Prevalence and Risks of Hepatitis E Virus infection in Blood Donors from the Western Cape, South Africa

by

Tatum Lopes

Thesis presented in fulfilment of the requirements for the degree of Master of Medical Science (Medical Virology) in the Faculty of Medicine and Health Sciences at Stellenbosch University

Supervisor: Dr. Monique I. Andersson
Co-supervisor: Prof. Wolfgang Preiser

March 2016

DECLARATION

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the sole author thereof (save to the extent explicitly otherwise stated), that reproduction and publication thereof by Stellenbosch University will not infringe any third party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

Signature

_____

Tatum Lopes

Date: _____ / _____ / _____

SUMMARY

Hepatitis E virus (HEV) is an important cause of enterically transmitted acute hepatitis worldwide and is a locally acquired disease in both developing and developed nations. Different genotypes in these two regions display the different characteristics of this interesting infection. HEV is classified into four major genotypes and it can present as two contrasting clinical entities. HEV genotypes 1 (HEV1) and 2 (HEV 2) are related to waterborne transmission and poor sanitation. Whereas genotypes 3 (HEV3) and 4 (HEV4) are associated with zoonotic transmission mainly through pigs, wild boar and deer. HEV infections in Africa are thought to be caused by HEV1 and HEV2. The seroprevalence of HEV has been described in Southern Africa, but all more than 10 years ago when assays were not well developed. South Africa has three HEV reports, describing a hospital outbreak, and the seroprevalence in specific communities of South Africa. The seroprevalence from these studies ranged from 2% to 10.7% however no genotyping was done.

Researchers have reported evidence of direct and indirect transfusion-transmitted HEV infection being a potential risk to recipients of blood transfusions. Asymptomatic HEV infections in blood donors increase the likelihood that blood or blood products are contaminated with HEV viral particles. Hence, there is a greater chance of infecting high-risk recipient groups with compromised immune systems. Therefore, the main aim of this study was to determine the prevalence of past and active HEV infection in blood donors from the Western Cape. We also investigated which risk factors are associated with infection.

Our study population consisted of 10,250 blood donors that were tested as two sub-studies. For study group 1 we recruited 250 donors to complete an HEV risk questionnaire. Thereafter these donors were tested using an indirect Wantai ELISA (Fortress Diagnostics) for anti-HEV IgG detection. Statistical analysis was done to determine which demographics and risk factors were associated with past HEV infection. In addition, to this, their plasma donations were pooled, prior to extraction and amplified with an in-house real-time reverse-transcriptase quantitative polymerase chain reaction (RT-qPCR) to detect HEV RNA. The 10,000 blood donors of study group 2 were tested as individual donations using a commercial Procleix HEV nucleic acid testing (NAT) assay to qualitatively detect HEV RNA by transcription-mediated amplification (TMA). Thereafter repeat-reactive donations were quantified using our in-house real-time RT-qPCR.

The total anti-HEV IgG seroprevalence of our study was found to be 42.4% in blood donors (study group 1). Risk analysis revealed that eating turkey (p=0.001) and organ meat (p=0.026)

and canoeing (p=0.017) were significantly associated with past HEV infection. Whereas direct contact with rabbits (p=0.045) or chickens (p=0.020) were statistically significantly different means of HEV exposure associated with HEV3 and HEV4. Furthermore, we found that the total HEV RNA prevalence was 0.009% (1/10,250). Studies are needed to further assess the risk of HEV blood-borne transmission and to understand the epidemiology of HEV in our setting.

OPSOMMING

Hepatitis E virus (HEV) is een van die grootste oorsake van akute hepatitis wêreldwyd en is algemeen bekend as 'n plaaslike siekte in baie ontwikkelende lande. Maar HEV infeksie wat oorgedra word in ontwikkelde lande word nie net meer verbind met reise na ander lande waar HEV infeksie dikwels voorkom nie. HEV word geklassifiseer in vier hoof genotipes en dit kom voor as twee kontrasterende kliniese entiteite. HEV genotipes 1 (HEV1) en (HEV 2) is verwant aan water oordrag en swak sanitasie, terwyl genotipes 3 (HEV3) en 4 (HEV4) geassosieer word met soönotiese oordrag wat hoofsaaklik plaasvind deur varke, wilde varke en takbokke. HEV infeksies in Afrika is vermoedelik veroorsaak deur HEV1 en HEV2. Verskeie gevalle is beskryf in Suider-Afrika, maar dit was reeds meer as 10 jaar gelede, toe toetse nog nie goed ontwikkel was nie. Suid-Afrika het drie HEV uitbrake gerapporteer; 'n hospitaal uitbraak, en twee serologiese voorkoms verslae in spesifieke gemeenskappe van Suid-Afrika. Die serologiese voorkoms van hierdie studies het gewissel vanaf 2% tot 10,7% en geen genotipering was gedoen nie.

Navorsers het bewys dat direkte en indirekte-bloedoortapping oordraagbare infeksie van HEV moontlik is en 'n potensiële risiko vir ontvangers van bloedoortappings beskryf. Asimptomatiese HEV infeksies in bloed skenkers verhoog die waarskynlikheid dat die bloed of bloed produkte geïnfekteer is met HEV virale partikels. As gevolg daarvan is daar 'n groter kans dat hoë-risiko ontvanger groepe met verswakte immuunstelsels geïnfekteer mag word. Daarom was die hoofdoel van hierdie studie om die voorkoms van vorige en aktiewe HEV infeksie in bloed skenkers van die Wes-Kaap te bepaal. Ons het ook ondersoek watter risiko faktore verband hou met infeksie.

Ons studie het bestaan uit 10,250 bloed skenkers wat getoets was as deel van twee sub-studies. Vir studie groep 1 het ons 250 skenkers gewerf om 'n HEV risiko vraelys te voltooi. Hierdie skenkers was getoets met behulp van 'n indirekte Wantai toets (Fortress Diagnostics) vir die identifisering van anti-HEV IgG. Daarna was statistiese analise gedoen om te bepaal wat die demografie en risiko faktore is wat verband hou met die voorkoms van vorige HEV infeksie. Benewens hierdie was hul plasma monsters saamgevoeg, voor ekstraksie en amplifikasie met 'n in-huis intydse omgekeerde transkriptase-kwantitatiewe polimerase kettingreaksie (PKR) om HEV RNS te spoor. Die 10,000 bloed skenkers van studie groep 2 was getoets as individuele skenkings met die gebruik van 'n kommersiële Procleix HEV NST toets om HEV RNS deur transkripsie amplifikasie te kwantifiseer. Daarna is herhaalde reaktiewe skenkings

gekwantifiseer met behulp van ons in-huis intydse omgekeerde transkriptase-kwantitatiewe PKR.

Die totale serologiese voorkoms van antiliggame teen HEV vir ons studie was 42,4% in bloedskenkers (studie groep 1). Risiko analise het aan die lig gebring dat om kalkoen (p=0.001) en orgaan vleis te eet (p=0.026) wat aanleiding gee tot ‘n voedselverwante oordrag van HEV3, en kanovaart (p=0.017) wat deur middel van water lei tot oordrag van HEV1, hou beduidend verband met die voorkoms van vorige HEV infeksie. Direkte kontak met hase (p=0.045) of hoenders (p=0.020) was beduidend statisties ‘n metode van HEV blootstelling wat verband hou met HEV3 of HEV4. Verder het ons gevind dat die totale HEV RNS voorkoms vir ons studie 0,009% (1/10,250) was. Studies is nodig om die risiko van HEV stygende oordrag verder te evalueer en om die epidemiologie in ons omgewing te verstaan.

ACKNOWLEDGEMENTS

Firstly, I would like thank God for this opportunity that I've been given to further my studies and for blessing me with amazing people during my postgraduate course at Stellenbosch University.

Secondly, to my supervisor, Dr. Monique I. Andersson, my experience as a one of her students can be summarised in the following words; "I could not have asked for a better supervisor". I appreciate all her effort, support and excellent leadership in helping me to grow as an individual both personally and professionally.

To my co-supervisor, Prof. Wolfgang Preiser I am very grateful for his valuable feedback and constant encouragement. I have truly learnt a lot from him as an expert in the field and will apply this knowledge as an aspiring researcher.

I would like to thank the funders who have made it possible to conduct this research project; Poliomyelitis Research Foundation (PRF) (Research grant and Bursary 2014 and 2015), Harry Crossely Foundation (HCF) (Research grant 2014 and 2015), National Health Laboratory Service (NHLS) Research Trust, Belgian Technical Corporation (BTC) scholarship received in 2014.

A big thank you to the staff from Western Province Blood Transfusion Service (WPBTS) for providing me with my project samples. I especially want to thank the WPBTS blood donation clinic staff for assisting me during the project survey and the virology laboratory staff for performing the automated molecular HEV testing; Mr Russell Cable, Ms Charlotte Pistorius, Ms Nadia Pietersen, Ms Amanda Visser and Ms Raeghaana Matthews. I would also like to thank the staff of Ilex SA; Ms Emily Tlale, Mr Piet Roux and in particular Mr Willem Hecther for liaising with Grifols to sponsor the Procleix HEV NAT kits and for my Panther system training at WPBTS.

I would like to say thank you to, Dr. Corena De Beer, the Division of Medical Virology postgraduate programme coordinator for always having an open door to assist and to Mr Tongai Maponga for advising me on my laboratory procedures. A sincere thank you to all the staff and fellow students of the Division of Medical Virology FMHS, SU, for the role they have played in the success of this research project.

Lastly, a special thanks to my mother Myra-Nisa, my sisters Amber and Zeta and my best friend Precious for their support and belief in me and also to all my family and friends who have motivated me throughout my studies.

CONFERENCE PRESENTATIONS

1. Lopes T, Andersson MI, Maponga TG, Preiser W, Cable R, Pistorius C. Hepatitis E virus infection in blood donors: Seroprevalence and risks.

   Presented at: Pathology Research and Development (PathReD) Congress in the Infectious Diseases Track (oral presentation), 14-16 April 2015, Emperors Palace, Johannesburg, South Africa. **Received the award for best oral presentation in the Infectious Diseases Track.**

2. Lopes T, Andersson MI, Maponga TG, Preiser W, Cable R, Pistorius C. The seroprevalence of HEV in blood donors in the Western Cape.

   Presented at: The 33rd South African National Blood Transfusion Congress as one of the speakers for the symposium (oral presentation), 24-27 August 2015, Champagne Sports Resort, Drakensberg, South Africa.

TABLE OF CONTENTS

Declaration ii
Summary iii
Opsomming v
Acknowledgements vii
Table of contents x
List of abbreviations xiv
List of tables xvii
List of figures xviii
CHAPTER ONE: INTRODUCTION 1
1.1 Background 1
1.1.1 HEV: The virus 1
1.1.2 Transmission of HEV 3
1.1.2.1 Waterborne 3
1.1.2.2 Zoonotic foodborne 3
1.1.2.3 Blood-borne 4
1.1.2.4 Perinatal 4
1.1.2.5 Possible HEV transmission routes and genotypes expected in South Africa 5
1.1.3 Clinical presentation and natural history 6
1.1.4 Diagnosis of HEV 7
1.2 Problem statement 8
1.2.1 Hypothesis 9
1.2.2 Aims and objectives 9
CHAPTER TWO: LITERATURE REVIEW 10
2.1 Epidemiology of HEV 10
2.1.1 HEV worldwide 10
2.1.2 HEV in Africa 11
2.1.3 HEV in South Africa 13
2.2 HEV in blood donors 14
2.2.1 Prevalence of HEV in blood donors 14

2.2.1.1 HEV prevalence in blood donors from developed countries 15
2.2.1.2 HEV prevalence in blood donors from developing countries.....18
2.2.1.3 HEV prevalence in blood donors from Africa 19
2.3 Risks and consequences of HEV in blood donors 21
2.3.1 HEV infection associated with blood transfusion.....21
2.4 Prevention of HEV TTIs.....23
2.4.1 HEV screening of blood donations.....23
2.4.1.1 Current screening in South Africa.....23
2.4.2 HEV surveillance 23
2.4.3 HEV vaccine.....24
2.5 Treatment of HEV infection 25
CHAPTER THREE: MATERIALS AND METHODS.....27
I. Materials 27
3.1 Ethical aspects.....27
3.2 Study design 27
3.3 Sample collection.....27
3.3.1 Study group 1: blood donors that donated blood and completed the questionnaire.....27
3.3.2 Study group 2: blood donors that only donated blood 28
3.4 Study questionnaire.....29
3.4.1 Questionnaire formatting 29
3.4.2 Selection process for blood donation clinics 29
3.4.3 Conducting the survey at the blood donation clinics.....30
II. Methods.....31
3.5 Study group 1: HEV serology testing 31
3.5.1 HEV IgG ELISA 31
3.5.2 Anti-HEV IgG test procedure 32
3.5.3 Quality control and interpretation of results.....34
3.6 Study group 1: HEV molecular testing 34
3.6.1 Pooling procedure 35
3.6.2 Controls.....35
3.6.3 Standards.....35
3.6.4 HEV RNA extraction 37
3.6.5 Principles of the in-house real-time RT-qPCR to detect HEV RNA 37

3.6.5.1 In-house real-time RT-qPCR procedure 37
3.7 Study group 2: HEV molecular testing 39
3.7.1 Panther system....40
3.7.2 Procleix HEV NAT assay 40
3.7.3 Principles of HEV RNA detection by TMA....41
3.7.3.1 Automated TMA procedure 41
3.8 Donor look back and notification system....43
3.8.1 Follow up procedure in case of active HEV infection(s) 43
3.9 Statistical analysis 43
3.9.1 Sample size....44
3.9.1.1 Study group 1....44
3.9.1.2 Study group 2....44
CHAPTER FOUR: RESULTS....45
4.1 Demographics of study participants 45
4.2 Study group 1: HEV serology....46
4.2.1 Anti-HEV IgG seroprevalence....46
4.2.2 Demographics of study group 1 according to anti-HEV IgG result....46
4.2.3 Anti-HEV IgG seroprevalence according to area....47
4.2.4 HEV questionnaire....49
4.2.4.1 Bivariate analysis 49
4.2.4.2 Multivariate analysis....51
4.3 Study group 1: HEV molecular....51
4.3.1 HEV RNA prevalence 51
4.4 Study group 2: HEV molecular....51
4.4.1 HEV RNA prevalence 51
CHAPTER FIVE: DISCUSSION 53
5.1 Anti-HEV IgG seroprevalence of study group 1 53
5.2 Anti-HEV IgG seroprevalence according to the demographics of study group 1....53
5.3 Anti-HEV IgG seroprevalence according to potential risk factors in study group 1 56
5.4 HEV RNA prevalence 58
5.4.1 Study group 1 58
5.4.2 Study group 2 59
5.5 Summary of the main findings....60
5.6 Study strengths and limitations....60

5.7 Outlook and future recommendations.....61

CHAPTER SIX: CONCLUSION.....62

REFERENCES 64
ADDENDA.....74

LIST OF ABBREVIATIONS

AFSSAPS – Agence Française de Sécurité Sanitaire des Produits de Santé

ALT – Alanine transaminase

anti-HAV– Antibody to hepatitis A virus

anti-HEV - Antibody to hepatitis E virus

ARVs – Antiretroviral drugs

AST – Aspartate transaminase

CBD – Central business district

CDC - Centre for Disease Control and Prevention

cDNA – Complementary deoxyribose nucleic acid

DNA – Deoxyribonucleic acid

dNTPs – Deoxyribonucleotide triphosphate

DKA - Dual Kinetic Assay

EFS – Establissement Français du Sang

EIA – Enzyme immunoassay

ELISA – Enzyme linked immunosorbent assay

GBD – Global Burden of Diseases

HAV – Hepatitis A virus

HBV – Hepatitis B virus

HCC - Hepatocellular carcinoma

HCV – Hepatitis C virus

HEPES – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HEV – Hepatitis E virus

HEV1 – Hepatitis E virus genotype 1

HEV2 – Hepatitis E virus genotype 2

HEV3 – Hepatitis E virus genotype 3

HEV4 – Hepatitis E virus genotype 4

HIV – Human immunodeficiency virus

HRP – Horseradish peroxidase

IC – Internal control

ID – Individual-donation

IS – International standard

LOD – Limit of detection

MMLV - Moloney Murine Leukemia Virus

MP – Minipool

MS – Microsoft

NANB – Non-A, non-B hepatitis

NAT – Nucleic acid testing

NHS – National Health Service

NHSBT – National Health Service Blood and Transplant

NHP – Normal human plasma

NTPs - Nucleoside triphosphate

ORF – Open reading frame

OD – Optical density

PBS – Phosphate buffered saline

PCR – Polymerase chain reaction

PHE – Public Health England

PI - Principal investigator

RNA – Ribonucleic acid

RT – Reverse transcriptase

RT-qPCR – Reverse transcriptase quantitative polymerase chain reaction

SANBS – South African National blood service

S/CO – Sample cut-off

SDP - Solvent-detergent-treated plasma

SHOT - Serious Hazards of Transfusion

TAT – Turnaround time

TMA – Transcription-mediated amplification

TMB –Tetramethylbenzidine

TTI(s) – transfusion-transmitted infection(s)

TTP - Thrombotic thrombocytopenic purpura

UK – United Kingdom

VL – Viral load

WPBTS – Western Province Blood Transfusion Service

LIST OT TABLES

Table 2.1 HEV prevalence in sub-Saharan Africa……14

Table 2.2 HEV prevalence in blood donors from high income (developed) countries……...17

Table 2.3 HEV prevalence in blood donors from low to middle income (developing) countries……..….21

Table 2.4 Haemovigilance systems in countries with HEV TTIs……24

Table 3.1 WPBTS blood donation clinics surrounding Cape Town……30

Table 3.2 Tenfold dilutions of the HEV WHO IS……...……36

Table 3.3 Tenfold dilutions of the HEV PHE standard……..36

Table 3.4 Components of the master mix per reaction……38

Table 3.5 Primer and probe sequences for real-time RT-qPCR……38

Table 3.6 Setup of real-time RT-qPCR plate……..……...39

Table 3.7 Real-time RT-qPCR cycling parameters……...39

Table 3.8 Sample size calculation for the questionnaire participants……...……...44

Table 4.1 Demographic data representing the donor profiles…..……....45

Table 4.2 Retested anti-HEV IgG borderline samples……46

Table 4.3 Anti-HEV IgG seroprevalence for each of the 25 blood donation clinics……48

Table 4.4 Bivariate analysis of donor demographics and anti-HEV IgG seroprevalence……..49

Table 4.5 Bivariate analysis between the HEV risk factors and anti-HEV IgG status……50

Table 4.6 Logistic regression model……...51

Table 4.7 Characteristics of the HEV RNA positive donor……...52

Table 5.1 Trend in the anti-HEV seroprevalence in blood donors from the Western Cape……55

LIST OF FIGURES

Figure 1.1 Structure of the HEV genome……..……..2

Figure 1.2 Phylogenetic tree of HEV strains isolated from humans and animals ……2

Figure 1.3 HEV routes of transmission……..……..6

Figure 1.4 Course of HEV infection and serology responses…….……...8

Figure 2.1 Global epidemiology of HEV infection and genotype distribution……11

Figure 3.1 Selection and sampling of study participants…….……..…….....28

Figure 3.2 Step by step procedure of the HEV IgG ELISA……..33

Figure 3.3 HEV serology testing for study group 1……34

Figure 3.4 Illustration of TMA……41

Figure 3.5 Dual kinetic assay used by the Panther system……..……...43

Figure 4.1 Anti-HEV seroprevalences according to age group and gender……..46

Figure 4.2 Racial profiles of anti-HEV IgG for all donors……..47

CHAPTER ONE: INTRODUCTION

1.1 Background

Hepatitis E virus (HEV) was first detected in the 1980s when a mysterious hepatitis outbreak occurred in Afghanistan at a military camp. The virus was identified in the stool of infected individuals by electron microscopy and was later, sequenced and designated as HEV. HEV is a hepatotropic virus, which has significantly impacted the health of individuals due to its potential to cause acute viral hepatitis (Kamar *et al*., 2012). According to the global burden of diseases (GBD) study which was conducted in 2010, approximately 20.1 million individuals were infected with HEV throughout the nine GBD regions during 2005. Of the 20.1 million HEV cases, it was reported that 3.4 million were symptomatic infections; 3,000 stillbirths in pregnant women and an overall 70,000 of these cases resulted in death (Kumar *et al*., 2013).

1.1.1 HEV: The virus

HEV is a non-enveloped, positive-sense single-stranded RNA virus which is 32nm to 34nm in diameter. It is a member of the Hepeviridae family with several mammalian HEV species which infect humans, domestic pigs, deer, wild boar, etc. In addition to this, the Hepeviridae family also contains avian HEV and cut-throat trout virus, which is representative of a novel genus that has not been linked to human infections (Kamar *et al*., 2012). The virus was initially part of the Caliciviridae family but was reclassified because its genome organization. The HEV genome consists of three open reading frames (ORF) that overlap each other. ORF1 encodes approximately 1,690 amino acids of a polyprotein which goes through post-translational cleavage into non-structural proteins required for virus replication. ORF2 is responsible for encoding the main structural protein and the capsid protein made up of 660 amino acids. There is speculation that ORF3 is involved with virus particle assembly because it encodes a phosphoprotein that has an association with the cytoskeleton. In the different HEV genotypes, ORF3 has a slight variation in its organization, but it retains the same functions (Teshale & Hu, 2011).

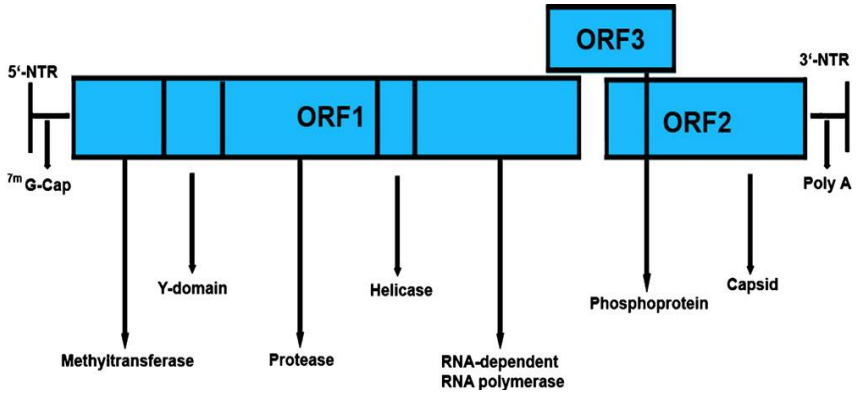


Figure 1.1 Structure of the HEV genome (Kumar *et al.*, 2013 with permission from Elsevier)

There are 4 HEV genotypes, 24 sub-types and 1 serotype (Teshale & Hu, 2011). Genotype 1 (HEV1) is prevalent in Asia and Africa, genotype 4 (HEV4) in Asia and genotype 2 (HEV2) in Mexico and West Africa whereas genotype 3 (HEV3) has mostly been reported in European countries. HEV1 and HEV2 are associated with large epidemics as well as endemics in areas where sporadic cases of hepatitis E occur. HEV3 and HEV4 have been detected in humans and in animals. It is thought that it is transmitted by consumption of undercooked meat from the infected animals (Teshale *et al.*, 2010a) such as wild boar, deer, and pigs (Vollmer *et al.*, 2012).

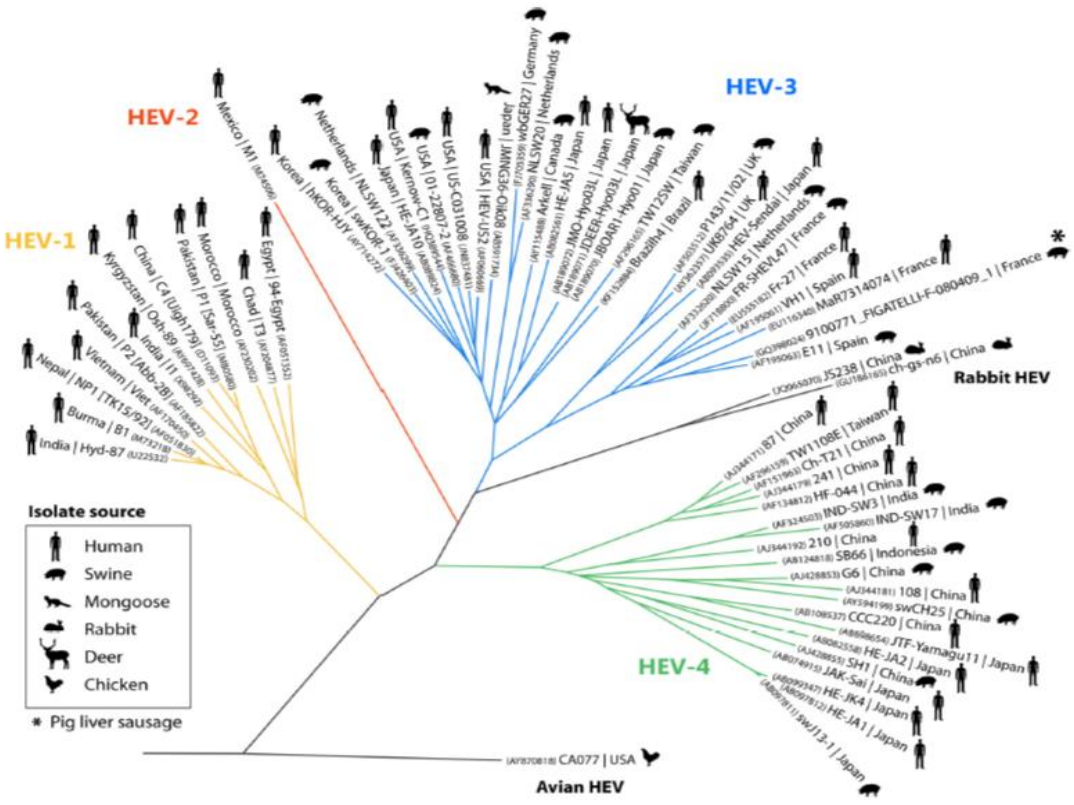


Figure 1.2 Phylogenetic tree of HEV strains isolated from humans and animals (Krain *et al.*, 2014 with permission from American Society for Microbiology)

1.1.2 Transmission of HEV

HEV has four known modes of transmission; waterborne, zoonotic foodborne, blood-borne and perinatal.

