IMMUNOGENICITY EVALUATION OF A133 AND Ss-IR: DEVELOPMENT OF VACCINE CANDIDATES AGAINST *Strongyloides stercoralis*

MATTHEW WONG TZE JIAN

UNIVERSITI SAINS MALAYSIA

2024

IMMUNOGENICITY EVALUATION OF A133 AND Ss-IR: DEVELOPMENT OF VACCINE CANDIDATES AGAINST *Strongyloides stercoralis*

by

MATTHEW WONG TZE JIAN

**Thesis submitted in fulfilment of the requirements
for the degree of
Doctor of Philosophy**

February 2024

ACKNOWLEDGEMENT

First and foremost, I would like to convey my heartfelt appreciation to my main supervisor, Associate Professor Dr Tye Gee Jun for his guidance in this doctoral research project. His patience, enthusiasm, and passion has been a refreshing source of inspiration and motivation. It was an honour working with him, a kind-hearted and open-minded supervisor. Next, I express my immense gratitude to my co-supervisor, Professor Dr Rahmah Noordin for her wisdom and guidance that were prudent towards ensuring that I grow and develop into an excellent researcher that can bring an impact to the world in the field of parasitology. It was a privilege working under her, a highly esteemed and reputable professor. I am also grateful to Ms Nor Suhada Anuar for her constant assistance in administrative procedures and inventory management for this research. Furthermore, I would also like to extend my thanks to the Institute for Research in Molecular Medicine (INFORMM), Universiti Sains Malaysia (USM) for the provision and maintenance of laboratory facilities that were essential to my work. Many thanks are also conveyed to the Animal Research and Service Centre (ARASC) for providing the necessary facilities for the mice study phase of the project, especially Dr Isma Suzyta Ismail and Ms Adilah Abdul Khalil for the incessant guidance and support. Besides that, I thank the Malaysian Institute of Pharmaceuticals and Nutraceuticals (iPharm), especially with the help of Ms Ilyana Hakimi, for giving me the wonderful opportunity to utilise their cutting-edge flow cytometry facilities. These research studies definitely cannot be performed and completed without the financial support by the Ministry of Higher Education (MoHE), Malaysia via the Fundamental Grant Research Scheme FRGS/1/2018/SKK08/USM/01/2 and the Higher Institution Centre of Excellence Grant HICoE:311/CIPPM/4401005.

Moreover, I extend my deep appreciation to my colleagues, Dr Sylvia Annabel Dass, Chin Ding Sheng, Jacqueline Mark Kar Kei, Liu Huaqiang and Ahmad Ismail Abdul Abdo, for their fantastic friendship and encouragement throughout my research study, and my juniors, Ding Yi En and Yap Zi Jian for providing me assistance when I needed it. A special mention goes to my senior, Dr Lew Min Han for his guidance and advice, particularly during the early phases of my research. Another honourable mention is to my high school biology teacher, Ms Koh Chian Rou for inspiring me to embark on this journey into biology with her passion in teaching. Furthermore, this research is a testament to the unconditional love for me from my parents, Wong Swee Fatt and Agnes Lee Suan Neo, my uncle and aunt, Wong Swee Peng and Teoh Lay Hoon, my sister, Anna Wong Tze Hui, and other family members, where their unwavering support has helped me to push through difficult times. Finally, above all else, this entire doctoral research and thesis is dedicated to the Lord God Almighty YHWH and Jesus Christ, my Lord and Saviour, for His everlasting love, faithfulness, blessings, and provisions towards me in my life.

"I can do all things through Christ that strengthens me."

– Philippians 4:13 (NKJV)

