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Evaluating the Risks Posed by Hepatitis E Virus to Blood Supply Safety

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Abstract

Transfusion is an important field of clinical medicine, responsible for saving the lives of millions of people. However, concerns remain for patients' safety from adverse reactions and transfusion transmissible infections (TTIs). While risks from well-known TTIs (such as human immunodeficiency virus, human T-lymphotrophic virus, hepatitis B virus, hepatitis C virus and *Treponema pallidum*) have been reduced in developed nations, developing countries struggle to maintain the minimum screening requirements for these infections. Introduction of stringent donor questionnaires and sensitive screening tests are strategies to minimise risk, however, there is a threat to the safety of the blood supply from emerging infectious diseases.

In 2005, the global annual disease burden for hepatitis E virus (HEV) was estimated to be 20.1 million incident infections, which resulted in an estimate of 3.3 million symptomatic cases, 70,000 deaths and 3,000 still births. This study represented 71% of world's population and was associated with genotype 1 and 2. An improved epidemiological understanding of autochthonous HEV through the adoption of improved tests and testing practices, together with evidence of the virus in blood donors and cases of transfusion transmitted HEV (TT-HEV), have raised concern for transfusion safety. Increasing HEV awareness in recent years in Australia, and limited prevalence and incidence data in the blood donor population, warranted investigation of the risk posed by this virus to the Australian blood supply. This study therefore aimed to evaluate HEV risk to blood safety.

Firstly, this study provided evidence that 5.99% of Australian blood donors had been exposed to HEV. HEV IgG was detected in international travellers (6.38%) and non-travellers (3.37%), indicating the possibility of both imported and locally-acquired HEV in Australia. The study also demonstrated prior HEV exposure was higher in donors with prior donation restrictions in relation to malaria and/or diarrhoea. This suggests the current blood donor management strategy in Australia in relation to malaria and diarrhoea are partially effective in minimizing risk of TT-HEV.

The rate of HEV RNA positivity among 14,799 blood donations was then assessed, with one donation testing positive. The risk of collecting an HEV infectious donation was estimated to be 1 in 14,799 donations (95% CI: 1 in 2,657 to 1 in 584,530). The one HEV

positive sample was HEV genotype 3, which suggests either the donor was infected overseas in a developed country where this genotype occurs, or within Australia via zoonotic transmission. The viral load in the HEV RNA positive sample was estimated to be 15,000 IU/ml. Viral loads between 400 and 250,000 IU/mL have been associated with TT-HEV in the United Kingdom, however, as this study was de-linked, risk of transfusion transmissibility from this donation was unable to be determined.

In addition, countries at higher risk for travel related HEV exposure were identified through a retrospective study of notified HEV cases in Australia. This analysis allowed an assessment of whether the current travel based considerations used by the Australian Red Cross Blood Service adequately manage this risk. This study demonstrated that the majority of notified overseas-acquired HEV infections in Australia were in travellers returning from South Asia, namely India, Bangladesh and Nepal. These countries are endemic for HEV as large water-borne outbreaks occur sporadically. The majority of these countries are also endemic for malaria. This study estimated that countries for which blood donations are restricted following travel due to malaria-risk accounted for 94%of overseas-acquired HEV cases in Australia.

HEV prevalence was also measured in Nepal, a developing nation endemic for HEV, allowing for a comparison between an endemic and presumed non-endemic country (Australia). This study measured HEV IgG prevalence of 41.90% in Nepalese blood donors. Current and recent HEV infection occurred in Nepalese donors, based on 0.11% and 2.98% of donors having HEV antigen and HEV IgM, respectively. Though the water-borne mode of HEV transmission is common in Nepal, this study suggests other modes of transmission including zoonotic transmission may occur in Nepal. HEV infection results in a relatively high mortality rate in pregnant women and can cause chronic infection in immunocompromised individuals. Hence a safe blood supply for these risk groups should also be of concern in developing countries. Priorities for safe blood transfusion vary between Australia and Nepal, based on how common infectious agents are in these countries, as well as the maturity of blood transfusion services, and the cost-effectiveness of screening.

This research has provided an evidence based assessment of the risk HEV currently poses to the safety of the blood supply in Australia. The research findings from this study

will be utilised to develop strategies for managing blood transfusion safety and form the basis of policies to manage the potential threat of TT-HEV. Prevalence and incidence data are also of importance to public health authorities to supplement existing data sets to assist with assessing the current burden of HEV infection in Australia.

Declaration by author

This thesis is composed of my original work, and contains no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

I have clearly stated the contribution of others to my thesis as a whole, including statistical assistance, survey design, data analysis, significant technical procedures, professional editorial advice, and any other original research work used or reported in my thesis. The content of my thesis is the result of work I have carried out since the commencement of my research higher degree candidature and does not include a substantial part of work that has been submitted to qualify for the award of any other degree or diploma in any university or other tertiary institution. I have clearly stated which parts of my thesis, if any, have been submitted to qualify for another award.

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Publications during candidature

Peer-reviewed papers

Shrestha AC, Seed CR, Flower RL, Rooks KM, Keller AJ, Harley RJ, Chan HT, Holmberg JA, Faddy HM. Hepatitis E virus and implications for blood supply safety, Australia. *Emerg Infect Dis*. 2014; 20: 1940-2.

Shrestha AC, Faddy HM, Flower RL, Seed CR, Keller AJ. Hepatitis E virus: do locally acquired infections in Australia necessitate laboratory testing in acute hepatitis patients with no overseas travel history? *Pathology*. 2015; 47: 97-100.

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Chapter 1: Hepatitis E Virus Epidemiology in Australia

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Chapter 3: Hepatitis E Virus RNA and Antigen Detection in Australian Blood Donations

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Keywords

hepatitis E virus, hepatitis, transfusion transmissible infections, emerging infectious diseases, risk, blood safety, Australia, Nepal

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List of Abbreviations

µl	Microlitre
ACT	Australian Capital Territory
Ag	Antigen
ALT	Alanine aminotransferases
ARCBS	Australian Red Cross Blood Service
AST	Aspartate aminotransferases
cDNA	Complementary Deoxyribo Nucleic Acid
CI	Confidence Interval
CMV	Cytomegalovirus
DENV	Dengue Virus
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetic acid
EIA	Enzyme Immuno Assay
ELISA	Enzyme Linked Immuno Sorbent Assay
ELISPOT	Enzyme Linked Immunosorbent Spot
ET-NANB	Enterically Transmitted Non-A Non- B
F	Female
GBV-C	Hepatitis G Virus
HAV	Hepatitis A Virus
HBsAg	Hepatitis B surface Antigen
HBV	Hepatitis B Virus
HCV	Hepatitis C Virus
Hel	Helicase
HEV	Hepatitis E Virus
HHV-8	Human Herpes Virus 8
HIV	Human Immunodeficiency Virus
HRP	Horse Radish Peroxidase
HTLV	Human T-lymphotrophic Virus
IC	Internal Control
IFN -α	Interferon Alpha
IFN-γ	Interferon Gamma
IgG	Immunoglobulin G

IgM	Immunoglobulin M
K_2EDTA	Dipotassium Ethylenediaminetetraacetic acid
M	Male
MeT	Methyl Transferase
mL	Mililitre
MTU	Multi Tube Units
NANB	Non-A Non-B
NAT	Nucleic Acid Testing
nm	Nanometer
NNDS	National Notifiable Diseases Surveillance System
NSW	New South Wales
NT	Northern Territory
OAD	Overseas Arrival Data
OR	Odds Ratio
ORF	Open Reading Frame
PCP	Papain like Cysteine Polymerase
PITs	Pathogen Inactivation Technologies
polyA	Poly Adenylated
QLD	Queensland
RdRp	RNA dependent RNA polymerase
RLU	Relative Lights Units
RNA	Ribonucleic Acid
RRV	Ross River Virus
RT-PCR	Reverse Transcription Polymerase Chain Reaction
S/Co	Sample to Cut-Off Ratio
SA	South Australia
SARs	Special Administrative Regions
STRR	Short Term Resident Return
STVA	Short Term Visitor Arrival
TAS	Tasmania
TMA	Transcription Mediated Amplification
TPHA	*Treponema pallidum* Haemagglutination
TT-HEV	Transfusion Transmitted Hepatitis E Virus
TTIs	Transfusion Transmissible Infections

UK	United Kingdom
USA	United States of America
UTRs	Untranslated Regions
vCJD	Variant Creutzfeldt-Jacob Disease
VIC	Victoria
WA	Western Australia
WHO	World Health Organisation
WNV	West Nile Virus
ZIKV	Zika Virus

Introduction

Transfusion medicine has evolved as a discipline that focuses on the use of blood, blood components and products (1). Blood transfusion is required for patients with various medical conditions such as cancer, blood diseases, anaemia, surgery, orthopaedic, obstetrics and trauma. In Australia, ~34% of all red blood cell transfusions are for haematological and oncological conditions, and ~4% are used for obstetric and gynaecological patients (2). The lifesaving process of transfusion, is however, challenged with adverse reactions (such as acute haemolytic reactions or allergic reactions) following transfusion and transfusion transmissible infections (TTIs) (3).

Introduction of stringent blood donor selection, sensitive screening assays and pathogen inactivation methods have reduced the threat from TTIs (4). However, there remains risk with the failure in quality management systems and also with the emergence of new pathogens that have adapted to the changing environment. Immigration and travel to disease endemic countries has exposed populations to new diseases. Emerging pathogens representing a potential threat to blood safety include West Nile virus (WNV), dengue virus (DENV), Zika virus (ZIKV), *Babesia spp.*, hepatitis E virus (HEV), human herpes virus-8 (HHV-8), *Trypanosoma cruzi* and prions (causing variant Creutzfeldt-Jacob Disease (vCJD)) (5-7). Re-emerging variants of human immunodeficiency virus (HIV), hepatitis B virus (HBV) and hepatitis C virus (HCV) (5), may also pose a threat to a safe blood supply. The risk of these infections to blood safety needs to be assessed separately for each disease, based on the myriad of disease-specific parameters. Depending on the risk, additional blood screening tests or new donation restriction policies may be warranted in the future.

Australia's blood supply is among safest in the world (8). Besides screening for well-known TTIs (HIV, HCV, HBV, *Treponema pallidum*), additional screening for exposure to *Plasmodium spp.* in donors returning from malaria endemic countries (9), and cytomegalovirus (CMV) for specific recipients (10), ensures safety for transfusion recipients. Regular dengue outbreaks in northern Queensland highlights the possible threat from emerging infectious diseases to the Australian blood supply (11).

The emergence of HEV in developed countries has gained global public health importance (12). The high rate of asymptomatic HEV infection, evidence of transfusion transmission and chronic hepatitis in immunocompromised individuals, has raised concern for the international blood transfusion community (13, 14). In Australia, hepatitis E is a nationally notifiable disease (15). However, the true incidence of the disease is not known due to its frequent subclinical infection and the limited published data. In addition, hepatitis E seems to be underdiagnosed as HEV testing was not recommended in non-travellers with acute hepatitis, as it was not thought to be a zoonosis in Australia. Reports of transfusion transmitted HEV (TT-HEV) have highlighted the risk of this virus to transfusion safety (16-18). Despite current management strategies at the Australian Red Cross Blood Service (Blood Service) to safeguard against TTIs, HEV may still pose a risk to the Australian blood supply if transmission increase in Australia, if unknowingly infected donors return from overseas, or if there is an increase in locally-acquired HEV. This thesis is therefore designed to address the knowledge gaps in relation to HEV and the risk posed by this virus to blood supply safety in Australia.

Maintaining a safe blood supply is an important aspect of medicine and an essential activity for the Blood Service. Surveillance studies for emerging infections in a population are important for the timely management and prevention of potential risk to blood safety, as well public health as a whole. Evidence based research via laboratory testing and data analyses and/or modelling are tools allowing for risk assessment. Risk assessment is required in order to determine if a particular emerging infectious disease is a risk to transfusion safety and if so, to identify new strategies to safeguard any risk posed by such a disease.

Thesis Structure

The accumulating reports of the detection of HEV RNA in blood donors and cases of TT-HEV have highlighted the risk posed by this virus to blood safety in the international transfusion community. This, together with increasing awareness globally with respect to this virus and limited prevalence studies on HEV in Australia, warranted an evidence-based evaluation of the risk posed by this virus to the Australian blood supply. The hypothesis of the research was: HEV poses a risk to the safety of the Australian blood supply. This hypothesis was addressed by the following research aims:

1. Determine if Australian blood donors were exposed to HEV
2. Estimate the risk of TT-HEV
3. Assess whether current Australian donor guidelines manage any TT-HEV risk
4. Compare HEV prevalence in Australia with an HEV endemic country

These aims were achieved in the following chapters of the thesis:

Chapter 1: Transfusion Transmissible Infections and Hepatitis E Virus: Literature Review

This chapter provides a comprehensive review on infectious agents transmissible through blood transfusion and introduces HEV as an emerging infectious agent. The chapter also gives an overview of HEV epidemiology in Australia and highlights the rationale for this research.

Chapter 2: Seroprevalence of Hepatitis E virus in Australian Blood Donors and Implications for the Safety of the Blood Supply

In this chapter, HEV seroprevalence in a population of Australian blood donors was measured and risk factors for HEV exposure assessed. These data were utilised to examine the effectiveness of current Australian blood safety strategies for the management of HEV.

Chapter 3: Hepatitis E Virus RNA and Antigen Detection in Australian Blood Donations

This section measured the rate of current HEV infection in Australian blood donations based on antigen and RNA detection. The risk of collecting an infectious donation based

on HEV RNA detection was also estimated. If any HEV RNA positive donations were identified, the infecting genotype of HEV was determined and the viral load measured.

Chapter 4: Overseas-Acquired Hepatitis E Virus in Australia and Assessing the Threat to Blood Supply Safety

In this chapter, trends in notified cases of HEV in Australia were analysed based on demographic details and place of acquisition. Countries considered at higher risk for HEV exposure were identified and the rate of importation estimated based on travel data. The study determined if existing Blood Service travel deferral policies assist with minimising the risk of TT-HEV from imported HEV infections.

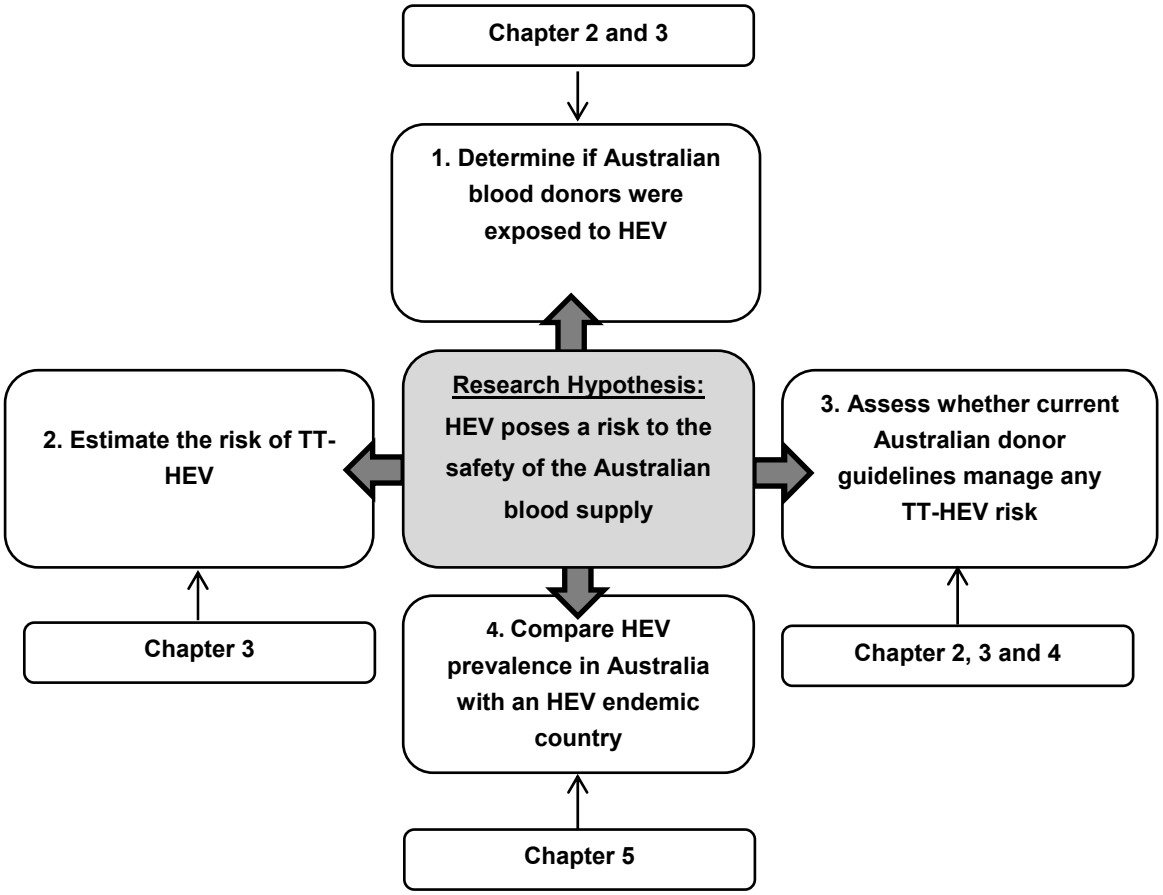
Chapter 5: Hepatitis E Virus Seroprevalence among Blood Donors in Nepal and Analysis of Variables as Possible Risk Factors

In this chapter, HEV seroprevalence in Nepal (a developing country endemic for HEV) was measured and variables as possible risk factors analysed. Differences in HEV exposure status between Nepalese and Australian blood donors were explored.

Chapter 6: Discussion, Conclusion, Risk Management Options and Future Research Directions

This chapter discusses the overall findings from the individual research chapters. Management options to reduce TT-HEV risk and future directions from this study are discussed with overall conclusions made.

Research Hypothesis, Aims and Chapters



Chapter 1. Transfusion Transmissible Infections and Hepatitis E Virus: Literature Review

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Context

This chapter provides a comprehensive review of blood transfusion and risks associated with transfusion. Specifically, the chapter discusses infectious agents transmissible through blood transfusion and introduces HEV as one of the emerging infectious agents of risk to blood supply safety. The chapter also gives an overview of hepatitis E disease in regards to clinical features, epidemiology and laboratory diagnosis. The chapter ends with a rationale for undertaking this study.

A section of this chapter (HEV Epidemiology in Australia) has been published in the journal *Pathology*:

Shrestha AC, Faddy HM, Flower RL, Seed CR, Keller AJ. Hepatitis E virus: do locally acquired infections in Australia necessitate laboratory testing in acute hepatitis patients with no overseas travel history? Pathology 2015; 47: 97-100. Wolters Kluwer Health Lippincott Williams & Wilkins.

1.1. Transfusion Medicine

Blood is a vital component of life. Blood donation is an important practice saving the lives of millions of people. Blood transfusion is the therapeutic use of blood, blood components (red blood cells, platelets, clinical plasma) or blood products (such as albumin, intravenous immunoglobulin, Anti-D, factor concentrates etc.) (1). Blood transfusion is routine for numerous clinical practices, from surgery, trauma, cancer, and anaemic care, to preventive measures such as haemolytic disease of the newborn. With scientific and clinical advances, transfusion practice has evolved as a separate discipline referred to as 'Transfusion medicine' (19).

Blood transfusion, however, is not without risk. The risks include adverse effects, incorrect component transfusions, transfusion reactions, such as transfusion related acute lung injury, and TTIs (20) (Figure 1.1). The benefits and relative risks associated with blood transfusion need to be communicated to patients whenever possible (3).

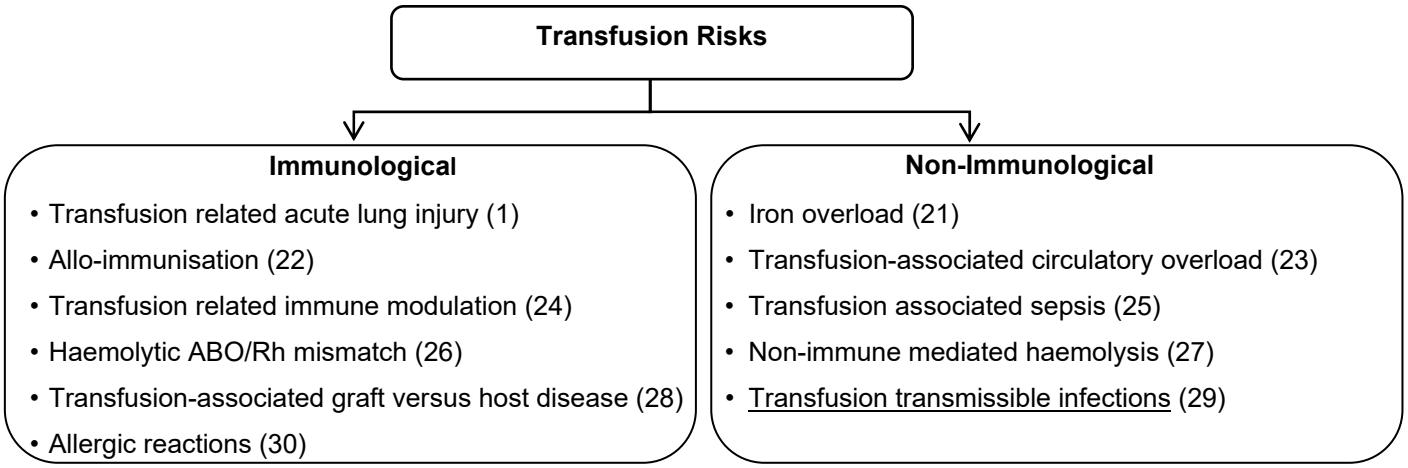


Figure 1.1: Possible risks associated with blood transfusion

1.2. Transfusion Transmissible Infections

TTIs are one of the potential threats to a safe transfusion (20). With the introduction of stringent donor selection guidelines, sensitive screening tests and pathogen inactivation methods, there has been a reduction in the associated risk (4, 20). Even though the risk of TTIs is lower than in the past, blood components and products are still subject to contamination with pathogens (4). However, risk of TTIs still varies widely between developed and developing countries. There is always a need to assess current risk, in order to prevent possible threat from the emergence of pathogens that are adapted to changing human behaviour and environmental conditions.

Some of the characteristics of microbial agents considered a risk to transfusion include (31):

- Presence and/or survival of the agent in one or more components of blood
- Transmission by the intravenous route
- Propensity for causing asymptomatic infections in the donor population
- Cause infection with prolonged incubation period and long-term carrier state
- Ability to cause symptomatic disease in transfusion recipient

Microbial agents that have been demonstrated to be transfusion transmissible are viruses, bacteria, parasitic protozoa and prions.

1.2.1. Viruses

The most commonly transmitted agents via blood transfusion are viruses. Transfusion-transmissible viruses include HIV-1/2, Human T-lymphotrophic Virus –I/II (HTLV-I/II), HCV, HBV, CMV and WNV (32-38). Other viruses that are transfusion transmissible include hepatitis A virus (HAV), HEV, hepatitis G virus (GBV-C), Epstein- Barr virus, DENV, human parvovirus B19, human herpesvirus 6, HHV-8, ZIKV and TT virus (38-48). HIV, HBV and HCV are considered to have the highest risk for transfusion safety and mandatory screening of blood donation is required for these agents in most countries.

1.2.2. Parasites

Parasitic infections that can be transmitted through blood transfusion include malaria caused by *Plasmodium spp.*, babesiosis caused by *Babesia spp.*, Chagas disease caused by *T. cruzi*, toxoplasmosis caused by *Toxoplasma gondii* and leishmaniasis caused by *Leishmania spp.* (49-53). The majority of these parasitic protozoa are transmitted via vectors, such as mosquitoes (*Plasmodium spp.*), ticks (*Babesia spp)* or sand flies (*Leishmania spp.)*. Hence, to ensure a safe blood supply in some countries, blood donors are also assessed based on their area of residence for parasites like *T. cruzi*, or travel to areas where such vectors and diseases are present.

1.2.3. Bacteria

Transfusion transmitted bacterial infections are most likely due to contamination of the blood component during collection (54). Bacterial contamination of platelets has most often been reported with many different species implicated (54), especially those that are part of the normal skin flora. The introduction of more stringent skin disinfection methods and

diversion pouches have reduced the risk of bacterial transfusion transmission in many countries (55). Bacterial TTIs although less common may also be possible from bacteraemic donors donating and include *T. pallidum,* the causative agent of syphilis (56). Bacteremia in blood donors resulting in TTIs have also been reported with *Yersinia enterocolitica* and *Staphylococcus aureus* (54).

1.2.4. Prions

It has been established with animal studies that vCJD is transfusion transmissible (57). Transfusion transmitted vCJD has been reported in the United Kingdom (UK) (58). Data from the UK suggest the risk of vCJD transfusion transmission is 14% from infected donors donating blood within 40 months of the onset of disease (58). To date, no other prion disease has been shown to be transfusion transmitted.

1.3. Screening for Transfusion Transmissible Infections

Screening of blood donations or donors is necessary for the maintenance of a safe blood supply. Medical history interview and evaluation of blood donors determines their eligibility to donate. Selection of healthy blood donors plays a crucial role in the exclusion of infected individuals with underlying disease, which can be either clinical or sub-clinical, and pose a potential risk to the blood supply. However, there still remains a residual risk of collecting an infectious blood donation from donors who are in the window period (duration from infection to detection by laboratory testing).

The World Health Organization (WHO) recommends mandatory screening of all blood donations for HIV, HBV, HCV and *T. pallidum*. However, according to a report published in 2011, blood donations are not screened for any TTIs in 39 countries (59). Additional screening for other infections is based on their epidemiological occurrence, such as seasonal screening for WNV in the United States of America (USA) or malaria testing in Australia for donors returning from endemic countries (60, 61). CMV testing and leucoreduction through filtration are strategies to reduce CMV transmission to high risk immunocompromised recipients in some countries (62).

Screening for TTIs generally involves testing for antigens and/or antibodies against the infectious agents or pathogenic nucleic acid. The introduction of nucleic acid amplification testing (NAT) has reduced the residual risk of HIV, HBV and HCV transmission through

the ability to detect infectious agent during the early stages of infection (63-65). Sensitive screening tests and medical examination with stringent questionnaires coupled with donor deferral minimises TT risk.

1.3.1. Screening for Transfusion Transmissible Infections in Australia

In Australia, all blood donations are tested for HIV-1 and -2, HTLV-I and -II, HBV, HCV and *T. pallidum* (66, 67). Screening tests based on immunoassays include testing for HBV surface antigen (HBsAg), antibodies to the following: HIV -1 and -2, HTLV-I, and II, HCV and *T. pallidum* using *T. pallidum* haemagglutination (TPHA). All donations are also tested for HIV-1 RNA, HCV RNA and HBV DNA (67), using a transcription mediated amplification (TMA) multiplex assay. Residual risk for HIV, HTLV, HBV, HCV and Syphilis transfusion transmission is decreasing and small (less than 1 in a million units transfused, except HBV with approximately 1 in 764,000) compared to the USA and European estimates (66, 68). The prevalence of HBV and HCV is higher in the blood donor population compared to HIV, HTLV and Syphilis, however, the prevalence of all infections in the donor population is still lower than the general population (66, 67).

Malaria is not endemic in Australia although local transmission and imported cases have been reported (69, 70). Malaria testing among the donor population in Australia is limited to those donors with a history of travel overseas or residency in countries endemic for malaria and/or a history of malaria infection (9). Donors are tested for malaria antibodies after 4 months from their return from malaria endemic countries or recovery from clinical infection (71). Donors reactive for anti- *Plasmodium spp.* antibodies are tested for parasitic antigens and DNA, and are eligible to donate only plasma for fractionation (61, 71, 72). In 2011, 2.1% of ‘at risk’ donors were reactive to malaria antibodies and none to either antigens or DNA (66).

CMV testing is performed to maintain an inventory of CMV seronegative cellular blood components required for specific groups of patients who are immunocompromised and are at risk for severe CMV disease. CMV management involves screening for CMV antibodies in selected donations, which is coupled with universal leucodepletion as an added safety precaution (10, 73).

1.4. Pathogen Inactivation Technologies

In addition to the stringent blood donor selection criteria and laboratory screening for TTIs, further safety can be achieved by leucoreduction and pathogen inactivation methods. Pathogen inactivation technologies (PITs) for red blood cells, platelets and clinical plasma have the potential to reduce the possible risk from emerging infections in these blood components, for which screening assays may not be available. At present, PITs for fresh blood components are available for treating platelet units as well as clinical plasma; no technologies are yet available for red blood cells. PITs are based on chemical inactivation and photo-inactivation methods (74). PITs have been adopted by different countries in Asia, Europe and the Middle-East (75). However, lack of cost-effectiveness and treatment related damage to a range of cellular functions in PIT treated components are some of the issues that prevent implementation of such technologies in other countries, including Australia.

1.5. Emerging Infectious Diseases and Blood Safety

Infectious diseases that have recently appeared in the population or have increased in incidence within the past two decades and still threaten to increase in the future are termed emerging infectious diseases (76, 77). The re-emergence of a disease refers to an increase in incidence of a disease that had previously declined (5). Emergence and re-emergence of infectious disease can be attributed to various factors related to changes in host range, virulence of pathogens, climatic conditions, vectors (if involved in transmission), improved detection capacities and travel/immigration of people (78, 79).

Although there is a decline in risk associated with well-known TTIs due to stringent donor selection and sensitive screening tests in many developed countries, threat to blood safety from emerging infectious diseases for which preventable measures may not be available still remains. Emerging pathogens currently of risk to blood safety include WNV, DENV, *Babesia spp*., HEV, HHV-8, *T. cruzi*, chikungunya virus, ZIKV and prion (causing vCJD) (5, 29, 48). Emerging infectious diseases may impact on transfusion safety with the reduction in donor attendance and hence blood sufficiency, or an increase in transfusion transmission risk if blood borne.

1.5.1. Emerging Infectious Diseases and Blood Safety in Australia

Surveillance for emerging infectious diseases that may impact on transfusion safety is of paramount importance to the Blood Service (66). Recent emerging infectious diseases include regular dengue outbreaks in northern Queensland, a reported case of human babesiosis in New South Wales (NSW) and Hendra virus cases in Queensland (66). Dengue is not considered endemic in Australia; however, both imported cases and indigenous outbreaks have occurred (80). Risk of collecting a dengue infected donation during the 2004 outbreak in Cairns was estimated to be 1 in 19,759, and during the 2008-2009 outbreak was approximated as 1 in 7,146 (11, 81). The risk of Ross River Virus (RRV) transfusion transmission was estimated to be 1 in 13,542 donations, during a 2004 outbreak in Cairns (82). A first case of probable transfusion transmitted RRV has been reported in Western Australia (82).

A case of locally-acquired HEV has been reported in an Australian liver transplant recipient, who also received blood transfusion (83). However, there was insufficient evidence to determine the mode of transmission. Reported cases of TT-HEV overseas (16, 17), the detection of HEV RNA in blood donors in other countries (13), warrants an investigation into the risk HEV poses to the safety of the Australian blood supply.