1.1.2.1 Waterborne

Waterborne transmission is the major cause of outbreaks in developing countries. HEV is spread by drinking water which has been contaminated in the course of waterborne epidemics which occasionally occur in tropical and subtropical regions. An investigation took place in Somalia and confirmed that HEV infection was waterborne due to a rise in the river's water level during the rainfall season. Consequently, the villagers that acquired their water from the river were at risk of being infected with contaminated water. However, the villagers getting water from wells displayed very severe cases which resulted in higher fatality rates. It was concluded that this inconsistency is an indication of the quantity of HEV required to induce the infection (Mushahwar, 2008).

1.1.2.2 Zoonotic foodborne

The zoonotic foodborne transmission of HEV has been reported to be widespread. The discovery of swine HEV has led to several studies investigating the homology of human and pig strains to determine whether a cross-species infection is possible. For the latter to be possible pigs would be a reservoir of the virus and humans would be infected by having direct contact with pigs. In addition to this, the consumption of undercooked pig livers has resulted in cases of hepatitis E with fatal outcomes reported in older or immunosuppressed individuals (Kato *et al*., 2004). Pig liver and other pig-derived food products have been reported to have detectable HEV at different stages in the pork production chain (Di Bartolo *et al*., 2012). Infected wild boar and deer are also known to cause zoonotic foodborne transmission of HEV. However unlike domestic pigs that usually live on farms, wild animals inhabit forest regions and could be exposed to various environmental sources of HEV (Lagler *et al*., 2014). The faecal-oral route may be associated with HEV infection in animals via faecal contaminated water or food sources and is probably due to defecation from an infected animal within the forest (Mushahwar, 2008). The latter suggests that the virus can be easily transmitted between a source and reservoir. Furthermore amongst other studies Takahashi *et al*. have shown that wild boar and deer have intra-species transmission of HEV and that humans have become infected by eating raw or undercooked contaminated meat from these species (Takahashi *et al*., 2004).

Wibawa *et al*., 2004 investigated the seroprevalence of HEV in Bali Indonesia in both pigs 72% (71/99) and blood donors 20% (54/276) and later in pregnant women with different dietary habits due to religious restrictions (Surya *et al*., 2005). They found that more than 50% of the pigs sampled in Bali had evidence of HEV exposure (Wibawa *et al*., 2004) hence possibly being a means of exposure to the majority of their Hindu residents. Residents who have pigs in their households are in direct contact with pigs i.e. through their faeces. Surya *et al*. compared the difference in HEV IgG seroprevalence of Hindu pregnant women (19%) with that of Muslim pregnant women (4%). They found that religious restrictions on eating pork may have influenced the lower prevalence in Muslim women; in that 2 of 50 were anti-HEV IgG positive. Whereas 149 of 749 of the Hindu women not prohibited to eating pork were seropositive (Surya *et al*., 2005).

1.1.2.3 Blood-borne

Transmitting HEV via blood products is a known risk however the prevalence of HEV transfusion-transmitted infection (TTI) varies and has limited data. In Spain a prevalence study was done among blood donors from Catalonia, Barcelona to investigate the safety of blood donations by screening 9,998 individual samples for HEV RNA. The assay which they used for nucleic acid testing (NAT) had a detection limit of 7.9 IU/ml (95% fiducial limits, 6.6-9.8 IU/ml) which was able to identify 3 HEV RNA positive individual donations with viral loads of 250, 564 and 2,755 IU/ml. Sequencing could only be done for the blood donor with the highest viral load and the HEV genotype was found to be 3f. The blood donors in Catalonia have a background HEV prevalence of 0.03% which is equivalent to approximately 1 in every 3,333 blood donations being viraemic. Hence, 25 to 225 of their annual 250,000 regional blood donations would be at risk of transmitting HEV. In addition to this depending on the presence of HEV antibodies in the blood donors, the amount of viral load and the remaining plasma present in their blood donation it could influence the transmission of the virus (Sauleda *et al*., 2015). Hence, it is difficult to establish when recipients will be infected with HEV contaminated blood products.

1.1.2.4 Perinatal

Pregnant women in developing countries have been greatly affected by HEV1 and HEV2 epidemics. The factors thought to be associated with an increased risk of mortality are altered immune response, hormonal changes associated with pregnancy and malnutrition (Kumara *et al*., 2004). The severity of maternal deaths caused by HEV infection occurs during the third trimester of pregnancy (Labrique *et al*., 2012).

1.1.2.5 Possible HEV transmission routes and genotypes expected in South Africa

HEV1 and HEV2 are expected to be prevalent in South Africa due to the geographical distribution of HEV (Figure 2.1). Most developing African countries have tropical climate conditions and seasonal rain that cause waterborne outbreaks which favour the faecal-oral transmission of HEV. Without proper housing, sanitation and safe drinking water, many individuals living in rural areas are easily exposed to enteric viruses such as HEV. However, faecal-oral transmission associated with HEV1 and HEV2 may not be the only cause of hepatitis E cases in developing countries. Several cases of HEV have been reported in South Africa (MI. Andersson personal communication, 2015) and suggest that the epidemiology of HEV in South Africa has changed.

A travel-associated case of HEV1 was diagnosed in a patient after returning from Pakistan (Andersson *et al*., unpublished data). The latter case is almost similar to the hepatitis E diagnosis made a few years ago that was reported to be an imported infection from India, but no genotyping was done due to limited HEV tests available at the time (Robson *et al*., 1992). However it was recommended by Robson *et al*. that hepatitis E should be suspected in individuals traveling to the Indian subcontinent, which was found in the patients returning from Pakistan (Andersson *et al*., 2015) and India (Robson *et al*., 1992).

In 2012, the production of South African meat products was under investigation for substituting animal species and incorrect labelling or omitting to label animal species that were present in meat products. The Department of Animal Sciences (University of Stellenbosch) and the Food & Allergy Consulting & Testing Services worked in collaboration to assess the processing and labelling of 139 meat products. These products were bought from retailers in the most populated provinces of South Africa; 38 from Western Cape, 28 from Eastern Cape, 38 from KwaZulu-Natal and 35 from Gauteng (Cawthorn *et al*., 2013). The abovementioned study revealed that there is indeed meat contamination which suggests that foodborne zoonotic transmission associated with HEV3 may occur through contaminated meat products sold in South Africa.

One of the HEV cases from South Africa involved an immunosuppressed HIV-infected patient from Cape Town that was diagnosed with chronic HEV infection (Andersson *et al*., 2012) which is associated with HEV3 (Kamar *et al*., 2012). The latter patients' phylogenetic analysis revealed HEV3 clustering and the patient cleared the virus infection when he was started on antiretroviral drugs (ARVs) (Andersson *et al*., 2012). Recently another case of

HEV3 was reported in a patient that received a renal transplant at a hospital in Cape Town (Andersson *et al*., 2015). The above-mentioned reports show that HEV3 seems to be prevalent in South Africa.

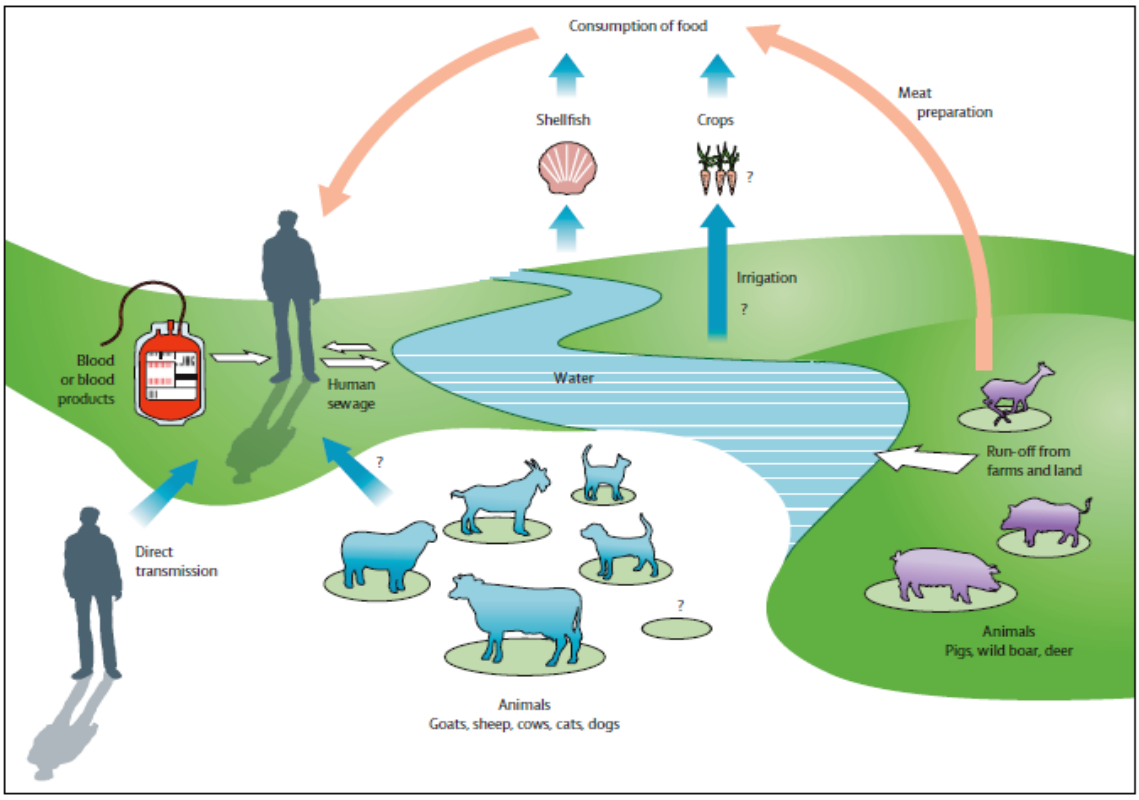


Figure 1.3 HEV routes of transmission (Kamar *et al.*, 2012 with permission from Elsevier)

1.1.3 Clinical presentation and natural history

The clinical presentation of HEV is comparable to other hepatotropic viruses which also induce acute viral hepatitis infections. Individuals infected with HEV display a broad range of clinical features ranging from fulminant to asymptomatic infections. The features are jaundice, anorexia, malaise, fever, pruritus and hepatomegaly. The laboratory results associated with these symptoms are increased levels of bilirubin in the serum, noticeable elevation of alanine transaminase (ALT) and aspartate transaminase (AST) and a slight increase in the alkaline phosphatase activity (Teshale *et al*., 2010a).

Hepatitis E is generally self-limiting, but it may potentially develop into the fulminant disease (Vollmer *et al*., 2012). HEV has an incubation period of 2 to 6 weeks. HEV infection is represented by an initial temporary HEV IgM response which is followed by a long-lasting HEV IgG response. Acute HEV infection displays a peak in HEV RNA during the incubation period and the early phases of HEV infection. After infected individuals start developing symptoms the HEV RNA in the blood becomes undetectable although it can still be detected

in faeces for further 10-14 days. The window period for detecting HEV RNA is thus narrow (Kamar *et al*., 2012).

1.1.4 Diagnosis of HEV

Acute HEV infection is diagnosed by detecting IgM or the seroconversion from IgG negative to positive using a serology based enzyme immunoassay (EIA). Anti-HEV IgM is detected first and anti-HEV IgG is detected shortly after the IgM response. Antibodies (IgM and IgG) produced against HEV are diagnostic markers that help to determine the stage of infection (Isaäcson *et al*., 2000), which could be either a recent or past infection. However serological kits use different antigens that are coated to the wells of the ELISA plate which has an influence on the sensitivity and specificity of the assay. Alternatively molecular testing is essential to detect replicating virus RNA, suggesting an active infection. Nucleic acid tests such as a polymerase chain reaction (PCR) assay detects HEV RNA in the blood and stool samples indicating the presence of the virus i.e. current infection. Detection of HEV RNA is possible earlier in blood than in stool samples (Teshale *et al*., 2010a).

Chronic HEV infection is defined by persistent HEV RNA in the serum or stool samples, for at least six months. Chronic HEV has been described in patients that received solid organ transplants, in HIV patients and those with haematological malignancies such as B-cell chronic lymphocytic leukaemia (Gauss *et al*., 2012). Furthermore prevention of chronic HEV has been studied in a cohort of patients that are co-infected with HEV and the following haematological malignancies; acute leukaemia, non-Hodgkin lymphoma, multiple myelomas and others (Tavitian *et al*., 2015). Hence, patients with suppressed immune systems are likely to develop chronic HEV infection, which may lead to cirrhosis. Only HEV3 has been reported to induce chronic HEV infection (Kamar *et al*., 2012).

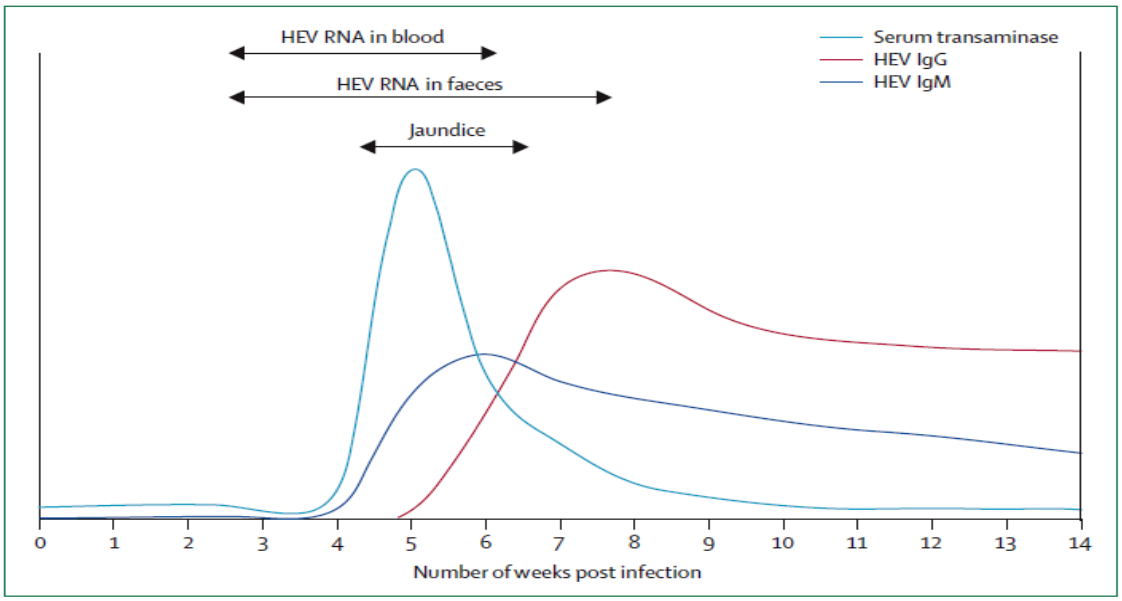


Figure 1.4 Course of HEV infection and serology responses (Kamar *et al.*, 2012 with permission from Elsevier)

1.2 Problem statement

The prevalence of HEV infection worldwide may have previously been under-reported. Data investigating the prevalence of HEV in blood donors is particularly sparse. Blood donors are carefully selected clinically healthy individuals and usually have asymptomatic infections which allow for the blood-borne transmission of HEV to immunosuppressed blood transfusion recipients. The latter is a concern because unlike blood donors who are able to clear the virus infection, immunosuppressed recipients of HEV contaminated blood could develop chronic HEV infection caused by genotype 3. Previous work from our laboratory (Lopes *et al*., unpublished data) has shown a seroprevalence of 25.3% in blood donors from the Western Cape, South Africa. Based on this data we hypothesize that, blood donors may be infected with HEV in the past or currently and may thus potentially infect recipients of blood and blood products.

1.2.1 Hypothesis

Blood donors with active HEV infection donate infected blood to recipients

- Null hypothesis:
  - There is no evidence of active HEV infection amongst blood donors

- Alternative hypothesis:
  - There is evidence of active HEV infection amongst blood donors

1.2.2 Aims and objectives

To determine

- the prevalence of HEV infection in blood donors from the Western Cape
- the risk factors associated with past HEV infection

CHAPTER TWO: LITERATURE REVIEW

2.1 Epidemiology of HEV

2.1.1 HEV worldwide

HEV is the most common cause of acute viral hepatitis in many developing nations (Kumar *et al*., 2013; Isaäcson *et al*., 2000). Reports have shown that HEV causes large outbreaks of hepatitis in low to middle-income countries. The first reported outbreak was in India during 1955 to 1956 and it resulted in 29,300 cases of hepatitis E (Viswanathan, 1957). However China had the largest outbreak of hepatitis E with 119,000 cases due to waterborne transmission (Zhang *et al*., 1991; Bi *et al*., 1993). During the mid-1990s, genotyping of HEV strains, led to the discovery that HEV1 was the primary cause of hepatitis E outbreaks in developing countries. HEV2 cases are the least reported and have been the cause of outbreaks in West Africa and Mexico (Teshale *et al*., 2010a). In developing countries people are frequently exposed to contaminated water and poor sanitary conditions, which causes waterborne outbreaks that are associated with HEV1 and HEV2.

Until relatively recently hepatitis E was thought to be an imported disease in the developed world. However recent data has shown in Europe HEV infection may be occurring more frequently than previously thought. HEV3 and HEV4 have been reported in developed (Slot *et al*., 2013) and developing countries (Ren *et al*., 2013). These genotypes are associated with sporadic cases of HEV. Infection is thought to occur mainly via animal-to-human transmission by eating undercooked meat and less frequently via human-to-human transmission by blood transfusion (Purdy *et al*., 2011). In the Netherlands, a study was done which showed that 64% of hepatitis patients were infected with HEV locally (Koot *et al*., 2015). The latter suggested that travel history to HEV endemic regions is not the only means of infection in developed countries. The estimated seroprevalence of HEV in developed countries has been reported to vary (Kamar *et al*., 2012) from less than 5% in the French blood donors (Boutrouille *et al*., 2007) up to 21% in civilians from the US (Kuniholm *et al*., 2009). In support of this, Table 2.2 indicates that the difference is most likely due to different ELISA assays being used in studies. The epidemiology of HEV is very diverse and may be influenced by a number of factors that still need to be studied.

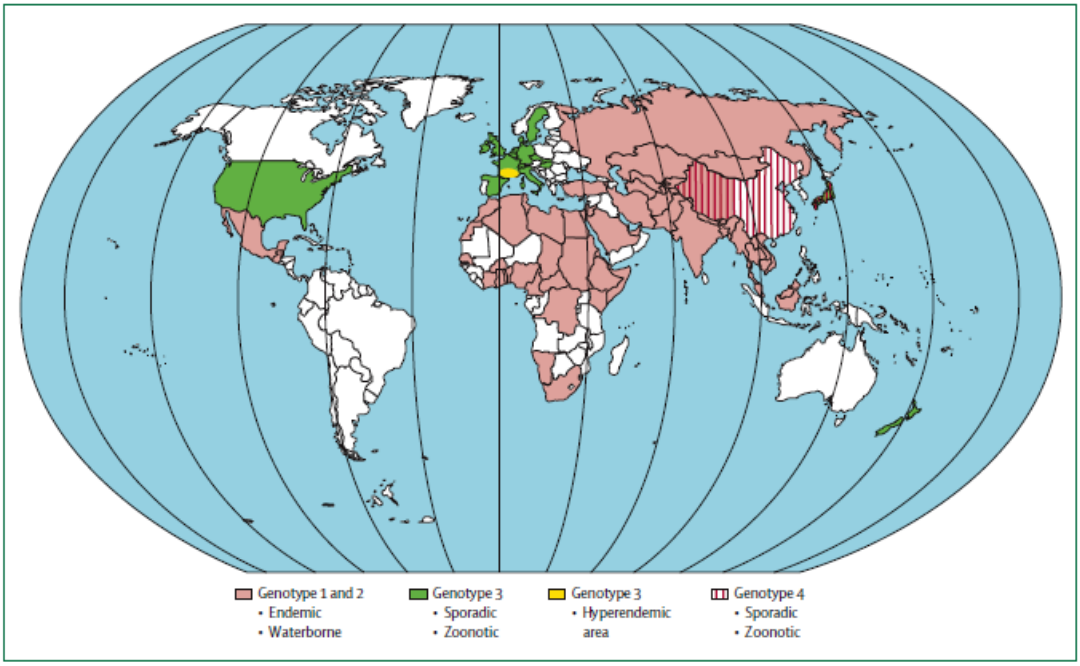


Figure 2.1 Global epidemiology of HEV infection and genotype distribution (Kamar *et al.*, 2012 with permission from Elsevier)

2.1.2 HEV in Africa

Various outbreaks of hepatitis E have been documented in Africa. One of the earliest documented outbreaks occurred in Namibia in 1983. Individuals residing in settlements with limited water supply and unsanitary living conditions were greatly affected by the outbreak. After the investigation, it was reported to be non-A, non-B (NANB) hepatitis waterborne outbreak. Thereafter samples were obtained from jaundiced patients and healthy residents that were living in an affected settlement; to later determine the cause of the hepatitis epidemic. Subsequently, in 1998, the archived samples were tested for HEV markers. The tests showed that HEV RNA was detected in 56% of the faecal samples that were obtained from the jaundiced patients. Furthermore, 42% of the serum samples from the healthy individuals had anti-HEV IgM and 25% had total immunoglobulin that was tested using an ELISA (Genelabs Technologies, Inc., Redwood City, CA). The serology evidence of HEV infection in the healthy individuals was used to confirm that HEV was the causative agent of the hepatitis outbreak in 1983 (Isaäcson *et al*., 2000). In addition to this, phylogenetic analysis showed that the HEV strain isolated from the Namibia 1983 outbreak was HEV1 (He *et al*., 2000). However a few years later in 1995, another outbreak occurred in the same region of Namibia and the four HEV strains from this outbreak belonged to HEV2 (Maila *et al*., 2004).

Madagascar

A study was done to determine the prevalence of HEV antibodies in slaughterhouse workers and pigs in Madagascar. The results of the study revealed that anti-HEV IgG (MP Diagnostics, Illkirch, France) is highly prevalent in 71.2% of the swine in Madagascar. In addition to this, 1.2% of the pigs had detectable HEV RNA and were further analysed using phylogenetic analysis. The latter revealed that the viruses were HEV3 strains. It was suggested that the transmission of HEV from pigs to humans may have occurred in Madagascar. Serum samples of the slaughterhouse workers were tested with an ELISA assay (EIAgen HEV Ab, Adaltis, Milan, Italy) and 14% had evidence of past HEV infection. However the authors recommend that other non-zoonotic routes of HEV infection in humans should also be investigated (Temmam *et al*., 2013).

Burkina Faso

The burden of acute hepatitis caused by HEV and hepatitis A virus (HAV) was investigated in central Burkina Faso. The study population consisted of blood donors and pregnant women; that were used as a representative of the general population. Serum samples were obtained to test for antibodies to HAV and HEV. The anti-HAV IgG seroprevalence was found to be 14.3% in blood donors and 23% in pregnant women using an immunochromatographic assay (SD Bioline IgG, Standard Diagnostics, Inc., Korea). While the anti-HEV IgG seroprevalence in the blood donors was reported to be 19.1% and 11.6% in pregnant women, using the Dia.Pro ELISA kit (Dia.Pro Diagnostic Bioprobes S.r.l., Italy). Furthermore, the seroprevalence of HEV in Burkina Faso was found to be similar to that of other African countries such as; Tunisia (22%), Burundi (14%) and South Africa (15.3%) (Traoré *et al*., 2012) (Table 2.1).

HEV and HAV share the faecal-oral route of transmission, which was also assessed during the above-mentioned study. However the findings showed that the prevalence of these two enteric viruses differed in immunocompetent i.e. blood donors and immunocompromised groups i.e. pregnant women (Traoré *et al*., 2012). A possible explanation was given for the higher HAV prevalence in pregnant women; it could have been caused by them having closer contact with children. The latter is as a result of children being susceptible to hepatitis A and could act as carriers of HAV to infect immunocompromised pregnant women (Smith *et al*., 1997).

Nigeria

Researchers in Nigeria took a different approach to identifying which risk factors are associated with HEV infection. HEV and HAV are known to be similar because of their enteric transmission and their potential to serve as food pathogens (Collier *et al*., 2014). It was found that external factors may serve as a means of foodborne zoonotic transmission of HEV3. For example, when crops are grown with manure made from faecal matter of HEV-infected animals or if the irrigation supply used to water the fruits and vegetables are from rivers contaminated with sewage, it may potentially contaminate the crops (Junaid *et al*., 2014). Consequently, if fruits and vegetables are not washed before consumption it may increase the risk of getting infected with HEV. Junaid *et al*. reported that the latter was a statistically significant behavioural risk factor associated with past HEV infection (p-value, <0.001). The study has provided valuable insight into the epidemiology of HEV in West Africa and assessed risk groups to assist with public health prevention strategies in Nigeria.

2.1.3 HEV in South Africa

In the mid-1990s, an overall seroprevalence rate of 10.7% for anti-HEV was observed amongst 767 black South Africans living in urban and rural areas. An enzyme-linked immunosorbent assay (Abbott HEV EIA; Abbott Laboratories) was used to detect HEV antibodies to antigenic regions coded from ORF2 and ORF3 (Tucker *et al*., 1996). However, the assay was less sensitive than the current assays and may have underestimated the seroprevalence of HEV infection in South Africa.

Two other reports of HEV infection in South Africa have previously been published: one report of a hospital outbreak caused by nosocomial transmission to medical staff who attended to a pregnant woman who had acquired hepatitis E while traveling aboard. The samples from this outbreak were tested for HEV antibodies using a western blot test performed at the CDC Hepatitis Laboratory in the US (Robson *et al*., 1992). The other report in medical students and canoeists used an EIA with synthetic peptides to detect HEV antibodies (Grabow *et al*., 1994). The results of these reports are shown in Table 2.1. These studies did not test for HEV RNA which indicates evidence of active infection, therefore, no genotyping was done. Since then advances have been made in molecular testing and current data have reported on the genotype of HEV strains found in South Africa. HEV3 has been found in an HIV-infected patient (Andersson *et al*., 2012) and more recently in a patient that received a renal transplant (Andersson *et al*., 2015). In addition, to this a case of HEV1 was isolated from a patient after visiting an HEV-endemic country (Andersson *et al*., 2015). Further investigations are required to better understand the epidemiology of HEV.