TABLE OF CONTENTS

ACKNOWLEDGEMENT .... **ii**

TABLE OF CONTENTS .... **iv**

LIST OF TABLES .... **x**

LIST OF FIGURES .... **xii**

LIST OF SYMBOLS .... **xv**

LIST OF ABBREVIATIONS .... **xvii**

LIST OF APPENDICES .... **xxiv**

ABSTRAK .... **xxv**

ABSTRACT .... **xxvii**

CHAPTER 1 INTRODUCTION **1**

1.1 *Strongyloides stercoralis* 1

1.1.1 History of the discovery of *S. stercoralis* and its abilities 1

1.1.2 Taxonomy of *S. stercoralis* 2

1.1.3 Life cycle and pathogenesis of *S. stercoralis* 3

1.1.4 Morphology of *S. stercoralis* 6

1.2 Strongyloidiasis 8

1.2.1 Overview of neglected tropical diseases and strongyloidiasis 8

1.2.2 Epidemiology of strongyloidiasis 9

1.2.2(a) Global epidemiology 9

1.2.2(b) Local epidemiology in Malaysia 12

1.2.3 Transmission of strongyloidiasis 13

1.2.4 Diagnosis of strongyloidiasis 15

1.2.4(a) Direct diagnostic methods 17

1.2.4(a)(i) Stool microscopy, and larvae concentration and culture methods 17

1.2.4(a)(ii) Deoxyribonucleic acid (DNA) amplification assays 18

1.2.4(a)(iii) Antigen detection assays 20

1.2.4(b) Indirect detection methods 21

1.2.4(b)(i) Absolute eosinophil count 21

1.2.4(b)(ii) Enzyme-linked immunosorbent assay (ELISA) 23

1.2.4(b)(iii) Immunofluorescence antibody test (IFAT) 24

1.2.4(b)(iv) Western blot 25

1.2.4(b)(v) Particle agglutination test 26

1.2.4(b)(vi) Lateral flow rapid test 27

1.2.5 Clinical manifestations of strongyloidiasis 28

1.2.5(a) Acute strongyloidiasis 29

1.2.5(b) Chronic strongyloidiasis 31

1.2.5(c) Severe strongyloidiasis 33

1.2.6 Risk factors for severe strongyloidiasis 35

1.2.6(a) Immunodeficiency diseases 35

1.2.6(b) Comorbidities 38

1.2.6(c) Corticosteroids usage 40

1.2.6(d) Organ transplantation 41

1.2.7 Treatment for strongyloidiasis 42

1.3 Immune response towards *S. stercoralis* 44

1.3.1 Overview of immune responses towards helminths and *S. stercoralis* 44

1.3.2 Innate immune responses 49

1.3.3 Adaptive immune responses 53

1.4 Vaccination 60

1.4.1 History of the invention and development of vaccines 61

1.4.2 Types of vaccines 62
1.5 Development of vaccine against *S. stercoralis* 64
1.5.1 History of strongyloidiasis vaccine development 64
1.5.2 Challenges in developing anthelminthic vaccines 72
1.6 Promising candidates for vaccine against *S. stercoralis* 72
1.6.1 *Strongyloides stercoralis* immunoreactive antigen (Ss-IR) 72
1.6.2 Alpha-133 (A133) 73
1.7 Problem statement and research aim 75
1.8 Research objectives 76
CHAPTER 2 MATERIALS AND METHODS 77
2.1 Rational design of the study 77
2.2 Materials 79
2.2.1 Biological materials and commercial kits 79
2.2.2 Chemical materials 81
2.2.3 Equipment 82
2.2.4 Apparatus 83
2.2.5 Glassware, plasticware and metalware 84
2.2.6 Consumables 84
2.2.7 Computational software 85
2.3 Methodology 86
2.3.1 Production of recombinant proteins A133 86
2.3.1(a) Expression of A133 in *Escherichia coli* 86
2.3.1(b) Bacterial cell lysis of A133/pET32a/C41(DE3) 87
2.3.1(c) Native nickel-nitriloacetic acid (Ni-NTA) purification of A133 87
2.3.1(d) Protein concentration and buffer exchange of A133 88
2.3.2 Production of recombinant protein Ss-IR 89
2.3.2(a) Expression of Ss-IR in *Escherichia coli* 89

2.3.2(b) Bacterial cell lysis of rSsIR/pET32a/C41(DE3) 90

2.3.2(c) Native Ni-NTA purification of Ss-IR 90

2.3.2(d) Protein concentration and buffer exchange of Ss-IR 91

2.3.3 Analytical and secondary purification procedures of recombinant antigens 91

2.3.3(a) Sodium dodecyl sulphate – polyacrylamide gel electrophoresis (SDS-PAGE).. 91

2.3.3(b) Western blot 93

2.3.3(c) Protein quantification by RC DC protein assay 95

2.3.3(d) Native SDS-PAGE (NSDS-PAGE) 95

2.3.3(e) Electro-elution.. 96

2.3.4 Preparation of vaccines.. 99

2.3.4(a) Preparation of priming vaccine 99

2.3.4(b) Preparation of booster vaccines 100

2.3.4(c) Preparation of challenge vaccines................................... 101

2.3.5 *In vivo* immunisation of BALB/c mice 101

2.3.5(a) Animal model and care 101

2.3.5(b) Immunisation regimen of A133 and Ss-IR 102

2.3.5(c) Method of vaccine administration 103

2.3.5(d) Mice sacrifice 104

2.3.6 Collection and preparation of biological samples for analysis......... 104

2.3.6(a) Whole blood.. 104

2.3.6(b) Serum 104

2.3.6(c) Peripheral blood mononuclear cells (PBMCs) 105

2.3.6(d) Splenocytes 106

2.3.6(e) Cell counting procedure and cell viability test 106

2.3.7 Analysis of mice antibody profiles upon immunisation................... 107

2.3.7(a) Ig ELISA protocol... 108

2.3.7(b) Optimisation of antigen coating concentration 109

2.3.7(c) Titration Ig ELISA 110

2.3.7(d) Time-course Ig ELISA 110

2.3.8 Analysis of immune cell responses upon vaccination via immunophenotyping 111

2.3.8(a) Fluorochrome-conjugated antibodies staining of PBMCs 111

2.3.8(a)(i) Surface staining of PBMCs 111

2.3.8(a)(ii) Intracellular staining of PBMCs 112

2.3.8(b) Operation and maintenance of flow cytometer 113

2.3.8(b)(i) Daily clean and top-up of sheath fluid of flow cytometer 113

2.3.8(b)(ii) Calibration of flow cytometer 113

2.3.8(b)(iii) Compensation of 4-colour fluorophores 114

2.3.8(b)(iv) Data acquisition on flow cytometer 114

2.3.8(c) Analysis and gating strategies of flow cytometry data 115

2.3.8(c)(i) Forward and side scatter analysis 116

2.3.8(c)(ii) $CD8^+$ T-cell population and its subsets 117

2.3.8(c)(iii) Regulatory T-cells (Treg) 118

2.3.8(c)(iv) Memory B-cells 119

2.3.9 Analysis of cytokine secretion by post-vaccinated mouse splenocytes when stimulated *in vitro* using antigens 120

2.3.9(a) *In vitro* stimulation of mouse splenocytes 120

2.3.9(b) Cytokine ELISA 121

2.3.10 Statistical analyses 122

CHAPTER 3 RESULTS 123

3.1 Production of recombinant antigens A133 and Ss-IR in *Escherichia coli* 123

3.2 Antibody profile of mice after immunisation with A133 and Ss-IR 131

3.3 CD8$^+$ T-cell, regulatory T-cell, and memory B-cell responses 143

3.4 Post-immunisation T-cell cytokine profile 145

CHAPTER 4 DISCUSSION 147

CHAPTER 5 CONCLUSION 175

REFERENCES 178

APPENDICES

LIST OF PUBLICATIONS

LIST OF TABLES

Page

Table 1.1 Taxonomic lineage of *S. stercoralis*………………………………….. 3

Table 1.2 Summary of recent studies about the prevalence of strongyloidiasis in various countries……………………………….... 12

Table 1.3 Summary of the symptoms for each stage of strongyloidiasis ……..... 30

Table 1.4 Previous studies on vaccines against *Strongyloides stercoralis* ……. 69

Table 2.1 List of antibodies used in this study ……………………………….. 79

Table 2.2 List of animals and immunological reagents used in this study ……. 80

Table 2.3 List of bacterial strains used in this study…………………………… 80

Table 2.4 List of cytokines and immunostimulants used in this study ……….... 80

Table 2.5 List of molecular reagents used in this study ……………………….. 80

Table 2.6 List of commercial kits used in this study ………………………… 81

Table 2.7 List of general chemicals used in this study ……………………….... 81

Table 2.8 List of reagents and media used for bacterial cell culture in this study ………………………………………………………… 82

Table 2.9 List of reagents and media used for animal cell culture in this study ………………………………………………………… 82

