working strength wash buffer (program setting: e.g. TECAN Columbus Washer "Overflow Modus"). Leave the wash buffer in each well for 30 to 60 seconds per washing cycle, then empty the wells. After washing (manual and automated tests), thoroughly dispose of all liquid from the microplate by tapping it on absorbent paper with the openings facing downwards to remove all residual wash buffer. Note: Residual liquid (> 10 µl) in the reagent wells after washing can interfere with the substrate and lead to false low extinction values. Insufficient washing (e.g., less than 3 wash cycles, too small wash buffer volumes, or too short reaction times) can lead to false high extinction values. Free positions on the microplate strip should be filled with blank wells of the same plate format as that of the parameter to be investigated.
Conjugate incubation: (2nd)	Pipette 100 µl of enzyme conjugate (peroxidase-labelled anti-human IgG) into each of the microplate wells. Incubate for **30 minutes** at room temperature (+18°C to +25°C).
Washing:	Empty the wells. Wash as described above.
Substrate incubation: (3rd)	Pipette 100 µl of chromogen/substrate solution into each of the microplate wells. Incubate for **15 minutes** at room temperature (+18°C to +25°C) (protect from direct sunlight).
Stopping the reaction:	Pipette 100 µl of stop solution into each of the microplate wells in the same order and at the same speed as the chromogen/substrate solution was introduced.
Measurement:	**Photometric measurement** of the colour intensity should be made at a wavelength of 450 nm and a reference wavelength between 620 nm and 650 nm **within 30 minutes of adding the stop solution.** Prior to measuring, slightly shake the microplate to ensure a homogeneous distribution of the solution.

Interference: Haemolytic, lipaemic and icteric samples showed no influence on the result up to a concentration of 10 mg/ml for haemoglobin, 20 mg/ml for triglycerides and 0.4 mg/ml for bilirubin in this ELISA.

Reproducibility: The reproducibility of the test was investigated by determining the intra- and inter-assay coefficients of variation using 4 sera. The intra-assay CVs are based on 20 determinations and the inter-assay CVs on 4 determinations performed in 6 different test runs.

Intra-assay variation, n = 20		
Serum	**Mean value (IU/ml)**	**CV (%)**
1	4.9	5.1
2	11.9	4.5
3	17.5	2.7
4	20.1	3.3

Inter-assay variation, n = 4 x 6		
Serum	**Mean value (IU/ml)**	**CV (%)**
1	5.0	7.6
2	11.4	9.1
3	17.5	5.1
4	20.7	5.3

Specificity and sensitivity: 22 patient samples precharacterized with a commercial Anti-HEV ELISA were examined with the EUROIMMUN Anti-Hepatitis E Virus (HEV) ELISA (IgG). The test showed a specificity of 100% and a sensitivity of 100%.

n = 22		Commercial ELISA		
		positive	borderline	negative
EUROIMMUN Anti-Hepatitis E Virus (HEV) ELISA (IgG)	positive	15	0	0
	borderline	0	0	0
	negative	0	0	7

Reference range: The levels of the anti-hepatitis E virus antibodies (IgG) were analyzed with this EUROIMMUN ELISA in a panel of 500 healthy blood donors (origin: Germany). With a cut-off of 2 IU/ml, 7.8% of the blood donors were anti-HEV positive (IgG).

Clinical significance

Hepatitis E virus (HEV) is an uncoated single (+)-stranded RNA virus of approximately 33 nm, which belongs to the monotypical family of Hepeviridae (previously called Caliciviridae). Several (>5) human pathogenic subtypes (genotypes) of HEV have been described. The most frequent zoonotic genotypes, HEV genotype 3 and HEV genotype 4, are widespread in humans: genotype 3 in the USA, Germany and East Europe and genotype 4 in South East Asia.

Hepatitis E, which was discovered in India in 1980, is a worldwide distributed infection in mammals and humans. Infection usually occurs via a faecal-oral route. Since hepatitis viruses are often detected in wild and domestic pigs, in rabbits, etc, it is assumed that hepatitis E virus can also be transmitted by the consumption of uncooked meat. Studies by the German Federal Institute for Risk Assessment show that, for example, around 15% of wild pigs and around 20% of domestic pigs in Germany are infected with this virus.