Table 2.1 HEV prevalence in sub-Saharan Africa

Reference	Country	Prevalence	Cohort	HEV genotype
Jacobs *et al.*, 2014	Zambia	42% (N=106)	Co-infected HEV-HIV Adults	Not reported (NR)
Teshale *et al.*, 2010a	Uganda	64% (N=475)	Residents from 2 sub counties in Uganda affected by the epidemic	1
Maila *et al.*, 2004	Namibia	(N=4)	Outbreak 1995	2
Isaäcson *et al.*, 2000; He *et al.*, 2000	Namibia	25% (N=24)	Healthy residents from a severely affected settlement	1
Tucker *et al.*, 1996	South Africa	10.7% (N=767) 407 urban adults (5.8%) 360 rural adults (19.1%)	Blacks from rural & urban areas	NR
Van Rensburg *et al.*, 1995	Swaziland	4% (Ndzevane) (N=NR) 2% (Malindza) (N=NR)	398 Mozambican refugees from Two camps	NR
Grabow *et al.,* 1994	South Africa	2.05% (N=782)	Canoeists & Medical students	NR
Byskov *et al.*, 1989	Botswana	1-2% (N=273)	Hepatitis & jaundiced adults	NR

2.2 HEV in blood donors

Blood safety is an important issue in the world and ensuring blood products are free from any infection is a priority. Over the past few years, there has been much attention paid to the risk of HEV transmission from blood products.

2.2.1 Prevalence of HEV in blood donors

The International Society of Blood Transfusion very recently published an International forum issue on Hepatitis E to address several questions on screening blood donations for HEV infection. They received a response from 18 institutions within 16 different countries; 12 developed and 4 developing (Petrik *et al*., 2015). This forum has summarised both

seroprevalence and RNA incidence data on HEV infection and reflects that the majority of the data is from anti-HEV IgG findings i.e. past HEV infection with very little or no data on anti-HEV IgM and HEV RNA indicating a recent or active HEV infection. The latter being crucial is determining the risk of HEV transmission via blood transfusion and also required for genotyping to determine the route of transmission. Subsequently by knowing the risk factors associated with acquiring HEV infection, it can help with prevention in the general population and effective screening strategies of blood donors and recipients.

2.2.1.1 HEV prevalence in blood donors from developed countries

England and Wales

In several developed countries such as England, serological and molecular tests were performed in a study cohort of English and Welsh blood donors. The cohort consisted of 262 donors from the National Health Service (NHS) Blood and Transplant and 333 samples from donors that were hepatitis B virus core antibody negative with past evidence of jaundice. Samples were tested for anti-HEV IgG and IgM; if the result was anti-HEV IgG positive. Additionally, an in-house TaqMan assay was used to test for HEV RNA. Overall the study showed that 10% of the blood donors were anti-HEV IgG positive which was linked to an increase in age with males and females. In addition to this, IgM reactive donors had no detectable HEV RNA which would have suggested recent active HEV infection (Beale *et al*., 2011).

France

Blood donors (N=512) from Midi-Pyrénées in France, completed a risk factor questionnaire and were tested for HEV antibodies. In addition to this, pig liver sausages from the local market in Midi-Pyrénées were tested for HEV RNA. According to the results, the seroprevalence of 52.5% using the Wantai assay [95%CI, 48.2-56.8] for HEV IgG antibodies is the highest reported for a high-income developed country. The HEV risk factor data showed that old age, rural residence, having direct contact with a cat and hunting game (wild boar or deer) was significantly associated (p-values <0.05) with the foodborne zoonotic transmission. HEV RNA was detected in some of the pig liver sausages and the strains belonged to HEV3 (Mansuy *et al*., 2011).

Another study reported on the seroprevalence of past HEV infection in blood donors from southern France and south-west France was found to be 39.1% with the Wantai assay [95%CI, 37.4-40.7] and 52.5% [95%CI, 48.2-56.8] respectively. Southern France has two regions; 1,897 of the blood donors from Midi-Pyrénées and 1,456 from Languedoc-

Roussillon had to complete a questionnaire. The four factors were found to be associated with HEV infection namely; eating raw pork liver sausages ($p<0.001$), offal ($p=0.003$) and shellfish ($p=0.02$). Hence, their results show that there is more than one main risk factor associated with HEV infection and many possible sources of contamination in southern France. However, more importantly, the incidence of HEV in blood donors from these two regions indicate that their annual number of blood donations has at least 148 of 184,000 donations that may be HEV RNA positive. Although not much is known about risks of transfusion-transmitted HEV infections in southern France (Mansuy *et al*., 2015).

Greece

In Athens, Greece 265 donors were tested for antibodies to HEV IgG using an ELISA kit (EIAgen, Adaltis Inc., Italy) and the seroprevalence was found to be 9.43%. All the HEV seropositive (25/265) blood donors were from urban areas with no recent history of traveling outside of Greece. No HEV molecular testing was done on the IgG positive samples to determine which strain of HEV is circulating in Greece. However judging by the literature it is most likely to be genotype 3 because it is frequently associated with infection in individuals from developed countries without any travel-history to developing countries. Further studies should test the animals in Greece for HEV RNA reveal whether the zoonotic transmission is occurring via direct contact with HEV-infected animals or consumption of undercooked infected meat (Pittaras *et al*., 2014).

Denmark

The seroprevalence of HEV in blood donors was monitored over time by comparing the data of three studies using an in-house ELISA developed at National Institutes of Health (NIH assay). The advantage of using the same assay to evaluate the prevalence at different time points in the same population has eliminated the problem of assay variation. To indicate the decline in anti-HEV IgG, three blood donors from the 2003 study were included in the 2013 study; two donors may have experienced a waning of their antibodies since initially they were seropositive and after a decade, their antibody levels were below the assay sample cut-off (S/CO) value. However, one of the three donors from the 2003 study remained seropositive. Therefore, they concluded that even though there has been a major decline in the prevalence of HEV from 32.9% in 1983 to 10.7% in 2013, the prevalence in Denmark is still fairly high and blood donors should be screening for HEV infection (Holm *et al*., 2015).

Table 2.2 HEV prevalence in blood donors from high income (developed) countries

Country	Sample size	HEV IgG seroprevalence	HEV RNA prevalence	Reference
United States (US)	18,829 blood donations	329 of 4,499 (7.3%) MP Biomedicals assay [95%CI,6.6-8.1]	2 of 18,829 (0.01 %) Procleix assay	Stramer *et al*., 2015
Southeast England	225,000 blood donations = 9,382 Minipools	23 of 79 (29%) Wantai assay	79 of 225,000 (0.04%)	Hewitt *et al*., 2014
Spain	9,998 blood donations	(19.96%) Wantai assay and (10.72%) Mikrogen assay	3 of 9,998 (0.03%) Procleix assay	Sauleda *et al*., 2015
France	53,234 blood donations = 558 Minipools	(23.6%) Wantai assay	24 of 53,234 (0.045%)	Gallian *et al*., 2014
Australia	3,237 blood donations	194 of 3,237 (5.99%) Wantai assay [95%CI,5.18‑6.81]	No RNA positives Procleix assay	Shrestha *et al*., 2014
Scotland	43,560 blood donations = 1,815 Minipools	73 of 1,559 (4.7%) Wantai assay [95%CI,3.6-5.8]	3 of 1,815 minipools (0.2%)	Cleland *et al*., 2013
Netherlands	45,415 blood donations	1,401 of 5,239 (27%) Wantai assay	17 of 45,415 (0.04%)	Slot *et al*., 2013
US	1,939 blood donations	364 of 1,939 (18.8%) Wantai assay [95%CI,17.0-20.5]	No RNA positives	Xu *et al*., 2013
Saudi Arabia	900 blood donations	168 of 900 (18.7%) Bioelisa assay	Not done (ND)	Johargy *et al*., 2013
England	42,000 blood donations = 880 Minipools	~13% (Ijaz et al.,2009)	6 of 880 minipools (0.7%)	Ijaz *et al*., 2012
Germany	16,125 blood donations *Minipools of 48	Donors with ↑ ALT (5.9%) RecomLine assay	13 of 16 125 (0.08%)	Vollmer *et al*., 2012
Switzerland	550 blood donations	27 of 550 (4.9%) MP Diagnostics assay	ND	Kaufmann *et al*., 2011
Japan	6,700 blood donations	479 of 6,700 (7.1%)	9 of 6,700 (0.1%)	Gotanda *et al*., 2007
Japan	5,343 blood donations	200 of 5,343 (3.7%) in-house assay	3 of 5,343 (0.06%)	Fukuda *et al*., 2004

2.2.1.2 HEV prevalence in blood donors from developing countries

There is little data describing the prevalence of HEV in blood donors from developing countries (Table 2.3).

China

In China, a study specifically looked at the risk of being infected with HEV through blood transfusion. The results showed that 2 of the 10,741 (0.02%) donors with normal ALT levels were possibly exposed to HEV and at risk of having undetectable viremia. Furthermore, in support of this 2 of the 4 HEV RNA positive donors had normal ALT levels and were most likely tested during the window period of HEV infection. Blood donors with elevated ALT levels had a significantly higher seroprevalence than normal donors, for both anti-HEV IgM and IgG, (Wantai Biopharmaceutical, Beijing,China) with a p-value of <0.01 for both these HEV markers. However there was no significant difference seen for the HEV antigen although it also had a higher prevalence in blood donors with elevated ALT levels, the p-value was equivalent to 0.10 > 0.05. Overall the results showed that two of the donors with elevated ALT levels had detectable HEV RNA, suggesting that it is not a very reliable marker for active HEV infection (Ren *et al*., 2013).

India

Four hundred and sixty male blood donors in India were tested for IgM antibodies to HEV (Diagnostic Bioprobes Srl, Milano, Italy). The findings indicated that they had a 4.78% anti-HEV IgM seroprevalence rate (Gajjar *et al*., 2014). However the 22 IgM HEV positive donors were not screened for HEV RNA but previously in Pune, India the HEV RNA prevalence was found to be 1.5% (3 of 200) (Arankalle *et al*., 2000). Gajjar *et al*. compared their HEV seroprevalence rate to several other HEV studies done in blood donors and had a similar prevalence as Switzerland of 4.9% (Kaufmann *et al*., 2011). The difference HEV seroprevalence of developing and developed countries was discussed by stating that it may be due to the different HEV routes of transmission. Waterborne transmission being most common in developing countries as opposed to foodborne zoonotic transmission however study population also plays a role.

Brazil

A seroprevalence study was done in a metropolitan area with a cultural preference for frequently eating pork meat products. Recent and past HEV infection was investigated in 300 blood donors tested for anti-HEV IgG and IgM. Thirty of the donors were positive for

anti-HEV IgG and these donors were subsequently tested for IgM antibodies (Wantai Biopharmaceutical, Beijing, China). One of the 30 seropositive IgG donors tested positive for IgM, suggesting that past HEV infection was more prevalent than recent infection. Furthermore, the presence of both IgG and IgM in the latter donor could indicate that seroconversion was taking place or it might have been a re-infection. There were no HEV RNA positive donors identified with active HEV infection (Passos-Castilho *et al*., 2015).

2.2.1.3 HEV prevalence in blood donors from Africa

Egypt

In the general population, it was found that Egyptians who live in rural villages have acquired natural immunity to HEV which is suggested by the prevalence of anti-HEV antibodies in 67.7% [95%CI, 66.7-68.6] using the Abbott HEV ELISA kit (Fix *et al*., 2000). However contrary to the high prevalence in the general population the findings from Ibrahim *et al*. suggested that there is a low seroprevalence of HEV among blood donors in Egypt. Nevertheless, the latter study has shown that there is evidence of recent and active HEV infection in blood donors. Anti-HEV IgM antibodies were detected in 3 of the 760 blood donors using the MP diagnostic assay and 2 of these 3 blood donors had detectable HEV RNA, (2/760) 0.26%. Hence, the general population, in particular, healthy individuals such as blood donors have asymptomatic HEV infection. In addition to this it is also evident that the prevalence of HEV will tend to vary because if individuals do not present with symptoms, or if they are not blood donors (Ibrahim *et al*., 2011) or recruited to participate in a community serosurvey (Fix *et al*., 2000) then the true seroprevalence will most likely remain underestimated.

Another study was done two years, later on, a smaller study population of blood donors (N=488). The total seroprevalence of the study was reported to be 20.9% (102/488) for both anti-HEV IgM and IgG. However, all the seropositive samples tested for HEV RNA had an undetectable viral load. The blood donors in Ismailia, Egypt had no active HEV infection, but they had a higher HEV seroprevalence (Tadesse *et al*., 2013) than the blood donors from Ibrahim *et al*. with 0.39% (3/760). Thus, the difference in HEV seroprevalence for blood donors from Egypt appears to vary and has reached as high as 45.2% in blood donors (Abdel Hady *et al*., 1998). Concluding that Egyptian blood donors have one the highest HEV seroprevalence rates worldwide and specifically in Africa.

Ghana

The seroprevalence in Ghana was observed to be 4.6% for anti-HEV IgG by using three ELISA assays [(Mikrogen, Neuried, Germany); (Fortress Diagnostics, Antrim, UK); (MP Biomedicals, Illkirch, France)]. Furthermore, molecular tests were done on all the reactive samples in individual reactions and in the non-reactive samples in pools of 10 donations. However none of the samples tested in the study were found to be HEV RNA positive (Meldal *et al*., 2013). Therefore, no genotyping could be done to investigate the possible routes of HEV transmission in Ghana. Nonetheless, it is suspected that the poor sanitary conditions together with the regular occurrence of floods in Ghana may induce the spread of HEV1 and HEV2. However, there is insufficient information to evaluate the number of outbreaks that may have been caused by the waterborne transmission (Meldal *et al*., 2013). Further studies are needed to confirm the current data that exists on the prevalence and epidemiology of HEV in blood donors from developing countries, such as Ghana.

South Africa

In 2013 we investigated a small sample of blood donors in the Western Cape, comprising 100 samples that were randomly selected from each of the three different race groups of blood donors, of different sex and different age groups. The blood donors were tested for anti-HEV IgG and IgM using an ELISA (Fortress Diagnostics, UK) and subsequently tested for HEV RNA in plasma mini pools of 10. Of the 300 samples 76 were positive for HEV IgG, with a total seroprevalence of 25.3% [95%CI, 20.1-29.9]. There was a trend toward a higher seroprevalence in coloured men with a statistical significance in those 26-45 years ($p<0.05$) and those older than 46 years ($p<0.001$). None of the samples were positive for anti-HEV IgM and none of the mini pools had detectable HEV RNA (Lopes *et al*., unpublished data). This allowed us to describe for the first time, demographic risk factors which may be associated with HEV infection. However, the sample size was not large enough to assess the risk of active HEV infection, but sufficient to indicate that there may be a problem which deserves further attention.

Table 2.3 HEV prevalence in blood donors from low to middle income (developing) countries

Country	Sample size	HEV IgG Seroprevalence	HEV RNA prevalence	Reference
Egypt	488 blood donations	102 of 488 (20.9%) Dia.Pro assay	No RNA positives	Tadesse *et al*., 2013
Ghana	239 blood donations	11 of 239 (4.6%) Wantai and recomWell assays	No RNA positives *Plasma pools of 10	Meldal *et al*., 2013
Iran	530 blood donations	76 of 530 (14.3%) Dia.Pro assay	ND	Ehteram *et al*., 2013
Burkina Faso	178 blood donations	(19.1%) Dia.Pro assay (14.6%) Wantai assay [95%CI,13.3-24.9]	ND	Traoré *et al*., 2012
Tunisia	687 blood donations	(5.4%) MP Diagnostics assay	ND	Houcine *et al*., 2012
China	44,816 blood donations	14,608 of 44,816 (32.6%) Wantai assay	30 of 44,816 (0.07%)	Gou *et al*., 2010
Brazil	996 blood donations	23 of 996 (2.3%) Abbott assay [95%CI,1.5-3.5]	ND	Bortoliero *et al*., 2006

2.3 Risks and consequences of HEV in blood donors

2.3.1 HEV infection associated with blood transfusion

Blood donations are crucial to saving the lives of patients in need of blood or blood-derived products. Hence, blood donation services have a selection criterion to ensure the safety of blood and blood products. However, the possibility of a recipient receiving blood or blood products that are contaminated with HEV is a growing concern.

In the United States (US) researchers have assessed the risk of HEV transfusion-transmitted infection (TTI) by testing blood donor samples (N=1,939) for active HEV infection and they monitored the recipients (N=362) of these blood donations. The recipients were tested for HEV markers (IgM and IgG antibodies and viral RNA) before and after having the blood transfusion. The researchers concluded that none of the blood donors had detectable HEV RNA and consequently the recipients had no cases of TTIs (Xu *et al*., 2013). This study is

one of the few studies that have included the recipients of blood donations to get a better understanding of the blood-borne transmission of HEV infection.

In addition to the above-mentioned study, a larger study was done in the UK to investigate the prevalence of HEV in donors and to determine the incidence of HEV TTIs in the blood recipients. Two hundred and twenty-five thousand individual blood donations from donors in southeast England were retrospectively screened during the study to detect blood donations containing HEV RNA. Follow-up was also done by the National Health Service Blood and Transplant (NHSBT) to identify, inform and monitor recipients of the blood components that were received from HEV viraemic donors. Of the 225,000 donations tested for HEV RNA, 79 had detectable HEV and 129 blood components were made. Furthermore, these HEV contaminated blood components had already been transfused to 60 recipients. Subsequently, follow-up was done and they were able to recruit 43 of which 18 recipients (42%) that had evidence of HEV transmission. Furthermore to confirm that it was transfusion-associated HEV infection, the sequences obtained from the viraemic donors (N=54) and recipients (N=12) were compared using a phylogenetic tree. As a result, all the sequences were found to be HEV genotype 3 and the donor and recipient sequences were identical (Hewitt *et al*., 2014).

In Canada efforts have been made to evaluate the risk of HEV transmission in thrombotic thrombocytopenic purpura (TTP) patients that received large volumes of plasma, up to 40 litres for treatment. The results indicated that 4 of 17 patients who received solvent-detergent-treated plasma (SDP) had seroconverted to IgG at six months post-treatment. Thus suggesting that plasma-derived blood products given as treatment may potentially permit the transmission of HEV. Due to the absence of the SDP residual samples, the researchers were unable to do sequencing analysis to check for homology between the SDP units used and the patient samples. The two patients with IgG seropositivity and detectable HEV RNA in the 1-month post-treatment samples received the same SDP unit hence the researchers concluded that there is indirect evidence of HEV TTI via plasma (Andonov *et al*., 2014). However further studies are required to confirm this by firstly having access to both the donor and patient samples to provide a direct link between the infected donor blood components causing infection in a patient if they have detectable viral RNA. Secondly to distinguish between actively and passively acquired antibody if a patient test antibody positive.

It is therefore requested that the blood donors report back to the blood donation clinic when they experience any signs and symptoms of viral hepatitis infection (Boxall *et al*., 2006). In the unlikely event that a blood donor is infected, there is a critical time period available in

which to prevent the transfusion of their infected blood or blood products. A recipient could be spared the fate of being infected and having their health deteriorate rapidly. Studies have reported that it is advantageous to have the records of both blood donors and their recipients. The latter is required to determine whether the blood donor and their recipient have sequence identity and to confirm whether HEV blood-borne transmission occurred (Khuroo *et al*., 2004).

2.4 Prevention of HEV TTIs

2.4.1 HEV screening of blood donations

HEV can be transmitted through transfusion of blood and blood products. Studies have reported that HEV3 has been detected in blood donors from developed countries (Cleland *et al*., 2013; Vollmer *et al*., 2012; Slot *et al*., 2013) as well as HEV4 in a transfusion-transmitted HEV case (Matsubayashi *et al*., 2008). Whilst highly sensitive HEV screening methods are available (Vollmer *et al*., 2012), it would be costly to individually screen the blood donor supply for HEV infection. Using a pooling strategy would be less expensive and could be suitable to screen large populations with low seroprevalence (Lui *et al*., 1997).

2.4.1.1 Current screening in South Africa

SANBS (Vermeulen *et al*., 2009) and WPBTS (Cable *et al*., 2013) routinely screen for HIV-1, HIV-2, HBV, HCV and also syphilis. Prior to each blood donation, the donor has to complete a confidential questionnaire with lifestyle and health related questions. Thereafter the donor may donate blood that gets tested for serology markers (anti-HIV, HBsAg and anti-HCV) on the Prism analyser (Abbott, USA) using the Ultrio assay (Cable *et al*., 2013) and for molecular markers (HIV RNA, HBV DNA and HCV RNA) on the Panther system (Hologic Inc., USA). The South African Blood transfusions do not routinely screen blood donations for HEV.

2.4.2 HEV surveillance

One of the most valuable prevention strategies for HEV TTI is haemovigilance. This is a surveillance system that aims to ensure the quality and safety of all blood transfusion products. Haemovigilance systems should, therefore, exist in all regions where blood transfusion procedures take place. However due to significant differences in the economy, infrastructure and governance of different countries, it is quite challenging to have standardised haemovigilance systems implemented worldwide. Nevertheless existing

haemovigilance systems are functioning well in certain countries irrespective of the variability in these systems which need to be addressed.

The two European programmes namely; the Agence Française de Sécurité Sanitaire des Produits de Santé (AFSSAPS) and Establissement Français du Sang (EFS) national system in France and the Serious Hazards of Transfusion (SHOT) national system in the UK, serve as haemovigilance models for other countries. These systems operate in a centralised national scheme which is ideal and report adverse transfusion reactions on either a mandatory (France) or voluntary basis (UK). This is reflected in the number of HEV TTIs that have been reported, from France (N=5) and UK (N= 19), summarised in Table 2.4.

Table 2.4 Haemovigilance systems in countries with HEV TTIs

Country	**Haemovigilance System / Surveillance**	**Notification of adverse blood transfusion incidents**	**Number of HEV TTIs reported in country**	**References**
France	Yes	Mandatory	5	
Germany	Yes	Mandatory	-	Juhl *et al.*, 2014
UK	Yes	Voluntary	1	Boxall *et al.*, 2006
UK	Yes	-	18	Hewitt *et al.*, 2014
Japan	Yes	Voluntary	5	
India	Yes	Mandatory	1	Arankalle *et al.*, 2000
Saudi Arabia	Yes	-	1	Khuroo *et al.*, 2004
China	Yes	-	1	Ren *et al.*, 2013

2.4.3 HEV vaccine

The development of an HEV vaccine is required to provide immunity to individuals that are at a higher risk i.e. particularly blood transfusion recipients that are immunosuppressed patients, the elderly, pregnant women and children (Kamar *et al*., 2012; Dreier *et al*., 2014). HEV vaccine trials done in Nepal and China have identified candidate vaccines which prove to be effective (Kamar *et al*., 2012). There are two HEV vaccines which have been manufactured; by GlaxoSmith Kline and Xiamen Innovax Biotech.

The Hecolin (HEV239) vaccine developed by Xiamen Innovax Biotech was registered after completing an initial 19-month efficacy study (Zhu *et al*., 2010) and has been administered in China since 2012. The vaccine was given at time points 0, 1 and 6 months and developed using HEV1. An extended efficacy study was done to observe the long-term effect and the following factors were assessed; the efficacy, safety and immunogenicity of the Hecolin vaccine. The antibody response against HEV was protective and lasted up to four and a half years (Zhang *et al*., 2015). However, the safety in high-risk groups has not been reported.

There are nevertheless certain things to take into consideration when developing an effective HEV vaccine and making it available worldwide. Firstly since HEV has different routes of transmission which are associated with HEV1 to HEV4 and this should be considered when determining the efficacy of the vaccine. Secondly, the time points when the vaccine should be given to the respective groups that are susceptible to HEV infection need to be established. Lastly there should be adequate funding to implement the HEV vaccination programme, especially in endemic developing regions and could it be part of the already existing viral hepatitis vaccines i.e. hepatitis A and hepatitis B to be given in combination as Twinrix which is licensed in the United States (Centre for Disease Control and Prevention, CDC).

2.5 Treatment of HEV infection

HEV infection is generally acute and self-limiting, however, immunosuppressed patients that are chronically infected need therapy to destroy the HEV-infected cells. The main concern of chronic viral hepatitis infection is the possibility of it leading to cirrhosis and eventually evolving to hepatocellular carcinoma (HCC).

There are two main types of treatment recommended for patients with chronic HEV infection. Ribavirin and pegylated interferon have been reported to be effective in solid-organ transplant recipients receiving therapy for 3 months (Kamar *et al*., 2014; Kamar *et al*., 2010a). According to Kamar *et al*. after consecutive studies on the treatment of chronic HEV infection in transplant recipients, ribavirin is appropriate to use in all solid-organ transplant recipients. However pegylated interferon- alpha has adverse effects on kidney transplant recipients and may induce acute kidney failure (Kamar *et al*., 2010b).

Treatment of chronic HEV infection aims to ultimately eradicate HEV replicating cells and to avoid retransplantation of solid-organs particularly the liver. However it has been reported that chronic HEV infection could reoccur in patients undergoing retransplantation of the liver (Haagsma *et al*., 2009). In some instances while patients are on treatment with either

ribavirin or pegylated interferon relapse occurs during or after the 3-month course of therapy (Kamar *et al*., 2010a; Kamar *et al*.,2010b; Kamar *et al*., 2014). Haagsma *et al*. had two case reports of chronic HEV infection in liver transplant recipients receiving pegylated interferon alpha-2b and during the study period, none of the patients had a recurrence of HEV infection. However it is important to bear in mind that patients respond differently to treatment and depending on their medical condition patients are given different optimal dosages of the prescribed therapy to ensure safety and efficacy (Kamar *et al*., 2014).

CHAPTER THREE: MATERIALS AND METHODS

I. Materials

3.1 Ethical aspects

Ethics approval was obtained for this project from the Health Research Ethics Committee (HREC) of Stellenbosch University on 23 June 2014 (reference number: S14/04/09) and renewed the following year.

3.2 Study design

This was a prospective study to determine the prevalence of past and active hepatitis E virus (HEV) infection in blood donors in the Western Cape and to assess risk factors associated with HEV status.

Two sub-studies were conducted: study group 1 consisted of 250 donors from 25 randomly selected blood donation clinics in Cape Town and its surrounding areas. Participants consented to completing a risk factor questionnaire (Addendum 1) and to being tested serologically for HEV IgG antibodies and molecularly for HEV RNA. Test results were correlated with demographic and behavioural factors as per questionnaire responses to identify risk factors associated with HEV infection (Addendum 2). All samples were tested individually for the presence of anti-HEV IgG antibodies as a marker of past infection. In addition 25 plasma minipools of 10 donations per pool were tested by an in-house real-time reverse-transcriptase quantitative polymerase chain reaction (RT-qPCR) for the presence of HEV RNA as a marker of active infection. Study group 2 consisted of 10,000 donors from blood donation clinics across the Western Cape who had their individual donations (ID) screened for HEV RNA using a qualitative transcription-mediated amplification (TMA) assay (Procleix HEV assay, Grifols Diagnostic Solutions Inc., Spain) on an automated machine (Panther system, Hologic Inc., USA) at the routine virology laboratory of the Western Province Blood Transfusion Service (WPBTS).