1.6. Viral Hepatitis and Transfusion Risk

Inflammation of the liver and necrosis of hepatocytes resulting from viral infection is referred to as viral hepatitis. These viruses in general include HAV, HBV, HCV, Hepatitis D virus (HDV), and HEV (84). HAV and HEV are mainly transmitted through the faecal-oral route causing self-limited infections and manifest as acute or asymptomatic hepatitis to fulminant hepatitis. However, there is evidence of transfusion transmission associated with both of these viruses (39, 40). Compared to HAV, the clinical course of HEV is more severe (85). HBV and HCV are transmitted parenterally, through sexual contact, perinatally and manifest as acute to chronic hepatitis leading to cirrhosis and hepatocellular carcinoma (86, 87). HBV and HCV are both transfusion transmissible and blood donations are screened routinely for these viruses in many countries (59). HDV replicates in the presence of HBV and co-infection increases the severity of disease (88). GBV-C is closely related to HCV and causes persistent infection, is parentally transmitted but is not known to be a primary pathogen (89). Given that HBV and HCV are routinely screened before transfusion and residual risk is therefore minimised, risk associated with

HAV and HEV still needs to be assessed. Although vaccines are available for HAV and HEV, the later poses a higher risk to individuals in terms of disease severity, and thus HEV is considered a current emerging infectious disease of possible threat to blood supply safety. Moreover, HEV has recently gained international significance in the transfusion medicine community.

1.7. Hepatitis E Virus (HEV)

The availability of serological tests for HAV and HBV distinguished a third virus, then referred to as non-A non-B (NANB) hepatitis virus, which was associated with waterborne outbreaks (90-92). It was not until 1983, that HEV was first observed under immune electron microscopy in stool samples from a volunteer experimentally infected with NANB hepatitis (93). Transmission to non-human primates (*Macacus cynomologus*) was also demonstrated (93). Hepatitis E was also known as enterically transmitted non-A non- B hepatitis (ET-NANB) (94). Isolation of cDNA from HEV further identified this virus as different from hepatitis A (95). This facilitated the development of serological assays to allow for an increased understanding of the epidemiology of this virus. HEV is classified in the genus *Hepevirus* in the *Hepeviridae* family (96), consisting of genotypes 1 to 4, infecting humans (13). The family also includes viruses that infect birds, bats, rodents and fish, and classification of HEV variants is still in progress (97, 98).

HEV epidemics occur periodically in developing countries and are associated with faecal contamination of water and poor sanitation (99). In developed nations, HEV is related to travel to countries endemic for HEV (100, 101). However, autochthonous HEV is increasingly being reported in many countries (102), indicating the possibility of local transmission. Interestingly, many cases in developed countries are from contaminated undercooked food or contact with infected animals rather than the classical faecal oral route seen in many developing countries (103-108). HEV transfusion transmission (17, 109) demonstrates that a blood borne phase exists, and may contribute to local transmission.

1.7.1. Morphology and Structure

HEV is an icosahedral virus with a diameter of 27-34nm (93, 110, 111). The absence of lipid envelope renders the virus stability in bile and thus excretion in faeces, providing access to the environment (112). The HEV genome is 7.5 kb in length, comprising of a

single stranded positive sense RNA with open reading frames (ORFs) capped at 5' and polyadenylated at 3' termini. ORF 1 closest to the 5' end codes for non-structural proteins, ORF 2 next to the 3' end codes for structural proteins and ORF 3 codes for cellular proteins with kinase activity, which overlaps ORF 1 and ORF 2 (Figure 1.2) (95, 112-115).

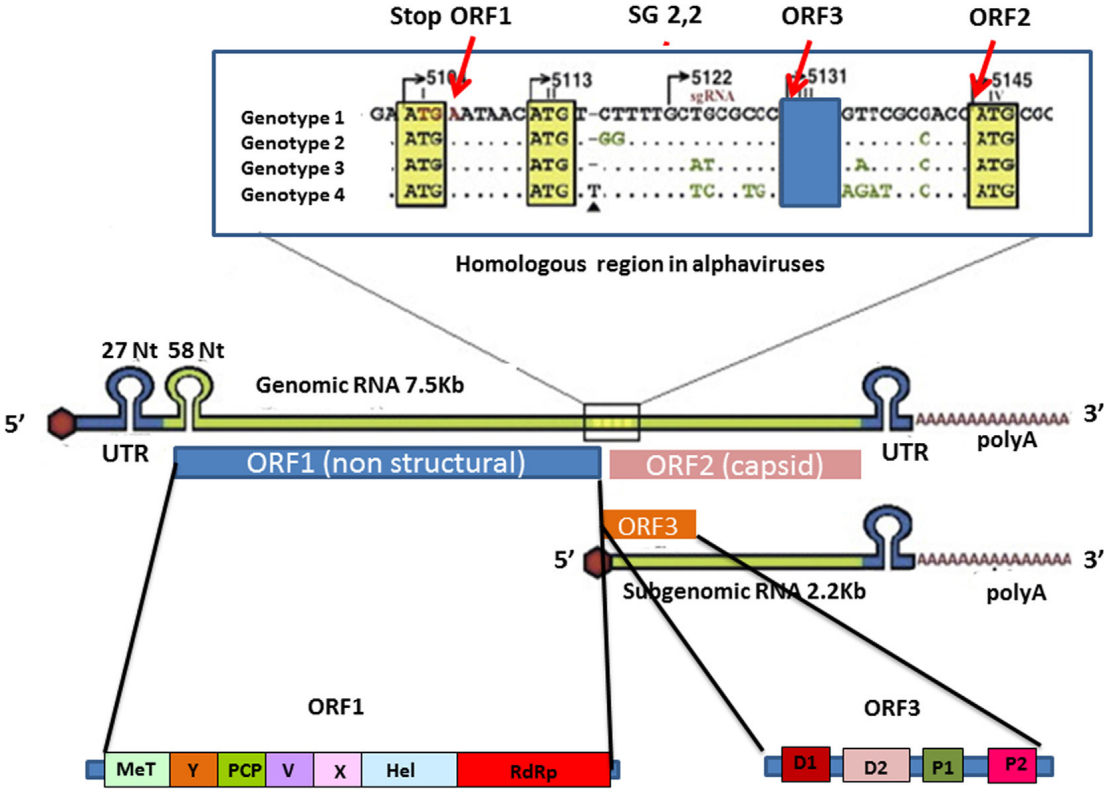


Figure 1.2: Schematic diagram of HEV genome [Open reading frame (ORF), Untranslated regions (UTRs), Methyl transferase (MeT), Y, V and X (domains), Papain like cysteine polymerase (PCP), RNA Helicase (Hel), RNA dependent RNA polymerase (RdRp), Poly Adenylated (polyA), D1 and D2 (hydrophobic domains), P1 and P2 (proline-rich domains)] (Reproduced with permission from the publisher (116))

1.7.2. Genotypes

Based on phylogenetic analyses of full length sequences, HEV is classified into 4 genotypes (1-4), which are further divided into subgenotypes 1 (a-e), 2 (a, b), 3 (a-j), 4 (a-g) (117). This classification into genotypes was previously based on differences in partial genome sequences, such as variation of more than 20% in the nucleotides in the ORF2 region (97, 118). Currently, HEV genotypes are characterised on the basis of sequences of ORF 1, ORF 2 as well as ORF 3 regions (118). The 371 base region of ORF 1, spanning nucleotides 80-450 has been used in phylogenetic analyses, which is considered to be consistent with analyses undertaken with full-length sequences (119). A region of

148 bases from ORF 2 between nucleotide positions 6322 and 6469 has also been considered in phylogenetic analysis (119). All HEV strains belong to a single serotype (13).

HEV genotypes vary in epidemiological distribution, mode of transmission (Figure 1.3), and pathogenicity of disease. Genotype 1 includes human HEV strains reported from Asia and Africa (120). Genotype 2 is also solely a human virus, with strains occurring in Mexico and some African countries (13). Genotype 3 infects both humans and swine, and strains are found globally (13). Genotype 4 also includes human and swine viruses, and is distributed in southeast Asia (117, 120, 121). HEV genotypes 3 has been isolated from deer, mongoose, rats and rabbits and genotype 4 from cattle and sheep (122).

Genotypes 1 and 2 are associated with the faecal oral transmission route in developing countries. Genotypes 3 and 4 occur predominantly in swine and are related to zoonotic transmission to humans via pork consumption or contact, in developed countries (13). Genotype 4 has also been isolated from pig livers in India (122), and genotype 3 from pigs in Thailand (123). HEV RNA (unknown genotype) has been detected in swine in Nepal (124).

Genotypes 1 and 2 cause self-limiting hepatitis in young adults with increasing mortality in pregnant women and immunocompromised patients (125). Genotypes 3 and 4 cause clinically apparent hepatitis in older individuals (125).

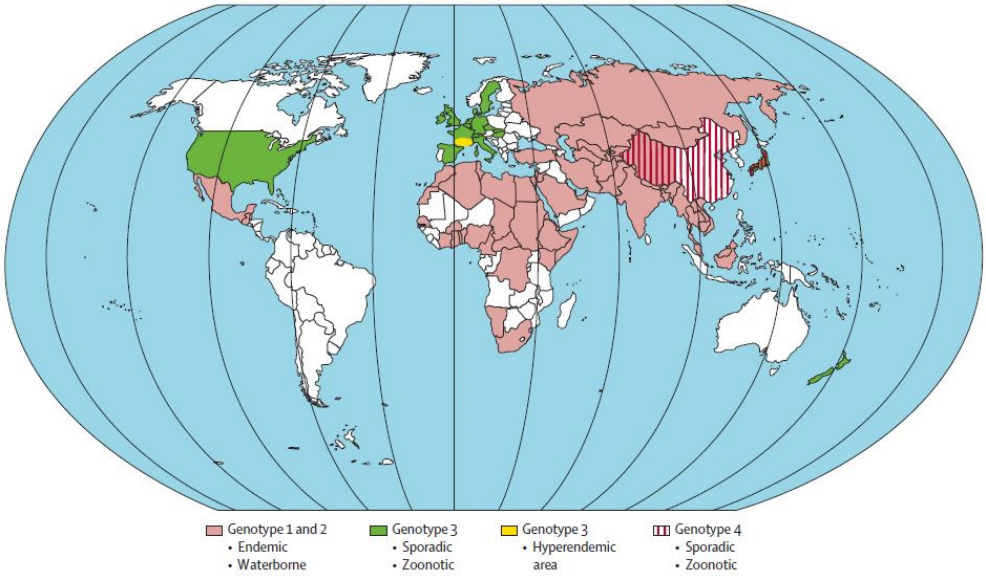


Figure 1.3: Geographical distribution of HEV cases and prevalence of genotypes (Reproduced with permission from the publisher (126))

1.7.3. Pathogenesis

The first attempt to understand HEV pathogenesis was initiated before HEV was completely identified. This involved infection of a human volunteer and non-human primates (*M. cynomolgous*) with an infectious inoculum (93). This study demonstrated that the infectious agent was transmitted by the faecal oral route. It has been proposed from an in vitro study that the truncated structural protein (p239) of HEV binds to the heparin sulphate proteoglycan receptors and penetrates susceptible cells (127, 128). The hepatic cell lines that have been used for propagation of HEV include PLC/PRF/5, Huh7A, HepG2/C3A (128). Other cell lines used include the lung carcinoma cell line A549, and the colon carcinoma line Caco-2 (13, 128). A proposed model of HEV replication is characterised by entry of the virus by endocytosis into the host cell, release of RNA after uncoating of capsid, translation and replication of the genome in the cytoplasm, synthesis of capsid protein that packages RNA to virions and subsequent release (129). Pathogenesis of HEV has been studied in *M. cynomolgous* with intravenous inoculation of bile or faeces containing HEV (130). The experimental animals were infected with HEV and after 2-3 weeks, a rise in serum alanine aminotransferases (ALT), histopathological changes in the liver, appearance of HEV antigen in the liver, virus in bile and HEV antibodies in serum were observed (130).

The ethical consequences of the following studies are questionable, however, are included in this review as they are in the published literature. HEV pathogenesis in a human volunteer was studied with oral administration of stool extracts from patients with presumed faeco-oral NANB hepatitis (93). The infected volunteer developed clinical features of acute hepatitis 36 days after inoculation. Virus-like particles were observed in stool collected on days 28, 43, 44 and 45 after inoculation (93). Another human volunteer based investigation was performed to understand the clinical picture of HEV infection with oral administration of a stool suspension from a patient diagnosed with ET-NANB hepatitis (131). This study involved the investigator himself ingesting the inoculum. Clinical features developed 30 days post-inoculation followed by an icteric phase (38 days post inoculation), rise in serum ALT and bilirubin (Figure 1.4) (131). Virus was detected in the serum after 22 days and in stool collected after 34 days post-inoculation. HEV antibody appeared on day 41 (131). In a report of TT-HEV, the virus was detected in blood after 5 weeks and for up to 12 weeks post transfusion (16). HEV IgM was detected after 9 weeks with decreasing reactivity after 19 weeks (16). HEV IgM is detectable 4 days after the onset of acute hepatitis, with a higher detection rate within 4 weeks (132). In general, HEV IgM declines after 3-6 months of illness (129, 133). Some studies indicate that HEV IgG persists for 1-2 years (94, 134), however, others demonstrate IgG to persist for 14 years after infection (135). In the general course of HEV infection, HEV RNA persists in blood for the duration of 4 weeks and in stool for 6 weeks (Figure 1.4).

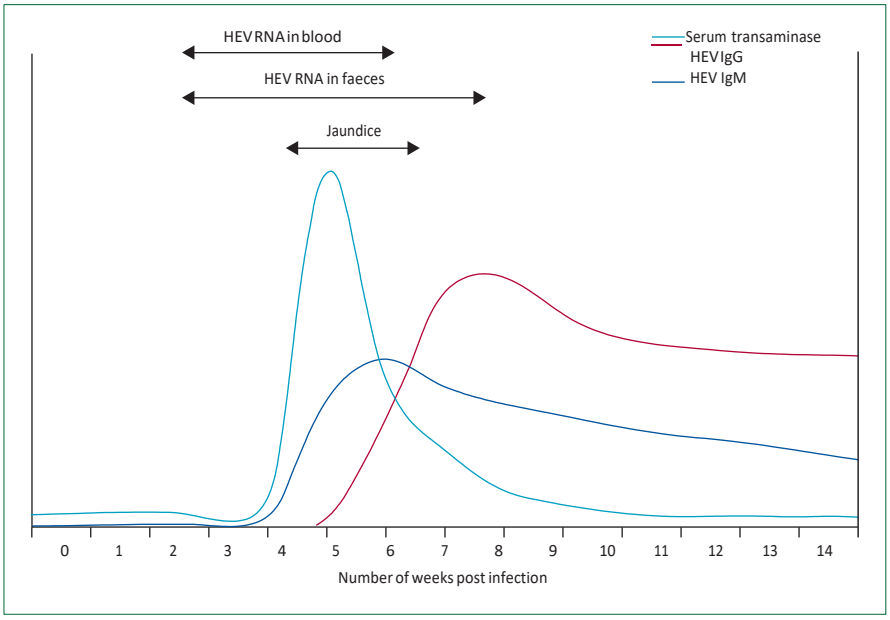


Figure 1.4: Diagrammatic representation of HEV infection with stages of RNA detection and serological responses (Reproduced with permission from the publisher (136))

1.7.4. Clinical Manifestations

HEV is associated with self-limited acute phase disease with recognized cases of chronic hepatitis (137). The incubation period is 15-60 days (mean of 40 days) (138). Clinical features include anorexia, epigastric pain, discoloured urine, nausea, vomiting, diarrhoea, fever, jaundice, elevation of serum transaminase and hepatomegaly (93, 131, 137, 139). The majority of HEV infections are asymptomatic, and rates vary between developing countries (where up to 50% of cases can be asymptomatic) and developed countries (where the asymptomatic rate can be 67-98%) (140). During outbreaks in developing countries, a case fatality rate of 0.5-4% has been reported (137). Incidence of disease and fulminant hepatitis failure is even higher in pregnant women during the third trimester (141). Maternal mortality in pregnancy also varies with geographical region, and reaches 10-25% in developing countries (13, 142). Cases of chronic HEV infection have occurred in solid-organ transplant (kidney, liver and pancreas) recipients (143). Chronic HEV disease in immune suppressive conditions like HIV (with HEV RNA persistence for 24 months), and reactivation in a lymphoblastic leukemia patient after stem cell transplantation have also been reported (144, 145). Non-hepatic manifestations of HEV such as pancreatitis, haematological manifestations (thrombocytopenia, hemolysis), autoimmune phenomena, neurological syndromes (Guillian-Barre syndrome, meningoencephalitis, pseudotumor cerebri, nerve palsies) have been reported (137).

1.7.5. Modes of Transmission

HEV is recognised as a food and water borne disease, transmitted through drinking contaminated water and through the consumption of raw or uncooked infected meat (12). There are other possible modes of transmission including, contact with animals, transfusion transmission and vertical transmission (Figure 1.5).

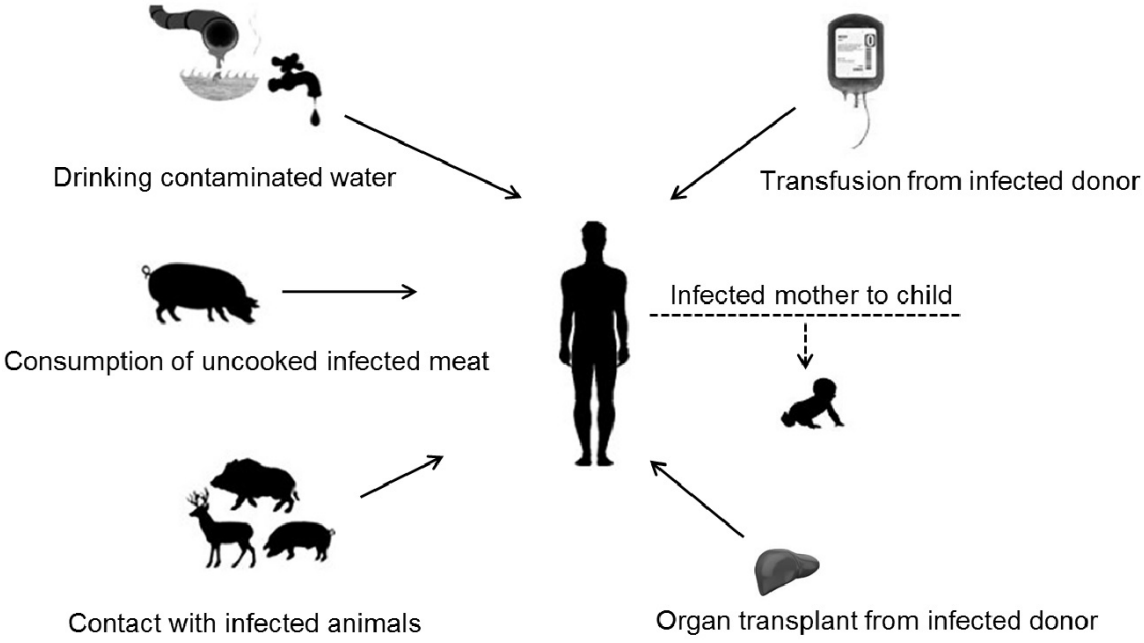


Figure 1.5: Different modes of HEV transmission

1.7.5.1. Faeco-oral Transmission

HEV outbreaks in developing countries have been associated with water contamination and the route of transmission is faeco-oral. This was demonstrated by detection of the virus in sewage and drinking water, and in the serum of patients in India (146). Waterborne HEV epidemics have occurred in many developing countries, including India (1955-1956, 1975-1976, 1978-1982, 1989), Nepal (1973, 1981, 1987) and Sudan (2004) (147, 148). HEV genotypes 1 and 2 are associated with such a mode of transmission (125).

1.7.5.2. Food Borne and Zoonotic Transmission

Locally-acquired HEV in developed countries has been associated with the consumption of raw or uncooked meat, such as pork, where HEV genotypes 3 and 4 are implicated (149). HEV genotype 3 has been detected in pig livers sold in grocery stores in the USA and Germany (150, 151). Pigs are natural reservoirs of HEV. Faeco-oral transmission in pigs can occur with shedding of infectious HEV in faeces and contamination of water sources in pig farms (122). A study in the USA has shown that pit manure slurry is a potential source of HEV infection and could contribute to contamination of the environment (152). However, in this study there was no evidence for contamination of drinking or surface water with HEV on or near the pig farms. Other animals (deer, boar, goats, sheep, bats, cows, camels, horses, rats, rabbits) have also been found to be infected with the virus (125, 153, 154). Higher HEV seroprevalence has been reported among workers in pig farms

compared to the general population, which indicates contact may be another possible mode of transmission (155).

1.7.5.3. Transfusion Transmission

The presence of HEV in blood allows for the transmission via blood and blood components. This has been demonstrated by detection of virus in the blood of HEV infected blood donors and recipients after transfusion. TT-HEV has been reported in France (17), the UK (16), Japan (109, 156) and Saudi Arabia (157). A retrospective study among blood transfusion recipients has shown a higher prevalence compared to control groups in an endemic country like India (157). A fatal case of HEV (genotype 3) after infusion of infected lymphocytes in a leukemia patient in Germany has been reported (158). HEV has been shown to be transmitted via clinical plasma treated with the Intercept system (synthetic psoralene amotosalen hydrochloride (HCL) and ultraviolet light A) demonstrating resistance of the virus to this type of PIT (18).

1.7.5.4. Vertical Transmission

There is evidence of HEV transmission from mother to child. HEV RNA has been detected in infants born to HEV infected mothers, with a variable rate of transmission, ranging from 33.33% in India (159) to 100% in United Arab Emirates (160).

1.7.6. Epidemiology

HEV causes acute hepatitis but in many cases the infection is subclinical (asymptomatic rate in developing countries is up to 50% while in developed countries is 67-98%) (140), therefore, the actual incidence of disease is not known. Hence, published data on disease prevalence as well as reported cases in such countries provide an estimate of the global burden. Prevalence varies in developing countries where HEV is considered endemic, with the occurrence of outbreaks associated with water borne transmission. In developed countries, the disease occurs sporadically and is related to food borne transmission, zoonotic transmission and travel to countries endemic for HEV. The global annual disease burden for HEV genotype 1 and 2 in 2005 was estimated to be 20.1 million incident infections, which resulted in an estimate of 3.3 million symptomatic cases, 70,000 deaths and 3,000 still births (161). This study represented 71% of world's population with incidence greatest in the younger age group (15 to 20 years) (161). The burden for HEV genotypes 3 and 4 is not known. HEV seroprevalence ranges from less than 5% to 53% in

different countries and also varies due to variability in sensitivity and specificity between assays (Table 1.1). A seroprevalence study conducted in southwestern France with different assays has demonstrated variable prevalence (162). Hence, comparison of the seroprevalence between regions needs to be interpreted with caution. HEV RNA has been detected in blood donors from China, the UK, Germany, Japan, the Netherlands and Scotland (Table 1.2). Genotypes 3 and 4 are reported from China, Japan and are emerging in developed countries related to food borne transmission (13, 163, 164).

Table 1.1: Global HEV IgG prevalence

Country	Year (published)	n (cohort)	HEV IgG %	Assay used	Refe-rence
Australia	1995	279 (blood donors)	0.4	Genelabs, Inc.	(165)
Bangladesh	2015	1,009 (blood donors)	49.8	Wantai	(166)
China	2009	44,816 (blood donors)	32.6	Wantai	(167)
France	2011	512 529 (blood donors)	52.5 16.6	Wantai Genelabs	(162) (168)
Germany	2012	4,422 (general population)	16.8	Mikrogen	(169)
Ghana	2012	239 (blood donors)	12.9 4.7 10	Wantai, Recomline, RecomWell	(170)
Hong Kong	2013	450 (archived sample)	28.7	Biotec laboratories	(171)
Hungary	2007	264 (hepatitis patients)	10.6	-	(172)
India	1998	200 (blood donors)	18.6	In-house	(173)

Country	Year (published)	n (cohort)	HEV IgG %	Assay used	Refe-rence
Iran	2013	530 blood donors)	14.3	Dia Pro	(174)
Japan	2010	22,027 (general population)	5.3	In -house	(175)
Korea	2006	147 (health check examinees)	23.1 14.3	Wantai Genelabs	(176)
Nepal	1997	757 (general population)	24.6	Diagnostic Biotechnology	(177)
Netherland	2013	5,239 (blood donors)	27	Wantai	(178)
New Zealand	2007	265 (blood donors)	4.2	Wantai	(179)
Scotland	2013	1,559 (blood donors)	4.7	Wantai	(180)
Spain	2006	1,536 (general population)	7.3	Biokit	(181)
Switzerland	2011	550 (blood donors)	4.9	MP Diagnostics	(182)
UK	2008	487 (blood donors)	15.8	Wantai	(183)
USA	2013	1,939 (blood donors)	18.8	Wantai	(184)

Table 1.2: HEV RNA prevalence in blood donors

Country	Year (published)	n (Blood donations/donors)	HEV RNA prevalence	Geno-type	Refe-rence
Austria	2015	58, 915 (donors)	0.012%	3	(185)
China	2009	44,816 (donations)	0.067%	1 & 4	(167)
France	2014	53,234 (donations)	0.041%	Unknown	(186)
Germany	2012	41,325 (donations)	0.081%	3	(187)
Japan	2013	~2,000,000 (donations)	0.012%	3 & 4	(188)
Netherland	2013	45,415 (donations)	0.037%	3	(178)
Scotland	2013	43,560 (donations)	0.007%	3	(180)
Spain	2014	9,998 (donors)	0.030%	3	(189)
UK	2014	225,000 (donations)	0.014%	3	(190)
USA	2016	18,829 (donations)	0.011%	Unknown	(191)

1.7.7. HEV Epidemiology in Australia

Data from the Commonwealth Department of Health indicate there were 378 HEV notified cases between 1999-2013, with an average of 25 cases per year (Figure 1.6) (192). Higher numbers of cases were reported from NSW and Victoria. Most cases were associated with travel to HEV endemic countries, mostly Asia (193), and few cases were reported to be locally-acquired. The true incidence of HEV and exposure status of the Australian population, however, is unknown due to subclinical infection.

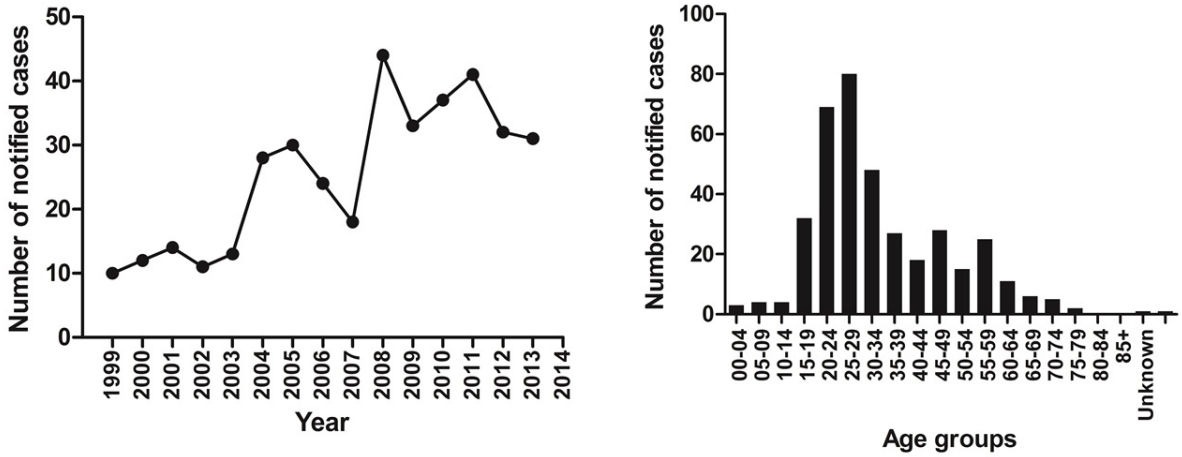


Figure 1.6: HEV notified cases in Australia from 1999–2013, by year (A) and age group (B) (Data Source: Australian Government Department of Health, National Notifiable Diseases Surveillance System) (192)

A seroprevalence study of HEV in 1995 among selected groups determined HEV IgG prevalence of 0.4% in blood donors, 2.2% in travellers and 7.7% in non-A, non-B, non- C hepatitis patients as well as refugees (165). Retrospective study of stored sera from patients in the 1970s (NANB hepatitis) have shown serological evidence of HEV IgG (194).

1.7.7.1. HEV in Animals

HEV IgG prevalence has been reported to be 17% in wild caught pigs and 30-95% in commercially raised pigs (195). This study used an in-house assay with unknown sensitivity and specificity (195). Transmission from pigs to humans is possible through ingesting infected undercooked meat or contact with infected animals. An avian HEV strain associated with big liver and spleen disease in chickens has been identified in Australia in 1980 (196). However, avian strains are not known to be transmitted to humans (140).

1.7.7.2. Locally-Acquired Infection in Humans

There are limited data on the extent of locally-acquired HEV in Australia and the burden of autochthonous HEV therefore requires investigation. The first case of locally-acquired HEV infection was reported in 1995 from the Northern Territory (NT) (197). The source of infection in this patient was not known (197). A case each in Victoria and Queensland were also notified as being locally-acquired in 2005 (198), however, neither case had been published in peer reviewed journals. 24 HEV cases have been reported in NSW in 2014,

all with no recent overseas travel history and likely to be linked to the consumption of undercooked pork (199-201). Domestically acquired HEV infection in Australia, associated with genotype 3, has recently been reported in a liver transplant recipient (83). Several possible modes of transmission were postulated, including via transplantation, blood transfusion, or through the consumption of contaminated food or water by the recipient. A study in 1999 has shown HEV IgG prevalence of up to 95% in Australian pig herds (195), which indicates that zoonotic transmission may be possible. Other modes of transmission such as person to person, congenital, transfusion and organ transplant have been documented overseas, (13, 108, 190) which could contribute to the occurrence of locally-acquired infection in Australia, and associated risks therefore need to be assessed.

1.7.8. HEV in Nepal - a Developing Country Endemic for HEV

In this study, Nepal was selected as a developing and HEV endemic country for a comparison with a developed and presumed HEV non-endemic country (Australia). HEV epidemics in Nepal have occurred in 1973, 1981-1982, 1987, 1995 and 2014 (128, 148, 202). HEV IgG prevalence in 1999-2000 was 38%, with the Kathmandu valley designated as hyper-endemic and rural areas as non-endemic for HEV (148). Epidemics have been associated with faecal contamination of water, and molecular characterization has shown genotype 1 as the cause of acute HEV infection (203). HEV during pregnancy is a concern, with 19% mortality and 5% of women having miscarriages (204). HEV antibodies and RNA have been demonstrated in farm swine from the Kathmandu valley (124), indicating the possibility of zoonotic transmission. HEV in Nepalese blood donors and the impact of this virus on blood safety has not yet been studied.