Table 2.10 List of equipment used in this study ……………………............... 82

Table 2.11 List of apparatus used in this study ………………………………… 83

Table 2.12 List of glassware, plasticware, and metalware used in this study……………………………………………………………. 84

Table 2.13 List of consumables used in this study …………………………….... 84

Table 2.14 List of computational software used in this study…………………... 85

Table 2.15 Recipe for SDS-PAGE gel………………………………………… 92

Table 2.16 Fluorochrome-conjugated antibodies used in this study to examine cell populations of interest ………………………………… 112

Table 3.1 Results for RCDC protein assay to determine protein concentration ……………………………………………... 125

Table 3.2 Results of the optimisation of antigen coating concentration for Ig ELISA ……………………………………………………….. 132

Table 3.3 Titration Ig ELISA cut-off values and antibody titres …………….. 139

Table 3.4 Results of titration ELISA for A133 ……………………………… 140

Table 3.5 Results of titration ELISA for Ss-IR ……………………………… 141

Table 3.6 Calculation of cut-off value from titration ELISA results ………… 142

LIST OF FIGURES

Page

Figure 1.1 Life cycle of *S. stercoralis*.. 4

Figure 1.2 Microscopic images and schematic morphology of different stages of *S. stercoralis*... 7

Figure 1.3 Raster map of the global distribution of *S. stercoralis* infection.. 11

Figure 1.4 Graphical summary of common and popular diagnostic methods for strongyloidiasis.. 16

Figure 1.5 Immune reaction towards *S. stercoralis* according to the chronological order of infection.. 47

Figure 1.6 Overview of immune response against helminths........................ 48

Figure 1.7 Innate immune response against *S. stercoralis*............................ 49

Figure 1.8 Adaptive immune response against *S. stercoralis*....................... 54

Figure 1.9 Serological profile of immunocompetent individuals infected with *S. stercoralis* 59

Figure 2.1 Experimental design flowchart 78

Figure 2.2 The arrangement of the glass tube, silicone adaptor, Frit and membrane cap... 97

Figure 2.3 Milky-white emulsion of CFA with PBS............................... 100

Figure 2.4 Immunisation regimen against recombinant A133 and Ss-IR proteins.. 102

Figure 2.5 Vaccine administration routes 103

Figure 2.6 Formula for calculating cell concentration, cell viability, and absolute cell count from a haemocytometer adapted from Strober (2015) 107

Figure 2.7 Formulae used to calculate the cut-off value for titration ELISA, adapted from Frey et al. (1998) 110

Figure 2.8 Example of identifying the target population using unstained and FMO controls by Bushnell (2015) 116

Figure 2.9 Identification of singlet lymphocytes in mouse PBMC population ...117

Figure 2.10 Gating strategy for acquiring CD8+ T-cell population and its subsets 118

Figure 2.11 Gating strategy for acquiring regulatory T-cell population 119

Figure 2.12 Gating strategy to acquire memory B-cell population 120

Figure 3.1 Purification profile of histidine-conjugated recombinant (A) A133 and (B) Ss-IR protein 124

Figure 3.2 Standard curve of protein concentration against absorbance at 750 nm for RC DC protein assay 125

Figure 3.3 SDS-PAGE gel (10%) of protein leakage in washing buffers of histidine-conjugated recombinant (A) Ss-IR and (B) A133 protein 127

Figure 3.4 SDS-PAGE gel (10%) of extended incubation time of cell lysate with purification resin during the purification of histidine-conjugated recombinant (A) Ss-IR and (B) A133 protein 129

Figure 3.5 Expression of histidine-conjugated recombinant A133 and Ss-IR proteins 130

Figure 3.6 Total IgG antibody responses generated after immunisation with recombinant A133 and Ss-IR, respectively ………………….. 134

Figure 3.7 IgG1 antibody responses generated after immunisation with recombinant A133 and Ss-IR, respectively ………………….. 136

Figure 3.8 IgG2a antibody responses generated upon immunisation with recombinant A133 and Ss-IR, respectively ………………….. 138

Figure 3.9 Immune cell response after immunisation with recombinant A133 and Ss-IR …………………………………………………… 144

Figure 3.10 Cytokine secretion of mouse splenocytes stimulated by recombinant A133 and Ss-IR protein …………………………….... 146

Figure 4.1 Influence of cytokines in the Ig class switch recombination (CSR) in mature B-cells ……………………………………………………. 162

Figure 4.2 B-cell maturation, activation, and class switch recombination …… 163

Figure 4.3 The quartet model of murine IgG function ……………………….... 164

Figure 4.4 The immunological synapses between antigen presenting cells, CD4+ T-cells, and B cells ………………………………………… 168

Figure 4.5 Crosstalk between Th1, Th2, Treg, and Th17 cells in response to helminthic infections and subsequent elimination of larvae, drawn using BioRender …………………………………………… 174

LIST OF SYMBOLS

%	Percent
~	Approximately
+	Add
<	Less than
=	Equal to
>	More than
±	More or less
× g	Multiply by gravitational value
°	Degree angle
A_{405nm}	Absorbance at wavelength 405 nm
℃	Celsius
cm	Centimetre
g	Gram
h	Hour
kDa	Kilodalton
L	Litre
M	Molar
mA	Milliampere
mg	Milligram
min	Minute
mL	Millilitre

mm	Millimetre
mM	Millimolar
n	Number of
ng	Nanogram
nm	nanometre
RT	Room temperature
s	Second
™	Trademark
V	Volt
α	Alpha
β	Beta
γ	Gamma
μg	Microgram
μL	Microliter
μm	Micrometre

LIST OF ABBREVIATIONS

A133	Alpha-133
AAM	Alternatively activated macrophages
ABTS	2,2’-azino-bis (3-ethylbenzothaizoline-6-sulfonic acid)
ADCC	Antibody-dependent cellular cytotoxicity
AIDS	Acquired immunodeficiency syndrome
Amp	Ampicillin
ANOVA	Analysis of variance
APC	Allophycocyanin
APCs	Antigen presenting cells
APS	Ammonium persulfate
ARASC	Animal Research and Service Centre
ATL	Adult T-cell leukaemia
BCG	Bacille Calmette–Guerin
BCR	B-cell receptor
BSA	Bovine serum albumin
CASAC	Combined Adjuvant for Synergistic Activation of Cellular immunity
CD	Cluster of differentiation
CDC	Centres for Disease Control and Prevention
CFA	Complete Freund's adjuvant
CO_2	Carbon dioxide
COPD	Chronic obstructive pulmonary disease