3.3 Sample collection

3.3.1 Study group 1: blood donors that donated blood and completed the questionnaire

These 250 donors were recruited and consented over a period of three months from September 2014 to December 2014. We randomly selected and attended blood donation clinics from the WPBTS clinic schedule in Cape Town and the surrounding areas. We received the corresponding blood samples from the 250 questionnaire donors after the

WPBTS virology laboratory had tested the blood for HIV, HBV, HCV and syphilis as per routine screening protocol. The blood samples that we received were the primary donor tubes which remained after the donors' blood had been tested by WPBTS. The 250 blood samples were tested at our division for anti-HEV IgG and then pooled to test for HEV RNA.

3.3.2 Study group 2: blood donors that only donated blood

We tested 10,000 blood samples from donors at WPBTS blood donation clinics across the Western Cape. These samples were collected from all blood donation clinics that were scheduled from October 2015 to November 2015. The criteria for inclusion in the study were as follows: any blood donor who donated a full pint of blood and underwent the WPBTS routine screening. Following the routine screening, the 10,000 samples were tested for HEV RNA on the Panther system (Hologic Inc., USA) at the virology laboratory of WPBTS. Only if a donor sample tested positive for HEV RNA, he or she was identified by WPBTS, counselled and given the option to complete our study HEV risk questionnaire.

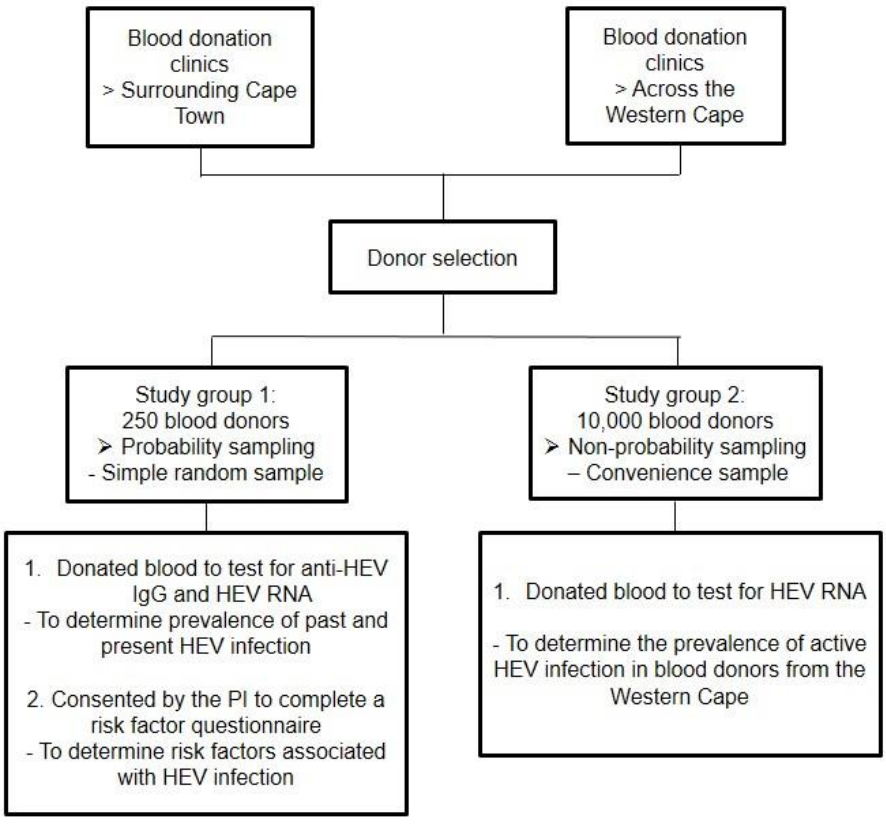


Figure 3.1 Selection and sampling of study participants

3.4 Study questionnaire

The study questionnaire (Addendum 2) was designed to assess the risk factors associated with HEV exposure in volunteer donors from the WPBTS blood donations clinics surrounding Cape Town. It contained 59 variables within 6 categories namely: demographics, medical history, travel history, food consumption, water consumption and contact with surface water and animal contact. After the data was captured in an Excel spreadsheet it was combined with the serology results to identify risk factors associated with HEV infection.

3.4.1 Questionnaire formatting

The questions in our study questionnaire were sourced from validated HEV risk assessment questionnaires namely; the Dutch questionnaire (Verhoef *et al*., 2012), the Public Health England (PHE) national HEV questionnaire and the Hepatitis E National Case-Control Study Case questionnaire (S. Ijaz personal communication, 2014). We integrated the questions from these questionnaires and adapted the questionnaire to be more specific to determine the possible risk factors in our study participants. The questions were translated into Afrikaans and both the English and Afrikaans questionnaires trialled using the staff of our Division of Medical Virology. We received valuable feedback from this and subsequently made changes to clarify certain questions to avoid ambiguity. We also decided to administer the questionnaire in an electronic format to prevent human error and bias when the data was captured and to not have to decipher handwriting. The developer tab of Microsoft (MS) Word 2010 was used to format the questionnaire as a fillable document by using check boxes, text boxes and etcetera. Another trial was done on blood donors at N1 City Mall WPBTS blood donation clinic in Cape Town on 22 September 2014 prior to starting the actual study.

3.4.2 Selection process for blood donation clinics

We used an online randomization tool (Random Integer Generator, http://www.random.org/integers/) to select 25 of the total 57 WPBTS blood donation clinics situated in Cape Town and the surrounding areas. Our aim was to get 10 donors from each of the 25 randomly selected clinics that are highlighted in grey in Table 3.1, to voluntarily complete the study questionnaire. The randomization was done to prevent selection bias by the principal investigator (PI) and also amongst the questionnaire participants.

Table 3.1 WPBTS blood donation clinics surrounding Cape Town

1. Athlone	29. Epping I
2. Belhar	30. Epping II
3. Bonteheuwel	31. Goodwood
4. Grassy Park	32. Kenridge
5. Lotus River	33. Kraaifontein
6. Mitchells Plain	34. Kuils River
7. Ravensmead	35. Maitland
8. Steenberg	36. Milnerton
9. Vanguard Estate	37. Monte Vista
10. Cape Town	38. Panorama
11. CBD	39. Parow
12. Kensington	40. Plattekloof
13. Maitland	41. Richwood
14. N'dabeni	42. Stellenberg
15. Observatory	43. Bergvliet
16. Pinelands	44. Claremont
17. Salt River	45. Constantia
18. Sea Point	46. Kenilworth
19. Woodstock	47. Lakeside
20. Bellville	48. Lansdowne
21. Blackheath	49. Newlands
22. Bothasig	50. Ottery
23. Brackenfell	51. Plumstead
24. Bellville South	52. Retreat
25. Durbanville	53. Tokai
26. Edgemead	54. Wetton
27. Eerste River	55. Wynberg
28. Elsies River	56. Rondebosch

3.4.3 Conducting the survey at the blood donation clinics

The PI travelled with the WPBTS staff to the 25 randomly selected blood donation clinics on the days that the clinics were scheduled. All blood donors have to complete the standard WPBTS consent form which states that they give permission for their blood to be tested and used for research purposes (Addendum 4). At each of the blood donation clinics, the following approach was used to voluntarily recruit donors for the questionnaire: The donor firstly had to meet all the WPBTS donation criteria and qualify as an eligible donor. Subsequently, any donor who was seated on a donation bed and had already been seen by a nursing sister was approached by the PI. The PI then briefly explained the purpose of the study and the questionnaire and asked the donors if they wanted to participate. All the blood donors that volunteered to participate completed a consent form (Addendum 1) in their

language of preference, i.e. English or Afrikaans. One of the WPBTS clinic staff members or a relative of the donor if present signed as a witness. Participants were given a copy of the signed form. Thereafter the PI conducted an interview to fill in the questionnaire on an individual basis. While the donors were being bled on the donation beds they were unable to complete the questionnaire themselves. Hence, the PI assisted the donors by reading the questions out loud to the donor. The majority of the questions were in a check box format and only required the donor to verbally motivate some of the “Yes” selected answers. All the interviews were conducted by the PI, who filled in the questionnaires on behalf of the blood donors on an electronic device using MS Word 2010. Each questionnaire was saved as an individual PDF file.

II. Methods

3.5 Study group 1: HEV serology testing

The 250 blood donors in study group 1 filled in the HEV risk factor questionnaire and were tested for anti-HEV IgG and HEV RNA. No HEV serology was done for study group 2.

3.5.1 HEV IgG ELISA

The Wantai anti-HEV IgG enzyme-linked immunosorbent assay (ELISA) (Fortress Diagnostics, Antrim, UK) was used. This is a qualitative indirect ELISA method which uses labelled anti-human globulin conjugate to detect the IgG antibodies present in the plasma samples. The kit included a negative and positive control and in addition, to this we included an in-house HEV positive control patient sample from Tygerberg Hospital (Andersson *et al*., 2015).

The assay was conducted according to manufacturer's specifications (Fortress Diagnostics Antrim, UK). First, sample diluent provided with the kit is added to each well and incubated. The samples are diluted to stabilize the serum proteins that are present in the biological samples and reduce non-specific binding (http://www.immunochemistry.com/products/elisa-solutions-1/elisa-sample-diluents.html).

The anti-HEV specific antibodies in the plasma samples bind to the pre-coated HEV ORF2 antigens in each well during the incubation. Thereafter the unbound serum proteins are washed off and horseradish peroxidase (HRP) conjugated rabbit anti-human IgG antibody is added and incubated. After the incubation, another wash step is done to remove all the unbound conjugate. Hence, only the conjugate that is bound to the antigen-antibody immunocomplexes formed in the previous incubation remains. Thereafter chromogen

solutions A and B are added to the wells. The urea peroxide from the chromogen solution A is reduced to water and Tetramethylbenzidine (TMB) from chromogen solution B is oxidised to a diimine that produces a blue colour in the presence of the antigen-antibody-anti-IgG (HRP) immunocomplex. Finally, sulphuric acid is added as a stop reagent, which changes the blue colour in the positive wells to yellow due to a drop in pH from alkaline to acidic (HEV-IgG [package insert]. Antrim, UK: Fortress Diagnostics; 2008). The absorbance values are measured with the ELx800 Absorbance Microplate Reader (BioTek Instruments Inc., USA).

The Wantai (Wantai Biological Pharmacy Enterprise Co., Beijing, China) assay was used for our study because it is considered to be one the best anti-HEV IgG tests that are currently available (Yan *et al*., 2008; Bendall *et al*., 2010; Schnegg *et al*., 2013). The Wantai assay has good sensitivity as a result of the PE2 protein which is made up of 221 of the 660 amino acids from HEV ORF2. The recombinant proteins (42 amino acids of ORF2) in MP Diagnostics assay (MP Biomedicals, Singapore) and synthetic peptides (88 amino acids of ORF2) used in the Dia.Pro (Diagnostics Bioprobes S.r.l., Milan, Italy) have shorter sequence lengths than the PE2 protein used in the Wantai kits. As a result, of the longer amino acid length of PE2 more conformational epitopes can be recognized by HEV antibodies in test samples. (Schnegg *et al*., 2013).

A comparative study done by Bendall *et al*. (2010) found that the Wantai assay 100% sensitivity compared to the MP Diagnostics assay with 50%. Recently another comparison study was done using a newer improved version of the MP Diagnostics kit which reported that it had a similar sensitivity rate as the Wantai (Avellon *et al*., 2015). Furthermore Avellon *et al*. stated that overall the Wantai assay appears to have better sensitivity than MP Diagnostics when using sample panels from blood donors (Bendall *et al*., 2010; Schnegg *et al*., 2013) and better than the Adaltis assay (InGen, France) using samples from patients with acute HEV infection (Abravanel *et al*., 2013; Avellon *et al*., 2015).

3.5.2 Anti-HEV IgG test procedure

The test was conducted as per manufacturer's instructions. The samples were tested in batches using half plates (48 of the 96 wells) at a time. Prior to starting the procedure all the samples that were stored at -20°C and the kit reagents stored at 4°C were thawed at room temperature for 15 to 30 minutes. A 5% wash buffer was freshly prepared in a 500 ml flask for each set of 48 wells which included the 6 controls and 42 donor samples tested as a 96 well half plate. The ELISA procedure is shown below in Figure 3.2.

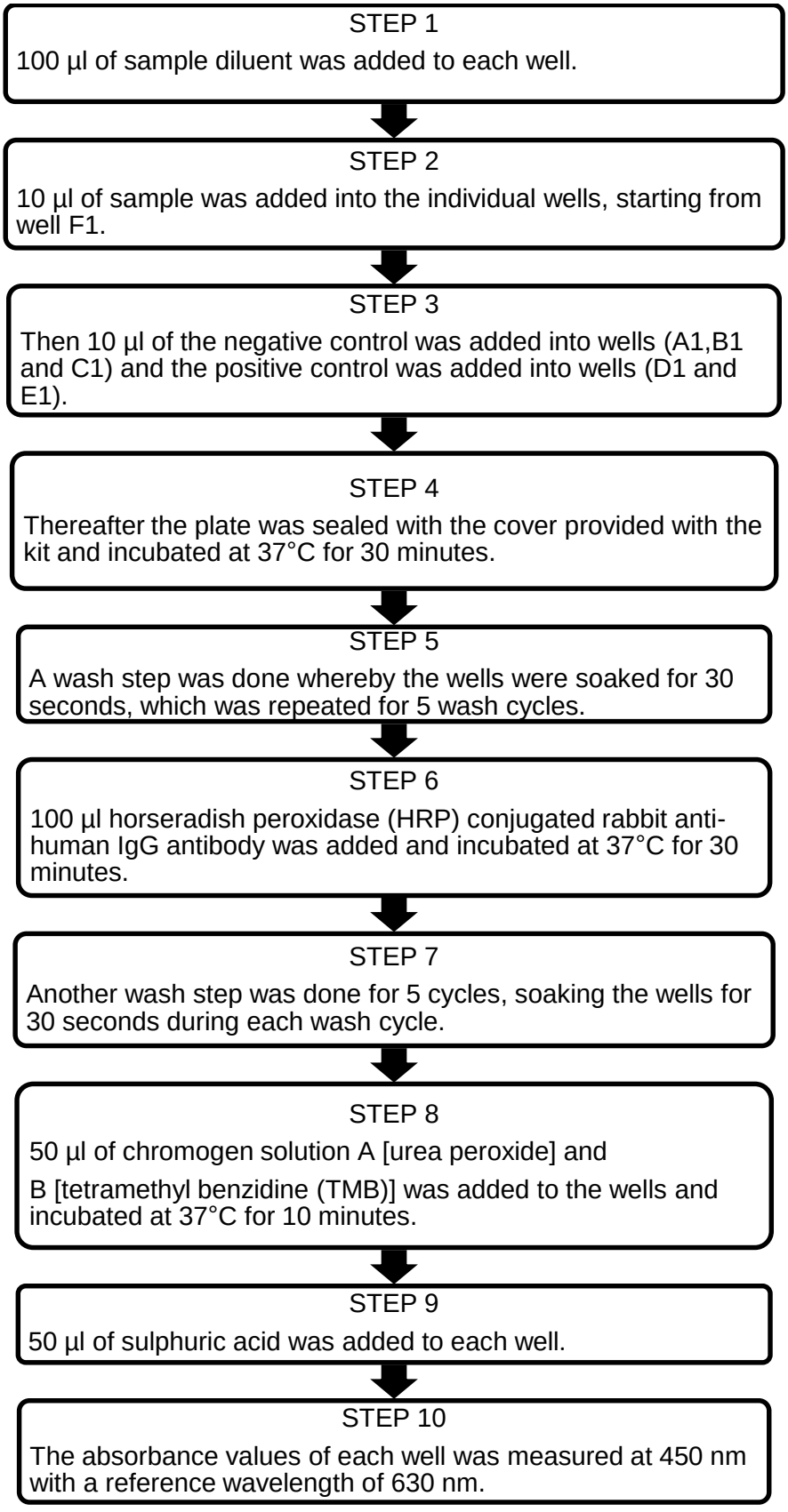


Figure 3.2 Step by step procedure of the HEV IgG ELISA

3.5.3 Quality control and interpretation of results

After the test was completed and the optical density (OD) values of the wells had been read, the cut-off value was calculated as follows: mean OD value of the three negative controls included in each run plus a constant of 0.16 which is used for every batch of kits as stipulated by the kit manufacturer.

Individual OD values for each sample were then divided by the cut-off value. If the ratio was <0.9 the result was negative; if it was >1.1 positive; and ratios between 0.9 and 1.1 were regarded as borderline. For a test to be valid the OD value of the negative control has to be <0.100 and the positive control has to be ≥ 0.800. According to manufacturer's instructions samples with borderline results should be re-tested in duplicate. We re-tested them in singlicate, using a previously unused kit to ensure that all reagents were fresh and the risk of possible contamination minimal.

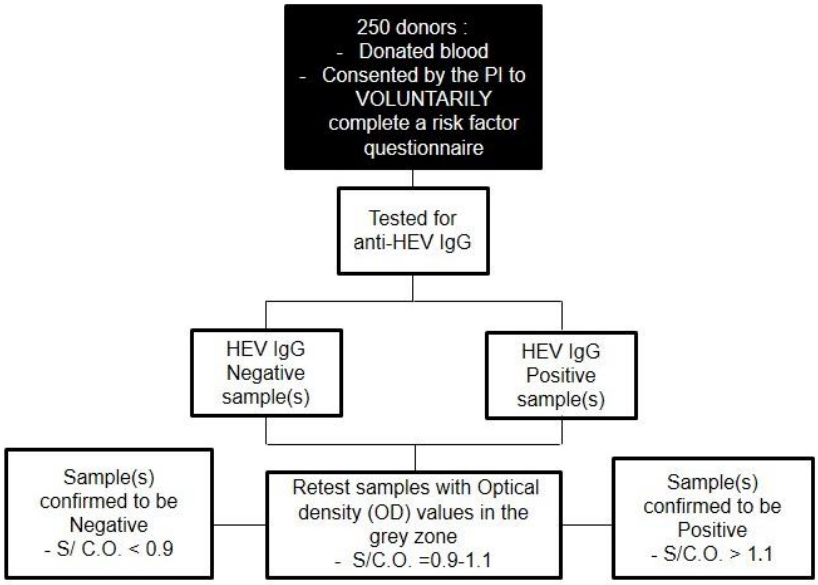


Figure 3.3 HEV serology testing for study group 1

3.6 Study group 1: HEV molecular testing

An in-house real-time RT-qPCR assay established at the Division of Medical Virology, Faculty of Medicine and Health Sciences (FMHS) of Stellenbosch University was used to screen for active HEV infections in study group 1. To save cost, and given the expected very low prevalence of HEV RNA positivity in this group, samples were pooled before RNA extraction and PCR testing.

3.6.1 Pooling procedure

We used a pooling strategy to test 25 minipools of ten samples each, instead of 250 individual samples. PCR pooling strategies are used to reduce the cost of screening for viral RNA detection (Lui *et al*., 1997). To generate the minipools, 100 µl of plasma from each of 10 donor samples was added to a 2 ml tube, with each pool having a total volume of 1 ml. Pooled samples were mixed thoroughly using a vortex for 1 minute and thereafter stored at 4°C until extraction of viral RNA the following day. Our pooling strategy of 10 samples per minipool (Ijaz *et al*., 2012) has previously been validated during a pilot study; the limit of detection (LOD) was 3,000 IU/ml as the lowest detectable viral load per minipool tested which is equivalent to a LOD of 30,000 IU/ml per individual donation (Lopes *et al*., unpublished data).

3.6.2 Controls

One negative control was included during the simultaneous extraction of all 25 minipools. The negative control was made up of 200 µl of normal human plasma (NHP) that was topped up to 1 ml with 800 µl of phosphate buffered saline (PBS). Including a negative control helps to monitor that no contamination occurs during the extraction and amplification procedure.

MS2 bacteriophage (Roche Applied Science) is an RNA virus used as an internal control (IC) to validate several RT-PCR assays that detect human RNA viruses (Dreier *et al*., 2005). The stock material was diluted with PBS and carrier RNA was added to each dilution. The dilutions were stored at -20°C as 10^{-5}, 10^{-4} and 10^{-3}. The 10^{-5} dilution was used as the MS2 working concentration which was prepared as a master mix with carrier RNA and then added to the samples during the extraction. The viral RNA of the IC and target HEV was then amplified and served as a non-competitive IC in our in-house TaqMan real-time RT-qPCR assay.

3.6.3 Standards

We used the World Health Organization (WHO) international standard (IS) for HEV RNA so that the HEV RNA concentrations of positive samples could be quantified. Establishing this IS has reduced the nucleic acid amplification technique assay variability previously observed in laboratories testing for HEV RNA (Baylis *et al*., 2012). We used the HEV WHO IS that was made by the Paul-Ehrlich-Institut (PEI) using plasma from a blood donor infected with HEV3 (1st World Health Organization International Standard for Hepatitis E Virus RNA Nucleic Acid Amplification Techniques-Based Assays [Version 1.0]. Langen, Germany: PEI; 2011).

The lyophilised HEV WHO IS was reconstituted with 500 µl of nuclease free water. Thereafter the stock concentration of $2.5x10^5$ IU/ml was diluted with NHP from $2.5x10^4$ IU/ml to 2.5x10 IU/ml according to Table 3.2. After the dilutions were made, 200 µl of the total 300 µl (30 µl + 270 µl) final volume was used to extract RNA from the HEV WHO standards.

Table 3.2 Tenfold dilutions of the HEV WHO IS

In-house Control	Composition
HEV WHO IS $2.5x10^5$	200 µl HEV WHO IS (stock material)
HEV WHO IS $2.5x10^4$	30 µl HEV WHO IS $2.5x10^5$ + 270 µl NHP
HEV WHO IS $2.5x10^3$	30 µl HEV WHO IS $2.5x10^4$ + 270 µl NHP
HEV WHO IS $2.5x10^2$	30 µl HEV WHO IS $2.5x10^3$ + 270 µl NHP
HEV WHO IS 2.5x10	30 µl HEV WHO IS $2.5x10^2$ + 270 µl NHP

These dilutions were used to quantify the in-house HEV RNA positive samples namely the HEV PHE standards 10^7 – 10^2. After establishing the viral loads of the in-house HEV RNA positive samples using the HEV WHO IS, it was subsequently used as the positive control. The HEV PHE standards were made using of a stock concentration of 10^8 and NHP to make serial dilutions from 10^7 to 10^2 according to Table 3.3.

Table 3.3 Tenfold dilutions of the HEV PHE standard

In-house Control	Composition
HEV PHE Std 10^8	200 µl HEV PHE standard (stock material)
HEV PHE Std 10^7	30 µl HEV PHE Std 10^8 + 270 µl NHP
HEV PHE Std 10^6	30 µl HEV PHE Std 10^7 + 270 µl NHP
HEV PHE Std 10^5	30 µl HEV PHE Std 10^6 + 270 µl NHP
HEV PHE Std 10^4	30 µl HEV PHE Std 10^5 + 270 µl NHP
HEV PHE Std 10^3	30 µl HEV PHE Std 10^4 + 270 µl NHP
HEV PHE Std 10^2	30 µl HEV PHE Std 10^3 + 270 µl NHP

3.6.4 HEV RNA extraction

All the samples were centrifuged at 3,000 rpm for 10 minutes before starting the extraction. The QIAamp UltraSens Virus kit (Qiagen, Germany) procedure was used to extract all the samples, controls and standards according to manufacturer's instructions. The purified viral RNA from each extract was eluted in a final volume of 65 µl and stored at -20 °C to stabilize the RNA until the real-time RT-qPCR was done.

3.6.5 Principles of the in-house real-time RT-qPCR to detect HEV RNA

An in-house HEV Taqman real-time RT-qRNA assay amplifies the ORF3 region of the HEV genome (Garson *et al*., 2012). This assay is able to quantify HEV RNA positive samples and has a LOD of 22 IU/ml (Hewitt *et al*., 2014). A dual well format was utilized to amplify the HEV RNA and the MS2 RNA in separate wells. Both targets were detected using labelled TaqMan probes unique to HEV and MS2, respectively. The HEV probe was labelled with FAM fluorescent dye and a minor-groove binder (MGB) modified quencher and the MS2 probe with JOE fluorescent dye with a blackhole quencher (BHQ) (Garson *et al*., 2012). The primer sequences for HEV and MS2 amplification are shown in Table 3.5. The ABI 7900HT Fast PCR system (Applied Biosystems, California, USA) was used and data analysed using SDS software (version 2.3). The following parameters were required for a valid PCR run: A standard curve slope between -3 and -3.6 and an R2 value of at least 0.9 (Standard operating procedure for detection of Hepatitis E Virus RNA by real-time PCR. Colindale, UK: PHE; 2011; Hewitt *et al*., 2014; Andersson *et al*., 2015).

3.6.5.1 In-house real-time RT-qPCR procedure

The reaction mixture was prepared on ice using the components listed in Table 3.4. The sequences of primers and probes are shown in Table 3.5. Of the prepared master mix, 15 µl each was added to the assigned HEV and MS2 wells of the 96 well plate. 10 µl of RNA from each extracted sample was added to each well, thus, the total volume of each well was 25 µl.

Table 3.4 Components of the master mix per reaction

Reagent	HEV Mix (n=1)	MS2 Mix (n=1)
QuantiTect Q RT-PCR master mix	12.5 µl	12.5 µl
QuantiTect RT enzyme	0.25 µl	0.25 µl
HEV TaqMan primer/probe mix	0.3 µl	-
MS2 TaqMan primer/probe mix	-	0.4 µl
Nuclease free water	1.95 µl	1.85 µl

Table 3.5 Primer and probe sequences for real-time RT-qPCR

Name	Sequence
HEV Primer 1 (F)	5'- GGTGGTTTCTGGGGTGAC-3'
HEV Primer 2 (R)	5'- AGGGGTTGGTTGGATGAA-3'
HEV Probe	5'FAM-TGATTCTCAGCCCTTCGC-MGB-3'
MS2 Primer 1	5'- TGGCACTACCCCTCTCCG TATTCACG-3'
MS2 Primer 2	5'- GTACGGGCGACCCCACGATGAC-3'
MS2 Probe	5'-JOE- CACATCGATAGATCAAGGTGCCTACAAGC -BHQ1-3'

*(F) = Forward primer *(R) = Reverse primer

The plate setup shown in Table 3.6 was saved as a default setting on the software. For each new run, the sample type (i.e. unknown =U, standard = 10^7 to 10^2, negative = NHP or negative template control (NTC) = nuclease free water) and the sample name had to be entered in each well. All the wells marked with a "U" contained the samples that were being tested, in addition to this the wells containing no samples were cleared.