1.8. Prevention

HEV infection can be prevented by avoiding exposure to the virus. This may include improvement of sanitation and the supply of clean drinking water. Zoonotic transmission is preventable by the proper handling of uncooked meat and its proper cooking before consumption. A study on thermal stability of HEV has shown that heating to 56^0C for one hour resulted in an inactivation rate of ~80% (205). However, this varied with genotype of HEV.

HEV is also preventable by vaccination. Two HEV vaccines have been developed, but neither is licensed worldwide. A recombinant vaccine was derived from the Sf9 insect cell

line using baculovirus, encoding ORF2 of a Pakistani HEV strain (206). This prototype vaccine was trialled in Rhesus monkeys (206). A Phase I clinical trial of this vaccine (produced by DynCorp, Rockville. Md., USA) was conducted at the Walter Reed Army Institute of Research and it was determined this prototype was safe and immunogenic, but required further evaluation (207). A Phase II trial of the vaccine was planned with the Nepalese community, but approval to commence the trial was not given for ethical reasons (208). However, the trial was performed with the Nepalese Army, and efficacy of three doses of vaccine was found to be 95.5%, with unclear information on durability of immunity (209).

A second vaccine, referred to as HEV 239, is based on the expression of a recombinant protein encoded by ORF 2 (HEV genotype 1) in *Escherichia coli* (210). A clinical phase II trial conducted in China determined this prototype vaccine was safe and immunogenic (211). A Phase III trial of HEV 239 (Hecolin : Xiamen Innovax Biotech, Xiamen, China) determined an efficacy of 100% (212). This vaccine has recently been approved for use in China (213). As of May 2015, WHO has not recommended routine use of an HEV vaccine in populations where HEV is epidemic and sporadic (214) .

Passive immunisation with immunoglobulin preparations in animal studies have been shown to be effective at preventing infection (139). Passive immunoprophylaxis with low titre human serum immunoglobulin has not been successful in humans (139). Monoclonal antibodies against HEV for use in humans has not been tested (139).

1.9. Treatment

Acute cases of HEV that are self-limiting do not require treatment. Generally, severe cases of HEV are treated with ribavirin (215). Treatment of chronic cases of HEV includes treatment with ribavirin at doses of 200 mg for 3 months (216). In addition, treatment with 1,000 mg ribavirin per day divided in two doses for 10 days (depending on the renal function of the patient) has been reported to be successful (216). A dose of 135 µg/week of pegylated interferon (IFN)–α 2a for 3 months has been shown to clear HEV RNA in chronic HEV patients (217). Likewise, pegylated IFN–α 2b is reported to be useful in chronic HEV patients (218). The use of ribavirin is contraindicated during pregnancy (13).

1.9.1. Diagnosis

HEV is similar to other hepatitis viruses in terms of clinical features. In majority of developed countries diagnosis is based on risk factors, such as recent travel history and risk of zoonotic or food borne transmission. A case is confirmed based on laboratory diagnostic tests and the patient's clinical features. Different methods for HEV diagnosis and blood donor screening are outlined below and summarised in table 1.3.

1.9.1.1. Immune Electron Microscopy

Immune electron microscopy can provide direct evidence of the virus, as was used to demonstrate the causative agent of NANB hepatitis transmitted by the faeco-oral route (93, 219). In this technique, a stool sample was treated with serum containing antibodies to HEV, which agglutinated HEV if present and enabled detection under electron microscope. The need for an expensive microscope and its non-sensitivity makes it less applicable for clinical diagnosis (220).

1.9.1.2. Serological Tests

Serological-based tests include the detection of HEV IgG, IgM, IgA and HEV antigen in serum or plasma. Enzyme immuno assay (EIA) based tests are widely used for laboratory diagnosis of patients. Antibody testing assays are generally based on detection of antibodies against epitopes of the gene products of ORF2 and ORF3 (221). EIA kits with antigens from one genotype of HEV are able to detect antibodies against a different genotype (222).

HEV IgG Assays

Detection of HEV IgG in an individual indicates a previous infection with HEV. This antibody may persists in an infected individual for years (135). Thus, the acute phase of HEV infection cannot be differentiated by detection of HEV IgG. However, these assays have important practical value in determining the exposure status of an individual. Studies with different commercial EIAs have shown variability in sensitivity (170, 223, 224). Seroprevalence determined with different assays therefore needs interpretation with caution. The Wantai HEV IgG ELISA manufactured by Beijing Wantai Biological Pharmaceutical Enterprise Co., Ltd is considered to be the most specific and sensitive assay available at present (184). This commercial assay has also been widely used for

HEV seroprevalence studies (162, 178, 184, 225), and is therefore preferred for the current study to allow a comparison with these other studies.

HEV IgM Assays

The acute phase of HEV infection can be diagnosed by the detection of HEV IgM. This class of antibody is detectable after the onset of acute hepatitis, and can last for up to 3-6 months post-infection (129). Both ELISA and immune chromatographic assays have been developed. Evaluation of HEV IgM commercial assays has shown variability in sensitivity and specificity (226).

HEV IgA Assays

HEV IgA has been detected in acute HEV patients, with a more prominent response against HEV genotype 1 compared to genotype 3 (227). It is considered that HEV IgA based ELISAs are more specific than HEV IgM assays, and diagnostic accuracy increases with use of both types (228). Such an assay can be used as a supplementary tool for diagnosis of acute HEV with no IgM response (229). However, the diagnostic application of this assay still requires further investigation.

HEV Antigen Assays

HEV antigens appear in the blood prior to the appearance of specific antibodies and the antigen assays are used for the direct detection of the virus (230). HEV antigen detection assays based on an indirect sandwich EIA have been developed using monoclonal antibodies produced against gene products of ORF2 of HEV genotype 2 (230). Concordance of HEV RNA and antigen detection with this assay has been shown to be 81% in clinical patients (230).

1.9.1.3. Nucleic Acid Amplification Assays

After HEV infection, peak viremia occurs during the incubation period of 2-6 weeks, and viral RNA is not detectable in blood until 3 weeks after the onset of symptoms (13). Viral nucleic acid detection is a sensitive and specific method of diagnosis. However, such assays have a high cost associated with reagents, instruments and the requirement for trained personnel. NAT provides an added advantage in immunocompromised patients who fail to develop antibody responses or to monitor responses to antiviral treatment (220).

NAT methods for HEV RNA detection includes real-time reverse transcription polymerase chain (RT-PCR). Nested RT-PCR with primers designated to three ORFs of specific HEV strains has been described to allow rapid detection of the virus in serum (231). RT-PCR based assays targeting ORF2 have been validated to be efficient for the detection and quantification of all the four HEV genotypes (232). Amplified PCR products are detected by gel electrophoresis or within the real time PCR system. Real-time RT-PCR has been demonstrated to be more sensitive than nested PCR. Though specific there is variability in sensitivity of PCR based assays (233). The need for standardization of HEV RNA NAT assays led to a collaborative approach that has established genotype 3a HEV strain with a unitage of 250,000 IU/ml as international standard for HEV RNA (234). Loop mediated isothermal amplification assays have demonstrated rapid detection of genotypes 1 and 2 with high sensitivity, without the requirement of thermocycling equipment (235).

TMA technology has been developed by Gen-Probe and the Procleix® system uses this technology, which is a fully integrated, automated nucleic acid testing system for blood screening for infectious diseases. TMA can amplify either DNA or RNA and produces an RNA amplicon (236). A prototype HEV assay based on this technology has been developed (Appendix III.d) (189). The limit of detection for this assay has shown to be 7.9 IU/ml (189).

1.9.1.4. Biochemical Tests

An increase in serum levels of bilirubin, ALT and aspartate aminotransferases (AST) is observed in acute HEV infection. However, none of these are specific as these biochemical markers increase in other forms of liver injury and viral hepatitis (220). Higher prevalence of HEV serological markers have been demonstrated with elevated levels of ALT (225).

1.9.1.5. Cellular Immune Response

Enzyme linked immunosorbent spot (ELISPOT), has been developed to test peripheral blood mononuclear cells for IFN-γ secreted in response to HEV infection (220). ELISPOT assays have been shown to correlate with the enzyme immune assays for the detection of past exposure to HEV (220). The utility of ELISPOT for HEV diagnosis is under evaluation (220, 237).

Table 1.3. Different testing methodologies for HEV diagnosis and blood donor screening

Test methods	Diagnosis	Screening	Sensitivity /specificity
Immune electron microscopy	Direct detection of virus indication of current infection	Unsuitable	Insensitive
IgG ELISA	Past exposure or indication of recent re-infection	Serosurveillance study to understand risk factors	Varies between commercial assays (238). Sensitivity: 65-98% Specificity: 74-100%
IgM ELISA	Evidence of recent infection	Surveillance study to determine recently infected donors	Varies between commercial assays (239). Specificity:84->99%, Sensitivity: 52-81%
Antigen ELISA	Indication of current infection	Early virus detection	Variable concordance: RNA positive in 50% of antigen positive (225) Antigen positive in 40% of RNA positive (240)
RT-PCR	Gold standard for current infection	Confirmation of current/recent infection	Varies with commercial assays. Altona Diagnostics LOD 37.8 IU/ml (241)
TMA	Commercially unavailable	Screening for infected donations	LOD 7.9 IU/ml (189)
Biochemical	Liver function test as indicator of acute hepatitis	Unsuitable	Non-specific

1.10. Hepatitis E - An Emerging Infectious Disease

Since 1955, hepatitis E has been associated with a large number of water-borne disease outbreaks (242). However, the causative agent was not identified until 1983 (93). HEV outbreaks continue to occur in many developing countries including Bangladesh, Nepal and Sudan (202, 243, 244). HEV is considered endemic in many developing countries. In developed countries, HEV was traditionally associated with travellers returning from developing countries endemic for HEV. However, in recent decades, there have been increased numbers of reports of locally-acquired cases, mainly associated with zoonotic transmission (12, 104). Development of more sensitive serological and molecular techniques for laboratory testing of HEV have also facilitated increased identification and reporting of HEV (128).

In many developed countries, travel to countries endemic for HEV is a risk factor for HEV infection. As more people are likely to travel to such countries in the future (for example due to decreased cost of international travel) (245), the incidence of HEV infection may increase. Consumption of undercooked infected meat or contact with infected animals is another risk factor associated with HEV infection; which has the potential to expose a greater number of people to this virus (162, 246). These factors threaten to increase the incidence of HEV in the future. Hence, HEV is considered an emerging pathogen of public health importance.

1.11. Conclusion and Rationale for the Study

Hepatitis E is an emerging infectious disease of public health concern. As the majority of cases are asymptomatic, HEV RNA has been detected in blood donors and cases of TT-HEV have been reported in developed countries (17, 190, 247). HEV transmission to immunocompromised individuals can cause chronic infection (12). Therefore, there is an increasing concern for HEV among the international transfusion community, including Australia. With limited HEV prevalence data in Australia and unavailability of vaccine for HEV, there is a possible threat of an HEV outbreak from imported or locally-acquired infections. This has the potential to be a risk to the safety of Australia's blood supply. Hence, this study is designed to measure the prevalence of past and current HEV infection in Australian blood donors. The study will utilise available testing methods and data sets to estimate the risk of TT-HEV in Australia and to provide additional knowledge in relation to

the burden of HEV in Australia. This study will also determine if current blood donation guidelines manage HEV risk to ensure the safety of Australian blood supply.

Chapter 2. Seroprevalence of Hepatitis E Virus in Australian Blood Donors and Implications for the Safety of the Blood Supply

Context

Hepatitis E is nationally notifiable in Australia. However, an accurate estimate of HEV population exposure is unknown, due to the occurrence of subclinical infection and limited seroprevalence studies. This chapter aims to determine if Australian blood donors have been exposed to HEV. This is achieved by the measurement of HEV past exposure through the detection of HEV IgG in blood donors. Risk factors for HEV exposure are assessed and data utilised to examine the effectiveness of current Australian blood safety strategies for the management of HEV.

A section of this chapter has been published in the journal Emerging Infectious Diseases:

Shrestha AC, Seed CR, Flower RL, Rooks KM, Keller AJ, Harley RJ, Chan HT, Holmberg JA, Faddy HM. Hepatitis E virus and implications for blood supply safety, Australia. Emerg Infect Dis 2014; 20: 1940-2.

2.1. Introduction

HEV is an emerging public health concern for developed countries (12). In developing nations, epidemics occur periodically and are associated with faecal contamination of water (13). Although HEV in developed nations has been associated with travel to endemic countries, autochthonous HEV is increasingly being identified, mainly associated with ingestion of contaminated food or contact with infected animals (13, 248). Transmission through blood transfusion is also possible (17, 18). HEV can result in acute hepatitis, however, chronic hepatitis has been described in organ transplant and immunocompromised patients (249). The high rate of asymptomatic infection, together with documented cases of transfusion-transmission, highlights the potential risk to blood safety (13).

In Australia, hepatitis E is nationally notifiable, averaging 25 cases per year (1999-2013 (192). The majority of cases are associated with travel (193). However, an accurate estimate of HEV incidence and population exposure in Australia is unknown, due to subclinical infection and limited recent seroprevalence studies. HEV infection has been demonstrated in Australian pig herds and avian HEV has been isolated from Australian chicken flocks (153, 195).

Detection of HEV RNA in blood donations in the UK, Germany, the Netherlands, Japan and China, and accumulating reports of TT-HEV, including from plasma treated with PITs, highlight the potential risk to transfusion safety (13, 17, 18, 250).

2.2. Aims

This chapter aimed to:

- Measure HEV seroprevalence in Australian blood donors
- Assess risk factors for HEV exposure
- Examine the effectiveness of current Australian blood safety strategies for the management of TT-HEV

2.3. Materials and Methods

2.3.1. Study Design

This was a cross-sectional seroprevalence study based on a stratified sampling method. Samples were stratified based on the states/territory of residence of blood donors and selected randomly. Sample weighting was not used. Participants in this study were Australian blood donors. The sample size required was approximately 3,000, based on a 95% confidence interval (CI), and assuming prevalence of 4% as estimated in New Zealand (179) (Appendix I.a). This sample population included approximately 400 samples from each state/territory (Figure 2.1), with random selection for sex and age groups. Relevant donor details were collected from the Blood Service National Blood Management System database. Ethical approval was received from the Blood Service Human Research Ethics Committee and the University of Queensland, School of Medicine Low Risk Ethical Review Committee (Appendix II.a, II.b).

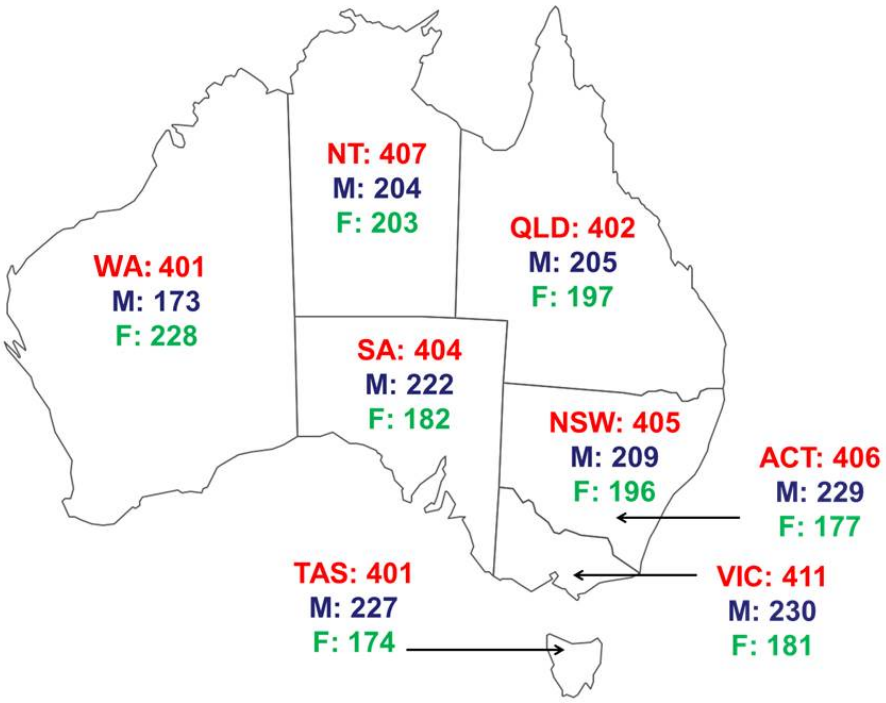


Figure 2.1: Numbers of male and female donors from each state and territory of Australia included in this study (ACT: Australian Capital Territory, NSW: New South Wales, NT: Northern territory, QLD: Queensland, SA: South Australia, TAS: Tasmania, VIC: Victoria, WA: Western Australia, M: Male, F: Female)

2.3.2. Sample Collection

Plasma samples were collected from 3,237 Australian donors between August and September, 2013. Blood samples were collected in EDTA tubes (BD Vacutainer® Whole Blood Collection tube with spray-coated K_2EDTA 6mL, Becton Dickinson, Plymouth, UK). Samples no longer required after routine viral screening at the Blood Service, were utilised for this study. All samples were stored at -20^{0}C until testing. Age, sex, state of residence, new/repeat donor status, and overseas travel disclosure, were obtained. Details of any relevant donation 'deferral' (malaria, diarrhoeal) applied on previous donation attempts were also collected. Application of a specific malaria deferral code is routine for donors disclosing travel to a malaria endemic country, and a diarrhoeal deferral applies where a donor reports having had diarrhoea (due to viral or unknown causes) one week prior to any attempted donation.

2.3.3. Sample Testing - HEV IgG and IgM

All samples were de-identified prior to testing. Samples were thawed at room temperature for 1 hour and centrifuged at 1,258 g for 5 minutes. Plasma samples were then tested in singlicate for HEV IgG (Wantai HEV-IgG ELISA, Beijing Wantai Biological Pharmacy Enterprise Co., Ltd.), as per the manufacturer's instructions (Appendix III.a). Any samples testing positive were re-tested in duplicate. Samples, two or three times reactive were reported as seropositive (Figure 2.2).

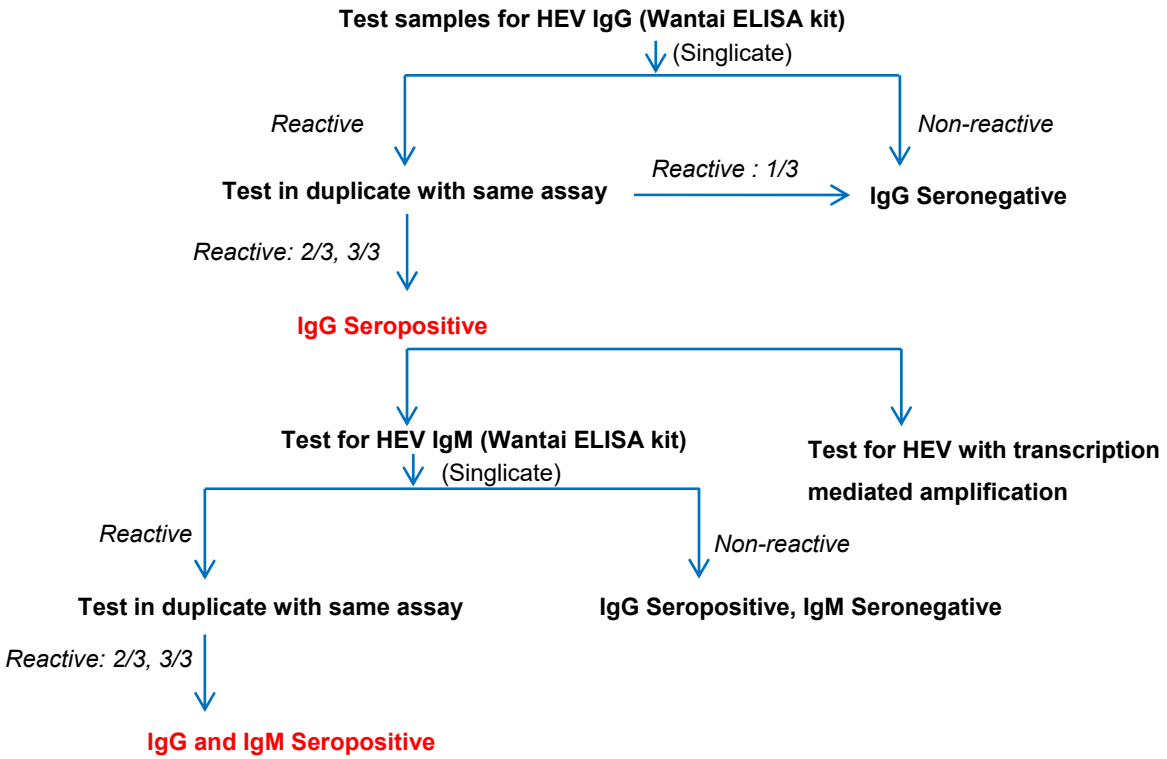


Figure 2.2: Algorithm for sample testing

HEV IgG confirmed positive samples were tested for HEV IgM (Wantai HEV-IgM ELISA, Beijing Wantai Biological Pharmacy Enterprise Co., Ltd.) as per the manufacturer's instructions (Appendix III.b), and results reported based on the similar algorithm as for HEV IgG (Figure 2.2).

Sample testing was performed using Tecan Evo ELISA processor (Tecan Australia Pty Ltd., Port Melbourne, Victoria, Australia). Optical density was measured at 450 nm using Hybrid Multi-Mode Microplate Reader (Bio Tek Instruments, Inc., Winooski, USA) and sample/cut-off ratio (S/Co) calculated.

2.3.4. Sample Testing - HEV TMA

All HEV IgG positive samples were also tested for HEV RNA in singlicate. The samples were tested by TMA using Procleix HEV assay (Appendix III.d) and the Panther system (Hologic Inc., San Diego, CA, USA). This testing was performed at Hologic laboratories in San Diego.

2.3.5. Statistical Analysis

Donor demographic details and risk factors were recorded in Microsoft Excel 2010 (Redmond, WA, USA). Data were analysed for chi-square and odds ratio measured. HEV seropositivity was considered as the dependent variable and risk factors independent variables for analyses. Logistic regression was used for factors significant in the univariate analyses, to determine association with HEV IgG seropositivity, using IBM SPSS Statistics 19 (IBM Centre, NSW, Australia).

2.4. Results

In this study, 55.11% of donors were male which was slightly higher than the normal donor population in 2013 (49.4%) (251). The age breakdown of the sample population was similar to the normal donor population.

Of the 3,237 blood donor samples tested, 194 tested positive for HEV IgG giving a seroporevalence of 5.99% (95% CI 5.18-6.81%) (see Appendix IV.a for S/Co of HEV IgG positive samples). The prevalence increased with age and was higher in donors of 45 years and above ($p<0.05$) (Table 2.1).

Table 2.1: HEV IgG prevalence, and demographics in Australian blood donors

Variable	n tested	HEV IgG seropositive		Univariate Analysis	
		N	% (95% CI)	Odds ratio (95% CI)	p value
Sex					
Female	1,453	78	5.37 (4.21-6.53)	*Reference group*	-
Male	1,784	116	6.50 (5.36-7.65)	1.23 (0.91-1.65)	>0.05
Age					**<0.05**
< 25 years	564	14	2.48 (1.20-3.77)	*Reference group*	-
25-34 years	569	13	2.28 (1.06-3.51)	0.92 (0.43-1.98)	>0.05
35-44 years	510	22	4.31 (2.55-6.08)	1.77 (0.89-3.5)	>0.05
45-54 years	666	40	6.01 (4.20-7.81)	2.51 (1.35-4.66)	**<0.05**
55-64 years	673	68	10.10 (7.83-12.38)	4.41(2.46-7.94)	**<0.05**
≥ 65 years	255	37	14.51 (10.19-18.83)	6.67 (3.54-12.58)	**<0.05**

Variable	n tested	HEV IgG seropositive		Univariate Analysis	
		N	% (95% CI)	Odds ratio (95% CI)	p value
State					>0.05
ACT	406	25	6.16 (3.82-8.50)	*Reference group*	-
NSW	405	23	5.68 (3.42-7.93)	0.92 (0.51-1.64)	>0.05
NT	407	26	6.39 (4.01-8.76)	1.04 (0.59-1.83)	>0.05
QLD	402	18	4.48 (2.46-6.50)	0.71 (0.38-1.33)	>0.05
SA	404	32	7.92 (5.29-10.55)	1.31 (0.76-2.25)	>0.05
TAS	401	20	4.99 (2.86-7.12)	0.80 (0.43-1.46)	>0.05
VIC	411	23	5.60 (3.37-7.82)	0.90 (0.50-1.62)	>0.05
WA	401	27	6.73 (4.28-9.19)	1.10 (0.63-1.93)	>0.05

CI: confidence interval; ACT: Australian Capital Territory; NSW: New South Wales; NT: Northern Territory; QLD: Queensland; SA: South Australia; TAS: Tasmania; VIC: Victoria; WA: Western Australia

Of the sample population, 87.14% (2,821) had travelled overseas and 6.38% of these donors were exposed to HEV. This rate was higher than in non-travellers (3.37%). HEV IgG prevalence was also higher in donors with a previous malaria deferral (7.73%) and diarrhoea deferral (15.52%) ($p<0.05$) (Table 2.2). The majority of samples in this study (90.51%) were from repeat donors (Table 2.2). Multivariate logistic regression analysis demonstrated only age, previous malaria deferral and previous diarrhoeal deferral were independent determinants of HEV IgG seropositivity (Table 2.3).

Table 2.2: HEV IgG prevalence and other risk factors in Australian blood donors

Variable	n tested	HEV IgG seropositive		Univariate Analysis	
		n	% (95% CI)	Odds ratio (95% CI)	p value
Overseas travel					
No	416	14	3.37 (1.63-5.10)	*Reference group*	-
Yes	2,821	180	6.38 (5.48-7.28)	1.96 (1.12-3.40)	**<0.05**
Malaria deferral					
No	1,684	74	4.39 (3.42-5.37)	*Reference group*	-
Yes	1,553	120	7.73 (6.40-9.06)	1.82 (1.35-2.45)	**<0.05**
Diarrhoeal deferral					
No	3,179	185	5.82 (5.01-6.63)	*Reference group*	-
Yes	58	9	15.52 (6.20-24.84)	2.97 (1.44–6.14)	**<0.05**
Donor Status					
New	307	13	4.23 (1.98-6.49)	*Reference group*	-
Repeat	2,930	181	6.18 (5.31-7.05)	1.49 (0.84-2.65)	>0.05

Table 2.3: Multivariate analysis of age, overseas travel, malaria and diarrhoea deferral and HEV IgG seropositivity

Variable	Multivariate Analysis	
	Adjusted Odds ratio (95% CI)	p value
Age		**<0.05**
< 25 years	*Reference group*	-
25-34 years	0.82 (0.38-1.77)	>0.05
35-44 years	1.72 (0.87-3.42)	>0.05
45-54 years	2.427 (1.30-4.52)	**<0.05**
55-64 years	4.18 (2.32-7.54)	**<0.05**
≥ 65 years	6.09 (3.21-11.55)	**<0.05**
Overseas travel (Yes/No)	1.24 (0.69-2.25)	>0.05
Malaria deferral (Yes/No)	1.80 (1.3 -2.47)	**<0.05**
Diarrhoeal deferral (Yes/No)	2.55 (1.22-5.33)	**<0.05**

Four donors tested dual IgM/IgG positive (Table 2.4) (see Appendix IV.b for the HEV IgM S/Co of positive samples). The donors were of age 45 years and above. All donors had

travelled overseas and 3 had travelled to malaria endemic countries in the past. HEV RNA was not detected in any of the HEV IgG positive samples.

Table 2.4: HEV IgM and IgG positive blood donors

Donor	State	Age	Sex	Overseas travel	Malaria deferral	Diarrhoeal deferral
1	NSW	60	M	Yes	Yes	No
2	NSW	72	M	Yes	No	No
3	SA	63	M	Yes	Yes	No
4	WA	45	F	Yes	Yes	No

NSW: New South Wales; SA: South Australia; WA: Western Australia; M: Male; F: Female

2.5. Discussion

In developed countries, HEV seroprevalence ranges from <5% to 53% (13, 162). In this study HEV IgG was demonstrated in 5.99% of Australian blood donors. Consistent with previous studies and as expected, an increase in IgG prevalence was observed with increasing age, indicating cumulative lifetime exposure (162). HEV seroprevalence among male and female donors were similar, indicating both the sexes were equally likely to be exposed to the virus. Based on concomitant detection of IgM, 2.06% of HEV IgG positive donors had been recently exposed. Comparing only with previous studies using Wantai ELISA (162, 179, 180, 184), the estimate is comparable with those reported from Scotland (4.7%) and New Zealand (4.2%), but lower than those from the USA (18.8%) and France (52.5%). There is considerable debate in relation to the sensitivity and specificity of HEV detection methods (13, 224); based on the studies in France and the UK, HEV seroprevalence in this study is measured accurately (162, 224).

Of donors who reported ever traveling outside of Australia, 6.38% were HEV IgG positive. IgG seropositivity was also higher (7.73%) in donors who were known to have travelled to a malaria endemic country, many of which are also endemic for HEV (252, 253). However, as exposure status before departure is unknown, the exact place of exposure cannot be determined.

Currently, management strategies to safe guard the Australian blood supply against TT-HEV are principally based on donor selection guidelines. In order to identify donors with

potential bacteremia/viremia, including HEV, a number of medical/travel-based questions are asked prior to donation. These include questions relating to general wellness, gastric upset, diarrhoea, abdominal pain or vomiting within the previous week. In addition, donations from donors who have travelled to malaria-risk countries are restricted to plasma for fractionation (which includes viral inactivation steps) for 4 months following their return. As many malaria endemic countries are also endemic for HEV (252, 253), this provides some protection against imported HEV infections. The higher HEV seroprevalence observed in donors with prior malaria or diarrhoeal deferral suggests some HEV risk reduction contribution by these screening questions. Hence, the current medical screening process for donor selection is likely to be effective in preventing the collection of HEV infected donations destined for fresh component manufacture.

Importantly, a small proportion of donors (3.37%) with evidence of previous HEV exposure that had not reported travel outside Australia was identified, and therefore may have acquired HEV locally. As subclinical infection is possible, such individuals may not be identified by the current screening questionnaire, and may pose a risk to blood safety if infectious at the time of donation.

Given the presence of HEV RNA in donated blood is considered the confirmatory marker for infectivity, some have proposed it be implemented for donor screening in HEV endemic countries in Europe (249). Others suggest that this is premature pending further studies, particularly assessing the clinical severity of TT-HEV infections (250). While it was encouraging that HEV RNA was not detected in the HEV IgG positive donors in this study, the sample size was insufficient to accurately determine the true rate of HEV RNA carriage among donors and a larger study was planned (Chapter 3). As noted, in addition to the rate of HEV viremia among donors, the clinical outcome in recipients of RNA positive blood components is critical when considering the need for additional risk mitigation (e.g. donation testing). Presently there are few data on the rate of transmission or longer-term clinical consequences of TT-HEV, emphasizing the need for hemovigilance.