CSR	Class switch recombination
Ctrl	Control
DC	Dendritic cell
dH20	Deionised water
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DOC-Ag	Antigen solubilised in deoxycholic acid
DOCSv	Antigen solubilised in deoxycholic acid derived from *S. venezuelensis*
E/S	Excretory/secretory
ECP	Eosinophil cationic protein
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EMA	European medicines agency
EPO	Eosinophil peroxidase
et al.	*Et alia* (and others)
FACS	Fluorescence-activated cell sorting
FBS	Foetal bovine serum
Fc	Fragment crystallizable region of antibody
FcR	Fc receptor
FcγR	Fc gamma receptor
FDA	Food and Drug Administration
FITC	Fluorescein isothiocyanate
FMO	Fluorescence minus one
FoxP3	Forkhead box protein P3

FSC	Forward scatter
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GPCR	G protein-coupled receptors
His	Histidine
HIV	Human immunodeficiency virus
HLTV-1	Human T-lymphotropic virus type-1
HPV	Human papillomavirus
HRM	High-resolution melting
HRP	Horseradish peroxidase
HSCT	Haematopoietic stem cell transplantations
HSD	Honest significant difference
IACUC	Institutional Animal Care and Use Committee
IFA	Incomplete Freund's adjuvant
IFAT	Immunofluorescence antibody test
IFN	Interferon
Ig	Immunoglobulin
IgA	Immunoglobulin isotype A
IgE	Immunoglobulin isotype E
IgG1	Immunoglobulin isotype G class 1
IgG2	Immunoglobulin isotype G class 2a
IgG3	Immunoglobulin isotype G class 3
IgG4	Immunoglobulin isotype G class 4
IgM	Immunoglobulin isotype M
IgY	Immunoglobulin isotype Y (Avian)
IL	Interleukin

iL3	Infective third-stage larvae
$iL3^+$	Infective larvae that conduct autoinfection
$iL3^{host}$	Host-adapted *S. stercoralis* larvae
IMAC	Immobilised metal affinity chromatography
IPTG	Isopropyl β-D-1-thiogalactopyranoside
ITS	Internal transcribed spacer
KC	Keratinocyte cytokine
L1	First stage larvae
L2	Second stage larvae
L4	Fourth stage larvae
LAMP	Loop-mediated isothermal amplification
LC-MS/MS	Liquid chromatography with tandem mass spectrometry
MBP	Major basic protein
MHC	Major histocompatibility complex
MIP-22	Macrophage inflammatory protein-2
MMR	Measles, Mumps and Rubella
MPO	Myeloperoxidase
mRNA	Messenger ribonucleic acid
mtDNA	Mitochondrial DNA
NaH_2PO_4	Sodium dihydrogen phosphate
$NaHCO_3$	Sodium bicarbonate
NaOH	Sodium hydroxide
NaOH	Sodium hydroxide
NC	Nitrocellulose
NET	Neutrophil extracellular DNA traps

Ni-NTA	Nickel-nitriloacetic acid
NK	Natural killer cell
NKT	Natural killer T-cell
NSDS-PAGE	Native SDS-PAGE
NTD	Neglected tropical diseases
PAL	Hydroalcoholic extracts of *Phlebodium pseudoaureum*
PALS	Periarteriolar lymphoid sheath
PAMP	Pathogen-associated molecular pattern
PB	Pacific blue
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PBS-T	Phosphate buffered saline with Tween-20 detergent
PCR	Polymerase chain reaction
PE	Phycoerythrin
PerCP-Cy5.5	Tandem fluorochrome that combines Peridinin-Chlorophyll-Protein with a cyanine
pF	Parthenogenetic female
pH	Potential of hydrogen
PMA	Phorbol myristate acetate
PMT	Photomultiplier tube
PPE	Personal protective equipment
QC	Quality control
qPCR	Real-time PCR
Qs	*Quillaja saponaria*
RBC	Red blood cells

rDNA	Ribosomal DNA
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
rpm	Revolutions per minute
RPMI-1640	Roswell Park Memorial Institute 1640 medium
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEM	Standard error of mean
SHM	Somatic hypermutation
SLE	Systemic lupus erythematosus
SOT	Solid organ transplantations
spp.	Species
SSC	Side scatter
Ss-IR	*S. Stercoralis* immunoreactive antigen
TB	Terrific broth
TBS	Tris-buffered saline
TBS-T	Tris-buffered saline with Tween-20 detergent
TEMED	Tetramethylethylenediamine
T_{FH}	Follicular T-helper cells
TGF	Transforming growth factor
Th	T-helper cell
Th1	T-helper 1 type immune response
Th17	T-helper 17 type immune response
Th2	T-helper 2 type immune response
TLR	Toll-like receptor
TNF	Tumour necrosis factor

Treg	Regulatory T-cells
Tris-HCI	Trisaminomethane hydochloride
UK	United Kingdom
USA	United States of America
USM	Universiti Sains Malaysia
UV	Ultraviolet
v/v	Volume over volume
w/v	Weight over volume
WHO	World health organization

LIST OF APPENDICES

APPENDIX A ANIMAL ETHICS APPROVAL

PENILAIAN IMUNOGENISITI A133 DAN Ss-IR: PEMBANGUNAN CALON VAKSIN TERHADAP *Strongyloides stercoralis*