Table 3.6 Setup of real-time RT-qPCR plate

TaqMan Mix:	HEV	HEV	HEV	HEV	HEV	HEV	MS2	MS2	MS2	MS2	MS2	MS2
	1	2	3	4	5	6	7	8	9	10	11	12
A	10^7	U	U	U	U	U	10^7	U	U	U	U	U
B	10^6	U	U	U	U	U	10^6	U	U	U	U	U
C	10^5	U	U	U	U	U	10^5	U	U	U	U	U
D	10^4	U	U	U	U	U	10^4	U	U	U	U	U
E	10^3	U	U	U	U	U	10^3	U	U	U	U	U
F	10^2	U	U	U	U	U	10^2	U	U	U	U	U
G	NHP	U	U	U	U	U	NHP	U	U	U	U	U
H	NTC	U	U	U	U	U	NTC	U	U	U	U	U

The plate was sealed with an optical adhesive film and centrifuged at 3,000 rpm for 1 minute to remove air bubbles. The protocol was set up on the PCR machine and saved for all the real-time runs that were done, see cycling parameters in Table 3.7.

Table 3.7 Real-time RT-qPCR cycling parameters

PCR step	Temp °C	Time	Cycles
Reverse transcription	50	30 min.	1
Initial denaturation	95	15 min.	1
Denaturation	95	15 sec.	45
Annealing / Elongation	60	60 sec.	45

3.7 Study group 2: HEV molecular testing

A commercial qualitative TMA assay was performed in the virology laboratory of WPBTS to test the samples from study group 2 for HEV RNA on the Panther system (Hologic Inc., USA).

3.7.1 Panther system

The Panther automated system was used to test the individual blood donations of study group 2 for HEV RNA with the Procleix HEV NAT assay. The platform has a non-batch continuous workflow with a capacity of 120 on board that was loaded with 118 samples to include two assay calibrators with each batch. To validate every set of 244 samples that were tested the positive and negative calibrators were run in triplicate and placed in no particular order or position. A new set of 6 calibrators were used with each run of 250 tests. Reagents from each kit were scanned by the Panther system using the barcodes on each tube to create an inventory. The platform is divided into 6 sections; reagents bay, sample racks, disposable tips, multi-tube units (MTUs), universal fluids and waste (Addendum 5). The Panther system has a luminometer in the Mid-Bay (Addendum 5) which utilized dual kinetic assay (DKA) to measure the chemiluminescent signals of the target virus and IC. The target virus and IC have acridinium ester labelled probes emitted different light signals which were measured as relative light units (RLU). The dual kinetic assay (DKA) then used the RLU to determine if the samples tested were valid, reactive or non-reactive using the range shown in Addendum 6.

3.7.2 Procleix HEV NAT assay

The Procleix HEV assay (Grifols Diagnostic Solutions Inc., Spain) is a commercial kit that uses transcription-mediated amplification (TMA). The Procleix HEV NAT kits for this study, a collaboration between the Division of Medical Virology and WPBTS were sponsored by Grifols through Ilex SA. Each Procleix HEV assay kit contains 250 tests with 4 sets (total of 1000 tests) of each reagent: an IC, target capture, amplification, enzyme, and probe and selection reagent. In addition, to this two calibrators were provided with the kit; an HEV negative control (HEPES buffered solution containing detergent, Procleix) and an HEV positive control (HEPES buffered solution containing detergent with an HEV RNA transcript, Procleix). Furthermore, the kit had the following reagents; auto detect R1 [Aqueous solution containing hydrogen peroxide (H2O2) and nitric acid (HNO3)] and R2 [1.6N sodium hydroxide (NaOH)], wash solution (HEPES buffered solution), oil (silicone oil) and DF [sodium bicarbonate (NaHCO3) buffered solution for deactivation fluid] which were developed for use on the Panther system. The kit was used to extract the individual plasma samples and amplify HEV RNA on the Panther system. The assay is qualitative and can detect all genotypes of HEV namely 1, 2, 3 and 4 with a 95% LOD of 7.89 IU/ml (6.63-9.83) which was determined with a HEV panel consisting of dilutions of the WHO IS (Procleix HEV Assay [package insert]. San Diego, CA: Hologic Inc.; 2014). Other studies were done in Europe, America and Australia have previously used this assay to determine the RNA

prevalence of HEV in their blood supply (Sauleda *et al*., 2015; Stramer *et al*., 2015; Shrestha *et al*., 2014).

3.7.3 Principles of HEV RNA detection by TMA

TMA is a qualitative method used by the Procleix HEV NAT assay (Grifols Diagnostic Solutions Inc., Spain) on the automated Panther system (Hologic Inc., USA). The TMA assay has the following three-step workflow: target capture, amplification and detection.

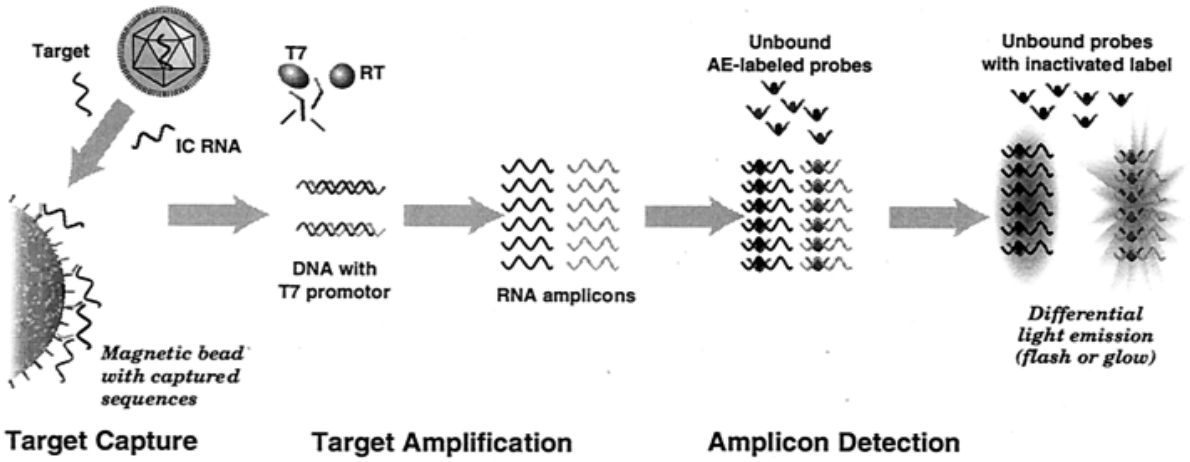


Figure 3.4 Illustration of TMA (Comanor *et al*., 2001 with permission from Nature Publishing Group)

3.7.3.1 Automated TMA procedure

An MTU was taken from the MTU Input Queue and moved by the distributor to the sample dispense slot. Then the 400 µl of the wTCR, placed in the TCR carousel (rotary mixer) was pipetted into the MTU. Each MTU consisted of 5 single reaction tubes. Each reaction tube has its own disposable tip used for adding the wTCR into the tubes. Liquid level sensing was done before adding the sample into a reaction tube which ensured that each tube contained the correct volume of wTCR (400 µl). Then 500 µl of the 525 µl aspirated volume was dispensed into each reaction tube. After the samples were added the pipettor that did level-sensing to check that each reaction tube contained 400 µl of wTCR plus 500 µl of sample. Thereafter the distributor moved the MTU to the sample mix station to mix the sample with the wTCR. After mixing the MTU was moved the HT incubator for target capture. During target capture, all the samples were treated to denature the proteins and isolate the nucleic acids. An internal control RNA transcript was added to the target capture reagent to monitor

the detection of each individual blood donation. After isolating the RNA oligonucleotides which correspond to the conserved regions of the HEV genome hybridized to the target HEV RNA present in the samples. The hybridized target in each sample was captured onto a magnetic particle. Once the target was captured a wash step was done to remove all the non-specific and unbound nucleic acids and to eliminate any potential inhibitors.

Exponential isothermal amplification took place in a single tube. Seventy-five microliters of the amplification reagent which consisted of primers, nucleotides (dNTPs and NTPs) and cofactors and 25 µl of the enzyme reagent which contained two enzymes, the MMLV (Moloney Murine Leukaemia Virus) reverse transcriptase and the T7 RNA polymerase, were added to the tube containing the captured nucleic acids. Subsequently, the first primer annealed to the target RNA sequence and the RT enzyme extended the sequence and formed cDNA. The cDNA copy is an RNA/DNA double-stranded intermediate and the RNase H activity of RT enzyme was used to degrade the RNA and resulted in a single-stranded DNA (ssDNA). The second primer then annealed to the ssDNA and the RT enzyme generated a dsDNA using its DNA polymerase function. The T7 promoter region of the first primer was transcribed into the dsDNA sequence. Transcription was initiated by the T7 RNA polymerase enzyme which transcribed DNA by binding to the promoter region and synthesized RNA amplicons. The RNA amplicons that were formed were then used as templates.

After amplifying the target RNA sequences single-stranded nucleic acid acridinium ester labelled probes (100 µl), which is complementary and highly specific for HEV RNA, was added to the reaction. Thereafter 250 µl of the selection reagent was added to the tubes to distinguish between the hybridized and unhybridized probes. The target and IC have different acridinium esters which produced different chemiluminescent signals. The IC RNA transcript has its own complementary acridinium labelled probe. IC-specific amplicon produced a rapid emission of light (termed a “flasher signal”). Whereas the amplicon specific to the target, HEV RNA produced a slower and longer lasting light emission (termed a “glower signal”). The Dual Kinetic Assay (DKA) analysed the chemiluminescent data to distinguish between the signals from the target virus and the IC (Procleix HEV Assay [package insert]. San Diego, CA: Hologic Inc.; 2014).

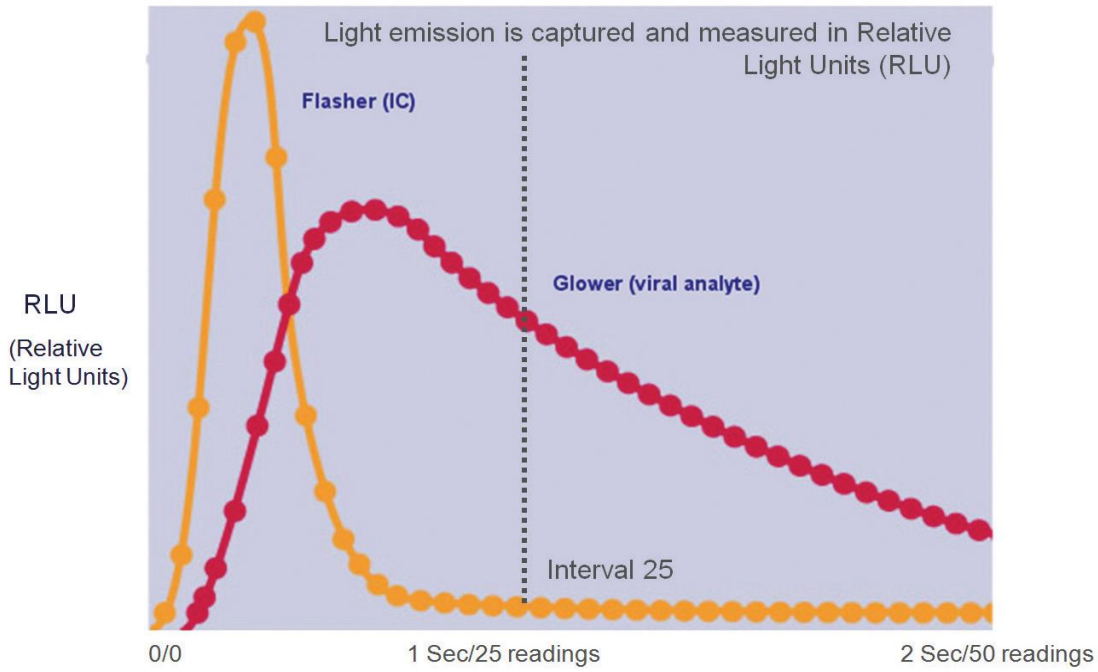


Figure 3.5 Dual kinetic assay used by the Panther system (GRIFOLS Procleix HEV Assay for Procleix Panther System – Operator Training Manual)

3.8 Donor look back and notification system

3.8.1 Follow up procedure in case of active HEV infection(s)

Blood donors are not routinely screened for HEV infection and may have previously donated HEV-infected blood. Hence, we wanted to identify donors found to have active HEV infection. If a donor had a reactive TMA test result, the WPBTS retested the sample in duplicate to confirm the result. Thereafter donors that were confirmed positive were identified and informed of the test result by WPBTS. All unused packs from donors that tested positive were blocked and discarded. Therefore, no known HEV contaminated blood or blood products were issued to recipients. The donors with active HEV infection were offered counselling by the WPBTS nurse and received an informational brochure on hepatitis E. Furthermore the donor was also informed about of our study HEV risk questionnaire and requested to complete it on a voluntary basis if the donor had no objections.

3.9 Statistical analysis

Descriptive statistics was performed and proportions calculated to include measures of spread and 95% confidence intervals. We compared the HEV IgG positive and HEV IgG negative donors with respect to demographic factors using the chi-squared test and Fisher's

exact test for categorical data. All p-values were two-tailed and the significance level was set at 0.05. Analyses were done using Stata (version 12).

3.9.1 Sample size

Our sample size was based on blood donor studies were done worldwide (Table 2.2 and Table 2.3) to estimate the background prevalence of active HEV infection in our blood donations. In addition, our pilot study done in 2013 had a 25.3% HEV IgG seroprevalence and sample size of 300 blood donors which suggested that we need a much larger sample size to detect active HEV infection in our setting.

3.9.1.1 Study group 1

In terms of the secondary outcome of our study, to identify risk factors associated with past HEV infection, we recruited 250 donors of whom 140 would have been expected to have been exposed to pork and pork products, as a primary risk factor. To determine the association of pork products with HEV infection with alpha =0.05 and 80% power, we have used the following calculation shown in Table 3.9:

Table 3.8 Sample size calculation for the questionnaire participants

Pork exposure	IgG Positive	IgG Negative	Sample size required
140	0.6	0.5	191
140	0.7	0.6	172

3.9.1.2 Study group 2

In terms of the primary outcome to determine the prevalence of active HEV infection in blood donors from the Western Cape, we had a fixed sample size of 10,000 blood donations and expected to identify at least 0.05% (95% CI, 0.0457-0.0543) active HEV cases, based on the RNA prevalence from other blood donor studies shown in Table 2.2 and 2.3.

CHAPTER FOUR: RESULTS

Two sub-studies were conducted to investigate the seroepidemiology and molecular prevalence of HEV infection in blood donors in the Western Cape. Study group 1 consisted of 250 blood donors who completed an HEV risk questionnaire and had their blood tested for HEV IgG antibodies and HEV RNA. Study group 2 consisted of 10,000 blood donors who were tested for HEV RNA, of whom only HEV RNA-positive donors were notified and asked to provide information on possible risk factors.

4.1 Demographics of study participants

The 250 blood donors were recruited at 25 randomly selected WPBTS blood donation clinics situated in Cape Town and its surrounding areas between September and December 2014. The 10,000 donors in study group 2 were from WPBTS blood donation clinics across the Western Cape during October and November 2015. The demographics of the two groups are summarized in Table 4.1.

Table 4.1 Demographic data representing the donor profiles

Characteristic	**Study group 1**	**Study group 2**
	N=250 (100%)	**N=10,000 (100%)**
Gender		
Males	122/250 (48.8)	5,114/10,000 (51.1)
Females	128/250 (51.2)	4,886/10,000 (48.9)
Age		
16-25 years	29/250 (11.6)	2,264/10,000 (22.6)
26-45 years	97/250 (38.8)	4,160/10,000 (41.6)
≥ 46 years	124/250 (49.6)	3,576/10,000 (35.8)
Race		
Asian	1/250 (0.4)	91/10,000 (0.9)
Black	12/250 (4.8)	500/10,000 (5.0)
Coloured	118/250 (47.2)	3,278/10,000 (32.8)
White	119/250 (47.6)	6,131/10,000 (61.3)

4.2 Study group 1: HEV serology

4.2.1 Anti-HEV IgG seroprevalence

Of the 250 samples that were tested for anti-HEV IgG, 106 samples (42.4%) had positive results. Four samples were in the grey zone with S/CO values between 0.9-1.1 on initial testing and were re-tested (in singlicate), yielding a positive result in each case (Table 4.2).

Table 4.2 Retested anti-HEV IgG borderline samples

Donor serial number	1st test OD value	S/CO value	Initial Result	2nd test OD value	S/CO value	Final Result
23 -10599131	0.185	0.97	Grey zone	0.259	1.36	POS
199 -10608289	0.195	1.03	Grey zone	0.448	2.36	POS
217 -10668018	0.197	1.04	Grey zone	0.843	4.44	POS
219 -10668030	0.193	1.02	Grey zone	0.417	2.19	POS

*OD = Optical density *S/CO = Sample cut-off ratio

4.2.2 Demographics of study group 1 according to anti-HEV IgG result

The anti-HEV IgG seroprevalence for the 250 interviewed blood donors was 42.4% (106/250). Figures 4.1 and 4.2 show the breakdown of the 250 donors HEV IgG result according to age, gender, ethnicity and area.

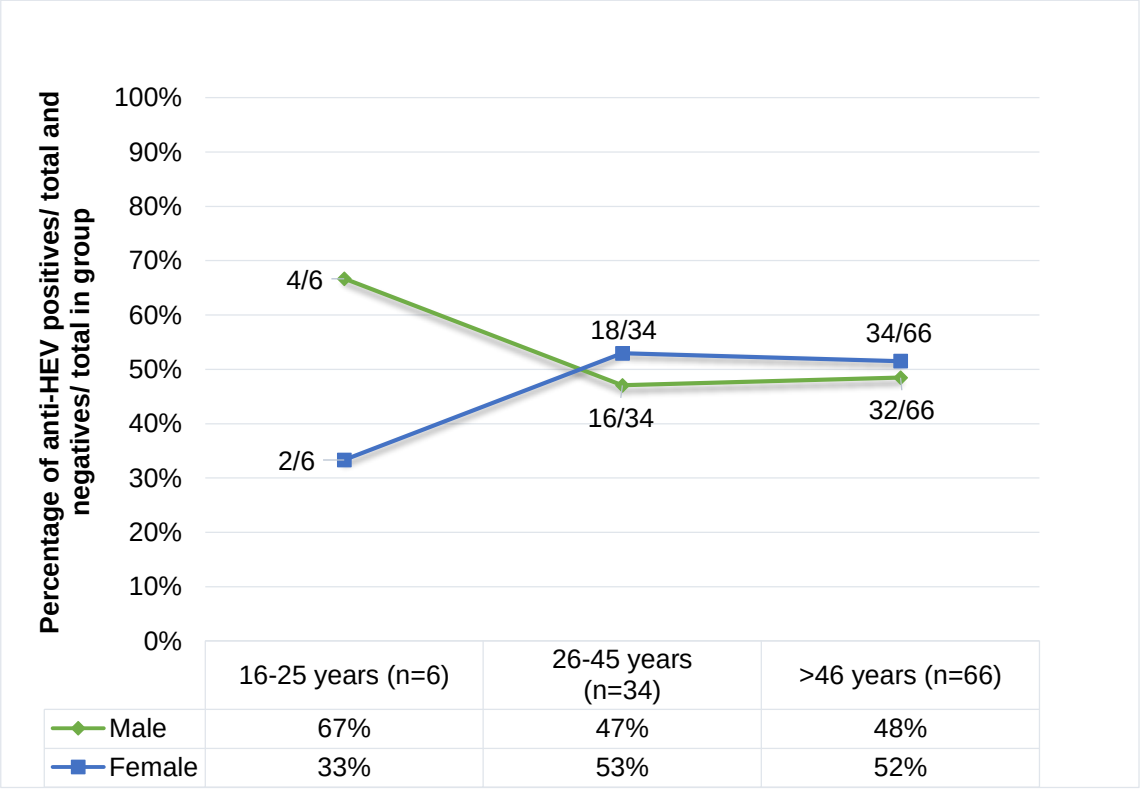
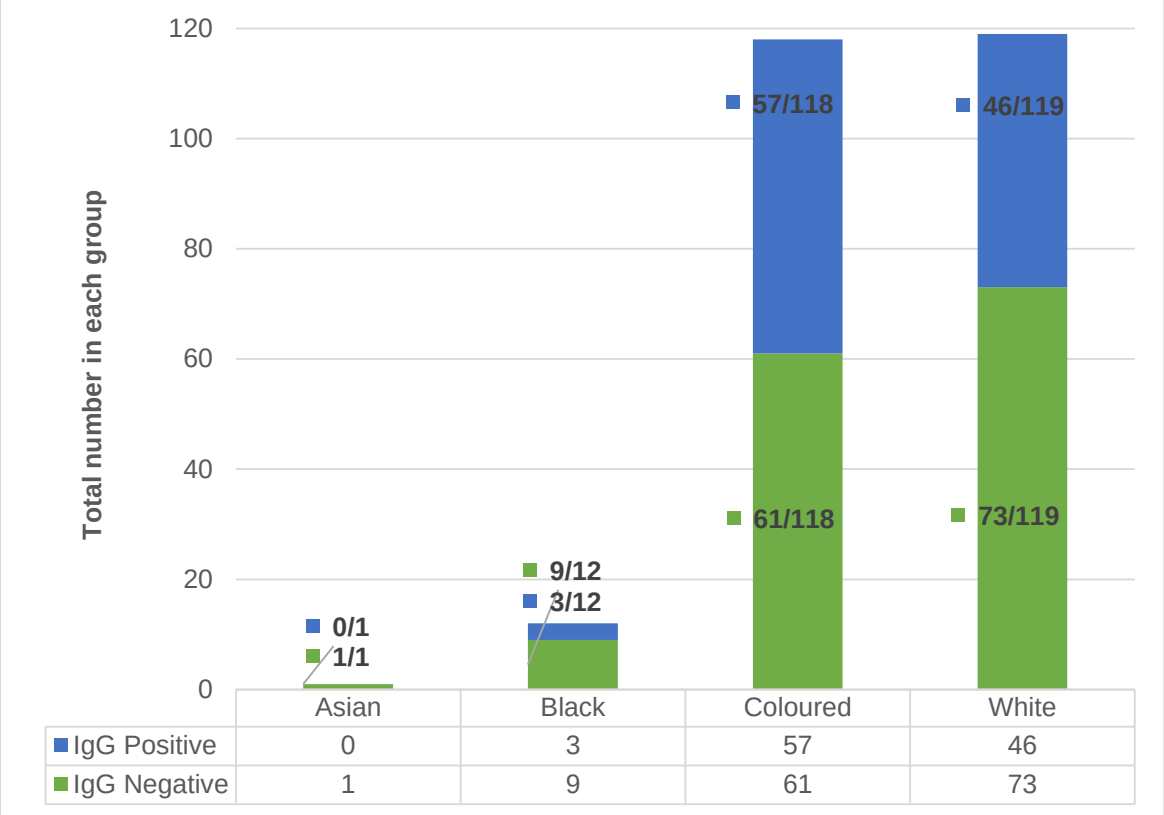


Figure 4.1 Anti-HEV IgG seroprevalences according to age group and gender



	Asian	Black	Coloured	White
IgG Positive	0	3	57	46
IgG Negative	1	9	61	73

Figure 4.2 Racial profiles of anti-HEV IgG for all donors

4.2.3 Anti-HEV IgG seroprevalence according to area

The anti-HEV IgG seropositivity rate was found to be the highest in the South Eastern suburbs with 51.8% (29/56) while the Southern suburbs had the lowest anti-HEV IgG seroprevalence with 35.1% (20/57). Donors residing in the Northern suburbs had an anti-HEV IgG seroprevalence of 43.1% (47/109) and the Central Business District (CBD) had 35.7% (10/28). The difference in the seroprevalences of the suburbs may be reflective of the population composition which varies between suburbs. Census data that is supplied by Statistics South Africa indicates that certain race groups are more represented than others in certain suburbs of Cape Town (City of Cape Town – 2011 Census Suburb Profiles, [s.a.]). The racial distribution of the different areas was taken into consideration when the anti-HEV IgG results were interpreted. A detailed representation of the donor seroprevalence is seen in Table 4.3, showing the number of anti-HEV IgG positive blood donors from each individual blood donation clinic.

Table 4.3 Anti-HEV IgG seroprevalences for each of the 25 blood donation clinics

Surburb	Site of blood donation clinic	Positive anti-HEV IgG as (%) of total number recruited per site
South Eastern	Bonteheuwel	6 of 10 (60)
South Eastern	Belhar	1 of 8 (13)
South Eastern	Ravensmead	6 of 8 (75)
South Eastern	Athlone	4 of 10 (40)
South Eastern	Mitchells Plain	6 of 10 (60)
South Eastern	Vanguard Estate	6 of 10 (60)
Northern	Monte Vista	4 of 9 (44)
Northern	Brackenfell	2 of 13 (15)
Northern	Kenridge	5 of 9 (56)
Northern	Bellville	5 of 15 (33)
Northern	Bothasig	4 of 10 (40)
Northern	Elsies River	6 of 10 (60)
Northern	Durbanville	2 of 10 (20)
Northern	Eerste River	7 of 13 (54)
Northern	Edgemead	6 of 10 (60)
Northern	Stellenberg	6 of 10 (60)
CBD	Cape Town	5 of 13 (39)
CBD	Sea Point	2 of 5 (40)
CBD	Pinelands	3 of 10 (30)
Southern	Wynberg	1 of 10 (10)
Southern	Tokai	4 of 9 (44)
Southern	Bergvliet	6 of 10 (60)
Southern	Steenberg	0 of 8 (0)
Southern	Lansdowne	4 of 10 (40)
Southern	Plumstead	5 of 10 (50)

4.2.4 HEV questionnaire

4.2.4.1 Bivariate analysis

Bivariate analysis showed that the demographic characteristics of the donors were not statistically significant as regards to their risk of being HEV IgG positive (Table 4.4).