2.6. Contribution of the Chapter to the Research Question

This chapter has addressed the research question of this thesis in the following ways:

- Demonstrated that Australian blood donors have been exposed to HEV.
- Assessed variables such as sex, age, state of residence and frequency of blood donation associated with HEV previous exposure.
- Assessed whether donor parameters (overseas travel history, deferrals for malaria and diarrhoea) were associated with prior HEV infection.

Chapter 3. Hepatitis E Virus RNA and Antigen Detection in Australian Blood Donations

Context

The previous chapter (Chapter 2) showed that Australian blood donors were exposed to HEV. However, the risk of TT-HEV could not be measured, which required a larger sample size for the detection of HEV RNA. Thus, this chapter aims to estimate the risk of TT-HEV, achieved through measurement of the rate of current HEV infection in Australian blood donations based on detection of HEV RNA. The risk of collecting an infectious donation is determined based on the rate of HEV RNA detection.

A section of this chapter has been accepted for publication in the journal Transfusion:

Shrestha AC, Flower RL, Seed CR, Keller AJ, Harley RJ, Chan HT, Hoad V, Warrilow D, J Northill, Holmberg JA, Faddy HM. Hepatitis E Virus RNA in Australian blood donations. (Accepted: 17.07.2016)

3.1. Introduction

HEV is a non-enveloped, single stranded RNA virus, in the genus *Hepevirus*, family *Hepeviridae* (13, 96). The four genotypes of HEV (1-4) differ in mode of transmission and geographical distribution (125). Genotypes 1 and 2 are transmitted by the faecal-oral route in developing countries due to poor water sanitation (12, 13). In developed countries, genotypes 3 and 4 are associated with zoonotic transmission (13, 125). These genotypes have also been identified in developing countries (122, 123).The occurrence of genotypes 1 and 2 in these countries is often associated with travellers to developing countries endemic for HEV (254). Genotypes 3 and 4 are also known to be transmitted via blood transfusion (16, 17, 247).

The clinical presentation of acute HEV infection is similar to that caused by other hepatotrophic viruses (140), and asymptomatic infections may be more common than symptomatic infection (13). The viremic phase begins 2-3 weeks after infection, and can last for 10 weeks (12, 255). Chronic HEV infection with genotypes 3 have been documented in solid organ transplant and blood transfusion recipients (143, 256, 257), and in such cases, viral RNA can persist for more than 3 months (258). HEV IgM appears after 3-4 weeks and is detectable for 6 months, while HEV IgG is detectable 4-5 weeks post infection (12) and can be present for more than 12 years (12). Re-infection with HEV is possible, and has been described in solid organ recipients with low titre HEV IgG (259).

Given that HEV infection may result in asymptomatic viraemia and that transfusion transmission has been documented, this virus is a subject of interest in the transfusion medicine community. HEV RNA has been detected in asymptomatic blood donors from the UK, Japan, Germany, Saudi Arabia, Scotland, Spain, Austria, the Netherlands and China (15, 180, 185, 187, 189, 190, 225, 255, 260). Moreover, cases of TT-HEV have been reported in the UK, Japan, France and Germany (17, 190, 260, 261). A study in the UK has demonstrated a transfusion transmission rate for HEV of 42% from infected donations (190). Following cases of TT-HEV, HEV NAT has been implemented in the Hokkaido region of Japan (260). In order to provide ‘HEV safe’ components for high risk patients, blood donation screening for HEV RNA has been proposed in the UK (262), and the Republic of Ireland (263), and has been implemented for plasma pools in France (264). Donations used for the production of solvent/detergent-treated plasma are also screened for HEV RNA in the Netherlands with an in-house real time RT-PCR (255). Non-enveloped

viruses, like HEV, may be resistant to many pathogen inactivation methods. Indeed, HEV has been transmitted to a recipient from infected plasma after treatment with the Intercept system (synthetic psoralen amotosalen HCL treatment and ultraviolet light A) (18). Given that HEV infection in immunocompromised individuals, such as many transfusion recipients, can lead to chronic infection (258), it is essential to assess HEV risk to blood supply safety.

In Australia, hepatitis E is a notifiable disease, with an average of 25 cases annually (15). The majority of diagnosed cases are acquired overseas in South Asian countries, with a smaller proportion of autochthonous cases (265). In 2014, there was an outbreak of HEV genotype 3 in northern Sydney, associated with the consumption of undercooked pork (201). HEV IgG prevalence was 5.99% in a cohort of Australian blood donors (266). Although HEV IgM was reported in 2.06% of the IgG seropositive donors, no HEV RNA was detected (266). To date, there have been no published case reports of TT-HEV in Australia. A case of HEV genotype 3, thought to be associated with transfusion was reported, but there was insufficient evidence to determine the mode of transmission (83).

In Australia, HEV risk to blood supply safety is managed through routine medical history examination and travel history questionnaire. Individuals with a current HEV infection are excluded from donating for 12 months from the date of their recovery (71). In the case of post donation notification of an HEV infection by a donor, blood components, where available, collected 2 months prior to a donor becoming ill are recalled (71). Donors in sexual/mucosal contact with an HEV infected person are also deferred from donation (71). The current policy of deferring donations from donors returning from travel to countries endemic for malaria is also likely to reduce the risk of TT-HEV as such donors are excluded from donating fresh components for 4 months following their travel (71). Indeed, 94% of HEV notifications in Australia were acquired in countries covered by such a travel deferral policy (265). Despite these strategies, there is still a need to assess whether HEV poses a risk to the safety of the Australian blood supply based on the detection of markers of current HEV infection in Australian blood donations.

3.2. Aims

This chapter aimed to:

- Determine the risk of collecting an HEV infectious donation
- Determine rate of HEV antigen prevalence

3.3. Materials and Methods

3.3.1. Study Population

This was a cross sectional study based on stratified sampling method. Samples from blood donations were collected from Australian blood donors between September and October, 2014. A total of 14,799 samples were included in this study, based on a sample size calculation assuming a similar rate of HEV RNA detection to that observed in Japanese blood donors (0.012%) (188), and with an absolute precision of 0.009% (Appendix I. b). Samples were collected from the Brisbane, Melbourne, Perth and Sydney processing centres to represent cross-sectional Australian blood donations (see Appendix V.a for breakdown of sample numbers by collection centre). Ethical approval for this study was obtained from the Blood Service Human Research Ethics Committee and the University of Queensland Human Research Ethics Committee (Appendix II.a, II.b).

3.3.2. Sample Collection

Samples were collected in EDTA tubes (BD Vacutainer® Whole Blood Collection tube with spray-coated K_2EDTA 6mL, Becton Dickinson, Plymouth, UK) then centrifuged at 1,258 g for 5 minutes as per routine procedures. Samples were recovered after routine screening for infectious diseases was complete. Samples were made available for this study and the plasma aliquoted into 5 ml Corning® Cryogenic Vials (Corning Incorporated, New York, USA). All samples were de-identified and a given unique study number. Samples were processed and stored at -20^{0}C until required.

3.3.3. HEV RNA Testing - Transcription Mediated Amplification

All samples were tested for HEV RNA by TMA (Figure 3.1). Plasma samples were tested with a research use only Procleix HEV assay on the Procleix Panther System (Grifols Diagnostic Solutions, Inc., Emeryville, CA, USA) as per the manufacturer's instructions (Appendix III.d), which included positive, negative and internal controls. The Procleix HEV assay can detect all four genotypes of HEV (189). The 95% limit of detection is reported to be 7.89 IU/ml (95% fiducial limits 6.63-9.83 IU/ml), with a specificity of 99.99% (95% CI,

99.94%-100.00%) (189). Samples testing initially reactive and with adequate volume were retested with the same assay.

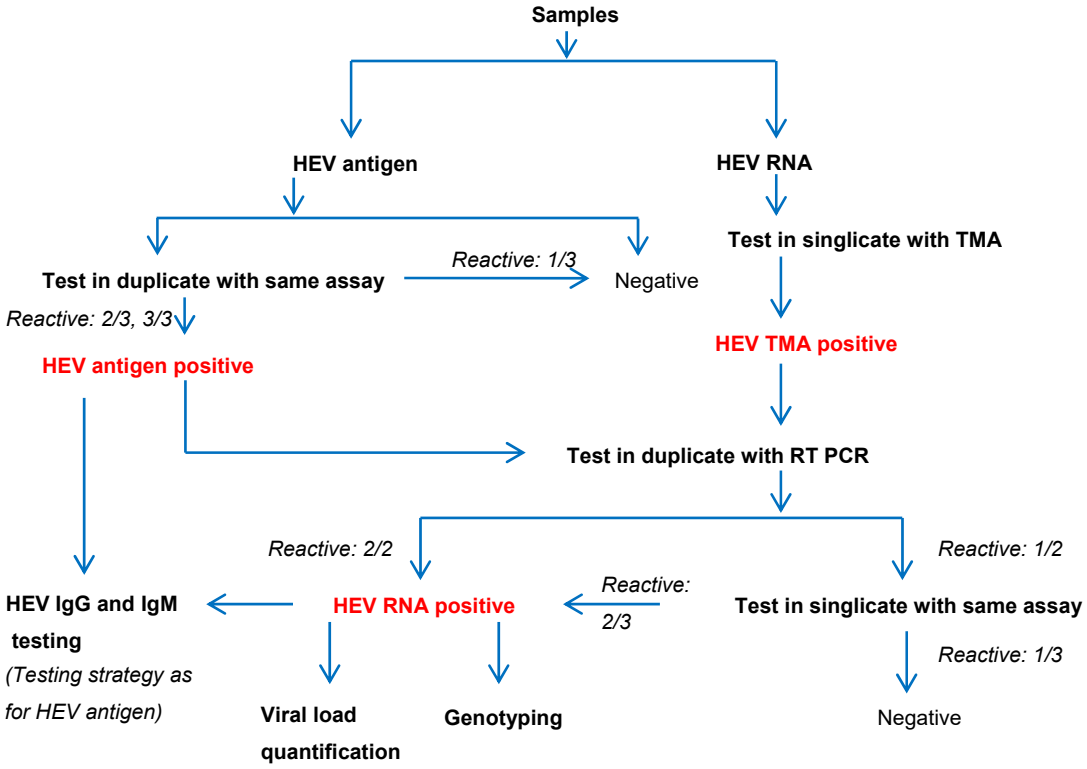


Figure 3.1: Algorithm for sample testing

3.3.4. HEV RNA Testing - RT-PCR

All HEV TMA initial reactive samples were tested for HEV RNA by RT-PCR (Figure 3.1). Viral RNA was extracted from each sample (140 µl) using QIAamp Viral RNA Mini Kit (Chadstone, Victoria, Australia) (Appendix III.e). An internal control (IC) from the RT-PCR kit described below was added to the lysis buffer during RNA extraction. RNA (25 µl) was tested in duplicate with the Realstar HEV RT-PCR kit 1.0 (Altona Diagnostics, Hamburg, Germany) using the Rotor-Gene 6000 (QIAGEN, Don Caster, Victoria, Australia) as per the manufacturer's instructions (Appendix III.f), which included positive and negative controls. The following conditions were used: 50^0C for 10 minutes, 95^0C for 10 minutes, then 45 cycles of: 95^0C for 15 seconds, 55^0C for 45 seconds and 72^0C for 15 seconds. Samples testing positive in duplicate were referred to as HEV RNA positive (Figure 3.1). The 95% limit of detection of the HEV RT-PCR assay is reported to range from 20 IU/ml to 100 IU/ml for individual sample testing (240, 267). The assay targets HEV ORF3, and has been shown to detect all HEV genotype 3 subtypes (267).

3.3.5. HEV Antigen Testing

All samples were also tested for HEV antigen (Figure 3.1) with the Wantai HEV-Ag ELISA *Plus* (Beijing Wantai Biological Pharmacy Enterprise Co., Ltd, China) as per the manufacturer's instructions (Appendix III.c), which included positive and negative controls. The assay is based on a monoclonal antibody against HEV ORF2 and can detect capsid protein of genotypes 1 and 4 within the window period and for 3-4 weeks post-infection (230, 240). According to the manufacturer, the specificity of the assay was 99.93% and positive agreement with PCR was 66.70% (268). Absorbance was measured using Hybrid Multi-Mode Microplate Reader (Bio Tek Instruments, Inc., Winooski, United States) at 450 nm and sample to cut-off ratio was calculated (Appendix IV.c). Initially reactive samples were re-tested in duplicate with the same assay, and considered positive if reactive at least twice (Figure 3.1).

3.3.6. HEV IgG and IgM Testing

HEV antigen positive or RNA positive samples were tested for HEV IgG with the Wantai HEV IgG ELISA (Beijing Wantai Biological Pharmacy) and for HEV IgM, with the Wantai HEV IgM ELISA (Beijing Wantai Biological Pharmacy), as per the manufacturer's instructions (Appendix III.a, III.b). Positive and negative controls were always included when performing an assay. The IgG assay uses a recombinant HEV PE2 protein derived from HEV genotype 1, and has been shown to detect antibodies against genotype 3 (223, 224). The IgM assay uses a recombinant protein derived from HEV ORF2 (specific details not disclosed) (269). According to the manufacturer, the sensitivity of the HEV IgG assay was 97.96%, while the HEV IgM assay was 97.10% (270, 271). Samples initially reactive for HEV IgG or HEV IgM were re-tested in duplicate with the same assay, and considered positive if reactive at least twice.

3.3.7. Viral Load Measurement

For samples confirmed HEV RNA positive, HEV quantification was performed in duplicate with 10-fold serial dilutions of the WHO International Standard for HEV (Paul-Ehrlich-Institut, Germany) using the RT-PCR conditions described above. HEV viral load in the RNA positive sample was measured in IU/ml by comparing the Ct value of the sample with the obtained standard curve (Appendix VI.a).

3.3.8. Sequencing and Phylogenetic Analyses

For samples confirmed HEV RNA positive, RT-PCR targeting the HEV methyltransferase and ORF2/3 regions was performed (272), and the PCR products were sequenced. A phylogenetic tree was constructed on a nucleotide sequence alignment with other HEV GenBank submissions. Evolutionary analyses were conducted in MEGA5 (273), using the Maximum Likelihood method based on the Tamura-Nei model and performing 1000 bootstrap replicates. Sequencing and phylogenetic analyses were performed by D. Warrilow and J. Northill at the Public Health Virology Laboratory, Queensland Health, Australia.

3.3.9. Statistical Analysis

Data analysis was performed using Microsoft Excel 2010 (Redmond, WA, USA). Exact CI were calculated for individual proportions (HEV RNA positivity) using a standard method (274).

3.4. Results

Of the 14,799 samples tested, 9 were TMA initial reactive; 8 of these were negative on repeat testing, and one could not be re-tested due to inadequate sample volume (Table 3.1). The 8 samples that were negative on repeat testing also tested negative by RT-PCR and were negative for HEV antigen. However, the sample that was initially TMA reactive, but could not be TMA re-tested, was confirmed positive by RT-PCR (Figure 3.2). This sample was also HEV antigen positive, however, was negative for HEV IgG and IgM. This gave a rate of HEV RNA of 0.0068% (95% CI: 0.0002 - 0.0376%), and resulted in a risk of collecting an HEV viraemic donation of 1 in 14,799 Australian blood donations (95% CI: 1 in 2,657 to 1 in 584,530). Of the total sample tested, 52 were positive for HEV antigen (0.35%, 95% CI 0.26-0.45%) (Appendix IV.c for S/Co of HEV antigen positive samples, Appendix V.b).

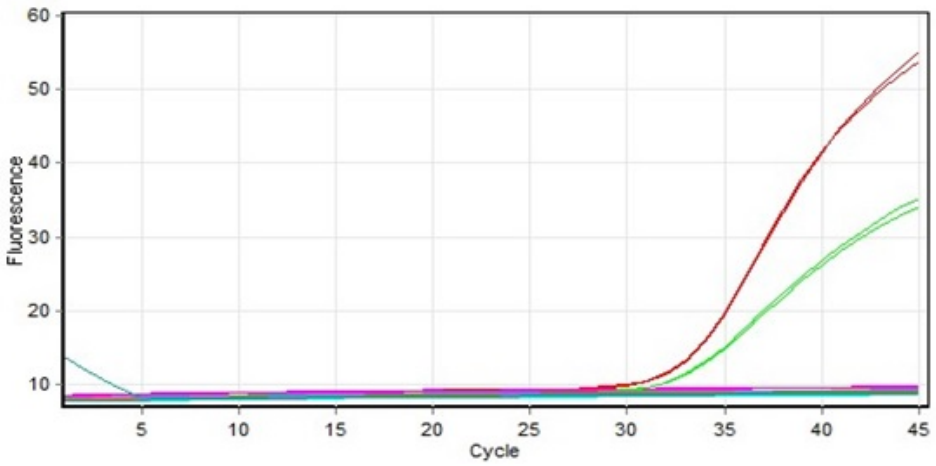
The viral load in the confirmed HEV RNA positive sample was estimated to be 15,000 IU/ml (Appendix VI.a). Phylogenetic analysis revealed that this sample (ARCBS 2015) was most closely related to genotype 3 isolates from Japan, Canada, Germany and USA (Figure 3.3). Next generation sequencing performed on the sample generated a 890 nt fragment, which gave a best match to HEV genotype 3 by Blastn (87% identity) (Appendix

VI.b), confirming the phylogenetic analysis. Together these observations indicate the isolate was genotype 3.

Table 3.1: HEV RNA detection in Australian blood donations

Sample	HEV antigen	HEV TMA initial	HEV TMA repeat	HEV RT-PCR
1	-	+	-	-
2	+	+	NT	+
3	-	+	-	-
4	-	+	-	-
5	-	+	-	-
6	-	+	-	-
7	-	+	-	-
8	-	+	-	-
9	-	+	-	-

- : Negative; + : Positive; NT: Not tested due to inadequate volume



Colour	Description	Ct values	Result
	Positive Control	31.09	Positive
	Positive Control	31.02	Positive
	Negative Control	-	Negative
	Negative Control	-	Negative
	ARCBS 2015	31.88	Positive
	ARCBS 2015	31.71	Positive

Figure 3.2: PCR amplification curves for controls and HEV RNA positive sample

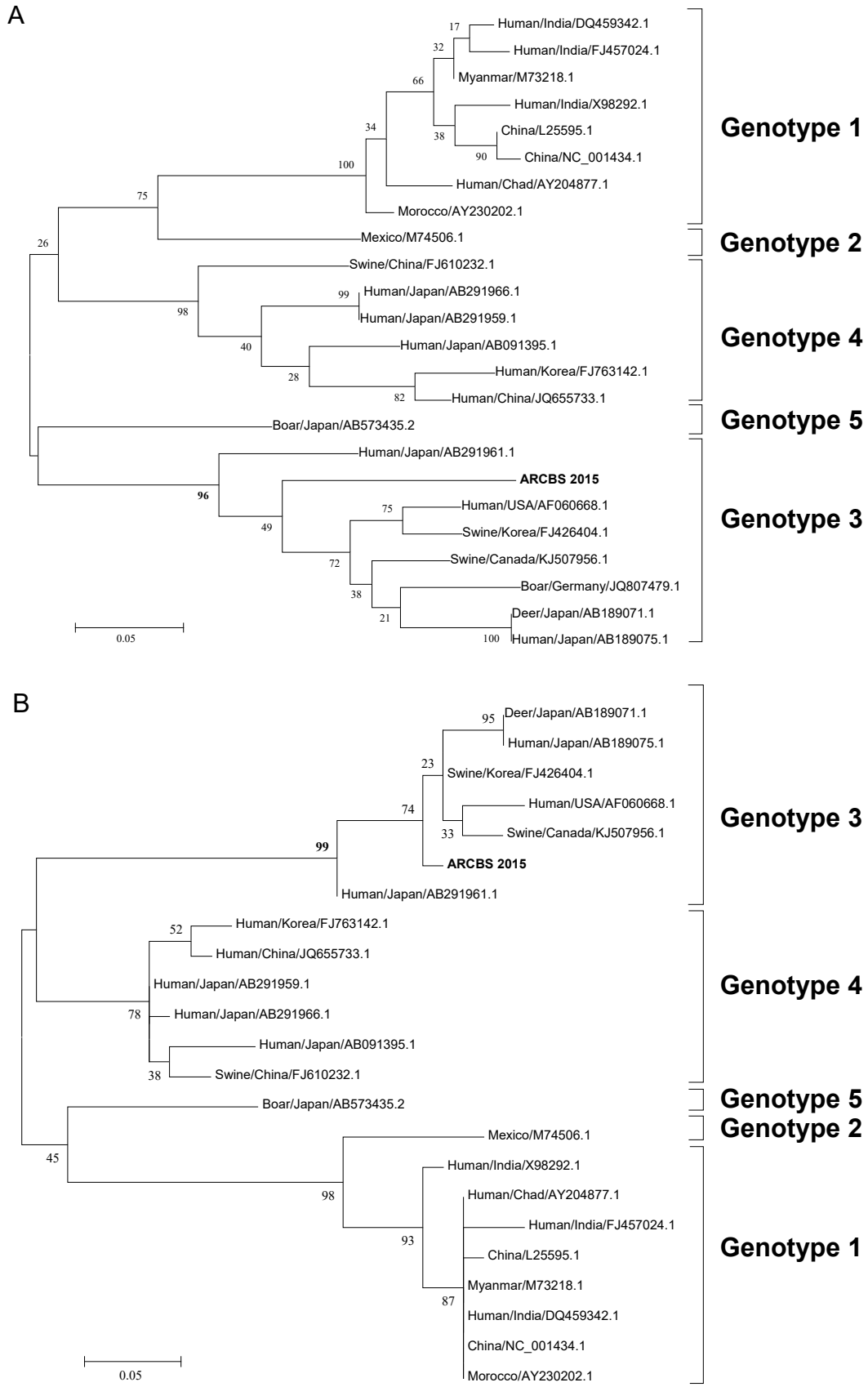


Figure 3.3: Phylogenetic analysis, based on MTase ORF1 (A) or ORF2/3 (B) of different HEV strains, including the HEV RNA positive sample identified in this study (ARCBS 2015)

3.5. Discussion

The detection of HEV RNA in asymptomatic blood donors and the demonstration of TT-HEV in immunocompromised individuals have resulted in HEV being the subject of much debate in the international transfusion community (14). In Australia, 5.99% of blood donors have been previously exposed to HEV (266). Although existing donation restrictions prevent many at-risk donors from donating (265), the residual risk of collecting an HEV infected donation remained to be investigated. The present study provided evidence of HEV RNA in Australian blood donations, which indicated a potential threat to blood supply safety.

In this study, the risk of collecting an HEV infected donation (based on detection of HEV RNA) was 1 in 14,799 donations (95% CI: 1 in 2,657 to 1 in 584,530). This rate of HEV RNA detection is similar to that reported in Scotland (1 in 14,520 individual donations) (180), however, it is lower than in other developed countries, including the UK (1 in 2,848 donors) (190), Spain (1 in 3,333 donations) (189), the Netherlands (1 in 1,322 donations) (275), Germany (1 in 1,240 donors) (187), Japan (1 in 8,658 donors) (188), USA (1 in 9,500) (191), and China (1 in 1,494 donors) (167). This reflects the HEV seroprevalence in these countries (108, 167, 169, 178, 180, 189), and is also consistent with the considerably lower HEV case notification data in Australia (265). However, notification data represent symptomatic cases, which are notified to a health authority following the patient seeking health care and confirmation of diagnosis (276), whereas RNA detection in blood donors is likely to represent asymptomatic cases. Despite these differences, both the rates of asymptomatic and symptomatic HEV in Australia are estimated to be low (15, 265). Further clinical studies exploring HEV in non-travellers with acute hepatitis in Australia could possibly provide additional evidence on disease burden.

The viral load in the HEV RNA positive sample was estimated to be 15,000 IU/ml. This was within the range reported among Dutch (less than 25 IU/ml to greater than 100,000 IU/ml) (178), Swedish (1,660-478,630 IU/ml) and German blood donors (1,820-223,872 IU/ml) (277), but higher than reported in a blood donor from the USA (14 IU/ml) (191). Viral loads ranging between 407 IU/ml and 257,039 IU/ml in blood donations have been associated with TT-HEV (190). This same study also demonstrated that only 42% of recipients were infected with HEV after transfusion from infected donations, with no association with viral load observed (190). In this study, the risk of transfusion

transmission from the HEV RNA positive donation could not be determined as this was a delinked study, so neither the donor nor the recipient were identified.

The HEV RNA positive sample was determined to be genotype 3. As HEV genotype 3 is predominately transmitted through the consumption of undercooked infected pork (12), this indicates the infected donor was likely to have acquired the infection through zoonotic transmission. The donor could have acquired HEV infection locally in Australia; indeed, the majority of autochthonous HEV in Australia are genotype 3 (83, 201). Alternatively, the donor may have been infected overseas in a developed country, where HEV genotypes 3 are commonly reported (125, 128). Given the majority of HEV infections with genotype 3 are asymptomatic (67-98%) (140), current infection of a blood donor, who must be well at the time of donation, is plausible.

Surprisingly, HEV antigen prevalence (0.35%) in Australian blood donors was higher than those reported in China (0.06%) (225), an endemic country with higher HEV RNA prevalence. With limited studies on HEV antigen prevalence and only one commercially available assay; interpretation of such differences is challenging. One of the samples testing positive by TMA and RT-PCR was also HEV antigen positive; however, all other antigen positive samples were negative for HEV RNA. This indicates a poor agreement between HEV antigen and RNA, which could be due to these markers occurring at different stages of infection, or non-specificity in the HEV antigen assay. Only a few studies have reported the use of HEV antigen assay as an alternative for HEV RNA detection (278, 279). A study in the Chinese blood donor population demonstrated non-concordance between antigen and RNA positivity (50% of HEV antigen positive being RNA positive), a study in German blood donors has revealed HEV antigen in 40% of HEV RNA positive samples (225, 240). Since the majority of HEV antigen positive samples (98%) were negative for HEV RNA and/or antibodies in this study, this could be due to false positive results with the antigen assay. With only one HEV antigen commercial assay available and one sample positive for HEV RNA obtained from this study, assay performance could not be determined.

Blood transfusion is one of the possible routes of HEV transmission (108), and the virus is known to cause acute and chronic hepatitis, especially in immunocompromised individuals (257). Given that the majority of HEV cases are asymptomatic and unexpectedly high

prevalence of asymptomatic viraemia has been detected in blood donors internationally (189, 190), screening of blood donations for HEV is widely debated in the transfusion community (14). This study provides inaugural data on HEV RNA prevalence in Australian blood donations. The rate of detection of HEV RNA (1 in 14,799 donations) in Australian blood donations appears to be lower than in many other developed countries, however, given the wide confidence intervals and lack of data on donation types, there is a need for future studies to more precisely evaluate the risk posed by this virus. HEV transmission may fluctuate in both human and animal reservoirs and novel genetic variants may emerge (280); the implications for transfusion safety are yet to be understood.

3.6. Contribution of the Chapter to the Research Question

This chapter has addressed the research question of this thesis in the following ways:

- Demonstrated Australian blood donors were infected with HEV.
- Estimated the risk of collecting HEV infectious donation based on the rate of HEV RNA detection in blood donations.

Chapter 4. Overseas-Acquired Hepatitis E Virus in Australia and Assessing the Threat to Blood Supply Safety

Context

The preceding chapters (Chapters 2 and Chapter 3) measured previous and current HEV exposure in Australian blood donations respectively. Further studies are required to assess whether current Australian donor guidelines manage any TT-HEV risk**.** This chapter therefore aims to determine if existing Blood Service travel deferral policies assist with minimising the risk of TT-HEV from imported HEV infections. Trends in notified cases of HEV in Australia are analysed based on demographic details and place of acquisition. Countries considered at higher risk for HEV exposure are identified and the rate of importation estimated based on travel data. The study provides evidence in relation to whether existing Blood Service travel-related exclusion policy for malaria manages the potential risk of TT-HEV from travellers.

This chapter has been published in the journal Blood Transfusion:

Shrestha AC, Flower RL, Seed CR, Keller AJ, Hoad V, Harley R, Leader R, Polkinghorne B, Furlong C, Faddy HM. Hepatitis E Virus Infections in Travellers: Assessing the Threat to the Australian Blood Supply. *Blood Transfus* 2016; DOI 10.2450/2016.0064-16

4.1. Introduction

HEV is a cause of acute hepatitis (190), associated with large outbreaks in developing countries resulting from faecal-oral transmission (12). In some developed countries, the majority of cases are associated with travellers returning from countries endemic for HEV (12, 108, 125). Autochthonous HEV infection transmitted via the consumption of undercooked contaminated meat or contact with infected animals, has recently emerged as a major transmission route in developed countries (140, 281, 282). Other modes of transmission include congenital transmission, as well as transmission through infected organs or blood (108, 283), which has led to HEV gaining the attention of the transfusion medicine community.

HEV is a single stranded positive sense RNA virus. The virus is the only genus of the *Hepeviridae* family (13, 96). There are four known genotypes (HEV 1, 2, 3 and 4) infecting humans, subdivided into 24 sub-genotypes (13, 128), which represent a common serotype (12). The genotypes vary in geographical distribution and mode of transmission (284). Genotype 1 has been reported in Asia and Africa while genotype 2 has been reported in Mexico, Nigeria and Chad (12). Both these genotypes infect humans only. Genotypes 3 and 4 infect humans and animals and are seen in Europe, and South-east Asia (108). HEV genotype 3 has recently been reported in the USA (285). HEV genotype 4 has been reported in Japan and China (12). The transmission route of genotypes 1 and 2 is faecal-oral, while genotypes 3 and 4 are predominantly transmitted via contact with infected animals or through the consumption of undercooked infected meat (primarily pork, but also deer and wild-boar) (108, 125, 286).

In 2005, it was estimated that there were 20.1 million incident HEV genotypes 1 and 2 infections globally, resulting in 3.4 million symptomatic cases, 70,000 deaths and 3,000 still births (161). In developing countries, the rate of mortality in pregnant women can be up to 25% (12). The majority of HEV infections are asymptomatic, however, there are differences in asymptomatic rates between developing countries (where up to 50% of cases can be asymptomatic) where genotypes 1 and 2 predominate and developed countries (where the asymptomatic rate can be 67-98%) where genotypes 3 and 4 predominate (140). This may be due to the infecting genotype, or the underlying health of the population. Symptomatic infection with HEV is similar to infection with other hepatitis viruses, including anorexia, vomiting, jaundice and hepatomegaly (12). Chronic HEV

infections, due to genotypes 3 and 4, with a viraemic phase of more than three months, have been reported in solid organ transplant recipients and in patients with immunosuppressive disorders (143).