ABSTRAK

Strongyloidiasis adalah satu penyakit yang disebabkan oleh cacing nematoda *Strongyloides stercoralis*, dan merupakan ancaman terhadap kesihatan awam sejagat. Sehingga kini, tiada vaksin terhadap strongyloidiasis wujud, dan tidak terdapatnya pembangunan vaksin yang ketara sejak penemuan protein rekombinan Ss-IR sebagai calon vaksin yang baik pada tahun 2011. Dari segi pembangunan terbaru diagnostik strongyloidiasis, terdapatnya penemuan A133 sebagai antigen diagnosis yang bagus pada tahun 2021, dan ini mencetuskan keingintahuan terhadap potensinya sebagai calon vaksin. Oleh itu, kajian ini bertujuan untuk menilai keberkesanan protein rekombinan A133, dibandingkan dengan Ss-IR, sebagai calon vaksin terhadap *S. stercoralis*, dengan menyiasat tindak balas imun sel dan humoral dalam tikus yang diimunisasikan. Antigen masing-masing telah dicampurkan dengan adjuvan Freund Lengkap (CFA) (dos primer) dan adjuvan Freund Tidak Lengkap (IFA) (dos galakan) dan diberi kepada tikus BALB/c betina menggunakan laluan suntikan intraperitoneum (dos primer) dan subkutanes (dos galakan). Untuk dos cabaran, hanya antigen tanpa sebarang adjuvan disuntikkan secara subkutanes. ELISA immunoglobulin telah dijalankan untuk menilai tindak balas khusus antibodi, bersama dengan sitometri aliran dan ELISA sitokin untuk memahami tindak balas sel imun. Keputusan kajian ini telah menunjukkan bahawa A133 dan Ss-IR merangsangkan penjanaan antibodi IgG1 dan IgG2a, dengan A133 menjana IgG2a yang lebih tinggi berbanding Ss-IR. Justeru, sitometri aliran telah menunjukkan peningkatan ketara dalam hasilan limfosit T CD8^{+}

dan sel ingatan B oleh A133 sahaja, dan penghasilan sel T regulatori tidak dipertingkatkan oleh immunisasi dengan antigen masing-masing. Di samping itu, ELISA sitokin menunjukkan bahawa tindak balas campuran Th1/Th2/Th17 telah dirangsangkan oleh imunisasi dengan A133 atau Ss-IR. Respon Th17 yang dirangsangkan oleh antigen masing-masing amat menarik, kerana Th17 membantu dalam perekrutan neutrophil yang sangat penting untuk pembunuhan larvae. Kesimpulannya, kajian pendahuluan ini menunjukkan potensi berharapan A133 sebagai calon vaksin yang lebih baik berbanding Ss-IR. Kajian ini juga menambah pengetahuan tentang mekanisme keimunan yang berkemungkinan dalam pertahanan keimunan oleh hos terhadap *S. stercoralis*.

IMMUNOGENICITY EVALUATION OF A133 AND Ss-IR: DEVELOPMENT OF VACCINE CANDIDATES AGAINST *Strongyloides stercoralis*

ABSTRACT

Strongyloidiasis, caused by the nematode *Strongyloides stercoralis,* remains a threat to global public health. To date, a vaccine against strongyloidiasis remains unavailable and there was no significant development in this area after discovering the potential of recombinant protein Ss-IR as a vaccine candidate in 2011. In light of recent developments in the diagnosis of strongyloidiasis, A133 emerged in 2021 as an excellent diagnostic antigen, prompting curiosity for its potential as a vaccine candidate. Therefore, this study aimed to evaluate the efficacy of recombinant protein A133 in comparison to Ss-IR, as a potential vaccine candidate against *S. stercoralis* by investigating humoral and cellular immune responses in immunised mice. Respective antigens were adjuvanted with Complete Freund's Adjuvant (prime) and Incomplete Freund's Adjuvant (boost) and administered intraperitoneally (prime) and subcutaneously (boost) to female BALB/c mice. For challenge doses, the antigens were injected subcutaneously without adjuvants. Ig ELISAs were conducted to assess specific antibody responses, along with flow cytometry and cytokine ELISA to elucidate cellular immune responses. The results showed that A133 and Ss-IR induced the production of IgG1 and IgG2a, with A133 generating a more robust IgG2a response than Ss-IR. Flow cytometry findings indicated that effector $CD8^+$ T-cells and memory B-cells activity were upregulated significantly for A133 only, and regulatory T-cells were not upregulated. Furthermore, cytokine ELISA demonstrated that a Th1/Th2/Th17 mixed cell response was triggered upon vaccination with either antigen.

The strong induction of the Th17 response by either antigen was interesting, because Th17 facilitates neutrophil recruitment which is essential in larval elimination. In conclusion, this preliminary study illustrates the promising potential of recombinant A133 being a better vaccine candidate against *S. stercoralis* compared to Ss-IR. This study also provided information on the probable immune mechanism involved in host defense against *S. stercoralis*.

CHAPTER 1

INTRODUCTION

1.1 *Strongyloides stercoralis*

1.1.1 History of the discovery of *S. stercoralis* and its abilities

The nematode *Strongyloides stercoralis* was first discovered in French soldiers returning from Vietnam in 1876, when it was still a French colony named Cochin China. The disease it caused was known as the Diarrhoea of Cochin China (Normand, 1876). During that time, it was recognised as two distinct species, *Anguillula stercoralis,* for its larval form (Bavay, 1877a) and *Anguillula intestinalis* for its adult form (Bavay, 1877b). Later, in 1879, Grassi and Parona observed the hatching of *A. intestinalis* eggs in *A. stercoralis* and concluded that they were the same species (Grassi and Parona, 1879). At the same time, Grassi suggested the genus *Strongyloides*, naming the parasite *Strongyloides intestinalis* (Grassi, 1879). However, due to multiple researchers working on the pathogens, it gave rise to different names that caused confusion within the world of parasitology. Finally, in 1902, Stiles and Hassal proposed that *Strongyloides stercoralis* should be correctly named using its first species name (Stiles and Hassal, 1902), and this proposal was later accepted by the International Commission on Zoological Nomenclature (1915).

The discovery of this unique ability was pioneered by Looss, who demonstrated intradermal penetration of the infective larvae by infecting himself in 1904 (Looss, 1905). Subsequently, Friedrich Fulleborn was the first to uncover the ability of the parasite to establish an autoinfection cycle (Fulleborn, 1914). Disseminated strongyloidiasis was first reported by Gill and Bell and was observed among prisoners of war from the Far East, where they had been infected (Gill and Bell, 1979). Complete information about the life cycle, clinical manifestations, and pathogenesis of *S. stercoralis* was described only in the 1930s (Schär *et al.*, 2013).