Table 4.4 Bivariate analysis of donor demographics and anti-HEV IgG seroprevalence

Variable	**Anti-HEV IgG positive/ Total in each group (%)**	**Anti-HEV IgG negative/ Total in each group (%)**	**p-value**
Characteristic			
Age: 16-25	6/29 (20.7)	23/29 (79.3)	
26-45	34/97 (35.1)	63/97 (64.9)	Not determined (ND)
≥ 46	66/124 (53.2)	58/124 (46.8)	
Gender: Male	57/122 (46.7)	65/122 (53.3)	0.221
Female	50/128 (39.1)	78/128 (61.0)	
Race : Asian	0/1 (0.0)	1/1 (100.0)	0.232
Black	3/12 (25.0)	9/12 (75.0)	
Coloured	57/118 (48.3)	61/118 (51.7)	
White	46/119 (38.7)	73/119 (61.3)	
Area : Urban	101/232 (43.5)	131/232 (56.5)	0.617
Semi-urban	5/16 (31.3)	11/16 (68.8)	
Rural	1/2 (50.0)	1/2 (50.0)	

However the following variables identified through the questionnaire were found to be significantly associated with HEV infection: consumption of turkey meat; consumption of well-done or medium to well-done organ meats; recreational canoeing; contact with rabbits or chickens. These variables together with non-significant variables were categorized according to the different possible routes of HEV transmission (Table 4.5).

Table 4.5 Bivariate analysis between the HEV risk factors and anti-HEV IgG status

Variable	Anti-HEV IgG positive / Total in each group (%)	Anti-HEV IgG negative / Total in each group (%)	p-value
Foodborne and zoonotic risks			
Turkey consumption:			
Never	57/156 (36.5)	99/156 (63.5)	
1-3 times per week	2/10 (20.0)	8/10 (80.0)	0.001
Less than once a month	48/80 (60.0)	32/80 (40.0)	
Pork consumption:			
Never	14/44 (31.8)	30/44 (68.2)	
Once a week	18/45 (40.0)	27/45 (60.0)	
More than once a week	8/20 (40.0)	12/20 (60.0)	0.291
1-3 times per week	48/94 (51.1)	46/94 (48.9)	
Less than once a month	19/43 (44.2)	24/43 (55.8)	
Chicken consumption:			
Never	1/2 (50.0)	1/2 (50.0)	
Once a week	21/55 (38.2)	34/55 (61.8)	
More than once a week	80/178 (44.9)	98/178 (55.1)	0.875
1-3 times per week	3/9 (33.3)	6/9 (66.7)	
Less than once a month	1/2 (50.0)	1/2 (50.0)	
Consumption of organ meats prepared well-done:			0.026
Yes	57/112 (50.9)	55/112 (49.1)	
No	0/5 (0)	5/5 (100)	
Consumption of organ meats prepared medium to well-done:			0.026
Yes	0/5 (0)	5/5 (100)	
No	56/110 (50.9)	54/110 (49.1)	
Direct contact with a rabbit:			
Yes	6/25 (24.0)	19/25 (76.0)	0.045
No	101/225 (44.9)	124/225 (55.1)	
Direct contact with a chicken:			
Yes	3/18 (16.7)	15/18 (83.3)	0.020
No	104/232 (44.8)	128/232 (55.2)	
Waterborne risks			
Exposure to sewage while canoeing:			
Yes	10/38 (26.3)	28/38 (73.7)	0.017
No	75/157 (47.8)	82/157 (52.2)	

4.2.4.2 Multivariate analysis

For the multivariate analysis, we included all the variables with p-values more than 0.05 but less than 0.10 in a logistic regression model. The following variables were used; contact with a jaundiced person within past 2 years, p=0.061; beef consumption, p=0.085; and rinse raw vegetables before eating, p=0.097. The logistic regression analysis with odds ratios (OR), p-values and confidence intervals (CI) is reported in Table 4.6.

Table 4.6 Logistic regression model

Characteristic	**Odds Ratio**	**P-value**	**95% Confidence Interval [95%CI]**
Contact with a jaundiced person	2.01	0.07	0.96 to 4.20
Beef consumption	0.72	0.62	0.20 to 2.57
Eating rinsed raw vegetables	0.45	0.25	0.11 to 1.74

4.3 Study group 1: HEV molecular testing

4.3.1 HEV RNA prevalence

All 25 minipools tested by HEV PCR had negative results. The pooling strategy that was used meant that individual samples were tested at 1:10 dilution. Therefore, if an individual sample would have had a low HEV viral load this could have been diluted to below the LOD of the pooling assay (3,000 IU/ml) and thus gone undetected.

4.4 Study group 2: HEV molecular testing

4.4.1 HEV RNA prevalence

Of the 10,000 donor samples tested by WPBTS using the Procleix HEV NAT assay, there were 500 to 600 donations tested per day with approximately 25 invalid results. These invalid results were due to the IC RNA being too low to be detected. All samples with invalid ICs were retested in duplicate according to the manufacturer's instructions (Addendum 6) and confirmed to have a valid and non-reactive result. Overall there were 9,999 non-reactive (negative) samples and 1 sample with a reactive (positive) result. This, too, was retested in duplicate for confirmation. In addition, a plasma aliquot of the reactive donor sample was sent to the Division of Medical Virology for confirmatory testing using the quantitative in-

house PCR (see above). The reactive donor was a 51 years old white male from the Northern suburbs. The molecular results are summarized in Table 4.7.

Table 4.7 Characteristics of the HEV RNA positive donor

Donor serial number	TMA result - RLU	TMA result - S/CO	RT-qPCR result – cycle threshold (Ct)	RT-qPCR result - viral load
10840601	1,879 324	51.89	30.7	$7.9x10^4$ IU/ml

CHAPTER FIVE: DISCUSSION

In order to investigate the prevalence of and risks associated with HEV infection in blood donors from the Western Cape, we conducted a survey and did serological and molecular tests. The main findings were: study group 1 had substantial evidence of past HEV infection but no active infection was detected among 250 participants; one active HEV infection was however detected among study group 2 consisting of 10,000 blood donors. This points to a considerable risk of acquiring HEV infection in the population of the Western Cape, here represented by blood donors, and an existing, albeit probably small, risk of transmission of undetected acute HEV infection to recipients of blood transfusions.

5.1 Anti-HEV IgG seroprevalence of study group 1

The total HEV seroprevalence was 42.4% with 106 of 250 blood donors positive for anti-HEV IgG. This is higher than the 25.3% (76/300) seroprevalence which we previously obtained for our pilot study (Lopes *et al*., unpublished data). Possible reasons for the difference in the seroprevalence of the current study and the pilot study is discussed in section 5.2 with regards to the different demographic characteristics of the blood donors. Our IgG seroprevalence is similar to that reported in different regions of France, which ranges from 39.1 to 52.5% (Mansuy *et al*., 2015; Mansuy *et al*., 2011). We used the same serological test as Mansuy *et al*., i.e. the Wantai assay. Even within countries, HEV IgG seroprevalence rates tend to vary in spite of the same assay being used (Petrik *et al*., 2015). A seroprevalence rate exceeding 20% would classify our setting amongst the high seroprevalence regions and countries such as e.g. Italy with 46% (Lucarelli *et al*., unpublished) and the Netherlands with 27% (Slot *et al*., 2013).

5.2 Anti-HEV IgG seroprevalence according to demographics

The demographic profile observed in the blood donors of study group 1 were not statistically significant. The differences are described below, with regards to the higher seroprevalence associated with age, gender, ethnicity (race) and area (site of blood donation clinic).

Age association

In our study, there was an age-dependent effect, blood donors that were 46 years and older had a higher risk for past HEV infection than donors in the younger age groups. Furthermore, the age-dependent effect seen in this study correlates with the data from our pilot study (Lopes *et al*., unpublished data) where we observed a statistically significant difference in individuals that were 46 years and older ($p<0.001$). Another study done in

Nigeria showed that the HEV IgG seroprevalence increased with age and was the highest in participants aged 60 years and older with a significance of p=0.044 (Junaid *et al*., 2014). Previous studies in blood donors from developed countries such as Australia (Shrestha *et al*., 2014), France (Mansuy *et al*., 2011) and the US (Xu *et al*., 2013) have reported similar findings consistent with our results. It appears that older age is associated with an accumulative exposure to HEV that results in a higher anti-HEV IgG seroprevalence. In addition to this in Austria, Fischer *et al*. looked at the incidence and seroprevalence of HEV infection in blood donors and observed that there was a greater risk of HEV infection amongst donors in the older age groups i.e. 50 years and older (OR=21.62; 95%CI: 30.1-53.3; x2: p<0.01) compared to those in the younger age groups (Fischer *et al*., 2015).

Gender association

The seroprevalence of anti-HEV IgG was similar between males 28.4% (46/162) and females 21.0% (29/138) from the pilot study and also between males and females in the current study: 45.9% (56/122) and 39.1% (50/128) respectively. In the current study (p=0.221) and in the pilot study (p=0.14) there was no statistically significant association between gender and past HEV infection. Other studies were done in blood donors from China (Jia *et al*., 2014) and France (Mansuy *et al*., 2011) also reported that gender was not a significant risk factor for HEV infection.

Ethnic association

Very few studies have reported on the seroprevalence of anti-HEV IgG in different race groups. The Western Cape, South Africa, is a unique setting with regards to its racial demographics: 0.3% Asian, 14.8% White, 33.9% Black and 51% Coloured people (WPBTS Annual Report 2013/2014, [s.a.]). As a result of the provincial demographics the WPBTS receives 0.9% of its blood donations from Asian people, 12.9% from Blacks, 25.4% from Whites and 60.8% from Coloureds (WPBTS Annual Report 2013/2014, [s.a.]). This allowed us to assess ethnic background as a probable risk of HEV infection. Table 5.1 shows HEV seroprevalences according to race group. A similar trend is seen between our current and the prior pilot study (Lopes *et al*., unpublished).

Table 5.1 Trend in the anti-HEV IgG seroprevalence in blood donors from the Western Cape

	Anti-HEV IgG seroprevalence	
Race group	**2013 - Pilot study N=300**	**2014 – Current study N=250**
Black	20/100 (20%)	3/12 (25%)
Coloured	33/100 (33%)	57/118 (48.3%)
White	23/100 (23%)	46/119 (38.7%)
Asian	Not sampled	0/1 (0%)

In the current study, we had an uneven representation of race groups: a random selection of blood donors were recruited irrespective of their demographics. While for the pilot study (Lopes *et al*., unpublished) we had 100 blood donors from each race group (Table 5.1). We observed that Coloured donors had a higher infection rate compared to White, Black and Asian donors. The latter pointing to possible differences in the risk of infection of race groups, but not a statistically significantly different in the current (p=0.232) or pilot (p=0.09) study. In spite of the lack of statistical significance, we believe that there may be a trend in evidence of past HEV infection amongst ethnic groups in the Western Cape. The provincial demographic profile of the Western Cape may have influenced the racial prevalence seen in our study.

Area association

Healthy individuals such as blood donors are part of the general population that are exposed to many potential risk factors. The differences observed in the HEV seroprevalence rate of the urban suburbs, could be dependent on several factors e.g. economic status, type of household infrastructure and community services i.e. water supply, refuse disposal etc. According to the census data there are some prominent differences between the above-mentioned factors that are associated with area. For example, in a high socioeconomic area, such as Constantia 75.3% of the people of White ethnicity (9,380/12,452) while in a middle to low socioeconomic area like Athlone 2.0% of the people are White (880/45,049) (City of Cape Town – 2011 Census Suburb Profiles, [s.a.]).

Donors were recruited at the blood donation clinics in the CBD are more often than not commuters that work in Central Cape Town. Therefore the HEV seroprevalence that we observed in this area was not a representative of individuals residing in the CBD.

Previously it was reported by Tucker *et al*. that people living in rural areas had a higher anti-HEV seroprevalence than individuals from urban areas in the Western and Eastern Cape provinces in South Africa (Table 2.1). However, we found a high anti-HEV IgG seroprevalence in donors recruited from blood donations clinics in suburbs (Table 4.3). Only 2 of the 250 donors indicated that they lived in a rural area (Table 4.4). However in France blood donors from a rural area, with residents that commonly hunt and eat undercooked meat, had a high anti-HEV IgG seroprevalence of 52.5% (Mansuy *et al*., 2011). These findings suggest that rural and urban areas may pose an equal risk of acquiring HEV infection. Further studies are needed to investigate the seroprevalence of anti-HEV in individuals that live in rural areas of the Western Cape.

5.3 Anti-HEV IgG seroprevalence according to potential risk factors in study group 1

We included different categories in our questionnaire to identify which means of exposure were associated with past HEV infection. In our study the following risk factors were statistically significantly associated with past HEV infection: consuming turkey or organ meats, exposure to surface water during recreational water sport, and having direct contact with rabbits or chickens. Further multivariate analysis revealed that contact with a jaundiced person was also a statistically significant risk factor. The risk factors associated with an increased risk of anti-HEV IgG seroprevalence in our study cohort were previously described in terms of the well-known HEV routes of transmission (Table 4.5).

Foodborne zoonotic risks

HEV has a foodborne zoonotic route of transmission, associated with genotype 3 which has been reported in South Africa (Andersson *et al*., 2012; Andersson *et al*., 2015). This warrants the evaluation of meat products sold in South Africa. The risk data from the current study found no statistically significant association with eating pork. This could be because we did not ask the how the questionnaire participants if they ate pork-derived products: raw, cooked medium to well-done or cooked well-done. An investigation was done to test for the presence HEV RNA in pork sausages sold at supermarkets in Italy, Czech Republic and Spain. The researchers found no evidence of HEV RNA in the sausage samples from Italy (0/128) and Czech Republic (0/92), but 6% of the sausages from Spain (6/93) were HEV RNA positive (Di Bartolo *et al*., 2012). Their findings support our assumption that pork-derived products sold at local supermarkets may be a potential source of HEV meat contamination. Due to the heat resistance of HEV, it is possible that undercooked pork-derived products could contain infectious HEV particles even after the meat is cooked in an oven or prepared on a fire. In our setting consuming raw or undercooked pork is not

common practice, however preparing meat on a fire “braai” at home or flame-grill in restaurants is a dietary habit in South Africa. Preparing meat on a fire does not allow one to set the cooking temperature to 71°C, which is required heat inactivate HEV particles. Therefore, this may potentially be facilitating the foodborne zoonotic transmission of HEV in our setting.

As a complicating factor, a recent study (Cawthorn *et al*., 2013) reported that pork (37%) and chicken (23%) were the most substituted species found in allegedly non-pork and non-chicken meat products sold in South African supermarkets. This could perhaps explain why we observed no statistical significance associated with eating pork (p=0.291) and chicken meat (p=0.875) amongst anti-HEV IgG positive donors (Table 4.5). Consumption of undercooked pig-derived meat is a major concern in the resource-rich settings; cases of foodborne zoonotic HEV transmission through raw or undercooked pork-derived products such as liver sausages (Mansuy *et al*., 2011; Mansuy *et al*., 2015). Our risk data showed that turkey meat which was generally less consume, but it was the only meat product that was statistically significantly associated with anti-HEV IgG positivity (Table 4.5). There are no documented cases in the literature that support the association between eating turkey and having past HEV infection, therefore, our finding might have been a randomly significant effect that requires further investigation.

It has been reported direct contact with pigs and other HEV animal hosts (Krumbholz *et al*., 2014; Lagler *et al*., 2014) serve as a significant risk of acquiring HEV infection. In the current study, we observed statistically significant association in the IgG status of individuals who had direct contact with rabbits (Table 4.5). Possible means of direct contact with rabbits are most likely due to individuals having a pet rabbit living in their backyard or visiting a petting zoo that has rabbits. There is evidence of HEV infection in rabbits (Geng *et al*., 2011; Izopet *et al*., 2012; Birke *et al*., 2014) however this does not explain the significant effect, which we found in people with more exposed to rabbits, having a lower HEV infection rate. A similar significance was seen in people having direct contact with chickens. Generally, farmers have more contact with poultry and people living in informal rural settlements that have chickens living in their backyards.

Occupational risks

We investigated possible occupational exposure to HEV and none of the variables within this category had statistical significance (Table 4.5). However other studies conducted in Madagascar (Temmam *et al*., 2013) and in Europe (Krumbholz *et al*., 2014) have found that having direct contact with pigs, as farmers or slaughterhouse workers, has a statistically

significant association with HEV infection. Verhoef *et al*. conducted a survey in the Netherlands that was similar to our study and found that working with human patients or working with animals were in HEV IgG positive individuals.

Waterborne risks

Exposure to HEV contaminated surface water is associated with transmission of HEV1 and HEV2. We observed there was a higher risk of infection in participants that did canoeing (p=0.017) as a recreational water activity. The latter could suggest that the rivers in our setting may be contaminated with debris containing HEV particles. The river possibly serves as a source of enteric pathogens including HEV, causing infection in individuals that come into contact with runoff from household waste, as a result of improper wastewater disposal (Govender *et al*., 2011). However, Grabow *et al*. reported that the seroprevalence of HEV in canoeists (1.8%) was found to be less than that of medical students (2.6%) who did not have regular exposure to contaminated water (Table 2.1). It is possible that individuals more exposed to possibly HEV contaminated water are at a greater risk of getting infected. Govender *et al*. have reported that water sources are polluted by unsanitary lifestyle practices of the residents from communities located in poor resource settings. Furthermore unsanitary practices can contribute to environmental pollution of water sources that promotes transmission of waterborne disease.

The socioeconomic status of people living in urban suburbs may be higher than that of people living in poorer rural areas. Furthermore, the socioeconomic status of an individual affects their lifestyle, e.g. people of low economic status would generally eat chicken which is cheaper than Thus our study participants may have been less exposed to surface water contaminated with HEV via the faecal-oral transmission. However, we cannot conclude on this matter until further investigations are done testing water samples for HEV contamination to confirm whether the waterborne transmission is a potential route of infection in our setting. In addition, to this sequencing should be done on HEV RNA positive samples to determine if HEV1 or HEV2 strains are circulating in our water sources.

5.4 HEV RNA prevalence

5.4.1 Study group 1

We detected no HEV RNA reactive pools and thus no evidence of active HEV infection in study group 1. The fact that we found no detectable HEV RNA in study group 1 may have been due to the pooled testing not being sensitive enough, or there could genuinely be no donors positive for HEV RNA. Our pooling assay had a LOD of 3,000 IU/ml per minipool

which amounts to 30,000 IU/ml per individual blood donation. The Ghanaian blood donor study had the same pool size as ours, but their assay was more sensitive (Table 2.3). Their LOD was reported to be to 1000 IU/ml for the minipools and to 250 IU/ml for the individual donations (Meldal *et al*., 2013). We used the HEV WHO IS as a reference (1 IU/ml = 2.5 copies/ml) to convert the measuring unit of the Ghanaian study's copies/ml to IU/ml: so that we could compare it to our study (Baylis *et al*., 2013). However Medal *et al*. had no cases of active HEV infection, which suggests that the prevalence of HEV RNA in developed countries might be lower than we suspected in spite of the background seroprevalence data.

Nevertheless, it seems more cost-effective to screen blood donations as minipools. Other advantages of using a pooling strategy include: using fewer reagents, which is beneficial in resource-limited settings. The turn-around time (TAT) is also reduced because more samples are tested in a single reaction e.g. testing a pool composed of 6 samples as one reaction compared to having 6 reactions for each sample. However the background prevalence of a setting plays a critical role in deciding whether ID- or MP-NAT would be more feasible. For example in a country such as South Africa which is known to be endemic for blood-borne viral pathogens such as hepatitis E virus (HEV) it would be ideal to have an assay which is sensitivity enough to test minipool. Therefore, perhaps the pool size should be reduced to improve the LOD. This could help to test more blood donations with reduced cost. However, the asymptomatic self-limiting course of HEV infection results in many undetected cases which are tested during the window period and can potentially infect a transfusion immunosuppressed recipient.

5.4.2 Study group 2

We detected HEV RNA in 1 of 10,000 blood donors tested, indicating a 0.01% (99%CI, 0-0.04) prevalence of active HEV infection. Our finding is comparable to a study from the US (Stramer *et al*., 2015) with the same RNA prevalence (Table 2.2) in a larger cohort. Another study conducted in Spanish blood donors (Sauleda *et al*., 2015) had a slightly higher prevalence of 0.03% (Table 2.2). Nonetheless, the reactive donors in their study had VLs much lower than our reactive donor (Table 4.7). According to Hewitt *et al*. the three reactive Spanish donors did not have an infectious dose of HEV. A blood donation should have a VL 4.53 IU/ml to cause HEV blood-borne transmission (Hewitt *et al*., 2014). This suggests that the blood and blood products from our reactive donor with a VL of $7.9x10^4$ IU/ml could have potentially transmitted HEV via blood transfusion.

5.5 Summary of the main findings

The findings of this study confirm the findings of our pilot study: there is evidence of past HEV infection in blood donors in the Western Cape. There appeared to be a trend with anti-HEV IgG seroprevalence and the demographic characteristics: age, gender, race and area. However, this was not statistically significantly associated with a higher HEV infection rate. Furthermore, we found proof that blood donors may be viraemic at the time of donation and could be a threat to immunosuppressed blood transfusion recipients. Consequently safety of blood and blood products the viral load of blood donation is infectious it could reduce the.

We found that using the HEV risk questionnaire was not a very strong predictor of infection, but the risk data could help to identify which blood donations have a higher chance of causing HEV infection. We might have found a random significant effect that was associated with eating turkey and being HEV IgG positive that requires further investigation. The IgG screening helped to evaluate to what extent HEV infection may have been undiagnosed.

Testing for HEV RNA could be used to screen blood donations for active infection to prevent HEV blood borne transmission. However this would be an expensive exercise and it will have to be shown whether the extra expense could be justified in terms of cases of infection averted, especially since many recipients of blood transfusions would not suffer serious consequences even if they became infected with HEV.

5.6 Study strengths and limitations

Our study participants were prospectively recruited from WPBTS, which made sample collection convenient and gave us easy access to a large number of blood donations. We were able to test blood donors from local and provincial blood donation clinics. This allowed us to describe the seroprevalence, risk and RNA prevalence of HEV infection in blood donors from the Western Cape. In addition, to this our study was the first in Africa to report on the prevalence of active HEV infection in blood donors, using ID-NAT to detect HEV RNA.

A limitation of our study was the sensitivity of our PCR assay, the LOD for our pools of 10 samples was 3,000 IU/ml which would not able to detect an individual sample with a VL lower 30,000 IU/ml. Therefore, our assays' sensitivity needs to be improved to be capable of detecting individual low positive samples in a minipool with VLs below 3,000 IU/ml. The latter will help to increase the negative predictive value. Another limitation was that our risk questionnaire was only completed by participants recruited at WPBTS blood donation clinics in Cape Town (study group 1) and not at all the blood donation clinics across the Western

Cape. Our study questionnaire may have reduced the differences in risks between the exposed and unexposed groups. After analysing the risk data, we observed that some questions could have been more specific, e.g. asking about meat preparation could be a better indicator of foodborne zoonotic transmission than the frequency of meat consumption. Furthermore to detect differences in the risks we require a greater sample size and broader sampling frame to recruit participants from both urban and rural areas.

5.7 Outlook and future recommendations

The Japanese Red Cross Society was the first blood bank to start screening blood donations by HEV NAT. They started screening for HEV RNA as of 2006 and are currently the only blood bank that has implemented HEV NAT as part of their routine blood donation tests (Kiely *et al*., 2015). Our low RNA prevalence (1 in 10,000) indicated that 0.01% of the blood donations could contain infectious HEV, which may cause severe liver damage in blood recipients. However instead of screening all the blood donations for HEV NAT, it may be advisable to only screen the blood and blood products given to high-risk recipients, such as the a renal transplant patient from the Western Cape who was diagnosed with hepatitis E infection (Andersson *et al*., 2015).

An alternative approach would be serology testing to detect anti-HEV IgG positive donors and subsequently use their blood for high-risk recipients. The latter could be safer since these donors would have already cleared the HEV RNA when they test IgG positive, see Figure 1.4 (Kamar *et al*., 2012). However to find a suitable screening method for avoiding transfusion-transmitted HEV infections in our resource-limited setting, a cost-effectiveness analysis (CEA) is needed, possibly based on WHO's CHOosing Interventions that are Cost-Effective (CHOICE) project. The WHO-CHOICE can be briefly described as a tool used to measure the amount of money needed, i.e. the cost of an intervention and its effectiveness, i.e. the number of quality-adjusted life years (QALYs) gained by implementing the intervention (Marseille *et al*., 2015).

CHAPTER SIX: CONCLUSION

Our study found a high seroprevalence of anti-HEV IgG antibodies in the blood donor samples tested with a very low HEV RNA prevalence. Testing revealed a seroprevalence of 42.4% (106/250) in this and 25.3% in the pilot study (76/300), providing evidence of past HEV infection in blood donors from the Western Cape. Analysis of our questionnaire identified that there may be an HEV contamination risk associated with meat products and water sources which could suggest transmission of HEV3 (foodborne, zoonotic) and HEV1 (waterborne) occurring. As regards active HEV infection, we only found one HEV RNA positive donor among 10,250 donors tested, i.e.an overall HEV RNA prevalence of 0.009% (1/10,250). The positive case had a viral load of $7.9x10^4$ IU/ml. At this HEV viral load, the transfusion-transmitted infection may have occurred in a blood recipient. The sequencing analysis will be done to fully characterize the HEV genotype. Previously cases of HEV3 had been reported from South Africa (Andersson *et al*., 2012; Andersson *et al*., 2015). We have shown that blood donors in Cape Town, Western Cape, have evidence of past and current HEV infection. Further studies are urgently needed to assess the risk factors for HEV infection and the risk of transmission through blood and blood products.

The data from this study is a contribution towards a better understanding of the epidemiology of HEV in Southern Africa, more specifically to assessing the risk of HEV blood-borne transmission from blood donors from the Western Cape, South Africa. Furthermore, this is the first study to provide evidence for a potential risk of HEV contamination in the blood supply to South Africa.

Presently blood transfusion services do not routinely screen blood donations for HEV as part of the serology and molecular tests. In our study, we used MP-NAT (n=250) and ID-NAT (n=10,000) to detect HEV RNA in the blood donations of 10,250 donors. We used both these NAT strategies and found that although we did not detect any active HEV donations with our MP-NAT, it appears to be more cost-effective than ID-NAT based on the low frequency of 1 in 10,000 HEV RNA reactive donations. Nevertheless, because the blood donations were tested in real-time by WPBTS, they were able to immediately block and discard all blood and blood products of the one HEV reactive donor. No HEV contaminated blood was issued during our study. Thus to fully investigate the risk of HEV blood-borne transmission we would need to follow the recipients receiving blood transfusions.