Hepatitis E is nationally notifiable in Australia (287). HEV seroprevalence was estimated to be 5.99% among Australian blood donors, with a higher prevalence in donors reporting overseas travels (6.38%) compared to donors who had not travelled overseas (3.37%) (266). HEV cases have been associated with travel to countries endemic for HEV, including India, Sri Lanka, Vietnam and Thailand (193). Locally-acquired HEV infections are also reported in Australia, albeit more rarely. 24 HEV cases were reported from October 2013 to June 2014, from the state of NSW, all with no recent overseas travel history and all linked to the consumption of undercooked infected pork (201). 18 of these cases were associated with an outbreak from a single restaurant (201). Recently a case of locally-acquired HEV was reported in a liver transplant recipient who had also received a blood transfusion (83). There was insufficient evidence to elucidate the exact route of transmission, however, it was postulated to include contaminated food or transfusion-transmission (83).

There has been increasing concern in relation to HEV within the transfusion community, due to the high proportion of asymptomatic infections. HEV RNA has been detected in blood donors in the UK, Japan, Germany, the Netherlands, Spain and Scotland (13, 190). Moreover, TT-HEV has been reported in the UK, Japan and France (17, 156, 190), leading to symptomatic disease in some transfusion recipients (17, 109). HEV has also been transmitted via plasma treated with a PIT, which demonstrates resistance of the virus to that type of PIT (18).

To date there are no published case reports of TT-HEV in Australia. Blood donations are currently not screened for markers of HEV infection in Australia, however, such testing has been proposed in France and the UK (14, 262), and has been implemented in the Hokkaido region of northern Japan (14, 260). Given the risk for TT-HEV, the Blood Service manages this risk through medical and travel history examination via a mandatory pre-donation history questionnaire. This results in the total exclusion of individuals diagnosed with an HEV infection for 12 months from the date of recovery (71). Moreover, in instances where a donor notifies the Blood Service of an infection post donation, fresh components

(red blood cells, platelets and fresh frozen plasma) are recalled for up to two months prior to the date of the donor becoming ill (71). Potential donors are also deferred from all types of blood donation if they have had household or sexual/mucosal contact with an infected person (71). Donors are also excluded from donating fresh components for a minimum of 120 days after travel to countries endemic for malaria and until the donation tests negative on malarial antibody screening (71). This travel deferral may prevent the risk of collecting an HEV infectious donation as many of these countries are also endemic for HEV.

4.2. Aims

This chapter aimed to:

- Describe overseas-acquired HEV cases notified in Australia in order to determine whether infection in travellers poses a risk to Australian blood supply safety
- Provide evidence in relation to whether the existing Blood Service travel-related exclusion policy for malaria manages the potential risk of TT-HEV from travellers

4.3. Materials and Methods

4.3.1. HEV Surveillance System in Australia

Hepatitis E is classified as a gastrointestinal disease and there is a requirement for all cases to be notified to state and territory health departments under their public health legislation. States and territories forward de-identified notification data to the Australian Government Department of Health's National Notifiable Diseases Surveillance System (NNDSS) (276). Only confirmed cases of HEV are notified, and HEV has been nationally notifiable since 1999. Cases therefore represent only those for which healthcare was sought by the patient, a test conducted, diagnosis made and confirmed, followed by notification to a health authority.

4.3.2. Case Definition

A confirmed case of HEV infection refers to one confirmed by laboratory definitive evidence. During the period of this study, the evidence was based on detection of: HEV RNA by NAT; virus by electron microscopy; IgG seroconversion; or a four-fold or greater rise in antibody titre to HEV (287). A case was also considered to be confirmed if laboratory suggestive evidence (detection of HEV IgG or IgM) was supported by clinical evidence (a clinically compatible illness) and epidemiological evidence (travel to an HEV

endemic country 15-64 days prior to the onset of disease or an epidemiological link to confirmed case) (287).

4.3.3. HEV Cases

Details of all HEV cases notified to public health authorities based on diagnosis date between 2002 and 2014 inclusive were extracted from the NNDSS. These data included diagnosis date, age, sex, state/territory of residence, and country of acquisition. The age of an individual was as reported to the health authority or calculated at onset, using the difference between date of birth and diagnosis date. Place of acquisition was usually obtained through public health follow-up.

4.3.4. Overseas Travel Data

The number of short term resident returns and visitor arrivals in Australia were accessed from the Australian Government Department of Immigration and Border Protection website (288). Visitor arrivals were included in this analysis because all hepatitis E infections diagnosed in Australia are notified to NNDSS, including international visitors. Also, providing all other donation requirements are met, international visitors are able to donate in Australia, although this group likely represents a small proportion of total donations. These data were obtained from July 2004 to December 2014 for countries of relevance. The source of Overseas Arrival and Departure (OAD) data (arrival and departure data for Australian residents or overseas visitors, through Australian airports and seaports) was incoming and outgoing passenger cards, which were matched with data from passports and visas (289). OAD data describes the number of movements of travellers rather than the number of travellers (288).

4.3.5. Data Analysis

Firstly, HEV cases in Australia were separated based on place of acquisition (local, overseas, and unknown). As this study focused on HEV cases in Australia acquired overseas, only overseas-acquired infections were included in subsequent analyses. These cases were then described by age, sex, year and seasonality of acquisition, as well as country of acquisition. The estimated HEV importation rate was then determined for countries with five or more cases of overseas-acquired HEV, based on the number of people in Australia who had recently (within 1 year) travelled to such countries. Short term movement information was used rather than long term movement to capture recent travel

and to minimize inaccuracies due to travel to multiple countries (more likely with longer travel). Short term movements refer to movements within one year, which includes short term resident departure, short term resident return (STRR), short term visitor departure and short term visitor arrivals (STVA) (289). STRR data were used to capture travel of Australian residents, while STVA data used for travelling non-residents. The number of people (STRR and STVA) arriving in Australia following travel to countries where five or more HEV cases were acquired during the study period were calculated. Importation rates over the study period were calculated per 10,000 persons. Countries with overseas-acquired HEV were then compared to those where donations are restricted for travel due to malaria-risk as per the Blood Service guidelines for selection of blood donors. Only individuals between the ages 15-69 years, representing those who are eligible to donate blood in Australia, were included in this analysis.

4.3.6. Ethical Approval

Ethical approval for this study was obtained from ACT Health Human Research Ethics Committee, the University of Queensland School of Medicine Low Risk Ethical Review Committee and the Blood Service Human Research Ethics Committee (Appendix II.a, II.c. II.d).

4.4. Results

During the study period, there were 400 cases of HEV notified to Australian health authorities. Of these, 332 cases (83%) were acquired overseas, with 41 (10%) locally-acquired and 27 (7%) cases with an unknown country of acquisition (Table 4.1). Only those cases confirmed to be acquired overseas were included in subsequent analyses. No individual had more than one country of acquisition listed.

The highest number (13%) of overseas-acquired HEV cases occurred in 2008, however, trends by year were non uniform and no seasonality was observed (Figure 4.1). 65% of overseas-acquired HEV infections were in males, and 22% of cases were in individuals aged 25-29 years (Figure 4.2). Individuals residing in the state of NSW represented 40% of overseas-acquired HEV, with 32% from Victoria, 13% from Queensland and 13% from the remaining states/territories (Table 4.2).

Table 4.1: Acquisition of HEV cases notified in Australia between 2002 and 2014: Local or Overseas

Year	Acquisition of HEV		
	Overseas	Local	Unknown
2002	6	0	5
2003	7	2	3
2004	25	1	2
2005	29	1	0
2006	22	1	1
2007	16	1	1
2008	42	2	0
2009	30	2	1
2010	33	2	2
2011	35	3	3
2012	31	0	1
2013	27	5	2
2014	29	21	6
Total	332	41	27

Travel to India accounted for 48% of overseas-acquired HEV infections, followed by travel to Bangladesh (12%), Nepal (7%) and Pakistan (4%) (Table 4.2). Based on the number of travellers arriving into Australia, the risk of HEV acquisition was highest for travel to Nepal (18 per 10,000 arriving travellers), and Bangladesh (17 cases per 10,000 travellers) followed by Sudan (14 cases per 10,000 travellers) and Pakistan (5 per 10,000 travellers) (Table 4.3).

All these ‘higher-risk’ countries are also endemic for malaria (252); blood donors returning from these countries are unable to donate fresh components for 4 months following their return. Moreover, countries where donations are restricted following travel due to malaria-risk accounted for 94% (298/316) of overseas-acquired HEV cases, within the age range eligible to donate blood in Australia.

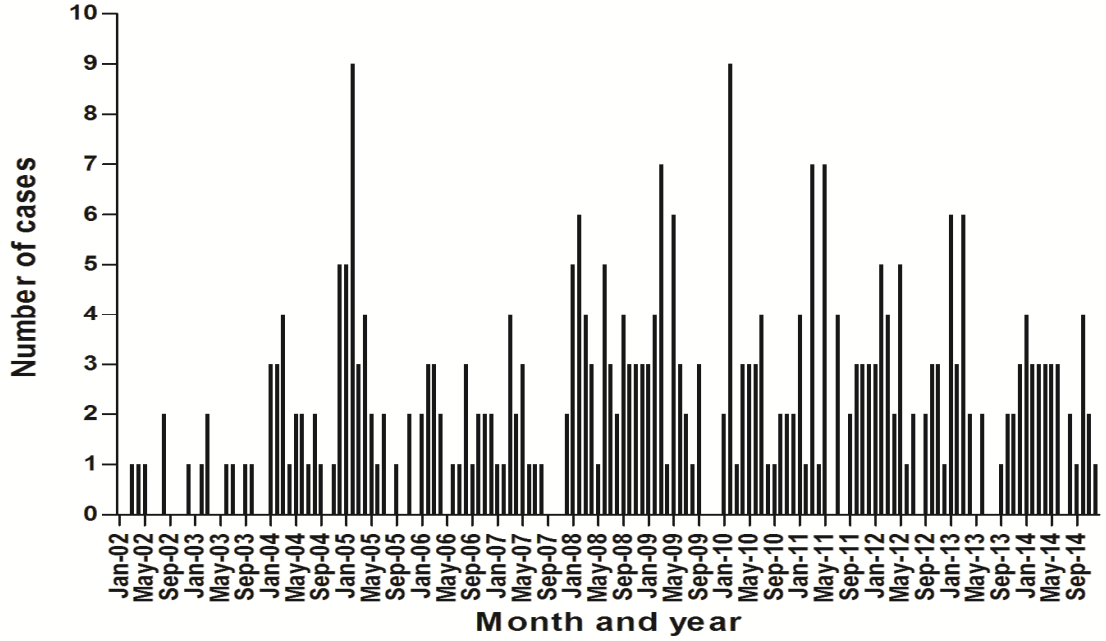


Figure 4.1: Overseas-acquired HEV cases notified in Australia between 2002 and 2014, by month and year (Data Source: Australian Government Department of Health, Communicable Diseases Network Australia)

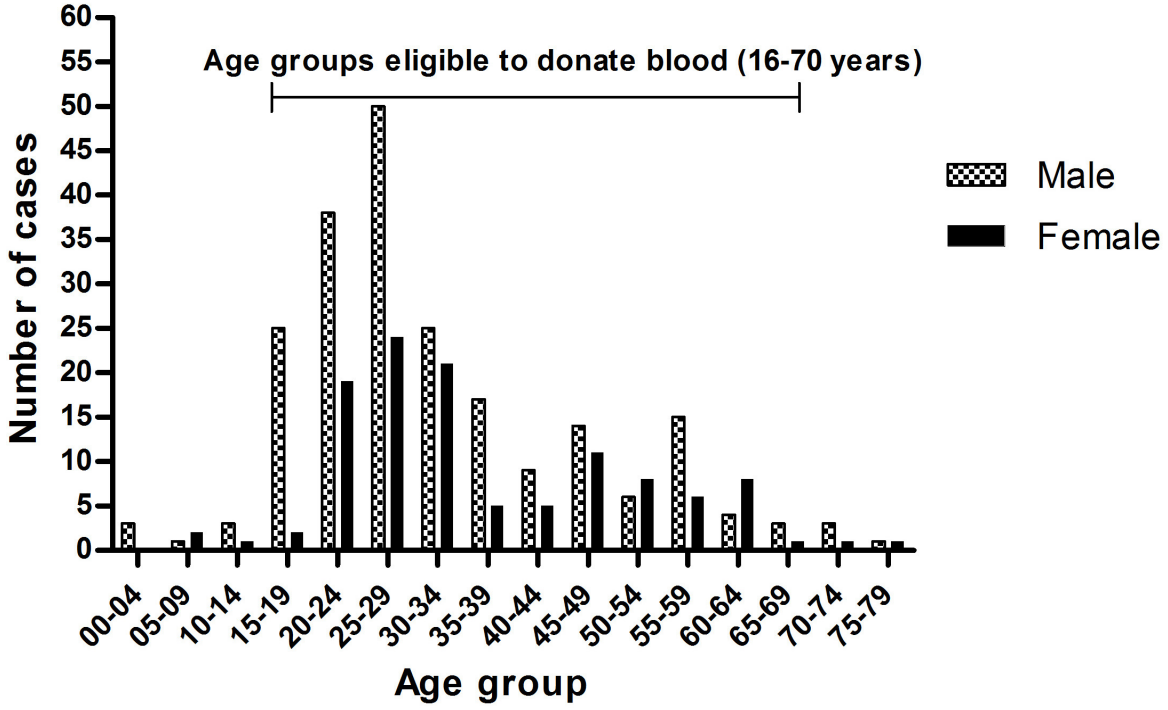


Figure 4.2: Overseas-acquired HEV cases notified in Australia between 2002 and 2014, by age group and sex (Data Source: Australian Government Department of Health, Communicable Diseases Network Australia)

Table 4.2: Country of acquisiton of overseas-acquired HEV cases notified in Australia between 2002 and 2014

Country	State of residence									Total
	ACT	NSW	NT	QLD	SA	TAS	VIC	WA	Unknown	
India	6	58	0	17	0	1	66	11	1	160 (48.19%)
Bangladesh	2	24	0	2	0	0	10	2	0	40 (12.05%)
Nepal	1	12	0	1	0	0	6	0	2	22 (6.63%)
Pakistan	2	7	0	0	0	0	5	0	0	14 (4.22%)
China[a]	0	7	0	4	0	0	3	0	0	14 (4.22%)
Thailand	0	4	0	1	0	0	3	4	0	12 (3.61%)
Vietnam	0	2	0	3	0	0	4	0	0	9 (2.71%)
Indonesia	0	4	0	0	0	0	0	2	1	7 (2.10%)
Sudan	0	2	0	1	0	0	2	0	0	5 (1.51%)

Country	State of residence									Total
	ACT	NSW	NT	QLD	SA	TAS	VIC	WA	Unknown	
Hong Kong	0	3	0	1	0	0	0	1	0	5 (1.51%)
Papua New Guinea	0	1	0	1	0	0	1	0	0	3 (1%)
Timor-Leste	0	0	3	0	0	0	0	0	0	3 (1%)
Other Countries[b]	2	9	0	12	0	2	7	4	2	38 (11.45%)
Total	13 (3.92%)	133 (40.06%)	3 (1.00%)	43 (12.95%)	0	3 (1.00%)	107 (32.23%)	24 (7.23%)	6 (1.81%)	332

[a]Excludes SARS (Special Administrative Regions) and Taiwan.

[b]Other Countries: Afghanistan (n=1), The Americas (n=1), Cambodia (n=2), Chinese Asia (includes Mongolia; n=1), Egypt (n=1), Greece (n=2), Iran (n=2), Italy (n=1), Mainland South East-Asia (n=1), Malaysia (n=2), Mozambique (n=1), Namibia (n=1), North Africa (n=1), North-East Asia (n=1), Peru (n=1), The Philippines (n=2), Singapore (n=3), South-East Asia (n=2), Southern Asia (n=1), Sri-Lanka (n=2), Turkey (n=1), United Arab Emirates (n=1), United Kingdom Channel Islands and Isle of Man (n=1), other unknown countries (n=6).

ACT: Australian Capital Territory, NSW: New South Wales, NT: Northern Territory, QLD: Queensland, SA: South Australian, TAS: Tasmania, VIC: Victoria, WA: Western Australia

Table 4.3: HEV importation rate per 10,000 travellers, July 2004-December 2014, by country of acquisition

Country	Number of imported cases	Total travellers	HEV importation rate
Bangladesh	40	23,227	17.22
China[a]	14	5,742,036	0.02
India	160	527,244	3.03
Indonesia	6	7,295,352	0.01
Nepal	22	12,404	17.74
Pakistan	14	28,226	4.96
Sudan	5	3,652	13.69
Thailand	12	6,320,903	0.02
Vietnam	9	1,264,056	0.07

*Excludes SARS (Special Administrative Regions) and Taiwan

4.5. Discussion

Due to accumulating reports of TT-HEV, this agent has gained the attention of the transfusion medicine community globally. In Australia, diagnosed autochthonous HEV is rare, with the majority of infections in overseas travellers. Indeed, it has been previously shown that 6.4% of Australian blood donors who had travelled overseas were previously exposed to HEV (266). The present study demonstrates that the majority of notified overseas-acquired HEV infections in Australia were in travellers returning from South Asia, namely India, Bangladesh and Nepal. These countries are endemic for HEV, where large water-borne outbreaks occur seasonally (13, 148, 243, 290). Thus, there is a potential risk to blood safety in Australia from donors after their return from such countries. However, the majority of HEV importations were acquired from countries where donation-related travel restrictions for malaria exist, demonstrating that existing Blood Service travel deferral policies assist with minimising the risk to fresh components from imported HEV infections.

Despite seasonal HEV outbreaks occurring in developing countries (140), no seasonality was observed in HEV cases in Australian travellers during the study period. Possible reasons for this could be due to ill travellers being diagnosed overseas, a lag between infection and notification in Australia, or be masked by under reporting given the high

asymptomatic rate. The number of travellers to endemic countries during seasonal outbreaks may influence cases diagnosed in Australia. However, data on travel period was not included in these analyses. In many developed countries, where HEV genotypes 3 and 4 occur, elderly males are predominantly affected (125). In this study, higher numbers of cases were among males and in younger aged individuals. This could be due to the different genotypes present in South Asian countries (13), which account for the majority of notified overseas-acquired HEV cases in Australia. The demographics of Australian travellers and/or food habits of younger travellers could also explain this.

Where known, the countries of acquisition of all cases in this study are endemic for HEV, with the majority in Asia and Africa categorised highly endemic and those in Europe (including UK, France) and America endemic (291). Specifically, 83% of overseas-acquired cases notified were acquired in India, Bangladesh, Nepal, Pakistan, Sudan, China (excluding SARs and Taiwan), Vietnam or Thailand. The year with the highest number of imported HEV cases was 2008; this may be linked to outbreaks of HEV in these countries. Indeed, in 2008 there were notable outbreaks for HEV in Bangladesh and India (243, 292). Despite the highest number of cases being from India, the rate of HEV importation was higher from Nepal, Bangladesh, Sudan and Pakistan, due to the large numbers of travellers to India and smaller numbers to Nepal, Bangladesh, Sudan and Pakistan. Therefore, both the number of cases and number of travellers returning from respective countries should be considered when determining which countries are ‘at-risk’. One of the limitations of this study is that OAD data represents number of movements rather than number of travellers; however, in the absence of data in relation to the latter, OAD estimates were used to approximate the rate of HEV importation.

HEV is transfusion-transmissible and hence importations of HEV into Australia have the potential to pose a risk to the safety of the Australian blood supply. Current strategies to mitigate the risk of TT-HEV include a medical and travel history examination and donor questionnaire. Symptomatic cases of HEV are managed by deferring potential donors from donating for 12 months from the date of recovery, however they may escape if in the incubation period (71). However, given cases are viraemic before the onset of symptoms and the majority of cases are asymptomatic, this strategy only has limited effectiveness in mitigating the risk.

The Blood Service guidelines for selection of blood donors list countries ‘at-risk’ for various infectious diseases to ensure transfusion safety. Travel risk is assessed via a questionnaire that asks whether a donor has travelled overseas in the past three years. In this study, 94% of notified overseas-acquired HEV cases were acquired from countries where donors are currently restricted from donating fresh components for 4 months after leaving such countries, due to risk of malaria (71). Countries not covered by malaria restrictions include the Americas (n=1), Greece (n=2), Hong Kong (n=5), Singapore (n=3), United Arab Emirates (n=1), and the UK, Channel Islands and Isle of Man (n=1), however, based on these very small numbers, travel to such countries is unlikely to pose a significant risk to transfusion safety in Australia. The typical length of HEV viremia of four to six weeks in most individuals (12) has recently been challenged in a study of asymptomatic viremic Dutch blood donors (255), where the authors estimated the mean duration of viremia to be 68 days. However, this calculation excluded donors with a shorter period of viremia whom the length could not be calculated, and repeat testing documented very low viral loads at levels where infectivity has not been determined. Therefore, these existing 4 month travel deferral policies assist with minimising the risk to fresh components from imported HEV infections. However, countries like Sri Lanka are progressing towards eliminating malaria (293), and this may necessitate reconsideration in the selection of blood donors in the future.

Underreporting of HEV is likely as most cases are asymptomatic (13). There is also a likelihood of misdiagnosis, as infection with HEV shares common clinical features with other hepatitis viruses and drug induced liver injury (294), or the possibility of under-diagnosis, perhaps due to limited knowledge among general practitioners concerning this disease. Since the majority of HEV infections with genotype 3 are asymptomatic (67-98%) (140), such cases are unlikely to be identified and notified. This is of particular significance for transfusion safety, given genotype 3 can cause chronic infection in patients with immunosuppressive disorders, who are disproportionately represented as fresh blood component recipients. Therefore, overseas acquired notification data may more likely represent genotype 1 and 2 infection and these data may not reflect the transfusion risk. Under-diagnosis is also possible as laboratory diagnosis for HEV is often considered only for overseas travellers in Australia (15), however, this message is actively being challenged by public health authorities. Moreover, the case data used in this study were after health care was sought, laboratory testing conducted and a confirmed diagnosis

made by a clinician, followed by notification to health authorities. Data analysed in this study therefore includes symptomatic cases of HEV in Australia only; to understand the real rate of HEV importation into Australia a study examining HEV prevalence in returned travellers is needed. In this study, no information was available on whether a notified case was HEV antibody and/or RNA positive, and if the latter, the infecting genotype. This therefore prevented hypothesising the mode of transmission.

Self-limiting acute cases of HEV do not require treatment. Chronic HEV cases are treated with ribavirin and pegylated interferon-α (140). A vaccine, HEV 239 (Hecolin; Xiamen Innovax Biotech, China), has been licensed in China (15), and may be used for the high risk groups in countries endemic for HEV, such as women of child bearing-age. In developed countries like Australia, HEV safety precautions should be advised to travellers, and should include general awareness of pathogens transmitted via the faecal-oral route, as well as a recommendation for the proper handling and cooking of pork, deer and wild boar. Transfusion from HEV infected donors can have potentially severe consequences in immunocompromised recipients, and hence the threat to the blood supply from such donors also needs to be assessed.

In Australia notified HEV infections predominantly occur in overseas travellers. This differs from other developed nations such as the UK where the incidence of diagnosed HEV infection based on notification data are considerably higher than occurs in Australia (approximately 6.5 times). In addition, the proportion of indigenously acquired infections in the UK are considerably higher than in Australia, with data from 2003-2012 indicating half of UK HEV infections are locally-acquired, with 71% in 2012 (280). Increase in locally-acquired HEV cases was observed in later years of this study, mainly during 2014, corresponding to an autochthonous HEV outbreak (201). Locally-acquired HEV may therefore contribute to disease burden in the future.

To determine the threat that HEV poses specifically to the Australian blood supply the rate of HEV viraemia in the Australian and blood donation populations needs to be established. However, notification data suggests locally-acquired HEV is a rare disease and the majority of HEV cases were acquired from countries where donation-related travel restrictions for malaria exist. Given the incubation period of up to 8 weeks and expected length of infectious HEV viremia (4-6 weeks) in most individuals, notification data indicates

that existing Blood Service travel deferral policies are effective in minimising the risk from imported HEV infections.

4.6. Contribution of the Chapter to the Research Question

This chapter has addressed the research question of this thesis in the following ways:

- Analysed overseas acquired HEV cases in Australia.
- Determined highest risk countries for HEV exposure.
- Assessed current blood donor guidelines for donors travelling to countries endemic for malaria.

Chapter 5. Hepatitis E Virus Seroprevalence among Blood Donors in Nepal and Analysis of Variables as Possible Risk Factors

Context

The previous chapter (Chapter 4) identified Nepal as one of the countries at risk for HEV, based on the HEV notification data in Australia. This chapter aims to measure HEV seroprevalence in Nepal (a developing country endemic for HEV) and identify possible risk factors. This study assists in identifying differences in HEV exposure status between Nepalese and Australian blood donors. The study also looks at the impact of the 2015 earthquakes in Nepal on the possible occurrence of hepatitis E outbreak.

This chapter has been submitted to a peer-reviewed journal:

Shrestha AC, Flower RLP, Seed CR, Hoad V, Rajkarnikar M, Shrestha SK, Thapa U, Faddy HM. Hepatitis E Virus Seroepidemiology: A Post-Earthquake Study among Among Blood Donors in Nepal.

5.1. Introduction

HEV has gained public health attention as one of the causative agents of viral hepatitis. The four genotypes of this non-enveloped RNA virus differ in mode of transmission and geographical occurrence (15, 138). In developing countries, major outbreaks of HEV with genotypes 1 and 2 are associated with transmission via the faecal-oral route (138, 140). In developed countries, HEV has traditionally been associated with travel to countries endemic for HEV (295), however, locally-acquired HEV infections, associated with zoonotic transmission, are increasingly reported in such countries (102, 103).

In 2005, the global burden of HEV with genotypes 1 and 2 was estimated to be 20.1 million incident infections, which resulted in an estimate of 3.4 million symptomatic cases, 70,000 deaths and 3,000 stillbirths [8]. This estimate was based on nine regions of Asia and Africa [8]. Of the incident infections, 72% occurred in East- and South-Asia [8].

In Nepal, regular HEV outbreaks have occurred during the previous 4 decades, with reported outbreaks in 1973, 1981-1982, 1987, 1995 and 2014 (128, 148, 202). During these outbreaks, a maternal mortality rate of 21-25% was reported (148). During an outbreak in Biratnagar in 2014, the case fatality rate was 0.2% (202). HEV IgG prevalence in 1999-2000 was estimated to be 38% among the general population of Nepal (148). Kathmandu was designated hyper-endemic for HEV, with rural areas non-endemic (148). A recent study has demonstrated HEV IgG prevalence of 47% among patients visiting a hospital in Kathmandu (166). HEV infections have also been reported in travellers to Nepal (265, 296, 297). Poor infrastructure development in terms of water supply and sewerage systems can facilitate the contamination of drinking water, especially during the summer monsoon season (13). Epidemics have been associated with faecal contamination of water, and molecular characterization has shown genotype 1 as a cause of acute HEV infection (203). HEV antibodies and RNA have been detected in farm swine from Kathmandu (124), indicating the possibility of zoonotic transmission in addition to the usual faeco-oral route.

In 2001, an HEV vaccine trial was conducted in Nepalese army recruits (209). A Phase II trial of the vaccine was planned with the Nepalese community, but approval to commence the trial was not given for ethical and political reasons (208). With the availability of a second vaccine candidate, HEV 239 (Hecolin : Xiamen Innovax Biotech, Xiamen, China),

there has been considerable debate in relation to the introduction of an HEV vaccine in Nepal (298-300).

The recent devastating earthquakes that occurred on 25th April and 12th May 2015 in Nepal raised concerns that the risk of an imminent HEV outbreak was very high, with HEV possibly causing up to 510 deaths in pregnant women (299). During these earthquakes and their aftermath, 8,891 people lost their lives (301), with many left homeless having to share common shelter under tents for months. Under such overcrowded living conditions poor sanitation and hygiene were likely and individuals no doubt had limited access to safe drinking water, contributing to increased potential for infectious disease outbreaks (299). The burden of HEV at this time was also expected to be high due to the approaching summer monsoon season and limited access to health facilities (299, 300). The study therefore sought to estimate the rate of previous and recent HEV infection in Nepalese blood donors in the months following the large earthquakes. This study aimed to provide surveillance data about HEV in Nepal, determine the possible impact of the recent earthquakes through serological evidence of recent HEV exposure, and analyse variables as possible risk factors for exposure to HEV.

5.2. Aims

This study aimed to:

- Measure previous, recent and current HEV infection among blood donors in Nepal (an endemic country)
- Identify variables associated with HEV exposure in Nepal
- Determine the possible impact of the recent earthquakes through serological evidence of recent HEV exposure

5.3. Methods

5.3.1. Sample Population

This was a cross-sectional study based on a convenience sampling method. A total of 1,845 blood donors eligible to donate blood as per the criteria of the Central Blood Transfusion Service, Nepal Red Cross Society, were included in this study. A cross section of samples was collected at blood transfusion services in Kathmandu (n=1,435), Chitwan (n=159), Bhaktapur (n=135), and Kavre (n=116) (Figure 5.1), during the months June-September, 2015. The required sample size for Kathmandu was estimated, using

standard methods (302), to be 1,448, based on the assumption of 38% HEV IgG prevalence (148), 95% confidence interval, and an absolute precision of 2.5% (Appendix I.c). Sample numbers from the other districts were based on accessibility to the donor population. Consent was obtained from participating blood donors, and details including age, sex and other variables were collected via additional questionnaire (Appendix VII.a, VII.b). Ethical approval was obtained from the Nepal Health Research Council and the University of Queensland Human Research Ethics Committee (Appendix II.e, II.f).

Figure 5.1: Selected districts for collection of blood donor samples, Nepal.

5.3.2. Sample Collection

Blood donor samples were collected in BD Vacutainer® PPT™ Plasma Preparation Tubes (Becton, Dickson and Company (BD) Biosciences, San Diego, USA). Samples were centrifuged at 1,258 g for 5 minutes before storage at -20^{0}C until testing.

5.3.3. Variables Obtained through Additional Questionnaire

In order to identify possible risk factors associated with HEV exposure, the following variables were included in the additional questionnaire:

1. Donor status: Donors who had previously donated were categorised as repeat donors and those who were donating for the first time as new donors.
2. History of jaundice: Jaundice was defined as any known feature of jaundice including yellow discolouration of skin and hepatitis, whether or not a donor required medical support. Family member jaundice referred to when any family member of a donor had jaundice, as defined above.

3. Source of drinking water: Donors were asked about their drinking water source, whether it be from a community tap (common tap in the community), municipality (water supplied at home by the local government authority), or underground (ground water source including hand-pumps and wells). Donors responding to two or more options were categorised as relying on multiple sources. Those reporting sources other than those mentioned above including bottled water were categorised as 'other'.
4. Drinking water treatment method: Donors were asked how they treated water for drinking purposes, which included: boiling (boiling water prior to drinking); filtering (filtration of water); chemical treatment (use of water purifier chemicals); or, no treatment (drinking directly from source). Donors with multiple options selected were categorised as multiple methods.
5. Vegetarianism: Donors who ate meat were categorised as non-vegetarian, while others were categorised as vegetarian.
6. Pork consumption: Non-vegetarians who ate pork were categorised as pork consumers and others as pork non-consumers.
7. International travel: Donors who had travelled to other countries were categorised as international travellers, while those who had not, as non-travellers.