Infected persons secrete the virus for several weeks via their faeces. In Germany, acute HEV infections are rare (237 in 2011), but have been increasing since 2009. The mortality rate is on average 0.4%-5%, but in pregnant women is up to 25%. HEV infections occur more frequently in other countries of the western hemisphere. Serological-epidemiological studies show that infections are particularly common in the Middle East, Central Asia, India, South East Asia and Central and South America. Hepatitis E is the second most frequent hepatitis in North Africa and southwest Asia. According to the WHO, 300 million people worldwide of all ages have symptomatic hepatitis E, with 300,000 deaths and 5,200 stillbirths per year.

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Test characteristics

Calibration: The controls of the Anti-Hepatitis E Virus ELISA were calibrated using the WHO reference serum (WHO Reference Reagents Hepatitis E Virus Antiserum, human, 1st IS NIBSC Code 95/584).

For every group of tests performed, the extinction values of the calibrators and the international units determined for the positive and negative controls must lie within the limits stated for the relevant test kit lot. A quality control certificate containing these reference values is included. If the values specified for the controls are not achieved, the test results may be inaccurate and the test should be repeated.

The activity of the enzyme used is temperature-dependent and the extinction values may vary if a thermostat is not used. The higher the room temperature during substrate incubation, the greater will be the extinction values. Corresponding variations apply also to the incubation times. However, the calibrators are subject to the same influences, with the result that such variations will be largely compensated in the calculation of the result.

Antigen: The antigen used is a mixture of recombinant target antigens of hepatitis E virus genotypes 1 and 3 expressed in E. coli.

Linearity: The linearity of the Anti-Hepatitis E Virus (HEV) ELISA (IgG) was determined by assaying 4 serial dilutions of different patient samples. The coefficient of determination R^2 for all sera was > 0.95. The Anti-Hepatitis E Virus (HEV) ELISA (IgG) is linear at least in the tested concentration range (1 IU/ml to 22 IU/ml).

Detection limit: The lower detection limit is defined as the mean value of an analyte-free sample plus three times the standard deviation and is the smallest detectable antibody titer. The lower detection limit of Anti-Hepatitis E Virus (HEV) ELISA (IgG) is 0.1 IU/ml.

Cross reactivity: The quality of the antigen used ensures a high specificity of the ELISA. Sera from patients with infections caused by various agents were investigated with the Anti-Hepatitis E Virus (HEV) ELISA (IgG).

Antibodies against	**n**	**Anti-Hepatitis E Virus (HEV) ELISA (IgG)**
Adenovirus	12	0%
Bordetella pertussis	12	0%
Bordetella FHA	12	0%
Chlamydia pneumoniae	12	0%
CMV	12	0%
EBV-CA	12	0%
HAV	8	0%
HBsAg	5	0%
HBV	14	0%
HCV	8	0%
Helicobacter pylori	12	0%
HSV-1	12	0%
Influenza virus A	12	0%
Influenza virus B	12	0%
Measles virus	12	0%
Mumps virus	12	0%
Mycoplasma pneumoniae	12	0%
Parainfluenza virus Pool	12	0%
Parvovirus B19	12	0%
RSV	12	0%
Rubella virus	12	0%
Toxoplasma gondii	12	0%
VZV	12	0%
Yersinia enterocolitica	12	0%

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The course of an HEV infection in the preliminary stages resembles hepatitis A and also other hepatitides. Hence, differential diagnostic analyses in both clinical and laboratory procedures are essential, particularly since a severe disease course can be fatal. Furthermore, an HEV infection must be differentiated from hepatitis as an accompanying disease of non-liver-specific infections with viruses (e.g. HSV, VZV, CMV, EBV, adeno-, coxsackie-, echo-, or Influenza virus), bacteria (e.g. Leptospirus, Brucella, Salmonella) or other pathogens.

The incubation period for hepatitis E is 3 to 9 weeks. Clinical signs in the acute phase are general malaise, loss of appetite and abdominal pain in 80% of cases, joint aches in 60%, fever, icterus hepatomegaly and dark-coloured urine in 40%, and vomiting and diarrhoea in 20%. The prognosis in pregnant women is poor, for example, development of gestosis. In a rare chronic course, liver transplantation may be necessary as a result of liver failure.