1.1.2 Taxonomy of *S. stercoralis*

The latest taxonomic lineages of *S. stercoralis* are listed in **Table 1.1** (Schoch *et al.*, 2020). *S. stercoralis* belongs a group of helminths called nematodes, whereby they have a narrow, long, non-segmented, threadlike body. Most parasitic nematodes, such as *S. stercoralis*, belong to the order Rhabditida, which has a study larval stage that can withstand harsh environmental conditions (Kiontke and Fitch, 2013). There are approximately 52 species belonging to the genus *Strongyloides*, all of which are obligate gastrointestinal parasites to a plethora of animal hosts, including birds, mammals, reptiles, and amphibians (Speare, 1989). In humans, only two species with one subspecies can cause the disease: *S. stercoralis*, *S. fuelleborni*, and *S. fuelleborni kellyi* (Schad, 1989). Of these three species, humans are primarily infected by *S. stercoralis* (Viney and Lok, 2015). *S. fuelleborni* prefers to infect non-human primates than humans. It is more commonly detected in African communities that have frequent contact with infected primates (Pampiglione and Ricciardi, 1972; Hira and Patel, 1980). Meanwhile, its subspecies *S. fuelleborni kellyi* is only found exclusively in humans living in Papua New Guinea, where it typically causes the swollen belly syndrome in neonates, which can be fatal (Ashford and Barnish, 1989; Ashford, Barnish and Viney, 1992).

Table 1.1 Taxonomic lineage of *S. stercoralis* (Schoch *et al.*, 2020).

Taxonomic rank	**Name**
Super-kingdom	Eukaryota
Kingdom	Metazoa
Clade	Ecdysozoa
Phylum	Nematoda
Class	Chromadorea
Order	Rhabditida
Sub-order	Tylenchina
Infra-order	Panagrolaimomorpha
Super-family	Strongyloidoidea
Family	Strongyloididae
Genus	*Strongyloides*
Species	*stercoralis*

1.1.3 Life cycle and pathogenesis of *S. stercoralis*

Figure 1.1 illustrates the life cycle of *S. stercoralis*. As shown in **Figure 1.1**, its life cycle can be separated into two smaller cycles, alternating between the free-living, sexual cycle in the soil environment and the parasitic, asexual cycle in a host like humans (Siddiqui and Berk, 2001). Like all nematodes, the full cycle of *S. stercoralis* has four larval stages (L1 to L4), and like all Rhabditida, *S. stercoralis* has specialised larvae for survival, dispersion, and infection, which are the infective third-stage filariform larvae (iL3), analogous to the dauer larva. For both cycles, it is important to know that iL3 is the main culprit responsible for host invasion, transmission, and autoinfection (Hotez, Hawdon and Schad, 1993; Kiontke and Fitch, 2013). Compared to other helminths, *S. stercoralis* is the only helminth that secretes larvae in stool, while other helminths usually secrete eggs in stool (Ganesh and Cruz, 2011). Because of that, the eggs of *S. stercoralis* cannot be found during stool microscopy.

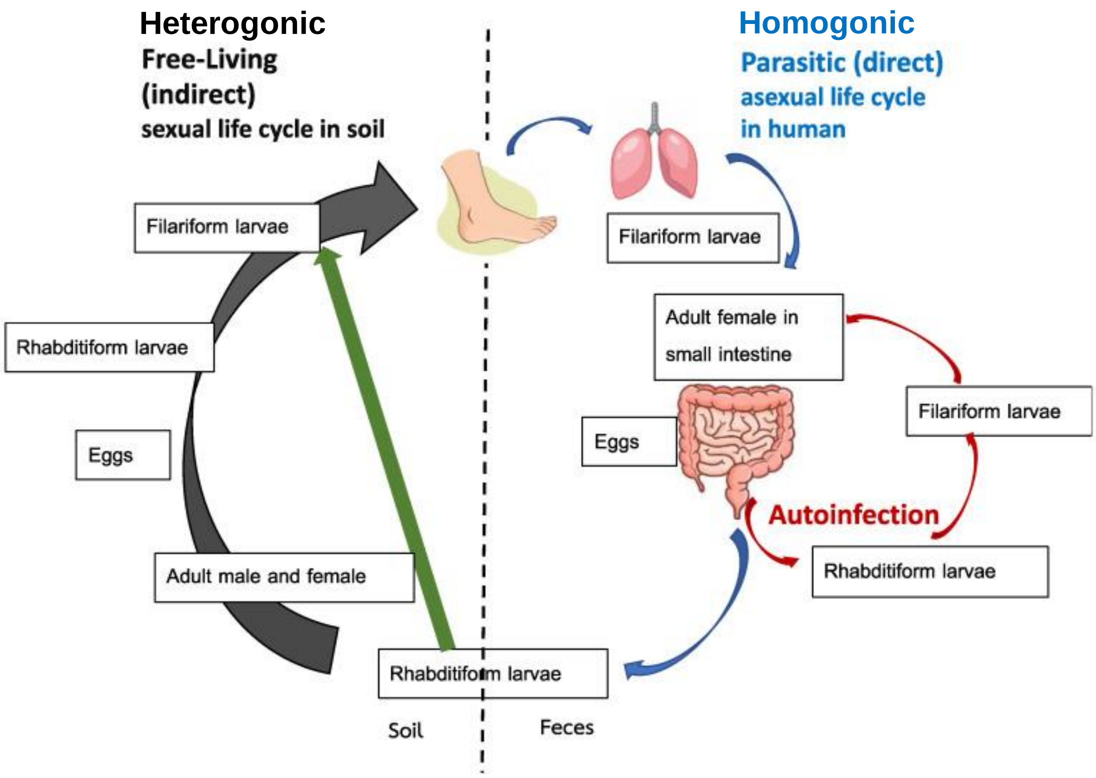

Figure 1.1 **Life cycle of *S. stercoralis*** (Siddiqui and Berk, 2001).

In the soil environment, the free-living cycle can be further subdivided into two routes: (1) direct, homogonic cycle and (2) indirect, heterogonic cycle. The first stage of rhabditiform larvae (L1) is usually found in contaminated faeces. In the homogonic cycle, L1 can directly moult into iL3, transitioning via the second stage (L2). iL3 cansurvive in suboptimal environments until it encounters a host to begin the parasitic cycle (Grove, 1996; Streit, 2008). On the other hand, the heterogonic cycle is also known as a sexual cycle, as it involves the development of free-living male and female adult worms. L1 goes through four moulting processes, transitioning between larval phases L2, L3, and L4, and finally develops into adult worms. Adult worms can only survive for one generation for approximately two to four days. During this period, male and female free-living adults need to mate to produce more eggs, which eventually develop into iL3s (Schad, 1989; Conway *et al.*, 1995; Streit, 2008). At this point, for both the homogonic and heterogonic cycles, the iL3s need to infect a host; otherwise, it will die and hence be unable to develop into adult worms. In suitable environments, the maximum period of the heterogenic cycle is three weeks (Schad, 1989; Yamada *et al.*, 1991). This free-living cycle helps the parasite survive in the environment for a while without a mammalian host (Iriemenam *et al.*, 2010).