5.3.4. Sample Testing

All samples were de-linked prior to testing. Plasma samples were tested individually for HEV IgM (Wantai HEV-IgM ELISA, Beijing Wantai Biological Pharmacy Enterprise Co., Ltd, Beijing, China), as per the manufacturer's instructions (Appendix III.b). Any samples testing positive were re-tested in duplicate. Samples that were reactive two or three times were reported as HEV IgM positive (Figure 5.2). All samples were also tested for HEV-IgG (Wantai HEV IgG ELISA, Beijing Wantai Biological Pharmacy Enterprise Co., Ltd) and HEV antigen (Wantai HEV-Ag ^{Plus} ELISA, Beijing Wantai Biological Pharmacy Enterprise Co., Ltd) as per the manufacturer's instructions (Appendix III.a, III.c) using the same testing algorithm as mentioned above for HEV IgM (Figure 5.2).

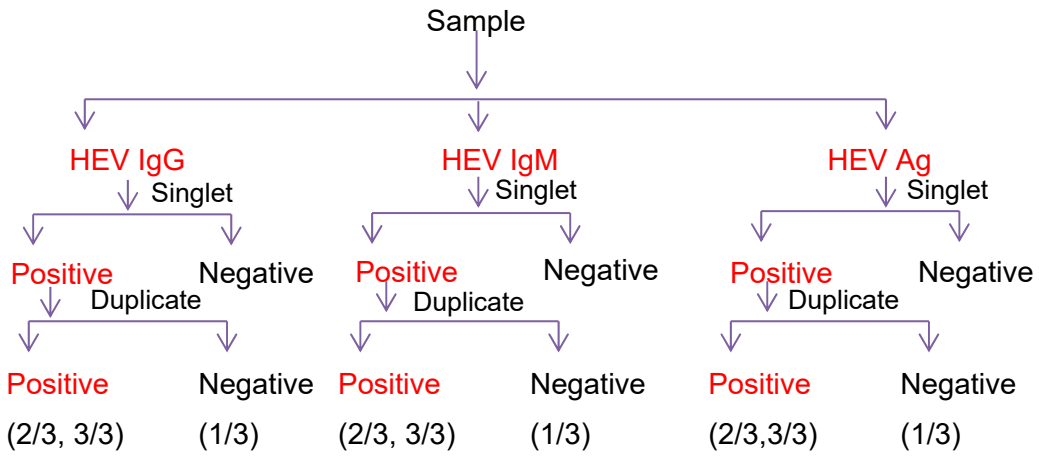


Figure 5.2: Algorithm for sample testing

5.3.5. Statistical Analysis

Donor data obtained from the questionnaire were entered in to a Microsoft Excel 2010 (Redmond, WA, USA) database. Proportions of donors HEV IgG, IgM or antigen positive were calculated overall and for each of the study variables, and 95% CIs estimated. IBM SPSS Statistics 23 (IBM Centre, NSW, Australia) was used to analyse for statistical inference (chi-square, odds ratio) and to determine association with variables. HEV IgG, IgM and antigen seropositivity were analysed as dependent variables and study variables as independent variables. Only those variables which were individually significant were included together in multivariate analyses. Some donors did not answer some of the questions on the questionnaire, resulting in missing data for some variables. In these instances, the missing data were subjected to multiple imputations prior to regression analyses. Thus, in the absence of data from donors not responding to the study variables, potential bias in the inference could not be excluded. In addition, donors' responses to the questions were based on recollection, introducing the risk of possible recall bias.

5.4. Results

Of the 1,845 samples tested, 55 (2.98%, 95% CI 2.21-3.76%) were positive for HEV IgM. The proportion of donors with both HEV IgM and IgG was 2.7%. HEV IgM prevalence was associated with a donor reporting having a history of jaundice or reporting pork consumption ($p<0.05$) (multivariate analysis) (Table 5.1 and 5.2). No associations were observed for the other factors investigated. Of the donors residing in earthquake affected regions (Kathmandu, Bhaktapur and Kavre), 3.20% (95% CI 2.36-4.04%) of donors were

HEV IgM positive, and two donors were positive for HEV antigen. These HEV antigen positive individuals were also HEV IgG positive, but negative for HEV IgM (Table 5.3). Both HEV antigen positive donors were from Kathmandu and 36 years of age.

Table 5.1: Univariate analysis of study variables and HEV IgM seropositivity

Variable	n tested	HEV IgM seropositive		Chi square	
		N	% (95% CI)	Odds ratio (95% CI)	p value
Sex					
Female	306	4	1.31 (0.03-2.58)	†	
Male	1,539	51	3.31 (2.42-4.21)	2.59 (0.93-7.21)	>0.05
Age					>0.05
< 25 years	735	18	2.45 (1.33-3.57)	†	
25-34 years	682	22	3.23 (1.90-4.55)	1.33 (0.71-2.50)	>0.05
35-44 years	315	10	3.17 (1.24-5.11)	1.31 (0.60-2.86)	>0.05
45-54 years	99	5	5.05 (0.74-9.36)	2.12 (0.77-5.84)	>0.05
55-64 years	14	0	0	0	>0.05
District					>0.05
Bhaktapur	135	6	4.44 (0.97-7.92)	7.35 (0.87-61.82)	>0.05
Kavre	116	3	2.59 (0-5.47)	4.20 (0.43-40.85)	>0.05
Kathmandu	1,435	45	3.14 (2.23-4.04)	5.12 (0.70-37.36)	>0.05
Chitwan	159	1	0.63 (0-1.86)	†	
Donor status					
Repeat	1,265	40	3.16 (2.20-4.13)	1.21(0.66-2.22)	>0.05
First time	580	15	2.59 (1.29-3.88)	†	
History of jaundice					
Yes	212	13	6.13 (2.90-9.36)	2.57 (1.35-4.88)	**<0.05**
No	1,633	42	2.57 (1.80-3.34)	†	
Family history of jaundice					
Yes	226	8	3.54 (1.13-5.95)	1.26 (0.58-2.72)	>0.05
No	1,619	47	2.90 (2.09-3.72)	†	

Variable	n tested	HEV IgM seropositive		Chi square	
		N	% (95% CI)	Odds ratio (95% CI)	p value
Sex					
Drinking water source					>0.05
Community tap	274	10	3.65 (1.43-5.87)	1.01 (0.33-3.04)	>0.05
Municipality	940	20	2.13 (1.21-3.05)	0.57 (0.24-1.37)	>0.05
Others	296	14	4.52 (2.20-6.83)	1.05 (0.21-5.29)	>0.05
Multiple sources	60	2	3.23 (0-7.62)	1.23 (0.49-3.09)	>0.05
Underground	250	9	3.47 (1.24-5.71)	†	
Drinking water treatment					>0.05
Boiling	315	13	4.13 (1.93-6.32)	†	
Filtering	1,030	30	2.91(1.89-3.94)	0.72 (0.36-1.46)	>0.05
Chemical treatment	50	1	2.00 (0-5.88)	0.50 (0.06-3.91)	>0.05
Multiple methods	202	4	1.98 (0.06-3.90)	0.53 (0.16-1.71)	>0.05
No treatment	248	7	2.82 (0.76-4.88)	0.70 (0.27-1.81)	>0.05
Vegetarianism					
Yes	1,663	48	2.89 (2.08-3.69)	†	
No	182	7	3.85 (1.05-6.64)	1.43 (0.64-3.22)	>0.05
Pork consumption					
Yes	700	29	4.14 (2.67-5.62)	1.89 (1.08-3.28)	**<0.05**
No	1,145	26	2.27 (1.41-3.13)	†	
International travel					
Yes	565	15	2.65 (1.33-3.98)	†	
No	1,280	40	3.13 (2.17-4.08)	1.16 (0.63-2.13)	>0.05

†: Reference group

Table 5.2: Multivariate analysis of study variables and HEV IgM seropositivity

Risk factor	Multivariate Analysis	
	Adjusted Odds ratio (95% CI)	p value
History of jaundice (Yes/No)	2.57 (1.35-4.88)	**<0.05**
Pork consumption (Yes/No)	1.89 (1.08-3.28)	**<0.05**

Table 5.3: HEV antigen positive donors

Variable	Sample 1059	Sample 1303
Collection Date	12/06/2015	13/06/2015
Collection District	Kathmandu	Kathmandu
Age	36	36
Sex	Male	Female
History of Jaundice	Yes	No
Family history of jaundice	No	No
Drinking water source	Municipality	Municipality
Vegetarianism	No	No
Pork consumption	No	No response
International travel	No	No
HEV IgG	Positive	Positive
HEV IgM	Negative	Negative
HEV antigen	Positive	Positive

HEV IgG was detected in 773 of the 1,845 samples tested (41.90%, 95% CI 39.65-44.15%). The prevalence was significantly higher ($p<0.05$) in Bhaktapur, Kavre and Kathmandu than the Chitwan district (Table 5.4). HEV IgG prevalence increased with increasing age and was highest (85.7%) in individuals above 55 years ($p<0.05$). HEV IgG prevalence was also higher in repeat blood donors, those with a history of jaundice and those reporting pork consumption ($p<0.05$) (multivariate analysis) (Table 5.5). Individuals who relied on drinking underground water were associated with having a lower HEV IgG prevalence ($p<0.05$) (multivariate analysis) (Table 5.5).

Table 5.4: Univariate analysis of study variables and HEV IgG seropositivity

Variable	n tested	HEV IgG seropositive		Chi square	
		n	% (95% CI)	Odds ratio (95% CI)	p value
Sex					
Female	306	109	35.62 (30.26-40.99)	†	
Male	1,539	664	43.14 (40.67-45.62)	1.37(1.06-1.77)	**<0.05**
Age					**<0.05**
< 25 years	735	155	21.09 (18.14-24.04)	†	
25-34 years	682	330	48.39 (44.64-52.14)	3.51 (2.78-4.43)	**<0.05**
35-44 years	315	203	64.44 (59.16-69.73)	6.78 (5.07-9.07)	**<0.05**
45-54 years	99	73	73.74 (65.07-82.41)	10.51 (6.49-17.00)	**<0.05**
55-64 years	14	12	85.71 (67.38-44.15)	22.45 (4.97-101.37)	**<0.05**
Districts					0.00
Bhaktapur	135	74	54.81(46.42-63.21)	10.84 (5.85-20.11)	**<0.05**
Kavre	116	52	44.83 (35.78-53.88)	7.26 (3.86-13.68)	**<0.05**
Kathmandu	1,435	631	43.97 (41.4-46.54)	7.01 (4.14-11.88)	**<0.05**
Chitwan	159	16	10.06 (5.39-14.74)	†	
Donor status					
Repeat	1,264	606	47.94 (45.19-50.70)	2.28 (1.84-2.82)	**<0.05**
First time	581	167	28.74 (25.06-32.42)	†	
History of jaundice					
Yes	213	119	55.87 (49.20-62.54)	1.91(1.43-2.55)	**<0.05**
No	1,632	654	40.07 (37.70-42.45)	†	
Family history of jaundice					
Yes	227	114	50.22 (43.72-56.72)	1.47 (1.10-1.97)	**<0.05**
No	1,618	659	40.73 (38.34-43.12)	†	
Drinking water source					
Community tap	273	103	37.72 (31.98-43.48)	1.19 (0.83-1.70)	>0.05
Municipality	940	423	45.00 (41.82-48.18)	1.62 (1.21-2.17)	**<0.05**
Others	309	135	43.69 (38.16-49.22)	1.31 (0.73-2.33)	>0.05
Multiple sources	63	25	39.68 (27.60-51.76)	1.54 (1.09-2.17)	**<0.05**
Underground	260	87	33.46 (27.73-39.20)	†	

Variable	n tested	HEV IgG seropositive		Chi square	
		n	% (95% CI)	Odds ratio (95% CI)	p value
Drinking water treatment					
Boiling	315	117	37.14 (31.81-42.48)	†	
Filtering	1,030	448	43.50 (40.47-46.52)	1.31 (1.01-1.70)	**<0.05**
Chemical treatment	49	18	36.73 (23.24-50.23)	1.00 (0.54-1.88)	>0.05
Multiple methods	203	91	44.83 (37.99-51.67)	1.39 (0.97-1.99)	>0.05
No treatment	248	99	39.92 (33.82-46.01)	1.13 (0.80-1.59)	>0.05
Vegetarianism					
Yes	1,662	701	42.18 (39.80-44.55)	1.13 (0.83-1.55)	>0.05
No	183	72	39.34 (32.27-46.42)	†	
Pork consumption					
Yes	701	323	46.08 (42.39-49.77)	1.32 (1.09-1.60)	**<0.05**
No	1,144	450	39.34 (36.50-42.17)	†	
International travel					
Yes	565	280	49.56 (45.43-53.68)	1.56 (1.28-1.92)	**<0.05**
No	1,280	493	38.52 (35.85-41.18)	†	

Table 5.5: Multivariate analysis of study variables and HEV IgG seropositivity

Risk factor	Multivariate Analysis	
	Adjusted Odds ratio (95% CI)	p value
Sex (Male/Female)	1.29 (0.96-1.73)	>0.05
Age		**<0.05**
< 25 years	†	
25-34 years	3.48 (2.71-4.48)	**<0.05**
35-44 years	7.60 (5.50-10.50)	**<0.05**
45-54 years	10.88 (6.47-18.30)	**<0.05**
55-64 years	24.57 (4.86-124.28)	**<0.05**
District		**<0.05**
Bhaktapur	13.49 (6.98-26.09)	**<0.05**
Kavre	7.01 (3.57-13.79)	**<0.05**
Kathmandu	8.02 (4.59-14.01)	**<0.05**
Chitwan	†	
Donor status (Repeat/First)	1.36 (1.07-1.74)	**<0.05**
History of Jaundice (Yes/No)	1.95 (1.39-2.72)	**<0.05**
Family history of jaundice (Yes/No)	1.23 (0.87-1.75)	>0.05
Drinking Water Source		**<0.05**
Community tap	1.40 (1.02-1.92)	**<0.05**
Municipality	0.94 (0.50-1.76)	>0.05
Others	1.18 (0.81-1.73)	>0.05
Multiple sources	0.83 (0.56-1.24)	>0.05
Underground	†	
Pork consumption (Yes/No)	1.30 (1.04-1.63)	**<0.05**
International travel (Yes/No)	0.92 (0.73-1.17)	>0.05

†: Reference group

5.5. Discussion

HEV outbreaks occur sporadically in developing countries due to faecal contamination of water and poor sanitation (108). Given a relatively high mortality rate (0.2-4%), which is particularly high in pregnant women (10-25%) (13) and there is potential for rapid increases in case numbers with limited duration of protective immunity, HEV is a topic of

public health concern in developing countries. Recent devastating earthquakes in Nepal could have facilitated an outbreak of HEV. In this study, we measured previous and current HEV infection in Nepalese blood donors after recent major earthquakes to provide surveillance data on HEV in Nepal and to determine possible risk factors for HEV exposure.

In this study, higher HEV IgG and IgM prevalence was observed in donors who reported eating pork, which is likely an indicator of zoonotic transmission (303). HEV RNA and antibodies have been detected in domestic swine in Kathmandu (124). This suggests zoonotic transmission via consumption of undercooked pork may also contribute to the burden of HEV in Nepal. However, to date, isolation of HEV genotype 3 from humans associated with swine has not been reported in Nepal.

HEV IgG prevalence in the blood donor population studied was relatively high. This is in a similar range to previous estimates in Nepal based on population studies (148, 166). HEV IgG prevalence was highest in Bhaktapur and lowest was in Chitwan, indicating HEV exposure varies between the different regions of Nepal. Geography and other factors, such as water supply systems, in these districts are likely to contribute to these observed differences. HEV IgG prevalence increased with age, which is in agreement with studies in other countries (162, 266), and indicates cumulative exposure. However, this observation differs from previous studies in Nepal, which have shown non-uniform increase with age (148, 166). The variation is likely to be due to differences in cohort selection between the studies.

Lower HEV IgG prevalence was associated with individuals relying on an underground water source. This could be due to less likelihood of faecal contamination of underground water compared to other sources. With the drinking water pipelines being adjacent to the sewer system in the Kathmandu district, there is a chance that the drinking water could be contaminated in the event of sewer leakage (148).

HEV antigen, indicative of current HEV infection, was detected in 2 donors from Kathmandu. Both of these donors were positive for HEV IgG, but negative for HEV IgM. This indicates HEV antigen is likely to persist for a short period and is undetectable by the time of appearance of HEV IgM. Concurrent detection of HEV antigen and IgG in both

these donors could indicate re-infection with HEV. In the absence of HEV RNA testing, the infectious state of these donors could not be determined.

This study was conducted during the months June-September, 2015, after the devastating earthquakes and the monsoon season period, when waterborne outbreaks of HEV were likely to occur (299, 304). Bhaktapur, Kavre and Kathmandu were among the earthquake-affected districts. In these regions, we report that 3.2% (54/1,686) of the healthy population demonstrated recent HEV exposure (through detection of HEV IgM) and we detected two donors with HEV antigen. This rate of HEV IgM prevalence was higher than in the non-earthquake affected region, Chitwan; however, a similar pattern was observed for HEV IgG, suggesting less HEV transmission in the Chitwan district. During an epidemic in Biratnagar, Nepal, 2014, HEV IgM prevalence was as high as 94-100% in acute hepatitis patients (202). In our study, subjects were blood donors, considered healthy and therefore would not capture the symptomatic group of the population. HEV IgM and antigen detection are likely to represent asymptomatic infections in blood donors. Since symptomatic HEV cases are unlikely to be included, studying blood donors may result in an inability to detect the full magnitude of an outbreak. Selecting well donors may additionally result in selection bias of those with pre-existing immunity in hyperendemic areas.

In the absence of complete population data during epidemic and inter-epidemic periods of HEV circulation, there is no definitive IgM positivity proportion that can be used to define a recent outbreak. The majority of serological studies in epidemics are done in acute cases, and not relevant to background population seroprevalence. However, population serosurveys during known large outbreaks indicate a higher prevalence of IgM positivity than detected in this study. In Sudan in 2012 a serosurvey performed before a large outbreak peak in refugee camps demonstrated an IgM positivity rate of 21.7% (305). Similarly, in a serosurvey of children aged 0-15 during an Ugandan outbreak, IgM positivity was 37.3% (306). In endemic areas, asymptomatic positivity in blood donors varies from 0.5% to 5% (242, 244, 307-309). In this study, finding of 3.2% IgM positivity in blood donors from earthquake-affected regions is consistent with ongoing endemic transmission. Therefore, this study did not find strong evidence of a large post-earthquake HEV outbreak.

HEV outbreaks in Nepal are either focal (where a large number of cases occur over days to weeks in a well-defined small population) or epidemic (148). This study did not have the power to detect a focal outbreak. The failure of this study to provide evidence of a large HEV outbreak in the months directly following the earthquakes reflects either inability of the study to detect the outbreak, or the absence of an HEV outbreak. It has been estimated that 390,000 individuals left the Kathmandu region immediately following the earthquakes, with movements into the area significantly below normal (310). These population flows may have decreased the HEV population susceptibility. If migrant populations with lower HEV immunity were disproportionately removed from the at risk population this would decrease the likelihood of an outbreak. The impact of earthquake relief support to public health threats such as provision of clean water and increased awareness of the risk may have also decreased the likelihood of an outbreak. Alternatively, given the lack of baseline HEV IgM positivity in Nepalese blood donors, it may be that 3.2% exposure represents a small outbreak. This argument is strengthened by the prolonged epidemic pattern that typically occurs in Kathmandu and the transient nature of IgM positivity (12). However, there are no recent published reports on HEV clinical cases that would indicate an outbreak in the general population post-earthquake.

Since HEV in developing countries is commonly associated with drinking contaminated water, there is less awareness of the potential risk of this virus to blood transfusion safety. A retrospective study in India has shown a higher prevalence of HEV infection markers among blood transfusion recipients compared to control groups (157). HEV is a possible risk to blood supply safety in developed countries (14, 162, 311). For developing countries, however, the main concerns are other modes of transmission, which are the major contributors to the burden of disease. However, HEV can cause chronic infection in immunocompromised individuals (13, 259), and contributes to a higher mortality rate in women during third trimester of pregnancy (140). Hence, a safe blood supply for these high-risk vulnerable patients should also be of concern in developing countries.

In summary, HEV infection in Nepalese blood donors is comparable to the general population. Past exposure to HEV was associated with multiple factors, including age, district of blood collection and consumption of pork. In developing countries like Nepal, where the main transmission route is faecal oral, other modes of transmission including zoonotic and transfusion may also occur. Detection of recent HEV infection in the donor

population demonstrates the risk of transfusion-transmission in vulnerable patients in Nepal. Unexpectedly, this study did not provide evidence of a sizeable HEV outbreak after the devastating earthquakes in 2015.

5.6. Contribution of the Chapter to the Research Question

This chapter has addressed the research question of this thesis in the following ways:

- Measured HEV exposure status in blood donors in an HEV endemic country.
- Assessed variables associated with HEV exposure in Nepalese blood donors.
- Provided data to discuss the differences in HEV prevalence between an endemic and a non-endemic country.

Chapter 6. General Discussion, Conclusion, Risk Management Options, Future Research Directions and Summary

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Context

This chapter discusses the major findings from the individual research chapters and how they address the overall research hypothesis. Management options to reduce TT-HEV risk are outlined and future research directions discussed.

6.1. General Discussion

TTIs are one of the risks for transfusion safety. Well-characterised TTIs are managed by stringent donor selection and sensitive screening tests. However, there is a threat to blood safety from emerging infectious diseases for which prevention strategies may not be in place. HEV is an emerging pathogen of possible threat to blood supply safety and has therefore gained significance in the international transfusion community. There are numerous reports describing the detection of HEV RNA in asymptomatic blood donors from developed countries (190, 255), which may lead to chronic infection in immunocompromised transfusion recipients (190). Given this, countries including the UK, Ireland and France have proposed the introduction of blood donation screening for HEV (262-264). It was therefore necessary to undertake an evaluation of the risk posed by HEV to blood supply safety in Australia. This is the first study to provide a quantitative assessment of HEV in Australia, to determine the risk posed by this virus to blood supply safety.

6.1.1. Seroprevalence of HEV in Australian Blood Donors

This study (chapter 2) measured HEV IgG prevalence of 5.99% in Australian donors, and demonstrated HEV exposure in international travellers and non-travellers, suggesting the possibility of both imported and locally-acquired HEV in Australia. The study also demonstrated prior HEV exposure was higher in donors who would have been temporarily excluded from donating on previous donation attempts for malaria and/or diarrhoea, suggesting the current management strategy in Australia is partially effective in minimizing any risk of TT-HEV. Occurrence of HEV IgG in donors who reported no overseas travel and/or no prior related deferrals coupled with the knowledge that asymptomatic infection is possible, suggests that additional safety precautions such as HEV RNA donation screening may be warranted.

6.1.2. Detection of Current Markers of HEV Infection in Australian Blood Donations

This study (chapter 3) provided evidence of current markers of HEV infection in Australian blood donations. This was the first study to measure prevalence of HEV antigen and HEV RNA in a large number of Australian blood donations. This study identified one HEV RNA positive donation, suggesting the rate of collecting an HEV infectious donations was 1 in 14,799 donations (95% CI: 1 in 2,657 to 1 in 584,530). In Australia, the risk of collecting an HEV infectious donation was lower than in most of other developed countries. The

infecting genotype was determined to be HEV genotype 3, suggesting the donor could have acquired HEV infection through zoonotic transmission, either in Australia or while travelling overseas to developed countries. The viral load in the HEV RNA positive sample was estimated to be 15,000 IU/ml, which was within the range associated with TT-HEV in the UK (400 and 250,000 IU/mL) (190). Unfortunately, as this study was de-linked, risk of transfusion transmissibility from this donation was unable to be determined. This study was also the first to measure HEV antigen in Australian blood donations. HEV antigen was detected in 0.35% of blood donations. HEV RNA was detected in only one of the above antigen positive samples, showing poor agreement between the screening tests and suggesting these markers may occur at different stages of infection. However, considering RNA detection as a 'gold standard' for HEV (240), the risk for this study was calculated based on the detection of HEV RNA in blood donations.

6.1.3. Overseas-Acquired Hepatitis E Virus in Australia and Blood Supply Safety

This study (chapter 4) demonstrated that the majority of HEV cases notified in Australia are acquired overseas (83%), especially in South Asian countries. This was in agreement with higher HEV IgG prevalence observed among blood donors who reported overseas travel compared to those who had not travelled (Chapter 2 as discussed above in section 6.1.1). However, notified cases represent symptomatic infections and seroprevalence in donors is likely to represent both asymptomatic and symptomatic cases (previously infected and recovered). The majority of HEV importations were acquired from countries where donation-related travel restrictions for malaria exist. This study showed that 94% of notified overseas-acquired HEV cases were acquired from countries where donors are currently restricted from donating fresh components for 4 months after leaving such countries. However, individuals remain eligible to donate plasma for fractionation during the restrictive period. In Australia, though overseas acquired HEV cases make up the majority of notifications, locally-acquired cases, associated with zoonotic transmission are also reported (201). HEV risk to blood safety should, therefore also be considered in international non-travellers. Currently, this is managed by donor screening through questionnaire, which is likely to prevent symptomatic donors from donating. Given that the majority of HEV cases are asymptomatic in developed countries (140), infected donors with no symptoms may escape screening prior to donation.

6.1.4. HEV Seroprevalence among Blood Donors in Nepal

This study (chapter 5) provided HEV surveillance data in Nepalese blood donors. HEV IgG prevalence in Nepalese donors (41.90%) was almost seven times higher than in Australian donors. Higher HEV prevalence in Nepalese blood donors was in agreement with the findings from chapter 4 (discussed in section 6.1.3), which showed a large number of imported HEV cases from Nepal in Australia. This study also demonstrated that the rate of HEV exposure in Nepal varied between the different regions studied. This observation was different to that in Australia with no significant difference observed between the states. HEV IgM was detected in 2.98% of Nepalese donors, indicating recent HEV infection. This study was conducted after the devastating earthquakes in 2015, when waterborne outbreaks of HEV were predicted to occur. In the studied earthquake affected areas, HEV IgM was measured to be 3.2%, which was within the range reported in blood donors from HEV endemic countries (0.5%-5%) (96, 119, 242, 307). This finding was consistent with ongoing endemic transmission and therefore did not provide strong evidence of a large post-earthquake HEV outbreak. Multivariate analyses revealed a number of variables associated with previous exposure to HEV in Nepalese blood donors, such as age, district of blood collection, history of jaundice, frequency of blood donation, source of drinking water and consumption of pork. In developing countries like Nepal, although the faecal oral route is a major transmission route, other modes of transmission are possible including zoonotic and transfusion. Given a fatality rate of 4% and maternal mortality rate of 25% in developing countries (13), risk of transfusion-transmission should also be considered for high risk groups including pregnant women and immunocompromised individuals in the absence of an approved vaccine.

6.2. Conclusion

This thesis provided a comprehensive evaluation of the risk posed by HEV to blood supply safety (summarised in figure 6.1). This work addressed knowledge gaps in relation to the burden of HEV in Australia and analysed HEV risk to transfusion safety.

This study showed that 5.99% of Australian blood donors have been previously exposed to HEV. HEV RNA prevalence was 0.006%, giving a risk of collecting a viremic donation of 1 in 14,799 (95% CI 1 in 2,657 to 1 in 584,530). Given the wide confidence interval, there is considerable uncertainty in this estimate, which may complicate future risk management modelling. The one HEV RNA positive sample was genotype 3, which indicates zoonotic

transmission, possibly acquired locally. Given that up to 98% of infections with genotype 3 and 4 are asymptomatic (140), and these genotypes occur in developed countries, locally-acquired HEV infection in Australian donors may pose a risk to blood supply safety.

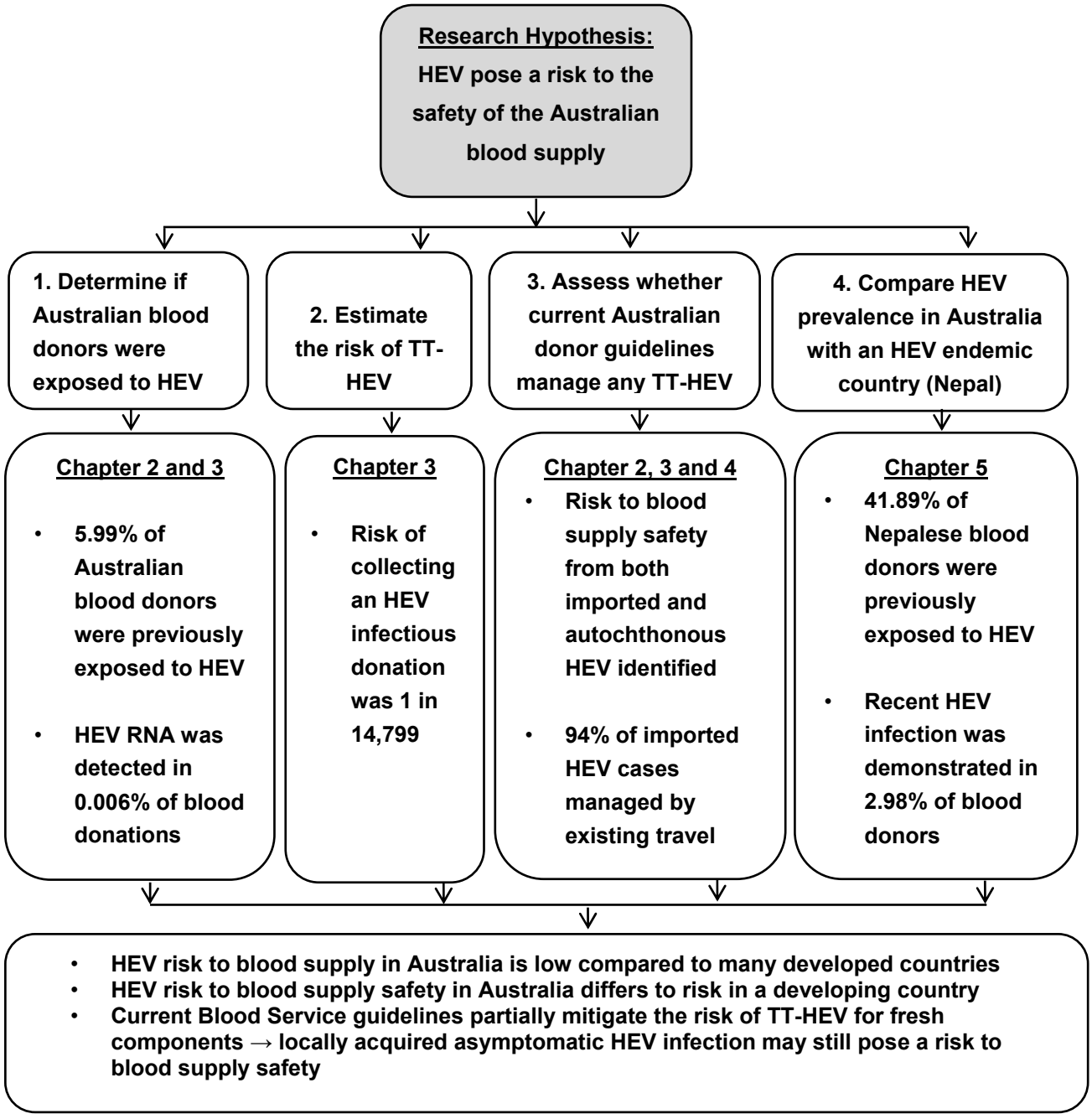

Figure 6.1: Summary of research aims, results and conclusions

This study provides evidence that the current Blood Service guidelines for donors travelling to malaria endemic countries mitigate the majority of the TT-HEV risk for fresh component donations. However, there remains a risk with plasma destined for fractionation. The fractionation process includes viral inactivation steps such as immunoaffinity chromatography, nanofiltration, cold ethanol fractionation and heat

treatment, which have been shown to reduce the infectivity of HEV by 3-4 log (48). Given these moderate reductions in HEV infectivity, the consequences of the residual viral infectivity in fractionated plasma products remain to be determined. Countries not covered by the malaria related travel restrictions contribute to only a small number of HEV imported cases in Australia, and are unlikely to pose a significant risk to transfusion safety with respect to fresh components.