The data from this study can be used to assist policy makers in the South African blood transfusion services, in their decision-making on whether blood donations in our setting should be routinely screened for HEV infection. In addition to this, the donor risk profile from

our study that was associated with HEV infection could be used to screen donors for risk factors for donating HEV contaminated blood. Based on our findings we recommend that MP-NAT would be more feasible as a tool to implement routine HEV screening for blood donations in our setting with limited resources.

Further studies are needed to better understand the risks associated with HEV infection and to understand the reservoir and sources of this infection. We have shown that the risk of an HEV PCR positive donor, causing HEV blod-borne transmission to be low. Further studies may assist in identifying cost-effective means to identify those who may be HEV RNA positive and so further reduce the risk of on-going infection to a vulnerable population.

REFERENCES

Abdel Hady SI, Al-Din MS, El-Din ME. A high hepatitis E virus seroprevalence among unpaid blood donors and hemodialysis patients in Egypt. J Egypt Public Health Assoc 1998; 73:165–179.

Andersson MI, Preiser W, Maponga TG, et al. Immune reconstitution hepatitis E: a neglected complication of antiretroviral therapy in Africa?. AIDS 2012; 27(3):487-9.

Andersson MI, Stead PA, Maponga T, van der Plas H, Preiser W. Hepatitis E virus infection: An underdiagnosed infection in transplant patients in Southern Africa? J Clin Virol. 2015; 70:23-5.

Andonov A, Rock G, Lin L, et al. Serological and molecular evidence of a plausible transmission of hepatitis E virus through pooled plasma. Vox Sang. 2014; 107(3):213-9.

Arankalle VA, Chobe LP. Retrospective analysis of blood transfusion recipients: evidence for post-transfusion hepatitis E. Vox Sang. 2000; 79(2):72-4.

Baylis SA, Koc O, Nick S, Blümel J. Widespread distribution of hepatitis E virus in plasma fractionation pools. Vox Sang. 2012; 102(2):182-3.

Baylis SA, Blümel J, Mizusawa S, et al. World Health Organization International Standard to harmonize assays for detection of hepatitis E virus RNA. Emerg Infect Dis. 2013; 19(5):729-735.

Beale MA, Tettmar K, Szypulska R, Tedder RS, Ijaz S. Is there evidence of recent hepatitis E virus infection in English and North Welsh blood donors? Vox Sang 2011; 100(3):340-2.

Bendall R, Ellis V, Ijaz S, et al. A comparison of two commercially available anti-HEV IgG kits and a re-evaluation of anti-HEV IgG seroprevalence data in developed countries. J Med Virol. 2010; 82(5):799-805.

Bi SL, Purdy MA, Mc Caustland KA, Margolis HS, Bradley DW. The sequence of hepatitis E virus isolated directly from a single source during an outbreak in China. Virus Res 1993; 28:233-247.

Birke L, Cormier SA, You D, et al. Hepatitis E antibodies in laboratory rabbits from 2 US vendors. Emerging Infectious Diseases. 2014; 20(4):693-6.

Bortoliero AL, Bonametti AM, Morimoto HK, Matsuo T, Reiche EM. Seroprevalence for hepatitis E virus (HEV) infection among volunteer blood donors of the Regional Blood Bank of Londrina, State of Paraná , Brazil. Rev Inst Med Trop Sao Paulo. 2006; 48(2):87-92.

Boutrouille A, Bakkali-Kassimi L, Crucière C, Pavio N. Prevalence of anti-hepatitis E virus antibodies in French blood donors. J Clin Microbiol. 2007; 45(6):2009-10.

Boxall E, Herborn A, Kochethu G, Pratt G, Adams D, Ijas S, Teo CG. Transfusion-transmitted hepatitis E in ‘‘nonhyperendemic’’country. Transfus Med 2006; 16:79–83

Byskov J, Wouters JS, Sathekge TJ, Swanepoel R. An outbreak of suspected water-borne epidemic non-A non-B hepatitis in northern Botswana with a high prevalence of hepatitis B carriers and hepatitis delta markers among patients. Trans R Soc Trop Med Hyg.1989; 83(1):110-6.

Cable R, Lelie N, Bird A. Reduction of the risk of transfusion-transmitted viral infection by nucleic acid amplification testing in the Western Cape of South Africa: a 5-year review. Vox Sang 2013; 104(2):93-9.

Cawthorn DM, Steinman HA, Hoffman LC. A high incidence of species substitution and mislabelling detected in meat products sold in South Africa. 2013; 1-14.

City of Cape Town – 2011 Census Suburb Profiles. [S.a.] [Online]. Available: https://www.capetown.gov.za/en/stats/Pages/2011-Census-Suburb-Profiles-land.aspx [28 January 2016].

Cleland A, Smith L, Crossan C, et al. Hepatitis E virus in Scottish blood donors. Vox Sang 2013; 105(4):283-9.

Collier MG, Khudyakov YE, Selvage D, et al. Outbreak of hepatitis A in the USA associated with frozen pomegranate arils imported from Turkey: an epidemiological case study. Lancet Infect Dis. 2014; 14(10):976-981.

Comanor L, Anderson F, Ghany M, et al. Transcription-mediated amplification is more sensitive than conventional PCR-based assays for detecting residual serum HCV RNA at end of treatment. Am J Gastroenterol. 2001; 96(10):2968-2972.

Di Bartolo I, Diez-Valcarce M, Vasickova P, et al. Hepatitis E virus in pork production chain in Czech Republic, Italy, and Spain, 2010. Emerg Infect Dis. 2012; 18(8):1282-9.

Dreier J, Störmer M, Kleesiek K. Use of bacteriophage MS2 as an internal control in viral reverse transcription-PCR assays. J Clin Microbiol. 2005; 43(9):4551-7.

Ehteram H, Ramezani A, Eslamifar A, et al. Seroprevalence of Hepatitis E Virus infection among volunteer blood donors in central province of Iran in 2012. Iran J Microbiol. 2013; 5(2):172-6.

Fischer C, Hofmann M, Danzer M. Seroprevalence and Incidence of hepatitis E in Blood Donors in Upper Austria. PLoS One. 2015; 10(3):e0119576.

Fix AD, Abdel-Hamid M, Purcell RH, et al. Prevalence of antibodies to hepatitis E in two rural Egyptian communities. Am J Trop Med Hyg. 2000; 62(4):519-523.

Fukuda S, Sunaga J, Saito N, et al. Prevalence of antibody to hepatitis E virus among Japanese blood donors: Identification of three blood donors infected with a genotype 3 hepatitis E virus. J Med Virol. 2004; 73:554–561.

Gajjar MD, Bhatnagar NM, Sonani RV, Gupta S, Patel T. Hepatitis E seroprevalence among blood donors: A pilot study from Western India. Asian J Transfus Sci. 2014; 8(1):29-31.

Gallian P, Lhomme S, Piquet Y, et al. Hepatitis E virus infections in blood donors, France. Emerg Infect Dis. 2014; 20(11):1914-7.

Garson JA, Ferns RB, Grant PR et al. Minor groove binder modification of widely used Taq Man probe for hepatitis E reduces risk of false negative real-time PCR results. J Virol Methods. 2012; 186(1-2):157-160.

Gauss A, Wenzel JJ, Flechtenmacher C, et al. Chronic hepatitis E virus infection in a patient with leukemia and elevated transaminases: a case report. J Med Case Rep. 2012; 6:334.

Geng Y, Zhao C, Song A, et al. The serological prevalence and genetic diversity of hepatitis E virus in farmed rabbits in China. Infect Genet Evol. 2011; 11(2):476-482.

Gotanda Y, Iwata A, Ohnuma H, et al. Ongoing subclinical infection of hepatitis E virus among blood donors with an elevated alanine aminotransferase level in Japan. J Med Virol 2007; 79:734–7.

Gou Q, Yan Q, Xiong J, et al. Prevalence of hepatitis E virus in Chinese blood donors. J Clin Microbiol. 2010; 48(1):317-8.

Govender T, Barnes JM. Contribution of water pollution from inadequate sanitation and housing quality to diarrheal disease in low-cost housing settlements of Cape Town, South Africa. Am J Public Health. 2011; 101:e4-e9.

Grabow WO, Favorov MO, Khudyakova NS, Taylor MB, Fields HA. Hepatitis E seroprevalence in selected individuals in South Africa. J Med Virol. 1994; 44(4):384-8.

Haagsma EB, Niesters HG, van den Berg AP, et al. Prevalence of hepatitis E virus infection in liver transplant recipients. Liver Transpl 2009; 15:1225-8.

He J, Binn LN, Tsarev SA, et al. Molecular characterization of a hepatitis E virus isolate from Namibia. J Biomed Sci. 2000; 7(4):334-8.

Hewitt PE, Ijaz S, Brailsford SR, et al. Hepatitis E virus in blood components: a prevalence and transmission study in southeast England. Lancet.2014; 384(9956):1766-73.

Holm DK, Moessner BK, Engle RE, et al. Declining prevalence of hepatitis E antibodies among Danish blood donors. Transfusion. 2015; 55(7):1662-7.

Houcine N, Jacques R, Salma F, et al. Seroprevalence of hepatitis E virus infection in rural and urban populations, Tunisia. Clin Microbiol Infect. 2012; 18(5):E119-21.

Ibrahim EH, Abdelwahab SF, Nady S, et al. Prevalence of anti-HEV IgM among blood donors in Egypt. Egypt J Immunol. 2011; 18(2):47-58.

Ijaz S, Szypulska R, Tettmar KI, Kitchen A, Tedder RS. Detection of hepatitis E virus RNA in plasma mini-pools from blood donors in England. Vox Sang 2012; 102(3):272.

Isaäcson M, Frean J, He J, Seriwatana J, Innis BL. An outbreak of hepatitis E in Northern Namibia, 1983. Am J Trop Med Hyg. 2000; 62(5):619-625.

Izopet J, Dubois M, Bertagnoli S, et al. Hepatitis E virus strains in rabbits and evidence of a closely related strain in humans, France. Emerg Infect Dis. 2012; 18(8):1274-1281.

Jacobs C, Chiluba C, Phiri C et al. Seroepidemiology of Hepatitis E Virus Infection in an Urban Population in Zambia: Strong Association with HIV and Environmental Enteropathy. J Infect Dis. 2014; 209(5):652-7.

Jia Z, Yi Y, Liu J, et al. Epidemiology of hepatitis E virus in China: results from the Third National Viral Hepatitis Prevalence Survey, 2005-2006. PLoS One. 2014; 9(10):e110837.

Johargy AK, Mahomed MF, Khan MM, Kabrah S. Anti hepatitis E virus seropositivity in a group of male blood donors in Makkah, Saudi Arabia. J Pak Med Assoc. 2013; 63(2):185-9.

Juhl D, Baylis SA, Blümel J, Görg, Hennig H. Seroprevalence and incidence of hepatitis E virus infection in German blood donors. Transfusion. 2014; 54(1):49-56.

Junaid SA, Agina SE, Abubakar KA. Epidemiology and associated risk factors of hepatitis e virus infection in plateau state, Nigeria. Virology (Auckl). 2014; 5:15-26.

Kamar N, Bendall R, Legrand-Abravanel F, et al. Hepatitis E. Lancet 2012; 379(9835):2477-2488.

Kato M, Taneichi K, Matsubayashi K. A mini-outbreak of hepatitis E infection in those who enjoyed Yokiniko party: One died of fulminant hepatitis. Kanzo. 2004; 45:1326–1332.

Kaufmann A, Kenfak-Foguena A, André C, et al. Hepatitis E Virus Seroprevalence among Blood Donors in Southwest Switzerland. PLoS One. 2011; 6(6):e21150.

Khuroo MS, Kamili S, Yattoo GN. Hepatitis E virus infection may be transmitted through blood transfusions in an endemic area. J Gastroenterol Hepatol. 2004; 19(7):778-784.

Koot H, Hogema BM, Koot M, Molier M, Zaaijer HL. Frequent hepatitis E in the Netherlands without traveling or immuno suppression. J Clin Virol. 2015; 62:38-40.

Krain LJ, Nelson KE, Labrique AB. Host immune status and response to hepatitis E virus infection. Clin Microbiol Rev. 2014; 27(1):139-165.

Krumbholz A, Joel S, Dremsek P, et al. Seroprevalence of hepatitis E virus (HEV) in humans living in high pig density areas of Germany. Med Microbiol Immunol. 2014; 203(4):273-282.

Kumar S, Subhadra S, Singh B, Panda BK. Hepatitis E virus: the current scenario. Int J Infect Dis. 2013; 17(4):e228-e233.

Kumara A, Beniwala M, Karb P, et al. Hepatitis E in pregnancy. Int J Gynecol Obstet. 2004; 85(3):240-4.

Kuniholm MH, Purcell RH, McQuillan GM, Engle RE, Wasley A, Nelson KE. Epidemiology of hepatitis E virus in the United States: results from the Third National Health and Nutrition Examination Survey, 1988-1994. J Infect Dis. 2009; 200(1):48-56.

Labrique AB, Sikder SS, Krain LJ, West KP, Jr, Christian P, Rashid M, Nelson KE. Hepatitis E, a vaccine-preventable cause of maternal death. Emerg Infect Dis.2012; 18:1401–4.

Labrique AB, Zaman K, Hossain Z, et al. An exploratory case control study of risk factors for hepatitis E in rural Bangladesh. PLoS One. 2013; 8(5):e61351.

Lagler H, Poeppl W, Winkler H, et al. Hepatitis E virus seroprevalence in Austrian adults: a nationwide cross-sectional study among civilians and military professionals. PLoS One. 2014; 9(2):e87669.

Liu P, Shi ZX, Zhang YC, Xu ZC, Shu HS, Zhang XY. A prospective study of a serum-pooling strategy in screening blood donors for antibody to hepatitis C virus. Transfusion. 1997; 37(7):732-6.

Maila HT, Bowyer SM, Swanepoel R. Identification of a new strain of hepatitis E virus from an outbreak in Namibia in 1995. J Gen Virol. 2004; 85(Pt 1):89-95.

Mansuy JM, Bendall R, Legrand-Abravanel F, et al. Hepatitis E virus antibodies in blood donors, France. Emerg Infect Dis. 2011; 17(12):2309-2312.

Mansuy JM, Saune K, Rech H, et al. Seroprevalence in blood donors reveals widespread, multi-source exposure to hepatitis E virus, southern France, October 2011. Euro Surveill. 2015; 20(19):27-34.

Marseille E, Larson B, Kazi DS, Kahn JG, Rosen S. Thresholds for the cost-effectiveness of interventions: alternative approaches. Bull World Health Organ. 2015; 93:118-124.

Matsubayashi K, Kang J, Sakata H, et al. A case of transfusion-transmitted hepatitis E caused by blood from a donor infected with hepatitis virus via zoonotic food-borne route. Transfusion. 2008; 48(7):1368-1375.

Meldal BHM, Sarkodie F, Owusu-Ofori S, Allain JP. Hepatitis E virus infection in Ghanaian blood donors the importance of immunoassay selection and confirmation. Vox Sang. 2013; 104(1):30-6.

Mushahwar IK. Hepatitis E Virus: Molecular Virology, Clinical Features, Diagnosis, Transmission, Epidemiology, and Prevention. J Med Virol. 2008; 80(4):646-658.

Passos-Castilho AM, de Sena A, Geraldo A, Spada C, Granato CF. High prevalence of hepatitis E virus antibodies among blood donors in Southern Brazil. J Med Virol. 2015 [Epub ahead of print]

Petrik J, Lozano M, Seed CR, et al. Hepatitis E. Vox Sang. 2015.

Petrović T, Lupulović D, Jiménez de Oya N, et al. Prevalence of hepatitis E virus (HEV) antibodies in Serbian blood donors. J Infect DevCtries. 2014; 8(10):1322-7.

Pittaras T, Valsami S, Mavrouli M, et al. Seroprevalence of hepatitis E virus in blood donors in Greece. Vox Sang. 2014; 106(4):387.

Purdy MA, Khudyakov YE. The molecular epidemiology of hepatitis E virus infection. Virus Res. 2011; 161(1):31-9.

Ren F, Zhao C, Wang L, et al. Hepatitis E virus seroprevalence and molecular study among blood donors in China. Transfusion. 2014; 54(3 Pt 2):910-7.

Robson SC, Adams S, Brink N, Woodruff B, Bradley D. Hospital outbreak of hepatitis E. Lancet. 1992; 339(8806):1424-5.

Sauleda S, Ong E, Bes M, et al. Seroprevalence of hepatitis E virus (HEV) and detection of HEV RNA with a transcription-mediated amplification assay in blood donors from Catalonia (Spain). Transfusion. 2015; 55(5):972-9.

Shrestha AC, Seed CR, Flower RL, et al. Hepatitis E virus and implications for blood supply safety, Australia. Emerg Infect Dis. 2014; 20(11):1940-2.

Slot E, Hogema BM, Riezebos-Brilman A, Kok TM, Moller M, Zaaijer HL. Silent hepatitis E virus infection in Dutch blood donors, 2011 to 2012.Euro Surveill. 2013; 18(31):pii:20550.

Smith PF, Grabau JC, Werzberger A, Gunn RA, Rolka HR, et al. The role of young children in a community-wide outbreak of hepatitis A. Epidemiol Infect. 1997; 118(3):243-252.

Stramer SL, Moritz ED, Foster GA, et al. Hepatitis E virus: seroprevalence and frequency of viral RNA detection among US blood donors. Transfusion. 2015 [Epub ahead of print]

Surya IG, Kornia K, Suwardewa TG, MulyantoTsuda F, Mishiro S. Serological markers of hepatitis B, C, and E viruses and human immunodeficiency virus type-1 infections in pregnant women in Bali, Indonesia. J. Med. Virol. 2005; 75:499–503.

Szypulska R. Health Protection Agency Microbiology Services Colindale Virus Reference Department Standard Operating Procedure: Detection of Hepatitis E Virus RNA by real time PCR.

Tadesse E, Metwally L, Alaa E. High prevalence of anti-hepatitis E virus among Egyptian blood donors. J Gen Mol Virol. 2013; 5(1):9–13.

Takahashi K, Kitajima N, Abe N, Mishiro S. Complete or near-complete nucleotide sequences of hepatitis E virus genome recovered from a wild boar, a deer, and four patients who ate the deer. Virology. 2004; 330:501-5.

Tavitian S, Peron JM, Huguet F, et al. Ribavirin for Chronic Hepatitis Prevention among Patients with Hematologic Malignancies. Emerg Infect Dis. 2015; 21(8):1466-9.

Temmam S, Besnard L, Andriamandimby SF, et al. High prevalence of hepatitis E in humans and pigs and evidence of genotype-3 virus in swine, Madagascar. Am J Trop Med Hyg. 2013; 88:329–338.

Teshale EH, Howard CM, Grytdal SP, et al. Hepatitis E Epidemic, Uganda. Emerg Infect Dis. 2010; 16(1):126-9.

Teshale EH, Hu DJ, Holmberg SD. The Two Faces of Hepatitis E Virus. Clin Infect Dis. 2010; 51(3):328-334.

Teshale EH, Hu DJ. Hepatitis E: Epidemiology and prevention. W J Hepatol. 2011; 3(11):285-291.

Traoré KA, Rouamba H, Nébie Y, et al. Seroprevalence of Fecal-Oral Transmitted Hepatitis A and E Virus Antibodies in Burkina Faso. PLoS One. 2012; 7(10):e48125.

Tucker TJ, Kirsch RE, Louw SJ, Isaacs S, Kannemeyer J, Robson SC. Hepatitis E in South Africa: Evidence for Sporadic Spread and Increased Seroprevalence in Rural Areas. J Med Virol. 1996; 50(2):117-9.

Van Rensburg EJ, Lemmer HR, Joubert JJ. Prevalence of viral infections in Mozambican refugees in Swaziland. East Afr Med J. 1995; 72(9):588-590.

Verhoef L, Koopmans M, Duizer E, Bakker J, Reimerink J, Van Pelt W. Seroprevalence of hepatitis E antibodies and risk profile of HEV seropositivity in The Netherlands, 2006-2007. Epidemiol Infect. 2012; 140(10):1838-47.

Vermeulen M, Lelie N, Sykes W, et al. Impact of individual-donation nucleic acid testing on risk of human immunodeficiency virus, hepatitis B virus, and hepatitis C virus transmission by blood transfusion in South Africa. Transfusion. 2009; 49(6):1115-1125.

Viswanathan R. Infectious hepatitis in Delhi (1955-56): a critical study-epidemiology. 1957. India J Med Res. 1957; 45[Suppl 12]:1-29.

Vollmer T, Diekmann J, Johne R, Eberhardt M, Knabbe C, Dreier J. Novel Approach for Detection of Hepatitis E Virus Infection in German Blood Donors. J Clin Microbiol. 2012; 50(8):2708-2713.

Wibawa ID, Muljono DH, Mulyanto, et al. Prevalence of antibodies to hepatitis E virus among apparently healthy humans and pigs in Bali, Indonesia: Identification of a pig infected with a genotype 4 hepatitis E virus. J Med Virol. 2004; 73(1):38-44.

WPBTS Annual Report 2013/2014. [S.a.] [Online]. Available: http://www.wpblood.org.za/village/wpbnew/sites/default/files/WPBTS_Annual_Report_2014_0.pdf [5 February 2015].

Xu C, Wang RY, Schechterly CA, et al. An assessment of hepatitis E virus (HEV) in US blood donors and recipients: no detectable HEV RNA in 1939 donors tested and no evidence for HEV transmission to 362 prospectively followed recipients. Transfusion. 2013; 53(10 Pt 2):2505-2511.

Yan Y, Zhang W, Shen Q, Cui L, Hua X. Prevalence of four different subgenotypes of genotype 4 hepatitis E virus among swine in the Shanghai area of China. Acta Vet Scand. 2008; 50:12.

Zhang H, Cao XY, Liu CB, et al. Epidemiology of hepatitis E in China. Gastroenterol Jpn. 1991; 26:135-8.

Zhang J, Zhang XF, Huang SJ, et al. Long-Term Efficacy of a Hepatitis E Vaccine. N Engl J Med. 2015; 372:914-922.

Zhu FC, Zhang J, Zhang XF, et al. Efficacy and safety of a recombinant hepatitis E vaccine in healthy adults: a large-scale, randomised, double-blind placebo-controlled, phase 3 trial. Lancet. 2010; 376(9744):895-902.

ADDENDA

Addendum 1 – Study consent form

PARTICIPANT INFORMATION LEAFLET AND CONSENT FORM

TITLE OF THE RESEARCH PROJECT: Prevalence and Risks of Hepatitis E Virus infection in Blood Donors from the Western Cape, South Africa

REFERENCE NUMBER: S14/04/091

PRINCIPAL INVESTIGATOR: Tatum Lopes

ADDRESS: Division of Medical Virology, Dept of Pathology
Stellenbosch University, Faculty of Medicine and Health Sciences
Francie van Zijl Ave, 8th Floor,
PO Box 19063, Tygerberg Campus, 7505
Western Cape Province
SOUTH AFRICA

CONTACT NUMBER: 021 938-9354

You are being invited to take part in a research project. Please take some time to read the information presented here, which will explain the details of this project. Please ask the study staff or doctor any questions about any part of this project that you do not fully understand. It is very important that you are fully satisfied that you clearly understand what this research entails and how you could be involved. Also, your participation is **entirely voluntary** and you are free to decline to participate. If you say no, this will not affect you negatively in any way whatsoever. You are also free to withdraw from the study at any point, even if you do agree to take part.

This study has been approved by the **Health Research Ethics Committee at Stellenbosch University** and will be conducted according to the ethical guidelines and principles of the international Declaration of Helsinki, South African Guidelines for Good Clinical Practice and the Medical Research Council (MRC) Ethical Guidelines for Research.

What is this research study all about?

- *The study will be conducted at the Division of Medical Virology, Faculty of Medicine and Health Sciences, Stellenbosch University in collaboration with Western Province Blood Transfusion Service.*
- *Our project aims to identify past and active hepatitis E virus (HEV) infection in blood donors because we don't know if blood donors in South Africa have active hepatitis E infection. Hepatitis E is an infection which is cleared by people who have a normal immune system. For those patients who have a very compromised immune system e.g. kidney transplant patients this infection can*

lead to liver damage. We also want to find out what the risks are that are associated with infection with hepatitis E.

- *We will be testing around 28 000 donors for this study. The 250 blood donors participating in the study questionnaire will be recruited and consented over a period of 3 months.*

Why have you been invited to participate?

- *You have been asked to participate in this study because you are a blood donor.*

What will your responsibilities be?

- *We would like you to complete a questionnaire and we will use 2 ml of your donated blood for the hepatitis E tests.*

Will you benefit from taking part in this research?

- *This study will help us assess the potential risk of HEV transmission in blood and blood products. In the event that a sample is positive for active hepatitis E infection we will inform WPBTS who will notify you. You will be retested to ensure that the infection has cleared. Once your infection has cleared you will be able to donate blood again with no restrictions. In addition to this the recipient of the blood or blood products will be traced and tested.*
  The long term benefit of the study is that we will be able to assess what sort of tests are needed if any to reduce hepatitis E transmission in South Africa and other endemic regions.

Are there any risks involved in your taking part in this research?

- *There are no risks involved with participating in this study.*

If you do not agree to take part, what alternatives do you have?

- *This will not alter your blood donation or your ability to donate blood at all.*

Who will have access to your questionnaire?

- *Your questionnaire will not have your name on it, only your donation number. These questionnaires are kept confidential and are only accessed by staff directly involved in the study. WPTBS does not reveal the identity of the blood donors. If a sample is found to be positive WPBTS will contact you.*

- *As a donor participating in this study questionnaire your research records may need to be inspected by the Human Research Ethics Committee (HREC).*

What will happen in the unlikely event of some form of injury occurring as a direct result of your taking part in this research study?

- *There are no risks.*

Will you be paid to take part in this study and are there any costs involved?

- No you will not be paid to take part in the study.

Is there anything else that you should know or do?

- You can contact **Dr Andersson** at **082-709 6152** if you have any further queries or encounter any problems.
- You can contact the **Health Research Ethics Committee** at **021-938 9207** if you have any concerns or complaints that have not been adequately addressed by your study doctor.
- You will receive a copy of this information and consent form for your own records.

Declaration by participant

By signing below, I ……..……. agree to take part in a research study entitled *(**Prevalence and Risks of Hepatitis E Virus infection in Blood Donors from the Western Cape, South Africa**)*.