As the name implies, the parasitic cycle occurs mainly within the host. It starts with skin penetration by iL3 when a host comes into contact with iL3-contaminated soil. The larvae then migrate via subcutaneous tissue into the circulatory system to the alveolar regions of the lungs, where they ascend the tracheobronchial branch and subsequently swallowed into the gastrointestinal tract (Mansfield *et al.*, 1996; Nutman, 2017). There, iL3 goes through the moulting process twice, transitioning into female parasitic adults. Adult females tunnel into the intestinal lining and produce eggs via parthenogenesis at the intestinal mucosal layer. The eggs hatch into L1 and travel through the intestinal lumen. At this stage, two things can occur: L1 will either be excreted to the external environment to start the free-living cycle or initiate autoinfection within the host and continue the parasitic cycle (Mansfield *et al.*, 1996; Viney and Lok, 2015). Autoinfection is the reason *S. stercoralis* can persist in the host for decades without being eradicated, as long as 75 years in an immunocompetent host (Prendki *et al.*, 2011). Within the genus of *Strongyloides*, this ability of autoinfection surprisingly only exists in *S. stercoralis*, and not other species such as *S. fuelleborni* which is capable of migration only (Lukeš *et al.*, 2014). This is extremely dangerous and worrying because an *S. stercoralis* infection cannot be completely cleared by the host's immune system owing to the extraordinary ability of the pathogen to establish repeated cycles of autoinfection, and thus exists in equilibrium with the host, unless the host's immune system is compromised which results in life-threatening severe strongyloidiasis, or intervention via anthelminthic medication is administered to the patient (Kalambay *et al.*, 2017; Luvira *et al.*, 2022). There are two types of autoinfection: (1) internal autoinfection, where iL3 (developed from L1 of the parasitic cycle) invades the intestinal mucosa, and (2) external autoinfection, where iL3 penetrates the skin in the perianal area (Neva, 1986; Siddiqui and Berk, 2001).

1.1.4 Morphology of *S. stercoralis*

Figure 1.2 shows microscopic images and schematic morphology of the various stages of *S. stercoralis*. The morphological features of *S. stercoralis* have been well established since the 1980s and described by several authors. Since then, there have been no significant publications or changes in the descriptions. The following paragraphs summarise the morphological characteristics of *S. stercoralis* based on the work of these authors (Little, 1966; Schad, 1989; Speare, 1989; Grove, 1996).

The eggs of *S. stercoralis* are small, oval-shaped bodies with a thin shell, measuring between 50 and 58 µm in length and 30 and 34 µm in width. They are partially embryonated during the two- to eight-cell stage of development. The eggs of parasitic and free-living females are similar, but those of parasitic females are rare and typically hatch in the crypts of Lieberkühn.

Parasitic female adults have a length between 2.1 mm and 2.7 mm, and a diameter between 30 mm and 40 mm. Its prominent characteristics are a lengthy filariform oesophagus, accounting for almost one-third of its body length, and a pointed blunt tail. The female is embedded in the submucosal layer of the anterior small intestine, particularly in the duodenum and upper jejunum, but can also be found in other areas of the gastrointestinal tract. In severe strongyloidiasis, they can be easily observed in the gut and faeces. Parasitic females reproduce through parthenogenesis, producing approximately 30 – 50 eggs per day, and can survive for up to 5 years.

Free-living male (**Figures 1.2A and 1.2E)** and female (**Figures 1.2B and 1.2F**) adults have a rhabditiform oesophagus which is characterised by a slender, worm-like structure. They are relatively small, up to approximately 1 mm in length and 85 μm in width. Males have two simple spicules, a gubernaculum, and a pointed tail which is ventrally curved. On the other hand, females are stout, with the vulva located near the centre of their bodies.