This study also provided evidence of higher HEV exposure in Nepalese blood donors compared to Australian donors. Nepal is a developing country, endemic for HEV. Priorities for the prevention of TT-HEV in such countries differ based on the overall burden of disease, modes of HEV transmission and risk reduction achieved for other conventional TTIs. Although, HEV in Nepal is mainly associated with the faecal-oral route, the risk of TT-HEV to vulnerable groups should also be considered. Nepal is also endemic for malaria, therefore, Australian donors travelling to Nepal would be deferred from donating fresh components for 4 months after their return to Australia. This suggests the risk of TT-HEV to the Australian blood supply from donors travelling to Nepal is adequately managed.

HEV risk to blood supply safety in Australia appears lower than in most developed countries. However, the rate of autochthonous HEV infection needs to be monitored and the risk of TT-HEV from locally-acquired cases remains to be assessed. Given asymptomatic infections are possible, in the absence of mandatory testing for this virus, locally-acquired HEV infections in blood donors could escape current screening methods. This study highlights the need for continual due diligence and hemovigilance in relation to transfusion-transmitted emerging infectious diseases.

6.3. Management Options to Reduce the Risk of TT-HEV

Risk of TT-HEV varies in different countries based on local epidemiology, including mode of transmission, risk behaviours of donors and existing strategies for transfusion-transmitted infectious disease management. In general, risk of TT-HEV can be managed or reduced by implementing the following, individually or in combination, based on the local situation:

- Increasing the awareness of environmental sources of HEV exposure among the general population for the prevention of infection and transmission.

- Increasing the awareness of HEV among clinicians particularly in relation to the occurrence of locally-acquired HEV.
- Recommending HEV testing in acute hepatitis patients with no international travel.
- Increasing the awareness of HEV among blood donors in relation to risk behaviours such as unsafe drinking water, consumption of undercooked pork and travel to HEV endemic countries.
- Developing additional questions on the donor questionnaire considering risk factors associated with HEV infection, such as including a question on preference for eating undercooked pork.
- Introducing pathogen inactivation (if level of inactivation is considered to be appropriate and if the technology is approved for use) to inactivate HEV in fresh components.
- Introducing an HEV screening assay either for universal testing, or for specific donations such as from donors with identified risk behaviours and/or for vulnerable groups of transfusion recipients (immunocompromised, pregnant women).

6.4. Future Research Directions

This thesis has evaluated the risk posed by HEV to blood supply safety based on testing donor samples and quantitative analysis of notified HEV cases in Australia. This study has opened doors for future research, for the management of HEV in Australia, which could include:

- A linked study designed to identify HEV infected donors. This would provide further information to understand risk factors for HEV exposure and prevent the possibility of TT-HEV.
- A cost-effectiveness analysis for the introduction of laboratory testing of blood donations for HEV. Such a study is necessary to determine the feasibility for the implementation of HEV testing of donation samples.
- A study on HEV in acute hepatitis patients with no international travel history. This would assist in understanding the extent of locally-acquired HEV.
- Studies on HEV in animals and food products derived from animals that could contribute to the occurrence of zoonotic transmission. This would identify possible sources of infection that may contribute to locally-acquired HEV in Australia.

- Studies, using animal models, to determine the infectious dose of HEV for transmission through blood transfusion. This would assist in determining the infective dose required for TT-HEV.

6.5. Summary

Knowledge of HEV pathogenesis, epidemiology and diagnosis has increased with the continual research over the past 30 years. Indeed, HEV is endemic in developing countries and genotype 1 and 2 are associated with water borne outbreaks (125). Zoonotic transmission with genotypes 3 and 4 is also plausible in such countries. In developed countries, hepatitis E was considered rare and reported in travellers returning from countries endemic for HEV. However, autochthonous HEV linked to zoonotic transmission is reported in developed countries (103, 163, 246), including Australia (201). This could be attributed to changes in risk behaviours among humans, such as increased consumption of undercooked pork.

HEV was considered to cause only self-limiting acute hepatitis, but there are a number of case reports of chronic infection in immunocompromised individuals (143, 258, 312). Surprisingly, the virus has been reported to cause extrahepatic illness, including neurological complications (such as Guillain-Barré syndrome, neuritis), glomerulonephritis and pancreatitis (13, 313).

HEV is also a pathogen of interest to the transfusion science community. Given that HEV causes asymptomatic infection, HEV RNA has been detected in healthy blood donors and cases of TT-HEV have been reported (190). Since, HEV can lead to chronic infection (258), transfusion transmission to immunocompromised individuals is a major concern in developed nations. Although the disease caused by HEV is not severe compared to other well-known viral TTIs (HIV, HBV and HCV), the residual risk of TT-HEV is higher in most developed countries (311). Safety precautions such as laboratory testing of blood donations for HEV seem to be necessary for at least vulnerable groups.

The occurrence of water-borne HEV outbreaks in developing countries together with locally-acquired zoonotic and TT-HEV cases in developed nations have led to increased awareness of hepatitis E as a pathogen of global significance. The development of sensitive laboratory testing for HEV has also contributed to identifying cases, and hence

reporting increased incidence and prevalence of HEV in recent years. Hence, HEV is an emerging pathogen of public health significance, which is likely to be a threat in the future in many countries, including Australia.

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Appendices

Appendix I: Sample Size Calculations

I. a. Sample Size Calculation for HEV Seroprevalence Study in Australian Blood Donors (Chapter 2)

Estimating a population proportion with specified relative precision (302)
Seroprevalence in reference region (p) =0.04 (Prevalence = 4% (New Zealand))
Precision/Standard error (SE) = 0.007 (0.7%)
Confidence Level =95%
Sample size (n) = 1.96^2 x p(1-p)/SE^2
= 1.96 ^2 x 0.04 (0.96)/0.007^2
n= 3, 011 (Sample number considered for study = 3,237)

I. b. Sample Size Calculation for HEV RNA Prevalence in Australian Blood Donations (Chapter 3)

Sample size calculation for rate of recent HEV infection (Given the very low expected prevalence, the method used is based on standard error without confidence level) (302)
Sample size, n = p(1-p)/SE^2
p = 0.00012 (RNA prevalence in Japan is 0.012%)
Absolute precision (SE) = 0.00009 (0.009%)
n = 14,813 (Sample size considered for the study = 14,799)

I. c. Sample Size Calculation for HEV Seroprevalence in Nepalese Blood Donors (Chapter 5)

Estimating a population proportion with specified relative precision (302)
Seroprevalence in reference region (p) =0.38 (Prevalence = 38% (Kathmandu)
Confidence Level = 95%,
Standard error (SE) = 0.025 (2.5%)
Sample size (n) = 1.96^2 x p(1-p)/SE^2
= 1.96 ^2 x 0.38 x 0.62/ 0.025^2 = 1,448 (Sample size considered for Kathmandu = 1,445)

Appendix II: Ethical Approvals

II. a. Ethical Approvals (Research Chapters 2, 3 and 4)

Australian Red Cross
BLOOD SERVICE

23 July 2013

Dr Helen Faddy
Research Development
Australian Red Cross Blood Service
Level 2, 44 Musk Ave, Kelvin Grove, Q
By email: HFaddy@redcrossblood.org.au

Dear Dr Faddy,

Re: Does Hepatitis E Virus Pose a Risk to the Safety of Australia's Blood Supply? : Low risk/negligible risk research

The Blood Service Ethics Committee is a Human Research Ethics Committee established and operating in accordance with the *National Statement on Ethical Conduct in Human Research*. In considering your application, it was therefore necessary to assess whether the aforementioned project meets these requirements.

I can confirm that this project meets the criteria of low risk/negligible risk research and therefore full committee review will not be required. This project will be added to a register maintained by the Committee Secretariat.

As for all projects, the Committee has a monitoring role. Therefore, it is required that the Committee be advised immediately of any changes to the aforementioned project that would alter the level of risk associated with it.

It would be appreciated if you could notify the Secretariat at the completion of this project so that the project can be removed from the register.

If you require any further information, please do not hesitate to contact me on 02 9234 2368 or via e-mail ethics@redcrossblood.org.au.

Yours sincerely,

B Turner

Brie Turner, PhD
Human Research Ethics Committee Secretary & Communication Officer
Research and Business Development
Australian Red Cross Blood Service

Australian Red Cross Blood Service
17 O'Riordan Street
Alexandria | NSW | 2015
P: +61 2 9234 2368 | F: +61 2 9234 2411
Web: www.donateblood.com.au

Appendix II

II. b. Ethical Approval (Chapter 2 and 3)

THE UNIVERSITY OF QUEENSLAND
AUSTRALIA

School of Medicine

The University of Queensland
Mayne Medical School
Herston Road
Herston Qld 4006 Australia

School of Medicine Approval Form for Research Involving Humans Including Behavioural Research

for Honours, MPhil & PhD Students in the School of Medicine

Chief Investigator (student)	Mr. Ashish Chandra Shrestha
Project Title	Does Hepatitis E Virus Pose a Risk to the Safety of Australia's Blood Supply?
Supervisor(s)	Dr Helen Faddy
Co-Investigator(s)	Dr Helen Faddy, Prof. Robert Flower
Research Centre/Institute/School	School of Medicine / Red Cross Blood Service
SOM Clearance Number	**2013-SOMILRE-0075**
Date of Issue	29 July 2013
Date of Expiry	29 July 2015

Comments:

Full approval from Red Cross Blood Service HERC 23 July 2013, and full UQ application

UQ School of Medicine Low Risk Ethical Review Committee

This project complies with the provisions contained in the National Statement on Ethical Conduct in Human Research (complies with the regulations governing research involving humans) and UQ ethical paragraphs concerning low risk research.

UQ School of Medicine Low Risk Ethical Review Committee Representative

Associate Professor Diann Eley

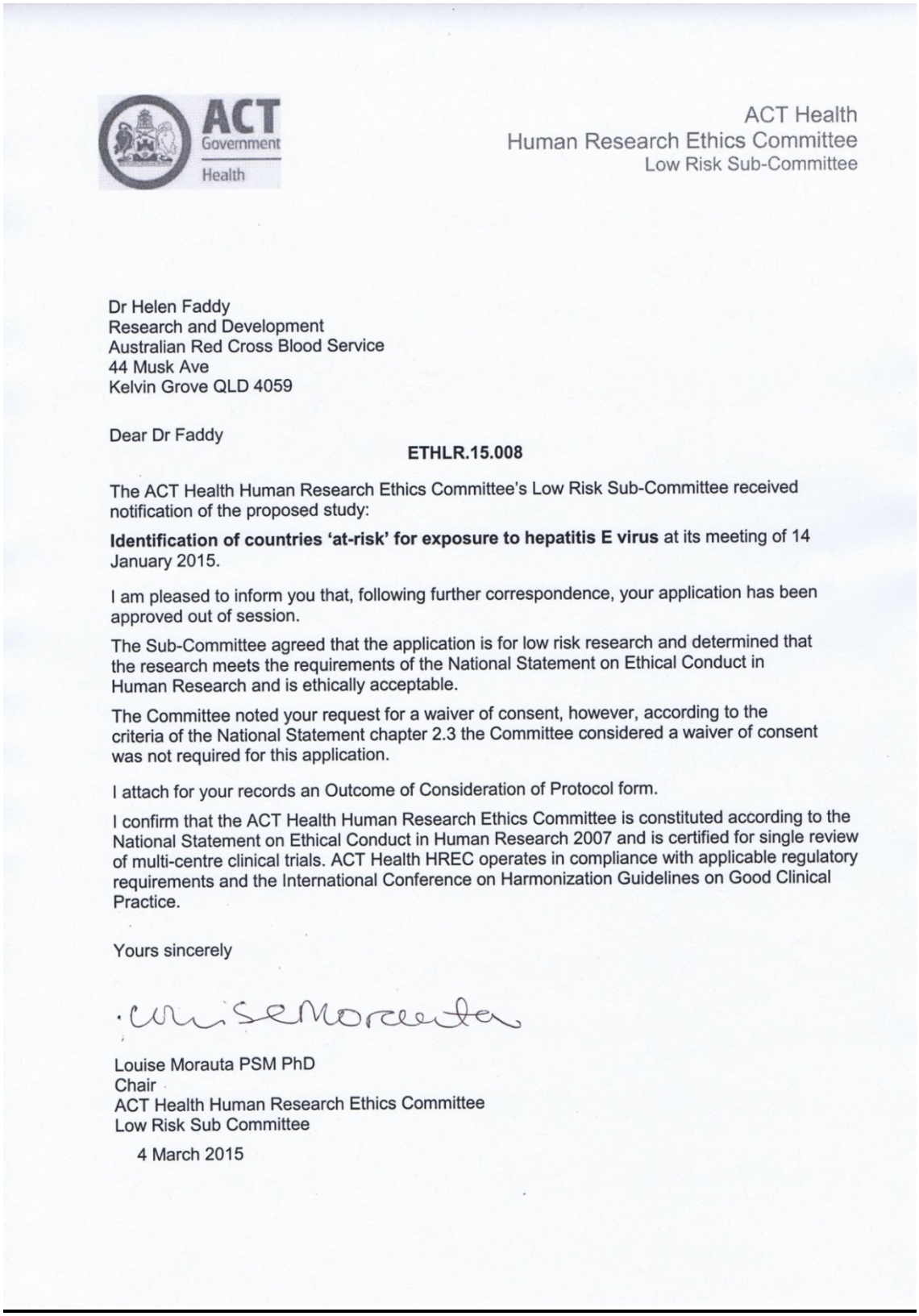
Signed

29 July 2013

Date

Appendix II

II. c. Ethical Approval (Chapter 4)



ACT Government
Health

ACT Health
Human Research Ethics Committee
Low Risk Sub-Committee

Dr Helen Faddy
Research and Development
Australian Red Cross Blood Service
44 Musk Ave
Kelvin Grove QLD 4059

Dear Dr Faddy

ETHLR.15.008

The ACT Health Human Research Ethics Committee's Low Risk Sub-Committee received notification of the proposed study:

Identification of countries 'at-risk' for exposure to hepatitis E virus at its meeting of 14 January 2015.

I am pleased to inform you that, following further correspondence, your application has been approved out of session.

The Sub-Committee agreed that the application is for low risk research and determined that the research meets the requirements of the National Statement on Ethical Conduct in Human Research and is ethically acceptable.

The Committee noted your request for a waiver of consent, however, according to the criteria of the National Statement chapter 2.3 the Committee considered a waiver of consent was not required for this application.

I attach for your records an Outcome of Consideration of Protocol form.

I confirm that the ACT Health Human Research Ethics Committee is constituted according to the National Statement on Ethical Conduct in Human Research 2007 and is certified for single review of multi-centre clinical trials. ACT Health HREC operates in compliance with applicable regulatory requirements and the International Conference on Harmonization Guidelines on Good Clinical Practice.

Yours sincerely

Louise Morauta PSM PhD
Chair
ACT Health Human Research Ethics Committee
Low Risk Sub Committee

4 March 2015

Appendix II

II. d. Ethical Approval (Chapter 4)

THE UNIVERSITY OF QUEENSLAND
AUSTRALIA

School of Medicine

The University of Queensland
Mayne Medical School
Herston Road
Herston Qld 4006 Australia

School of Medicine Approval Form for Research Involving Humans Including Behavioural Research

for Honours, MPhil & PhD Students in the School of Medicine

Chief Investigator (student)	Ashish Chandra Shrestha
Project Title	Hepatitis E virus infections in travellers: assessing the threat to the Australian blood supply: Project 1
Supervisor(s)	Prof. Robert Flower, Dr Helen Faddy
Co-Investigator(s)	Dr Helen Faddy, Prof. Robert Flower, Dr Clive R. Seed, Dr Anthony J. Keller, Dr Veronica Hoad, Dr Robert Harley, Dr Robyn Leader, Dr Ben Polkinghorne, Dr Catriona Furlong
Research Centre/Institute/School	Australian Red Cross Blood Service, Kelvin Grove
SOM Clearance Number	2015-SOMILRE-0151
Date of Issue	10 December 2015
Date of Expiry	10/12/2017

Comments:

Low risk approval: Australian Red Cross Blood Service HREC approval (HFaddy230713)

UQ School of Medicine Low Risk Ethical Review Committee

This project complies with the provisions contained in the National Statement on Ethical Conduct in Human Research (complies with the regulations governing research involving humans) and UQ ethical paragraphs concerning low risk research.

UQ School of Medicine Low Risk Ethical Review Committee Representative

Associate Professor Diann Eley

Signed Date 10/12/2015

Appendix II

II. e. Ethical Approval (Chapter 5)

Nepal Health Research Council

Estd. 1991

Ref. No.: 1519

19 March 2015

Mr. Ashish Chandra Shrestha
Principal Investigator
University of Queensland, Australia

Ref: **Approval of Research Proposal** entitles **Epidemiological study of Hepatitis E Virus among blood donors in selected districts of Nepal**

Dear Mr. Shrestha,

It is my pleasure to inform you that the above-mentioned proposal submitted on 19 January 2015 (**Reg. no. 17/2015** please use this Reg. No. during further correspondence) has been approved by NHRC Ethical Review Board on 18 March 2015 (2071- 12-4).

As per NHRC rules and regulations, the investigator has to strictly follow the protocol stipulated in the proposal. Any change in objective(s), problem statement, research question or hypothesis, methodology, implementation procedure, data management and budget that may be necessary in course of the implementation of the research proposal can only be made so and implemented after prior approval from this council. Thus, it is compulsory to submit the detail of such changes intended or desired with justification prior to actual change in the protocol.

If the researcher requires transfer of the bio samples to other countries, the investigator should apply to the NHRC for the permission.

Further, the researchers are directed to strictly abide by the National Ethical Guidelines published by NHRC during the implementation of their research proposal and submit progress report and full or summary report upon completion.

As per your research proposal, the total research amount is **US$ 3,604.00** and accordingly the processing fee amounts to **NRs- 9,930.00.** It is acknowledged that the above-mentioned processing fee has been received at NHRC.

If you have any questions, please contact the Ethical Review M & E section of NHRC.

Thanking you.

.....
Dr. Khem Bahadur Karki
Member-Secretary

Appendix II

II.f. Ethical Approval (Chapter 5)

THE UNIVERSITY OF QUEENSLAND

Institutional Human Research Ethics Approval

Project Title:	Epidemiological Study of Hepatitis E Virus Among Blood Donors in Selected Districts of Nepal
Chief Investigator:	Mr Ashish Chandra Shrestha
Supervisor:	Dr Helen Faddy, Prof Robert Flower
Co-Investigator(s):	Dr Helen Faddy, Prof Robert Flower, Dr Manita Rajkarnikar
School(s):	School of Medicine
Approval Number:	2015000593
Granting Agency/Degree:	Australian Red Cross Blood Service; Travel award from UQ Graduate School
Duration:	31st December 2015

Comments/Conditions:

Note: if this approval is for amendments to an already approved protocol for which a UQ Clinical Trials Protection/Insurance Form was originally submitted, then the researchers must directly notify the UQ Insurance Office of any changes to that Form and Participant Information Sheets & Consent Forms as a result of the amendments, before action.

Name of responsible Committee:
Medical Research Ethics Committee
This project complies with the provisions contained in the *National Statement on Ethical Conduct in Human Research* and complies with the regulations governing experimentation on humans.

Name of Ethics Committee representative:
Professor Bill Vicenzino
Chairperson
Medical Research Ethics Committee

Signature _____ Date 29/5/2015

Appendix III: Kit Instructions

III. a. Kit Instructions for Wantai HEV IgG ELISA

(The content of this appendix is based on the instructions for use of the commercial ELISA (270))

Principle

Wantai HEV IgG ELISA is an indirect method for detection of HEV IgG antibodies. Microwell strips are pre-coated with HEV recombinant protein. The kit uses a recombinant protein (PE2) containing 211 amino acids of ORF-2 derived from a Chinese isolate of HEV-1 (223, 224). HEV IgG in sample if present binds to the pre-coated HEV antigen. Unbound serum proteins are removed during washing. Rabbit anti-human IgG antibodies conjugated to horseradish peroxidase (HRP-conjugate) binds to the bound HEV IgG antibody if present. Unbound conjugate is removed after washing. Chromogen A (urea peroxide) and B (tetramethylbenzidine) are hydrolysed by bound HRP conjugate, changing colour to blue. The reaction is stopped by sulphuric acid and changes colour from blue to yellow. Colour intensity is measured with spectrophotometer. Samples negative for HEV IgG do not change colour and remains colourless

Procedure

1. All the reagents are brought to room temperature (18-30^{0}C).
2. Three wells are marked as negative control, two wells as positive controls and one as blank.
3. Specimen diluent (100 µl) is added each well except the blank.
4. Positive controls, negative controls and samples (10 µl) are added to respective wells.
5. ELISA plate is incubated for 30 minutes at 37^{0}C.
6. Each well is washed 5 times with wash buffer (diluted phosphate buffer saline with deionised water - 1:20). Microwells are soaked for 30-60 seconds in between subsequent washing.
7. The wells are blotted dry by tapping on paper towel.
8. HRP- conjugate is added to all the wells except the blank.
9. Each well is washed 5 times with wash buffer. Microwells are soaked for 30-60 seconds in between subsequent washing.

10. Chromogen A (50 µl) and chromogen B (50 µl) are added into each well included blank.
11. The plate is incubated at 37^0C for 15 minutes in dark.
12. Stop solution (50 µl) is added in each well including the blank.
13. Absorbance (A) is measured at 450nm within 10 minutes of adding stop solution.

Calculation and Quality Control (Assay Validation)

Calculation of the cut-off value (C.O) = Nc+ 0.16

Nc= mean absorbance value for negative controls after subtracting blank.

If mean absorbance value of the negative controls is lower than 0.03, NC is taken as 0.03

Quality Control (Assay Validation)

Absorbance value of the blank well is < 0.08 at 450 nm.

Absorbance values of the positive control is ≥ 0.8 at 450 nm after blanking.

Absorbance values of the Negative control is ≤ 0.1 at 450 nm after blanking.

If negative control absorbance values do not meet the quality control criteria, it is discarded, and the mean value is calculated by using the remaining two values.

If more than one Negative control absorbance values do not meet the quality control range specifications, the test is invalid and repeated.

Interpretations of the test results

Negative results (A/C.O < 1): Specimens giving absorbance value less than the cut-off value are negative.

Positive results (A/C.O ≥ 1): Specimens giving absorbance value equal or greater than the cut-off value are considered initially reactive.

Borderline (A/C.O. = 0.9 -1.1): Specimens with absorbance value to cut-off ratio between 0.9 and 1.1 are considered borderline)

Appendix III

III. b. Kit Instructions for Wantai HEV IgM

(The content of this appendix is based on the instruction for use of the commercial ELISA (271))

Principle

Wantai HEV IgM ELISA is an indirect method for detection of HEV IgM antibodies. Microwell strips are pre-coated with HEV recombinant protein derived from ORF2 (269). HEV IgM in sample if present binds to the pre-coated HEV antigen. Unbound serum proteins are removed during washing. Rabbit anti-human IgM antibodies conjugated to horseradish peroxidase (HRP-conjugate) binds to the bound HEV IgM antibody if present in sample. Unbound conjugate is removed after washing. Chromogen A (urea peroxide) and B (tetramethylbenzidine) are hydrolysed by bound HRP conjugate, changing colour to blue. The reaction is stopped by sulphuric acid and changes colour from blue to yellow. Colour intensity is measured with spectrophotometer. Samples negative for HEV IgM do not change colour and remains colourless.

Procedure

1. All the reagents are brought to room temperature (18-30^{0}C).
2. Three wells are marked as negative control, two wells as positive controls and one as blank.
3. Specimen diluent (100 µl) is added to each well except the blank.
4. Positive controls, negative controls and samples (10 µl) are added to respective wells.
5. ELISA plate is incubated for 30 minutes at 37^{0}C.
6. Each well is washed 5 times with wash buffer (diluted phosphate buffer saline - 1:20). Microwells are soaked for 30-60 seconds in between subsequent washing.
7. The wells are blotted dry by tapping on paper towel.
8. HRP-conjugate is added to all the wells except the blank.
9. Each well is washed 5 times with wash buffer. Microwells are soaked for 30-60 seconds in between subsequent washing.
10. Chromogen A (50 µl) and chromogen B (50 µl) are added into each well included blank.

11. The plate is incubated at 37^0C for 15 minutes in dark.
12. Stop solution (50µl) is added in each well including the blank.
13. Absorbance (A) is measured at 450nm within 10 minutes of adding stop solution.

Calculation and Quality Control (Assay Validation)

Calculation of the cut-off value (C.O) = Nc+ 0.16

Nc= mean absorbance value for negative controls after subtracting blank.

If mean absorbance value of the negative controls is lower than 0.03, NC is taken as 0.03

Quality Control (Assay Validation)

Absorbance value of the blank well is < 0.08 at 450 nm.

Absorbance values of the positive control is ≥ 0.80 at 450 nm after blanking.

Absorbance values of the Negative control is ≤ 0.10 at 450 nm after blanking.

If negative control absorbance values do not meet the quality control criteria, it is discarded, and the mean value is calculated by using the remaining two values.

If more than one Negative control absorbance values do not meet the quality control range specifications, the test is invalid and repeated.

Interpretations of the test results

Negative results (A/C.O < 1): Specimens giving absorbance value less than the cut-off value are negative.

Positive results (A/C.O ≥ 1): Specimens giving absorbance value equal or greater than the cut-off value are considered initially reactive.

Borderline (A/C.O = 0.9 -1.1): Specimens with absorbance value to cut-off ratio between 0.9 and 1.1 are considered borderline)

Appendix III

III. c. Kit Instructions for Wantai HEV- Ag ELISA ^{Plus}

(The content of this appendix is based on the instructions for use of the commercial ELISA (268))

Principle

Wantai HEV-Ag ELISA is a “sandwich’ ELISA in which microwell strips are pre-coated with anti-HEV antibodies against HEV antigen (Ag). In the presence of HEV antigen in sample, pre-coated antibodies binds to the viral antigen during the incubation step. Washing removes unbound sample proteins. Anti-HEV antibody conjugated to the enzyme horseradish peroxidase (HRP) binds to anti-HEV HEV Ag complexes on the wells during second incubation if the antigen is present in sample. Unbound HRP conjugate is removed during washing. Chromogen A (urea peroxide) and chromogen B (tetramethylbenzidine) added to the wells are hydrolysed by the bound HRP-conjugate to give a blue-coloured product. Stop solution (sulphuric acid) changes colour from blue to yellow. Colour intensity is measured with spectrophotometer. Samples negative for HEV Ag do not change colour and remains colourless.

Procedure

1. All the reagents are brought to room temperature (18-30^{0}C).
2. Three wells are marked as negative control, two wells as positive controls and one as blank.
3. Specimen diluent (20 µl) is added each well except the blank.
4. Positive controls, negative controls and samples (50 µl) are added to respective wells.
5. ELISA plate is incubated for 60 minutes at 37^{0}C.
6. HRP conjugate is added to all the wells except the blank.
7. Each well is washed 5 times with wash buffer (diluted phosphate buffer saline - 1:20). Microwells are soaked for 30-60 seconds in between subsequent washing.
8. Chromogen A (50 µl) and chromogen B (50 µl) are added into each well included blank.
9. The plate is incubated at 37^{0}C for 15 minutes in dark.
10. Stop solution (50 µl) is added in each well including the blank.

11. Absorbance (A) is measured at 450nm within 10 minutes of adding stop solution.

Calculation and Quality Control (Assay Validation)

Calculation of the cut-off value (C.O) = Nc+ 0.16

Nc= mean absorbance value for negative controls after subtracting blank.

If mean absorbance value of the negative controls is lower than 0.03, NC is taken as 0.03

Absorbance value of the blank well is < 0.08 at 450 nm.

Absorbance values of the positive control is ≥ 0.8 at 450 nm after blanking.

Absorbance values of the Negative control is ≤ 0.1 at 450 nm after blanking.

If negative control absorbance values do not meet the quality control criteria, it is discarded, and the mean value is calculated by using the remaining two values.

If more than one Negative control absorbance values do not meet the quality control range specifications, the test is invalid and repeated.

Interpretations of the test results

Negative results (A/C.O < 1): Specimens giving absorbance value less than the cut-off value are negative.

Positive results (A/C.O ≥ 1): Specimens giving absorbance value equal or greater than the cut-off value are considered initially reactive.

Borderline (A/C.O. = 0.9 -1.1): Specimens with absorbance value to cut-off ratio between 0.9 and 1.1 are considered borderline).

Appendix III

III. d. Kit Instructions for Procleix HEV Assay

(The content of this appendix is based on the instructions for use of the assay (314))

The Procleix HEV Assay is a qualitative nucleic acid assay used with an automatic Panther system for the detection of HEV RNA in plasma and serum specimens, tested individually or in pools.

Principle

The assay involves three steps in a single tube called multi-tube units (MTUs):

Target Capture

HEV RNA is isolated from specimens with the use of target capture in MTUs. Specimen is treated with a detergent to denature proteins and release viral genomic RNA. Capture oligonucleotides that are homologous to highly conserved regions of HEV are hybridised to the HEV RNA target, if present in the test specimen. The hybridised target is captured onto magnetic micro particles that are separated from the specimen in a magnetic field. Washing removes extraneous components from the reaction tube. Oil is added to the tube to prevent evaporation of the bound sample.

Target Amplification

The amplification utilises two enzymes, Moloney murine leukemia virus reverse transcriptase and T7 RNA polymerase. The reverse transcriptase generates a DNA copy (containing a promoter sequence for T7 RNA polymerase) of the target RNA sequence. RNAse H degrades original RNA strand. The T7 RNA polymerase produces multiple copies of RNA amplicon from the DNA copy template.