I declare that:

- I have read or had read to me this information and consent form and it is written in a language with which I am fluent and comfortable.
- I have had a chance to ask questions and all my questions have been adequately answered.
- I understand that taking part in this study is **voluntary** and I have not been pressurised to take part.
- I may choose to leave the study at any time and will not be penalised or prejudiced in any way.
- I may be asked to leave the study before it has finished, if the study doctor or researcher feels it is in my best interests, or if I do not follow the study plan, as agreed to.

Signed at (*place*) .....……..…….. On (*date*) ……..…….. 2014.

.....	.....
Signature of participant	**Signature of witness**

Declaration by investigator

I *(name)* ……..……. declare that:

- I explained the information in this document to ……..
- I encouraged him/her to ask questions and took adequate time to answer them.
- I am satisfied that he/she adequately understands all aspects of the research, as discussed above
- I did/did not use an interpreter. (*If an interpreter is used then the interpreter must sign the declaration below.*

Signed at (*place*) .....…….. On (*date*) ……..…….. 2014.

....
Signature of investigator **Signature of witness**

Declaration by interpreter

I *(name)* ……..…… declare that:

- I assisted the investigator (*name*) ……. to explain the information in this document to (*name of participant*) ……..…….. using the language medium of Afrikaans/Xhosa.

- We encouraged him/her to ask questions and took adequate time to answer them.
- I conveyed a factually correct version of what was related to me.
- I am satisfied that the participant fully understands the content of this informed consent document and has had all his/her question satisfactorily answered.

Signed at (*place*).....……. On (*date*) ……..……..2014.

....
Signature of interpreter **Signature of witness**

Addendum 2 – HEV study questionnaire

Questionnaire - hepatitis E virus infections in the Western Cape

Donor serial number: .....

1. Date of Birth: …../…../……... (dd/mm/yyyy)

2. Gender: ❑ M ❑ F

3. Please describe your ethnic group/culture background:

❑ Black ❑ White ❑ Coloured ❑ Other (please specify).....

4. Where have you lived in the past 10 years, is the area:

❑urban ❑rural ❑ coastal, distance from sea .....

4.1 If your residential area is/was rural, please specify……

5. Occupation: ……..

5.1 Work that involves:

❑handling food ❑exposure to blood/blood products ❑direct contact with animals

 none of the above

Name of interviewer …….

MEDICAL HISTORY

6. Are you being treated by a doctor for a long-term illness, disorder?

☐ yes ☐ no ☐ do not know

If yes, specify disease……

7. Are you on prescription medication?

❑ yes ❑ no ❑ do not know

If yes, what medication(s) ……

8.Have you undergone surgery in the past 2 years?

❑ yes ❑ no ❑ do not know

If so, for……

9.Have you had a blood transfusion (received donor blood) in the past 2 years?

❑ yes ❑ no ❑ do not know

If so, on …./…./…..…… (dd/mm/yyyy) Place:…….....

10. Have you had vaccinations / injections given in the past 2 years?

❑ yes ❑ no ❑ do not know

If so, on …./…./…..… (dd/mm/yyyy) Against……

11. Do you drink alcohol? ☐ yes ☐ no ☐ do not know

11.1 Applicable to males only, If yes do you drink 21 units or more per week?

❑ yes ❑ no

11.2 Applicable to females only, If yes do you drink 14 units or more per week?

❑ yes ❑ no

11.3 Do you binge drink?

❑ yes ❑ no

11.3.1 If so, how much alcohol do you drink on average over the weekend?

.....glasses of wine .....beer bottles .....tots of spirits

TRAVEL HISTORY

12. Have you travelled outside of the Western Cape in the past 2 years?

☐ yes ☐ no ☐ do not know

Number of occasions	**Where ?** (Place)	**When?** (mm/yyyy)
1.		
2.		
3.		
4.		

13. Have you had contact with one or more persons visiting the Western Cape in the past 2 years?

☐ yes ☐ no ☐ do not know

13.1 If so, from which place? ……..

13.2 If so, relation to the person?

- Family member ❑
- Friend ❑
- Colleagues ❑
- Others, namely …… ❑

14. Have one or more family members/ friends/ acquaintances had jaundice in the past 10 years?

☐ yes ☐ no ☐ do not know

If so,

Relation to patient	Disease	Start of disease
1.		
2.		
3.		
4.		

FOOD CONSUMPTION

15. How often do you eat in a restaurant/ fast-food outlet or have (take-out) delivered at home?

-More often than 1 time per week ❑
- 1 time per week ❑
- 1-3 times per month ❑
- Less than 1 time per month ❑
- Never ❑

16. How often do you eat one of the following products?

Product	More than 1 time per week	1 time per week	1-3 times per month	Less than 1 time per month	Never	Do not know
Beef						
Pork						
mutton						
chicken						
turkey						
wild meat						
organ meats						
Fish						
shellfish						
unpasteurized milk						

17. Before eating at home, do you normally wash the following foods with tap water?

17.1 lettuce ☐ yes ☐ no ☐ do not know
17.2 raw vegetables ☐ yes ☐ no ☐ do not know
17.3 fruit ☐ yes ☐ no ☐ do not know

18. If you eat game products, please specify:

Wild (which animal)	Which product	Welldone	Half-baked	Raw

19. If you eat organ meats, please specify:

19.1 from which animal?……....

19.2 from which organ? ……..……..

☐ raw ☐ half baked ☐ welldone

20. If you eat shellfish(eg. crab, lobster, prawns,etc.) please specify:

20.1 what kind? .....

☐ raw ☐ half baked ☐ welldone

21. Do you catch your own shellfish? ☐ yes ☐ no

21.1 If not, where do you buy your shellfish?

☐ at a supermarket ☐ fishmonger shop (fisheries) ☐ street vendor

☐ restaurant, where? .....

Water consumption and contact with surface water

22. How often do you consume the following type (s) of water?

Water type	Daily	1 time per week	1-3 times per month	Less than 1 time per month	Never	Do not know
Tap water						
Spring water (bottles)						
Spring water (pump)						
Water from a well						
Water from a river, stream or lake						

23. Do you have safe, clean ruuning tap water at home?

❑ yes ❑ no

24. Have you been exposed to surface water in the past 2 years?

❑ yes ❑ no ❑ do not know

24.1 If so, with:

-ditch water ❑
-recreational water ❑
-sewage ❑
-dirty water from stable with
(specify species)❑ ……..
- otherwise, namely ❑ ……

25. In the past 2 years, did you participate in any of the following recreational water activities?

- swimming ❑ yes ❑ no ❑ do not know
- sailing ❑ yes ❑ no ❑ do not know
- canoeing ❑ yes ❑ no ❑ do not know
- windsurfing/ water skiing ❑ yes ❑ no ❑ do not know
- fishing ❑ yes ❑ no ❑ do not know

- Other?.....

Animal contact

26. In the past 2 years have you had any direct contact (touch) with pigs?

❑ yes ❑ no ❑ do not know

26.1 If so, how often?

❑ more often than 1 time per week
❑ 1 time per week
❑ 1-3 times per month
❑ less than 1 time per month

27. Do you live at a residence (e.g. farm) where pigs are present?

❑ yes ❑ no

28. In the past 2 years have you visited a residence

28.1 where pigs were present at that time?
❑ yes ❑ no ❑ do not know
28.2 where animals other than pigs were present?
❑ yes ❑ no ❑ do not know

28.2.1 If so, which animal?
……
……

29 In the past 2 years have you had direct contact with the following animals?

Animal Category	Species	Yes	No	Do not know	Contact specification (E.g. touching, kissing/licking, etc.)
Wild	Deer				
	Wild boar				
	Hazen (rabbit)				
Farm animals	Sheep				
	Goats				
	Oxen				
	Equine (horse)				
	Poultry (chicken)				
Pets	Dog				
	Cat				
	Others……				

30 In the past 2 years, did you prepare any foods for your pets/or other animals?

❑ yes ❑ no ❑ do not know

30.1 If yes, which foods? (please specify how it was prepared)
.....
.....

Additional information and / or comments

Addendum 3 - Questionnaire terms and definitions

Urban area:

1. Most countries use a combination of total population and population density as a definition of urban area for statistical purposes. There are no international standard criteria for defining 'urban'; however, the concept of **a town with a population size of > 1000** is common.

   Some national statistical organisations **define as urban areas with a locality threshold population of > 1000**. Other countries use combined criteria of locality population and population density. The **urban area criteria** are **specific to each country, based on their population dynamics and settlement patterns**.

   The equivalent geographic classification for locality in the South African context is 'main place'. After several spatial iterations with the urban area criteria, it was determined that a combination of locality population and population density is required for the South African settlement patterns. The alternative proposed criteria are:
   - **Main place population >=1000** (greater than or equal to 1000) and **population density >= 500 per km²** at the main place and sub-places; or
   - **Main place population >=1000** and **population density >= 1000 per km²** at the main place and sub places.

   [Urban/rural discussion document Statistics South Africa, 2003]

Rural area:

1. ***Western Cape*** is using the measure of **less than 150 persons per sq. km**. www.**westerncape**.gov.za/.../**rural**-tourism-strategy-leononre-beukes.pdf

2. If potential subsidies or institutional arrangements, such as the housing subsidy and the proposed land reform Settlement Grant, or the type of local authority, differ between 'urban' and 'rural' areas, a legal definition is required. A formal definition is also required to ensure consistency in data used by the different actors in rural areas. This issue will be given serious attention under the National Information Project. However, historical complexities, cultural perceptions and modem needs for service delivery, cannot easily be simplified into a definition that suits South African purposes.

   As an interim measure, we argue for a continuum of **population sizes and densities** that affect service provision. **Rural areas**, then, are those **areas that have the lowest level of services, and the greatest average distance to the nearest service points**.

   - They include **large scale farming areas**, much - but not all - of the **ex-bantustan areas**, and **small municipalities with little potential to raise taxes sufficient to meet the costs of services**.

Peri-urban squatter camps, being tied to the economies of contiguous urban areas, do not count as rural, except in the context of sometimes being part of rural municipalities.

http://www.polity.org.za/polity/govdocs/rdp/rural1.html

Direct contact with animals: (think in terms of risk of transmission)

1. Touching the animal; example **feeding**, **washing**, **playing** or **training**.
2. Coming into contact with the animals' bodily fluids
   Saliva (being licked or bitten by the animal or being exposed to their drool)
   Blood (helping female animals give birth, nursing a wound of an injured animal)

Binge drink:

1. The National Institute on Alcohol Abuse and Alcoholism defines binge drinking as a pattern of drinking that brings a person's blood alcohol concentration **(BAC) to 0.08 grams percent or above.** This typically happens when:

   - **men** consume 5 or more drinks, and when
   - **women** consume 4 or more drinks, in about 2 hours.

   CDC - Fact Sheets-**Binge Drinking** - Alcohol

   www.cdc.gov/**alcohol**/fact-sheets/**binge-drinking**.htm

2. Binge drinking was defined as **having five or more drinks** on at least one occasion in the two weeks prior to the study.
   [Parry CDH et al. Trends in adolescent alcohol and other drug use: findings from three sentinel sites in South Africa (1997–2001). Journal of Adolescence, in press.]

   Risky drinking (on weekdays) was defined as: for **males** drinking five or more drinks per day, and for **females** as drinking three or more drinks per day. From the same survey the corresponding rates for risky drinking during weekends was 32.8% (males) and 32.4% (females).
   [South African Demographic and Health Survey. South African Department of Health, 1998.]

3. The WHO defines binge drinking as **consuming more than 60ml of alcohol per week** where one standard drink is equivalent to 10g of pure alcohol. This means that **consuming more than six drinks in a single sitting** would be considered binge drinking.

   [(WHO) report that tracks global drinking patterns reveals that South African women have the biggest appetite for alcohol on the continent, with a whopping 41,2% of women reported to be binge drinkers.]
   http://www.destinyconnect.com/2014/05/06/do-sa-women-have-a-drinking-problem/

4. While there is no universal scientific or medical definition of what constitutes 'binge' or 'extreme' drinking, it usually means **drinking excessive amounts of alcohol in a**

short period of time.
http://www.talkingalcohol.com/files/factsheets/body_bingedrinking.pdf

Unrecorded alcohol consumption

The unrecorded alcohol consumption in South Africa is estimated to be 2.2 litres pure alcohol per capita for population older than 15 for the years after 1995 (estimated by a group of key alcohol experts). [Alcohol per capita consumption, patterns of drinking and abstention worldwide after 1995. Appendix 2. European Addiction Research, 2001, 7(3):155–157.]

Contact with people: (think in terms of risk of transmission)

1. **Person to person transmission**
   This may occur by being in **close contact** with an individual or individuals for a long period of time or on multiple occasions for example **living or working together** which increases your risk of being exposed to pathogens that spread between hosts.
   - sharing eating utensils *saliva*
   - using the same bathroom
   - exposure to vomit if the person is feeling ill

2. **Faecal oral transmission**
   This can easily take place if you **share sanitary facilitates** or **your work exposes you to unclean water** and **direct contact with faeces**.
   - Washing or cleaning a baby or elderly persons' diaper or any person incapable of taking care of themselves
   - Burst sewage pipe

Surface water:

1. Water collecting on the ground or in a stream, river, lake, sea or ocean, as opposed to groundwater.

Recreational water:

1. **Recreational water** refers to **all inland water** which is used for recreational purposes. These include:
   - **full contact** recreation (**swimming**),
   - **intermediate-contact** recreation (**water skiing** and **canoeing**) and
   - **non-contact** recreation like **picnicking** and **hiking alongside water bodies**.

   http://www.waternet.co.za/policy/g_wq.html

Domestic water refers to water that is used in domestic environment and also refers to all uses water can be put to in this environment. These include water for drinking, food and beverage preparation, hot water systems, bathing and personal hygiene, laundry and gardening.

Recreational water illness: An illness that is spread by swallowing, breathing, or having contact with contaminated water from swimming pools, spas, hot tubs, decorative water fountains, lakes, rivers, or oceans.

http://www.medterms.com/script/main/art.asp?articlekey=33192

Unpasteurized milk:

1. Milk that has not been exposed to high temperatures to destroy microorganisms and prevent fermentation.

Addendum 4 – WPBTS Consent Form (page 2 of 4)

DECLARATION : Please read and sign before donating blood.

1. I have read and understood the pamphlet "Important Information for Blood Donors".
2. To the best of my knowledge all the information supplied is the truth.
3. I understand that if I have not answered these questions truthfully this could endanger the patient and lead to legal proceedings against me. I undertake that should I for any reason deem my blood not safe for use, I will immediately inform WPBTS.
4. I consent to my blood being tested for Syphilis, Hepatitis B, Hepatitis C and HIV.
5. I understand that I will be informed of any test results that are important to my health or affect my ability to donate blood.
6. I accept that samples of my blood and / or donation data may be used on occasion for scientific research, the objective of which is to improve the safety of the blood supply to patient and donor health and well being. On occasion the Service may permit researchers to request additional samples from me with my consent.
7. I confirm that I am 16 years of age or older.
8. I understand that the information on this form will be kept in a secure facility indefinitely under my donor code, not my name.
9. I understand the donation process and the possible risks involved as explained.
10. I consent to the administration of such fluids and medications as deemed necessary in the management of an untoward donor reaction.
11. I consent to the infusion of fluids, medications and re-infusion of my own blood components during apheresis collection procedures.

Please do not sign until you have answered all the questions and read the declaration.

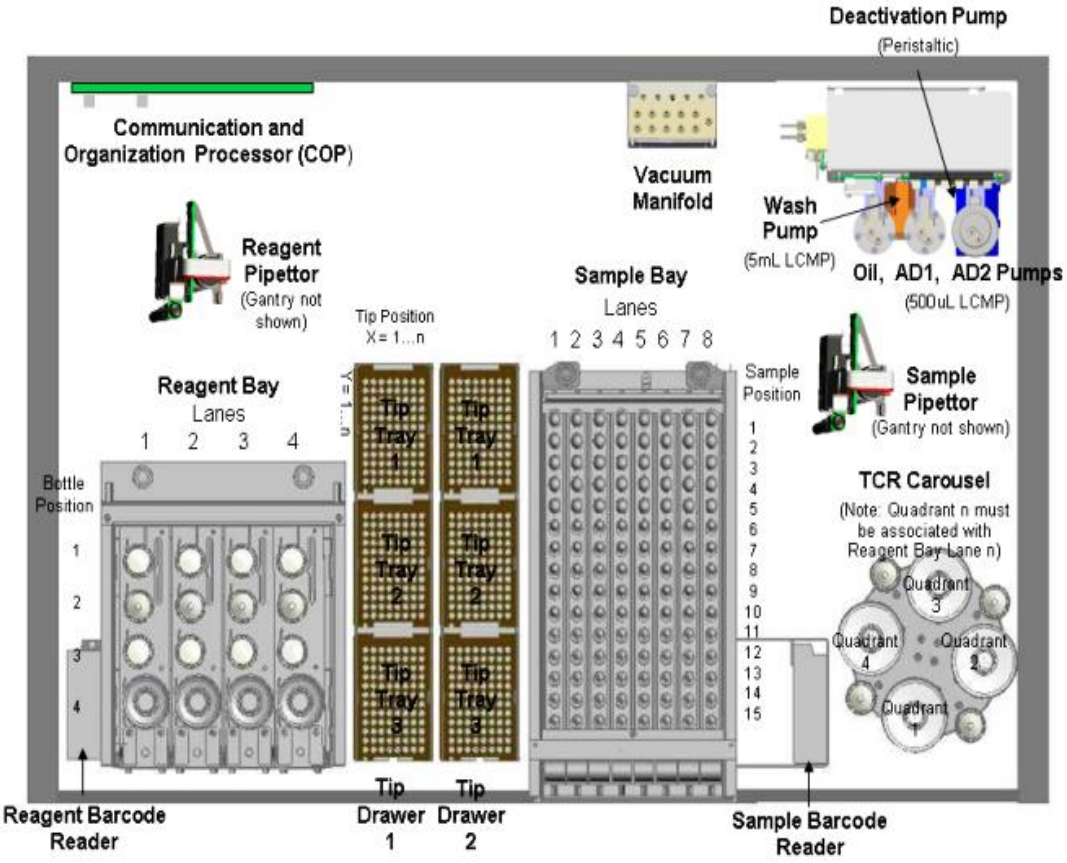
Cell number: Tel number:	
Name and surname:	
Date of birth:	
Donor's signature:	

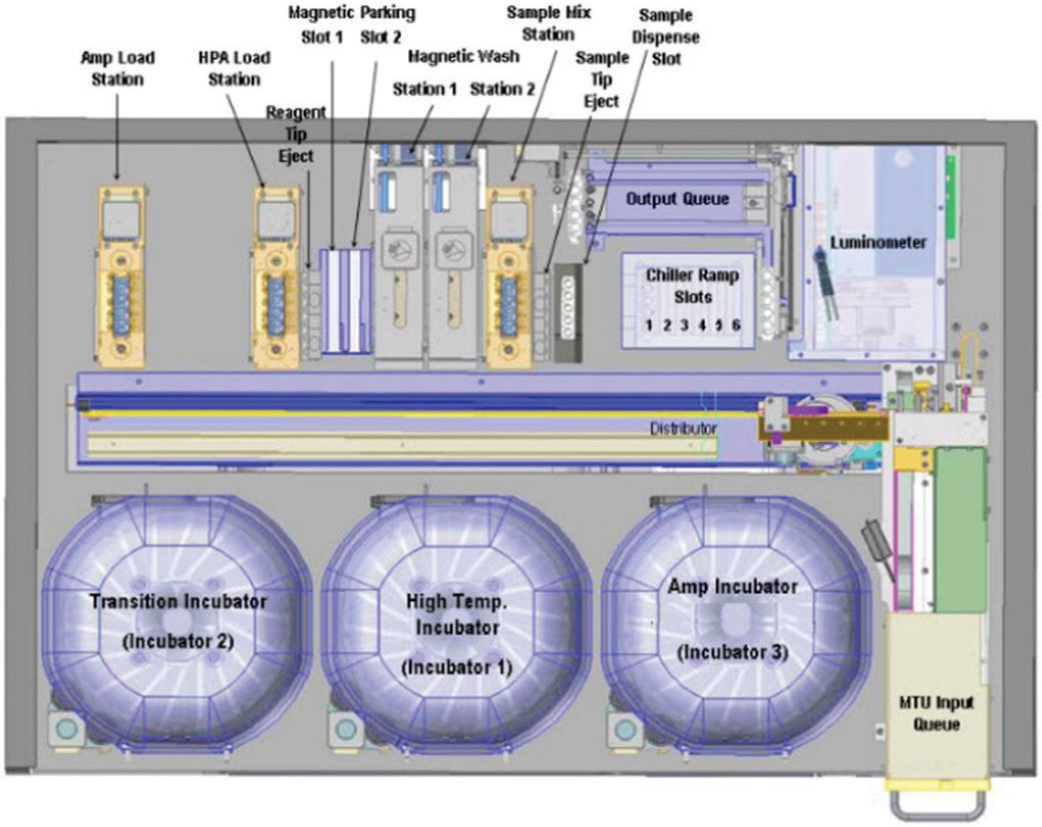
FOR OFFICE USE:				
Interview done	No		Yes	
Signature: Phlebotomist				
Signature: Interviewer (only if interview was done)				

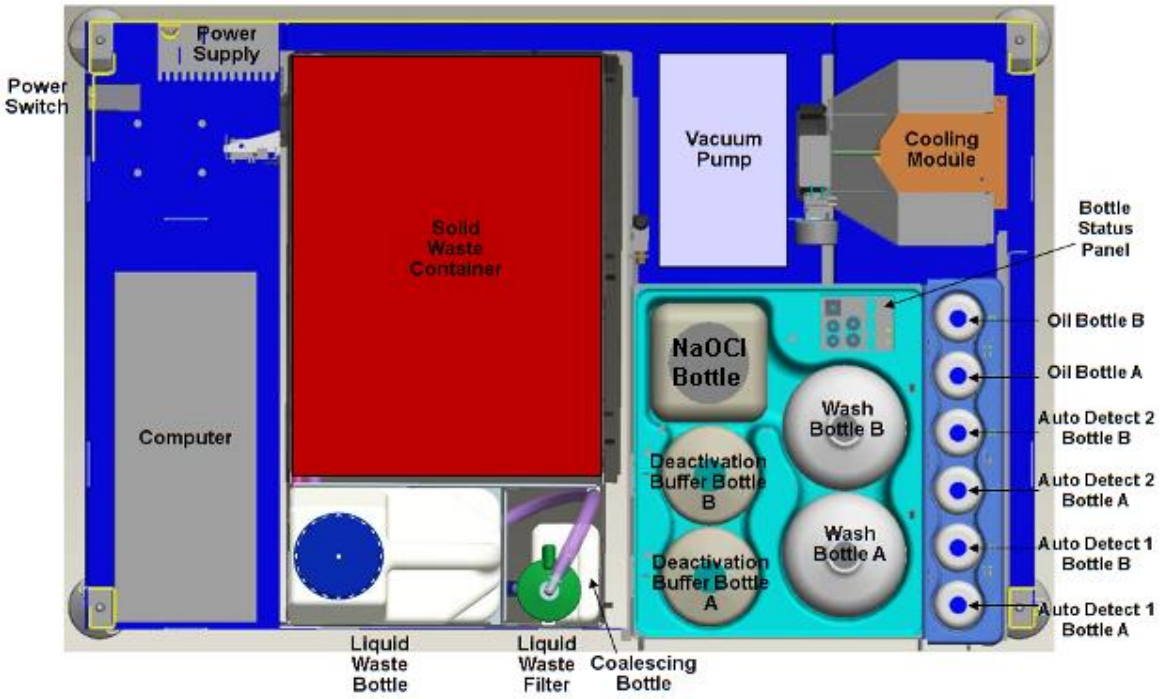
CLN 12 (01 Aug 13)

Addendum 5 – Panther System labelled diagrams of Upper, Mid and Lower Bays respectively

(GRIFOLS Procleix HEV Assay for Procleix Panther System – Operator Training)







Addendum 6 – Procleix HEV Assay results interpretation

(GRIFOLS Procleix HEV Assay package insert – page 13 of 21)

INTERPRETATION OF RESULTS

All calculations described above are performed by the Procleix Panther System Software. Two cutoffs are determined for each assay: one for the Analyte Signal (glower signal) termed the Analyte Cutoff and one for the Internal Control Signal (flasher signal) termed the Internal Control Cutoff. The calculation of these cutoffs is shown above. For each sample, an Analyte Signal RLU value and Internal Control Signal RLU value are determined. Analyte Signal RLU divided by the Analyte Cutoff is abbreviated as the Analyte Signal/Cutoff (S/CO) on the report.

A specimen is Nonreactive if the Analyte Signal is less than the Analyte Cutoff (i.e., Analyte S/CO <1.00) and the Internal Control (IC) Signal is greater than or equal to the Internal Control Cutoff (IC Cutoff) and less than or equal to 750,000 RLU. A specimen is Reactive if the Analyte Signal is greater than or equal to the Analyte Cutoff (i.e., Analyte S/CO ≥ 1.00), and the Analyte Signal is less than or equal to 5,100,000 RLU and the IC Signal is less than or equal to 750,000 RLU. Reactive results will be designated by the software. A specimen is invalid if the Analyte Signal is less than the Analyte Cutoff (i.e., Analyte S/CO <1.00) and the Internal Control Signal is less than the Internal Control Cutoff. Any specimen with Internal Control values greater than 750,000 RLU is considered Invalid and the reactive status cannot be assessed.

Cadaveric (non-heart-beating) blood specimens, when tested neat, may be invalid due to inhibitory substances within the specimen. These invalid samples may be diluted as explained in SPECIMEN COLLECTION, STORAGE, AND HANDLING, Cadaveric Blood Specimens, and repeated in singlet.

Summary of Specimen Interpretation:

Specimen Interpretation	Criteria
Nonreactive	Analyte S/CO < 1.00 and IC ≥ IC Cutoff and IC ≤ 750,000 RLU
Reactive	Analyte S/CO ≥ 1.00 and Analyte ≤ 5,100,000 RLU and IC ≤ 750,000 RLU
Invalid*	IC > 750,000 RLU or Analyte S/CO < 1.00 and IC < Cutoff
*For specimens with IC signal greater than 750,000 RLU, the specimen will be invalidated by the software and the reactive status cannot be assessed.	

A. Any specimen with an interpretation of Invalid in the Procleix HEV Assay must be retested.

B. Specimens with a valid Internal Control value and with an Analyte S/CO less than 1.00 in the Procleix HEV Assay are considered Nonreactive for HEV RNA.

C. Specimens with an Analyte S/CO greater than or equal to 1.00 with IC Signal less than or equal to 750,000 RLU are considered Reactive.