In laboratory tests a high transaminase titer indicates hepatitis. Augmented values of bilirubin and alkaline phosphatase are also found. The course of an acute HEV infection ranges from subclinical to fulminant. In 96% to 99.5% of affected persons hepatitis E heals spontaneously within 1 to 2 months. The majority of HEV infections proceed asymptomatically, as demonstrated by epidemiological-immunoserological studies in blood donors in Germany, which reveal a prevalence of 1.5% to 2.5% (up to 30% in butchers, meat inspectors, pig farmers, vets). For comparison, the prevalence in blood donors in Brazil is 2.3%-4%, in Switzerland 3.2%-4.9%, in the UK 3.6%-16.2%, in France 16.6%, in Denmark 20.6% and in the USA 18.3%.

Hepatitis E virus can be detected in faeces or in blood/serum using PCR. Immunological HEV diagnostics, which deliver reliable results only at a later stage (2nd to 4th week of infection), are based on the determination of specific antibodies of class IgA/IgG/IgM in serum by ELISA (sensitivity 98.5%-99.0%, specificity 99%) using recombinant viral structural proteins (expressed in E. coli). IgM anti-HEV titers drop rapidly after healing, while IgG antibody titers persist for more than 10 years and are an indicator for an existing immunity against reinfection.

There is currently no specific antiviral therapy available. Patients with acute HEV infections should be monitored to exclude enteral spread. A vaccine containing HEV 239 was approved in the People's Republic of China in April 2012. According to the German Infection Protection Act, hepatitis E (suspicion, disease or death or medical laboratory proof) is notifiable.

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HEV IgG kits


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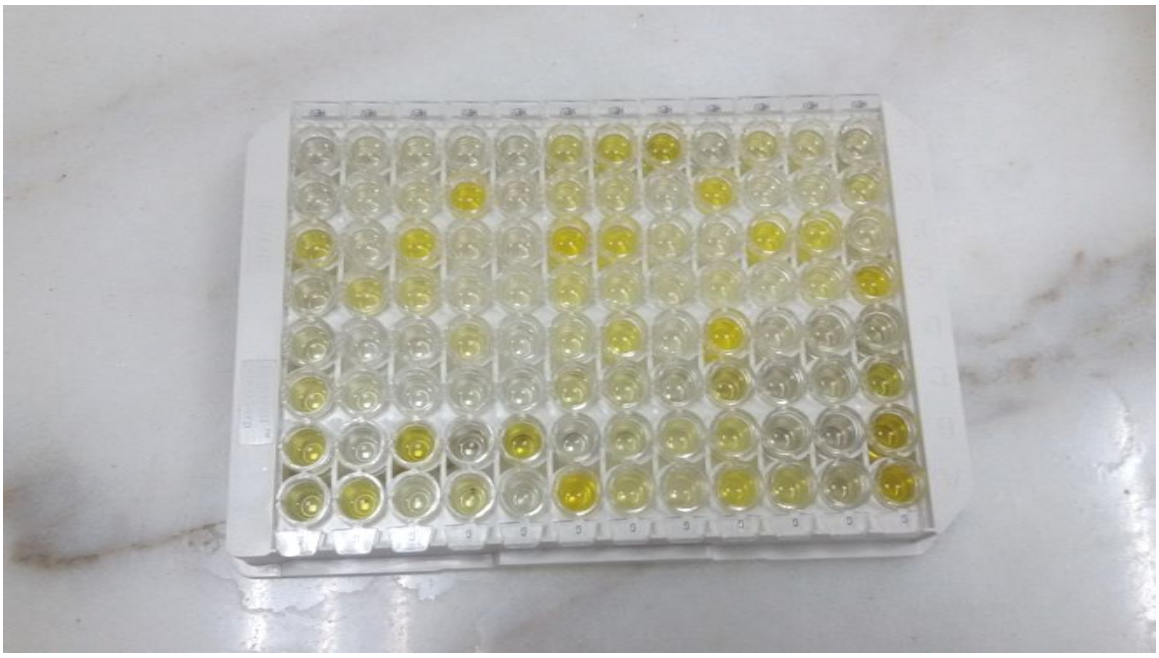
Anti-Hepatitis E Virus (HEV) ELISA (IgG)

Plate

Plate



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