Detection (Hybridization Protection Assay)

The process utilises DNA probe labelled with Acridinium Ester (AE) that hybridize specifically with amplicon. Selection reagent hydrolyses between hybridized and unhybridized probe by inactivating the label on unhybridised probes. Detection of the hybridized probe is done by chemiluminescence using autodetect fluid 1 and 2 added to MTU. Internal conrol is added to each reaction tube with the working target capture

reagent. The IC in target capture serves as a control for specimen processing, amplification and detection steps. Signal of IC is discriminated from HEV signal by the differential kinetics. Dual Kinetic assay technology allows detection of flasher (quick luminescence) and glower (slow luminescence) probes. Flasher signal is emitted by IC specific amplicon and glower signal by HEV specific amplicon. The chemiluminescent signal produced by the hybridized probe is measured by a luminometer and is reported as Relative Lights Units (RLU).

Deactivation

Sodium hyphochloride and buffer is used for deactivation of residual infectious material and newly formed amplicon. After 15 minutes, liquid aspirated from MTU and empty tubes are discarded.

Procedure

1. A new or specific set of reagents of HEV Procleix assay are used with the Panther system.
2. Target capture, amplification, enzyme, probe and selection reagents are incubated in reagent preparation incubator at 32^0C for 1 hour.
3. IC is added to target capture reagent to prepare working target capture reagent.
4. Working target capture is loaded in target capture carousel.
5. Amplification, enzyme, probe and selection reagents are loaded in the reagent bay.
6. System fluids (wash solution, auto detect 1 and 2, buffer for deactivation and oil) and Sodium hypochloride are loaded in universal fluids bay.
7. Consumables such as MTUs, disposable tips, cleaning solution and other disposable supplies (waste bag, cover) are loaded in respective bays.
8. Positive and negative calibrators are loaded in separate tubes with samples in the carousel.
9. Testing is performed in fully closed system with operator commands from the monitor.
10. 244 samples are tested with a set of reagents.
11. First result is obtained after 3.5 hours and every five minutes five additional results are obtained.

Procleix HEV Assay Results

Acceptance criteria

Analyte ≥ 0 and ≤ 40,000 RLU

IC ≥ 75,000 and ≤ 400,000 RLU

HEV Positive Calibrator

Analyte ≥ 400,000 and ≤ 3,000,000 RLU

IC < 75,000 RLU

Procleix HEV assay cut-off calculations

Analyte Cutoff = NC Analyte Mean RLU + [0.03 x (PC Analyte Mean RLU)]

IC Cutoff = 0.5 x (Negative Calibrator IC Mean RLU)

Procleix HEV assay specimen acceptable ranges and interpretation

Specimen interpretation	**Criteria for the Procleix HEV assay**
Non reactive	Analyte S/CO < 1 and IC ≥ IC Cutoff and IC ≤ 750,000 RLU
Reactive	Analyte S/CO ≥ 1 and Analyte ≤ 5,100,000 RLU and IC ≤ 750,000 RLU
Invalid	IC > 750,000 RLU or Analyte S/Co < 1 and IC < IC Cutoff

Limit of Detection of Procleix HEV assay is 7.9 IU/ml (189).

Appendix III

III. e. Instructions for QIAamp Viral RNA Mini kit

(The content of this appendix is based on the instructions for use of the commercial kit (315))

Principle

QIAamp Viral RNA Mini Kits is a viral RNA extraction kit. The kit uses selective binding properties of a silica based membrane with the speed of microspin. The sample is lysed under denaturing conditions to inactivate RNases for the isolation of intact viral RNA. Buffering conditions provide optimum binding of the RNA to the QIAamp membrane. The RNA of virus if present in sample binds to the membrane and contaminants are washed away with buffers. RNA is eluted in RNase-free buffer. Purified RNA is free of protein, nucleases, and other contaminants.

Preparation of reagents

Buffer AVE (310µl) is added to the tube containing 310 µg lyophilized carrier RNA to obtain a solution of 1 µg/µl.

Volume of buffer AVL-carrier RNA required is calculated as follows:

n x 0.56 ml = y ml

y ml x 10 µl/ml = z µl

where: n = number of samples to be processed simultaneously

y = calculated volume of Buffer AVL

z = volume of carrier RNA-Buffer AVE to add to Buffer AVL

Buffer AW1 is prepared with ethanol (96-100%) as follows:

Number of preparations	AW1 concentrate	Ethanol	Final Volume
50	19 ml	25 ml	44 ml

Buffer AW2 is prepared with ethanol (96-100%) as follows:

Number of preparations	AW2 concentrate	Ethanol	Final Volume
50	13 ml	30 ml	43 ml

Procedure

1. Prepared buffer AVL containing carrier RNA (560 µl) is pipetted into a 1.5 ml microcentrifuge tube.
2. Plasma/serum sample (140 µl) is added the buffer AVL-carrier RNA in the microcentrifuge tube.
3. The mixture is vortexed for 15 seconds and incubated at room temperature (15-20^0C) for 10 minutes.
4. The tube is centrifuged briefly.
5. Ethanol (96-100%, 560 µl) is added to the sample, and mixed by vortexing for 15 seconds and then centrifuged briefly.
6. Above solution (ethanol with sample) is added to the QIAamp Mini column in a collection tube (630 µl). The column is spun at 6000g for 1 min. QIAamp Mini column is placed into a clean 2 ml collection tube and tube containing filtrate is discarded.
7. Step 6 is repeated until remaining lysate has been loaded onto the spin column.
8. Buffer AW1 (500 µl) is added to the QIAamp Mini column and centrifuged at 6000g for 1 minute. The column is placed in a clean 2 ml collection tube and tube containing filtrate is discarded.
9. Buffer AW2 (500 µl) is added to the QIAamp Mini column. The column is spun at 20000g for 3 minutes.
10. The column is placed in a new 2 ml collection tube and the old collection tube with filtrate discarded. The column is centrifuged at 20000g for 1 minute.
11. QIAamp Mini column is placed in a clean 1.5 ml microcentrifuge tube. The used collection tube with filtrate is discarded. Buffer AVE (40 µl) is added to the column, incubated at room temperature for 1 minute and centrifuged at 6000g for 1 minute.
12. Additional buffer AVE (40 µl) is added to the column, incubated and eluted into the microcentrifuge tube.

Appendix III

III. f. Kit Instructions RealStar® HEV RT-PCR Kit 1.0

(The content of this appendix is based on the instructions for use of the assay (241))

Principle

The RealStar® HEV RT-PCR Kit 1.0 is a qualitative test for the detection of hepatitis E virus specific RNA, based on real-time PCR technology. The test utilises reverse-transcriptase (RT) reaction that converts RNA to cDNA and amplifies specific target sequences and target specific probes for the detection of the amplified DNA. The probes are labelled with fluorescent reporter and quencher dyes. Probes specific for HEV RNA are labelled with the fluorophore FAM. The probe specific for the target of the IC is labelled with the fluorophore JOE. Probes with distinguishable dyes enable parallel detection of HEV specific RNA and IC in the corresponding detector channels of the real-time PCR instrument.

The kit consists of two master reagents (master A and master B) which contains all components (buffer, enzymes, primers and probes) to allow reverse transcription, PCR mediated amplification and target detection (HEV and internal control).
The test consists of three processes in a single tube assay:

- Reverse transcription of target RNA to cDNA
- PCR amplification of target cDNA and internal control
- Simultaneous detection of PCR amplicons by fluorescent dye labelled probes

Procedure

- Extracted HEV RNA from plasma sample and reagents are thawed, mixed and centrifuged prior to use.
- The kit contains IC which is used either as RT-PCR inhibition control or as a control of the sample preparation procedure (nucleic acid extraction) and as a RT-PCR inhibition control.

IC used as a RT-PCR inhibition control

Number of reactions	1	12
Master A	5 µl	60 µl
Master B	20 µl	240 µl
Internal Control	2.5 µl	30 µl
Volume Master Mix	27.5 µl	330 µl

IC used as a control for the sample preparation procedure and as a RT-PCR inhibition control, IC is added during nucleic acid extraction procedure (10% of elution volume)

Number of reactions	1	12
Master A	5 µl	60 µl
Master B	20 µl	240 µl
Volume Master Mix	25 µl	300 µl

Reaction setup

- Master mix (25 µl) is pipetted into each optical reaction tube.
- Eluate sample from the nucleic acid extraction (25 µl), positive control (25 µl), negative control (25 µl) are added in respective reaction tubes with master mix.
- Samples and controls are mixed with the master mix.
- Reaction tubes with lids are set up in the thermal cycler.

Temperature profile and dye acquisition

	Stage	**Cycle Repeats**	**Acquisition**	**Temperature**	**Time**
Reverse transcription	Hold	1	-	50^0C	10:00 min
Denaturation	Hold	1	-	95^0C	10:00 min
Amplification	Cycling	45	-	95^0C	0:15 min
			√	55^0C	0:45 min
			-	72^0C	0:15 min

Interpretation of results

Sample	FAM Detection Channel	JOE Detection Channel	Result Interpretation
A	Positive	Positive	HEV specific RNA detected
B	Negative	Positive	HEV specific RNA not detected. Sample does not contain detectable amounts of HEV specific RNA.
C	Negative	Negative	RT-PCR inhibition or reagent failure. Repeat testing from original sample.

Limit of detection of the kit for pool screening is 4.7 IU/ml (95% CI 3.6-7.6) (240).

Limit of detection of the kit for individual sample screening is 37.8 IU/ml (95% CI 22.2-671.2) (240). Analytical sensitivity as per the kit is 0.31 IU/µl (95% confidence interval: 0.20 - 0.74 IU/µl) (241).

Appendix IV: Serological Analysis

IV.a. Serological Analysis (Chapter 2)

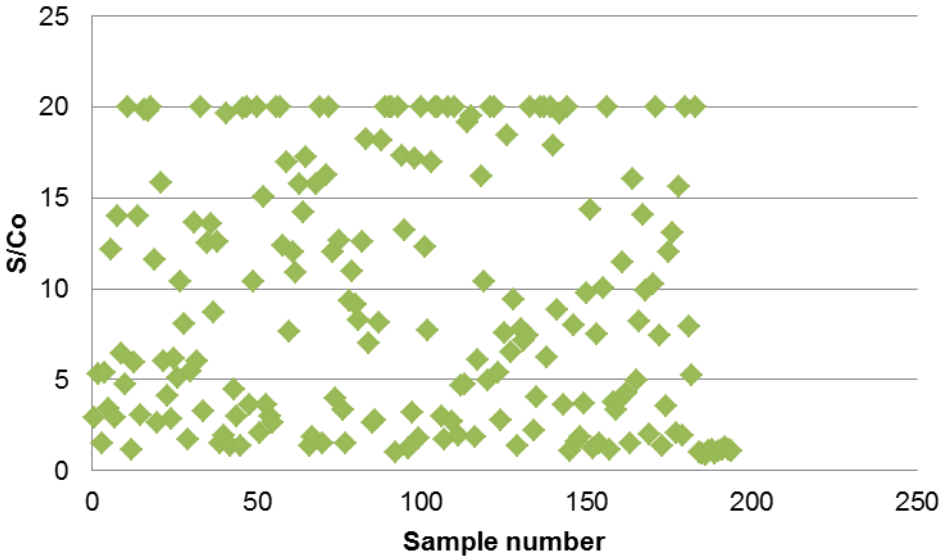


Figure IV.a. Mean S/Co of HEV IgG positive samples tested three times

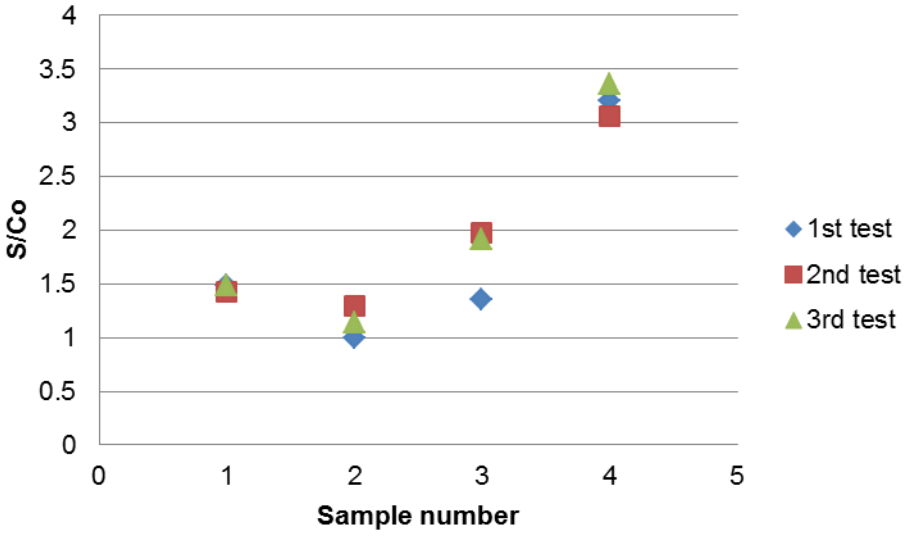


Figure IV.b. Mean S/Co of HEV IgM positive samples tested three times

Appendix IV

IV.c. Serological Analysis (Chapter 3)

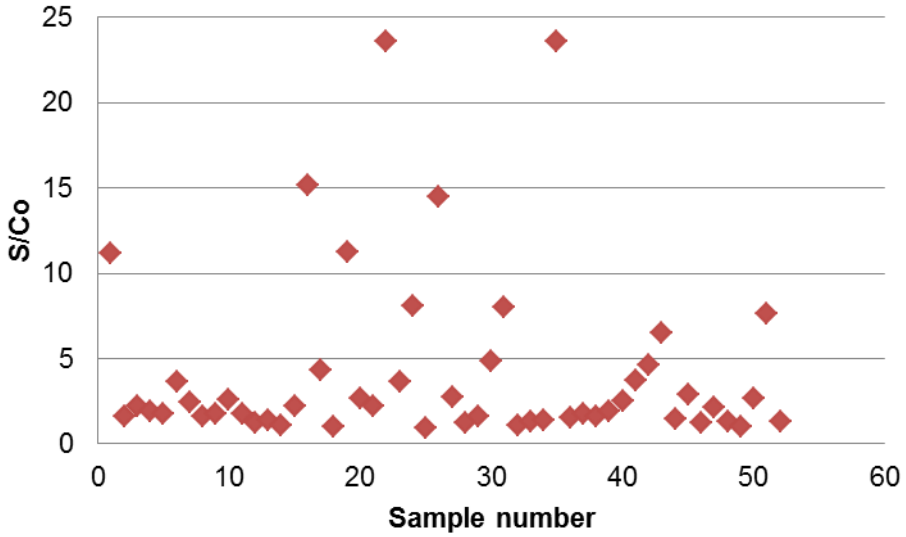


Figure IV.c. Mean S/Co of HEV Ag positive samples tested three times

Appendix V: Sample Breakdown and Results (Chapter 3)

Table V. a: Sample breakdown from each of the following processing centres

Processing centre	Total units of whole blood collection*	Sample number required	Actual number of sample collected and tested
BPC	248,448	2754	3,000
MPC	480,393	5325	5,499
SPC	430,490	4772	5,000
PPC	103,706	1150	1,300
Total	1,263,037	14000	14,799

BPC: Brisbane Processing Centre, MPC: Melbourne Processing Centre, SPC: Sydney Processing Centre, PPC: Perth Processing Centre

* 1 January 2013 to 4 August 2014 (Source: Report Centre, The Australian Red Cross Blood Service)

Table. V. b. HEV antigen and RNA detection in Australian blood donations

Processing centre	Samples tested	HEV Ag Positive	% (95% CI)	HEV RNA positive	% (95% CI)
Brisbane Processing Centre	3,000	13	0.43 (0.2-0.67)	-	-
Melbourne Processing Centre	5,499	14	0.25 (0.12-0.39)	1	0.0182 (0.0005-0.1013)
Perth Processing Centre	1,300	2	0.15 (0.00-0.43)	-	-
Sydney Processing Centre	5,000	23	0.46 (0.27-0.65)	-	-
Total tested	14,799	52	0.35 (0.26-0.45)	1	0.0068 (0.0002 -.0376)

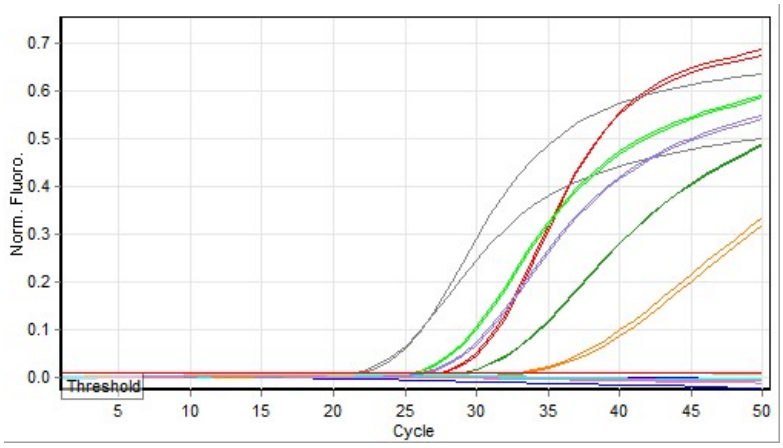
Appendix VI: HEV Quantification and Sequencing (Chapter 3)

VI. a. Viral load measurement of HEV RNA positive sample

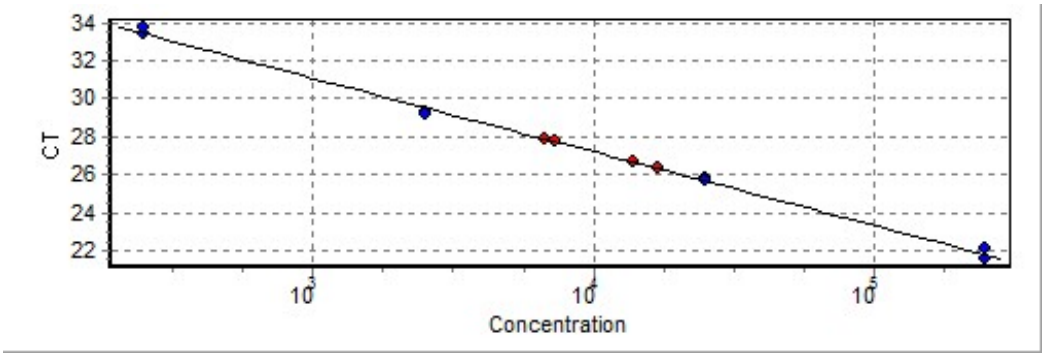
Quantitation Information

Threshold	0.0108
Left Threshold	1.000
Standard Curve Imported	No
Standard Curve (1)	conc= 10^(-0.259*CT + 11.059)
Standard Curve (2)	CT = -3.854*log(conc) + 42.624
Reaction efficiency (*)	(* = 10^(-1/m) - 1) 0.81738
M	-3.85437
B	42.62415
R Value	0.99847
R^2 Value	0.99694
Start normalising from cycle	1
Noise Slope Correction	Yes
No Template Control Threshold	% 0
Reaction Efficiency Threshold	Disabled
Normalisation Method	Dynamic Tube Normalisation
Digital Filter	Light
Sample Page	Page 1
Imported Analysis Settings	

Quantitation data for Cycling A.Green



Standard Curve



No.	Color	Name	Type	Ct	Given Conc (IU/ml)	Calc Conc (IU/ml)
1		POSITIVE CONTROL	Positive Control	27.89		6,661.9
2		POSITIVE CONTROL	Positive Control	27.73		7,310.5
3		NEGATIVE CONTROL	Positive Control			
4		NEGATIVE CONTROL	Positive Control			
5		WHO HEV Standard	Standard	21.61	250,000.0	283,824.9
6		WHO HEV Standard	Standard	22.11	250,000.0	210,417.6
7		Standard 1	Standard	25.85	25,000.0	22,490.8
8		Standard 1	Standard	25.68	25,000.0	24,946.5
9		Standard 2	Standard	29.24	2,500.0	2,965.5
10		Standard 2	Standard	29.22	2,500.0	2,997.5
11		Standard 3	Standard	33.37	250.0	252.4
12		Standard 3	Standard	33.73	250.0	203.0
13		Standard 4	Standard		25.0	
14		Standard 4	Standard		25.0	
15		Standard 5	Standard		2.5	
16		Standard 5	Standard		2.5	
17		Positive Sample	Unknown	26.32		16,989.6
18		Positive Sample	Unknown	26.66		13,821.8

Appendix VI

VI. b. Alignment of nucleotide sequences of HEV RNA postive sample idenfied in this study with a genotype 3 strain from swine.

Score	Expect	Identities	Gaps	Strand
996 bits(539)	0.0	776/893(87%)	6/893(0%)	Plus/Plus

```
Query  1     GCAATCTCTATTTCATTCTGGCCACAGACTACTACGACCCCTACATCTGTTGATATGAAC  60
             ||||||||||||||| ||||||||  |||||  |||||  ||||||||||| ||||||||||
Sbjct  5762  GCAATCTCTATTTCTTTCTGGCCTCAGACCACTACCACCCCTACATCCGTTGATATGAAT  5821

Query  61    TCCATTACCTCTACTGATGTTAGGATTTTGGTCCAGCCCGGCATCGCCTCTGAACTTGTT  120
             ||  |||||  ||||||||||  ||||||||||||||  |||||  ||  |||||  |||  |  ||
Sbjct  5822  TCTATTACTTCTACTGATGTCAGGATTTTGGTCCAACCCGGTATTGCCTCCGAATTGGTC  5881

Query  121   ATTCCTAGCGAGCGCCTCCATTACCGTAATCAGGGTTGGCGCTCTGTTGAAACATCGGGC  180
             ||  ||  ||||||||||||||  ||  ||  ||||||||||||||||||||||||  ||  ||  |||
Sbjct  5882  ATCCCCAGCGAGCGCCTCCACTATCGCAATCAGGGTTGGCGCTCTGTTGAGACCTCAGGC  5941

Query  181   GTTGCCGAGGAGGAGGCTACCTCTGGCTTGGTGATGCTCTGTATCCATGGCTCCCCCGTT  240
             ||  |||||  ||||||||||||||  ||  |||||  ||||||||||  ||||||||  ||  ||
Sbjct  5942  GTGGCCGAAGAGGAGGCTACCTCCGGTTTGGTAATGCTCTGTATTCATGGCTCTCCTGTC  6001

Query  241   AATTCTTACACTAATACCCCTTATACTGGGGCGTTGGGGCTTCTTGACTTTGCATTGGAA  300
             ||||||||  ||  ||||||||  ||||||||||||  |  |||||  ||||||||||||  |  |||
Sbjct  6002  AATTCTTATACCAATACCCCCTATACTGGGGCGCTTGGGCTCCTTGACTTTGCACTAGAA  6061

Query  301   CTTGAGTTTAGGAATTTGACACCTGGGAACACTAACACCCGTGTATCCCGGTATACAAGT  360
             ||||||||||||||||||||||  ||||||||||  ||  ||  ||  |||||||||  |||||
Sbjct  6062  CTTGAGTTTAGGAATTTGACACCCGGGAACACTAATACTCGCGTGTCCCGGTACACAAGC  6121

Query  361   ACAGCCCGTCACCGGCTGCGCCGCGGGGCCGATGGTACTGCTGAGCTTACCACCACGGCG  420
             ||||||||||||||||||||||||||  ||||||||||||||||  ||||||||||||||||
Sbjct  6122  ACAGCCCGTCACCGGCTGCGCCGCGGCGCCGATGGTACTGCTGAACTTACCACCACGGCG  6181

Query  421   GCCACGCGCTTCATGAAGGACTTGCATTTCACCGGCACGAATGGCGTCGGTGAGGTGGGT  480
             |||||  ||  ||||||||||  ||  |||||  ||  ||  ||||||||  ||||||||||  ||
Sbjct  6182  GCCACACGGTTCATGAAGGATTTACATTTTACTGGTACGAATGGGGTCGGTGAGGTCGGC  6241

Query  481   CGTGGCATTGCTCTGACATTGTTTAACCTTGCTGACACACTCCTTGGCGGTCTGCCGACA  540
             |||||  ||||||||||||||||||||  ||||||||  ||  ||  |||||  |||  ||||||||
Sbjct  6242  CGTGGTATTGCTCTGACATTGTTTAATCTTGCTGATACGCTTCTTGGTGGTTTGCCGACA  6301

Query  541   GAATTGATTTCGTCGGCTATAAGAGAC-A-GGTTCTACTCCCGCCCTGTCGTCTCAGCCA  598
             ||||||||||||||||||    |  |  |  |   ||  ||||||||||||||||||||||||||
Sbjct  6302  GAATTGATTTCGTCGGCT--GGGGGTCAACTATTTTACTCCCGCCCTGTCGTCTCAGCCA  6359

Query  599   ATGGCGAGCCGACTGTCAAGTTATATACATCTGTTGAGAATGCGCAGCAAGATAAGGGGA  658
             ||||||||||||||||  ||  ||||||||||||||||||||||||||||||  |||||  ||  |
Sbjct  6360  ATGGCGAGCCGACTGTTAAATTATATACATCTGTTGAGAATGCGCAGCAGGATAAAGGTA  6419

Query  659   TTGTTATCCCGCATGATATAGACCCTGTCTC-TTATAGTATTGTCATCCAGGACTATGAC  717
             |||  ||||||||||  |||||||  ||  |  ||  |  ||  |  ||||||||||  |||||
Sbjct  6420  TTGCCATCCCGCATGACATAGACC-TGGGTGATTCTCGTGTGGTCATCCAGGATTATGAT  6478

Query  718   AACCAGCATGAGCAGGATCGGCCTACTCCATCGCCCGCCCCCTCACGCCCCTTTTCTGTC  777
             ||  ||  ||||||||||||||||||||  ||  ||||||||||  ||||||||  |||||||||
Sbjct  6479  AATCAACATGAGCAGGATCGGCCTACCCCTTCGCCCGCCCCATCACGCCCATTTTCTGTC  6538

Query  778   CTTCGTGCTAATGATGTTTTGTGGCTTTCTCTAACTGCCGCTGAGTATGACCAGACCACA  837
             ||  ||||||||  ||||||||||||||||||||  ||  ||  ||||||||  ||  |||||  |||
Sbjct  6539  CTCCGTGCTAACGATGTTTTGTGGCTTTCTCTTACCGCTGCTGAGTACGATCAGACTACA  6598

Query  838   TATGGGTCGTCAACCAACCCAATGTATGTTTCAGACACTGTTACACTTGTTAA  890
             ||||||||||  ||||||||  ||||||||  ||  ||||||||  |||  |||||||
Sbjct  6599  TATGGGTCGTCCACCAACCCGATGTATGTCTCGGACACTGTAACATTTGTTAA  6651
```

Appendix VII: Participant Information Sheet and Questionnaire

VII. a. Participant Information Sheet (Chapter 5)

Nepal Red Cross Society Australian Red Cross BLOOD SERVICE The University Of Queensland AUSTRALIA

Participant Information Sheet and Consent Form

Epidemiological study of hepatitis E virus among blood donors in selected districts of Nepal

Background:

Hepatitis E virus (HEV) is emerging as a global public health concern. Most of the infections with this virus are asymptomatic. In symptomatic individuals, the virus causes jaundice and hepatitis. Recipients of blood transfusion are immunocompromised and blood from infected donors can cause such a disease in transfused patients. In order to assess the risk posed by this virus to blood safety, Nepal Red Cross (NRCS), Central Blood Transfusion Service with Australian Red Cross Blood Service, Research and Development and the University of Queensland are conducting this research project.

Details of participation:

- Your participation in this research is voluntary.
- A blood sample (5 ml) will be taken as part of the NRCS blood service routine practice for mandatory infectious disease screening, whether or not you participate in the research; if you do participate in the research, an extra sample (5 ml) will be collected.
- The blood sample will only be collected by NRCS staff.
- Mandatory routine screening at the NRCS, blood service includes testing for human immunodeficiency virus (HIV), hepatitis B virus (HBV), hepatitis C virus (HCV) and Syphilis
- As a part of this research, a sample will be tested for markers of previous and current infection with HEV
- This research testing is carried out in addition to, and separate from, the routine blood screening performed at NRCS, blood service.
- A sample will collected at the same time as the sample required for mandatory testing
- The blood donation program is organized by the NRCS, blood service
- The extra blood drawn for the purposes of the research is entirely voluntary and a decision to participate or not participate in the research (by giving the extra blood) does not affect your participation in the routine donation program.

You may simply participate in the routine program without giving the extra blood for the research project.

- If participating in the study, you are also requested to submit the questionnaire form *(Overleaf)*, prior to blood donation. Information provided will remain confidential.

- Your blood sample will be de-identified prior to the testing i.e. you cannot be identified once the tests are performed.

- You may withdraw at any time during your donation, however, it is not possible to withdraw after your donation has finished and your sample has been de-identified.

- After your participation, you are not required to give any more samples for this study.

We acknowledge your voluntary participation in this study.

This study adheres to the guidelines of the ethical review process of the Nepal Health Research Council and The University of Queensland and the National Statement on Ethical Conduct in Human Research (Australia). Whilst you are free to discuss your participation in this study with project staff (contactable on - *mobile number to be provided*), if you would like to speak to an officer of the University not involved in the study, you may contact the Ethics Coordinator on +61 073365 3924.

For further details, contact:

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PhD scholar
(The University of Queensland, Australia)

Kathmandu, Nepal
Contact no: 977 9860769879

Dr Manita Rajkarnikar
Director, Central Blood Transfusion Service
Nepal Red Cross Society, Pradarshani Marg,
Kathmandu, Nepal
Contact no: 977 4225344

Co-investigators/collaborators:

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Research and Development
Australian Red Cross Blood Service
Queensland, Australia

Dr Helen Faddy
Research and Development
Australian Red Cross Blood Service
Queensland, Australia

Appendix VII

VII. b. Questionnaire (Chapter 5)

Donation number:

Name: .....Date of Birth:..... Sex:☐Male☐Female

Residential District: .....Occupation: .....

1. Have you donated blood before? ☐ Yes ☐ No
2. Have you ever had jaundice (yellow eyes/skin) or hepatitis? ☐ Yes ☐ No
3. Has someone in your family had jaundice or hepatitis? ☐ Yes ☐ No

   If Yes, when? .....
4. Main source of drinking water at home: ☐ Municipality supplied ☐ Local tap in the community ☐Underground water ☐other.....
5. How do you treat your drinking water at home? ☐ Do no treat ☐ Boil ☐ Filter ☐ Add water purifier ☐other.....
6. I am ☐ Non-vegeterian ☐Vegeterian

   If non-vegeterian: Do you eat pork? ☐ Yes ☐ No
7. Have you travelled outside Nepal? ☐Yes ☐No

   If yes, which was the most recent country you visited? .....
8. Have you ever had malaria or dengue?

   ☐Malaria. If yes, when? .....

   ☐Dengue. If yes, when? .....

   ☐No

I have read the participation information sheet and agree that a part of my donation and information provided will be used for research purpose mentioned above. I have understood all the information in the consent form and am willing to volunteer / participate in the research.

....

Signature and Date Witness