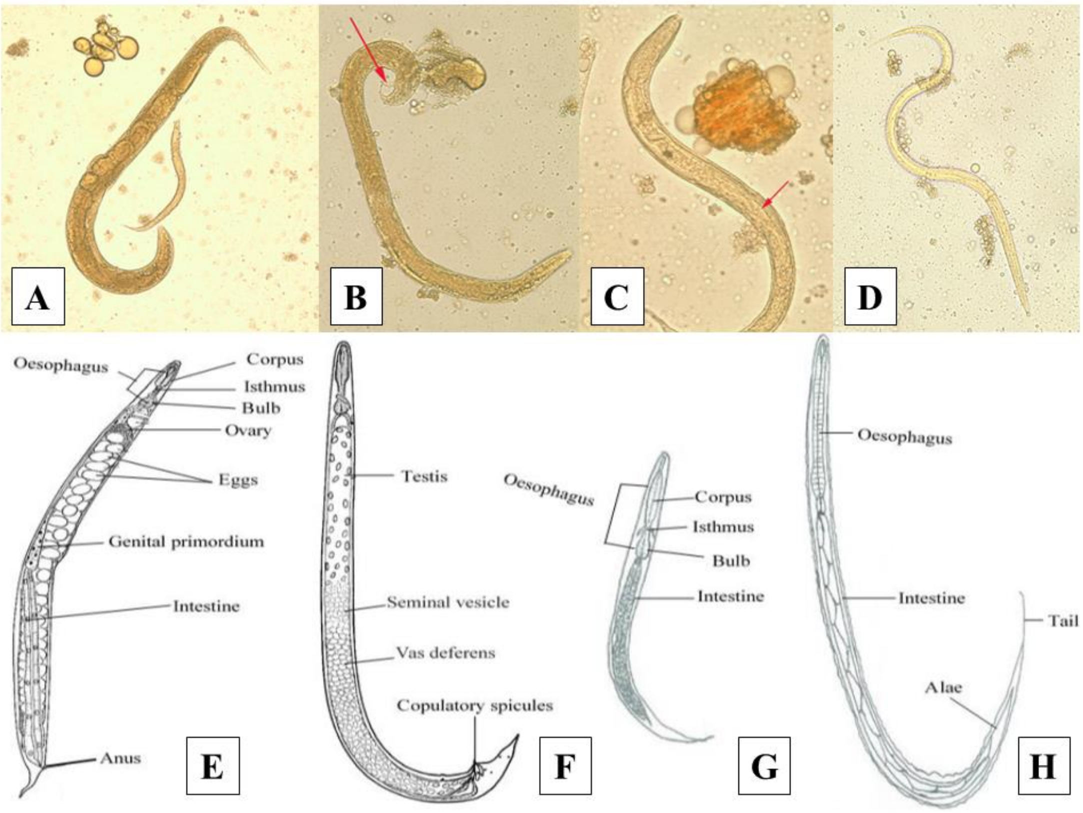

Figure 1.2 **Microscopic images and schematic morphology of different stages of *S. stercoralis*** (Little, 1966; Centres for Disease Control and Prevention, 2019)**.** (A) free-living female, (B) free-living male, (C) rhabditiform larva, (D) infective filariform larvae (iL3).

The L1 to L4 rhabditiform larvae (**Figures 1.2C and 1.2G**) typically had a length ranging from 180 to 240 µm and a width of 15 µm. They also have a rhabditiform oesophagus located in the front one-third of their body. L1 undergoes four moults up to the L4 and adult stages. L2 to L4 have similar characteristics to L1, and their sexual differentiation, head reorganisation, and progressive growth can be observed throughout the developmental process. There is little variation between the L1 rhabditiform larvae originating from free-living and parasitic adults.

Infective third-stage filariform larvae (iL3) (**Figures 1.2D and 1.2H**) can reach approximately 600 µm in length and 15 µm in width. This type of larva has a filariform oesophagus that accounts for 40% of its body length. In the soil, these larvae do not feed and are uncovered with a notched pointed tail and closed mouth. Two types of iL3 larvae have been proposed: (1) infective larvae originating from free-living adults and (2) infective larvae that conduct autoinfection ($iL3^+$) (Nolan *et al.*, 1997). Autoinfective iL3+ larvae have a shorter body, never exceeding 500 mm, and have a more strongyliform oesophagus compared to free-living iL3.

1.2 Strongyloidiasis

1.2.1 Overview of neglected tropical diseases and strongyloidiasis

Strongyloidiasis is a human intestinal parasitosis caused by the nematode *Strongyloides stercoralis and,* to a lesser extent, by *S. fuelleborni* (Siddiqui and Berk, 2001), with the former being the most pathogenic species. The disease does not pose a substantial risk for immunocompetent individuals; however, in immunocompromised or immunosuppressed individuals, it can be fatal and life-threatening despite treatment (Kandi and Bashir Bhatti, 2015; Nutman, 2017).

Strongyloidiasis is part of a group of diseases known as neglected tropical diseases (NTDs). NTDs are still rampant and pose a threat to global public health. NTDs are prevalent in the 'bottom billion' countries plagued by poverty, poor hygiene, and inadequate healthcare facilities and services (Ehrenberg and Ault, 2005; Truscott *et al.*, 2016; Jourdan *et al.*, 2018). Poor sanitation and sewage management systems in these countries encourage the survival of parasites and their eggs in the environment (Jia *et al.*, 2012). Many NTDs are soil-transmitted helminth infections, estimated to affect more than a billion people worldwide (Pullan *et al.*, 2014; Montresor *et al.*, 2020). Soil-transmitted helminth infections with high prevalence include the whipworm *Trichuris trichiura*, roundworms *Ascaris lumbricoides*, threadworm *Strongyloides stercoralis*, and hookworms *Necator americanus* and *Ancylostoma duodenale* (Montresor *et al.*, 2020; Zawawi and Else, 2020). Among these five pathogenic helminths, *S. stercoralis* is the least understood, but there has been steady progress in unravelling knowledge about the pathogen and the disease it causes (Nutman, 2017; Arifin *et al.*, 2019; Vasquez-Rios *et al.*, 2019).

1.2.2 Epidemiology of strongyloidiasis

1.2.2(a) Global epidemiology

The World Health Organization estimates that approximately 30 to 100 million people are infected worldwide, most commonly underdeveloped countries residing in tropical and subtropical regions (World Health Organization, 2023). However, this number is considered an underestimation as such countries do not have the required facilities and techniques to conduct high sensitivity and accuracy tests, such as polymerase chain reaction (PCR), immunofluorescence antibody test (IFAT), and enzyme-linked immunosorbent assay (ELISA). In addition, the infection is often asymptomatic, has symptoms similar to other common non-severe infections, and is difficult to diagnose accurately (Beknazarova, Whiley and Ross, 2016; Levenhagen, Conte and Costa-Cruz, 2016). Using a spatiotemporal statistical modelling approach, it was predicted that in 2017, the global prevalence of strongyloidiasis was 613.9 million (95% CI:313.1 million – 910.1 million) people, which is approximately 8.1% (95% CI:4.2–12.4%) of the global population. (Buonfrate *et al.*, 2020). Furthermore, in 2022, using ecological niche modelling and raster mapping, it was estimated that 2.6 billion people of the world's population are at risk of *S. stercoralis* infection (Fleitas *et al.*, 2022).

In terms of geographic distribution, strongyloidiasis is found in virtually every continent on this planet except Antarctica, but the most prevalent region is in the tropics and subtropics, such as Southeast Asia, Central and South America, sub-Saharan Africa, North Australia, and Sri Lanka which when merged, covers approximately 76.1% of the infected population around the globe. However, it has also been reported in other regions, including the Western Pacific and some parts of Southern Europe. A raster map of the latest estimated global distribution of *S. stercoralis* infection is shown in **Figure 1.3** (Buonfrate *et al.*, 2020; Fleitas *et al.*, 2022; World Health Organization, 2023). Monitoring the trend of global and regional prevalence of strongyloidiasis is extremely difficult due to the dearth of reports or statistics from affected countries, which are affected by various factors such as socioeconomic status, availability of adequate diagnostic technologies, and government public health policies (Beknazarova, Whiley and Ross, 2016).

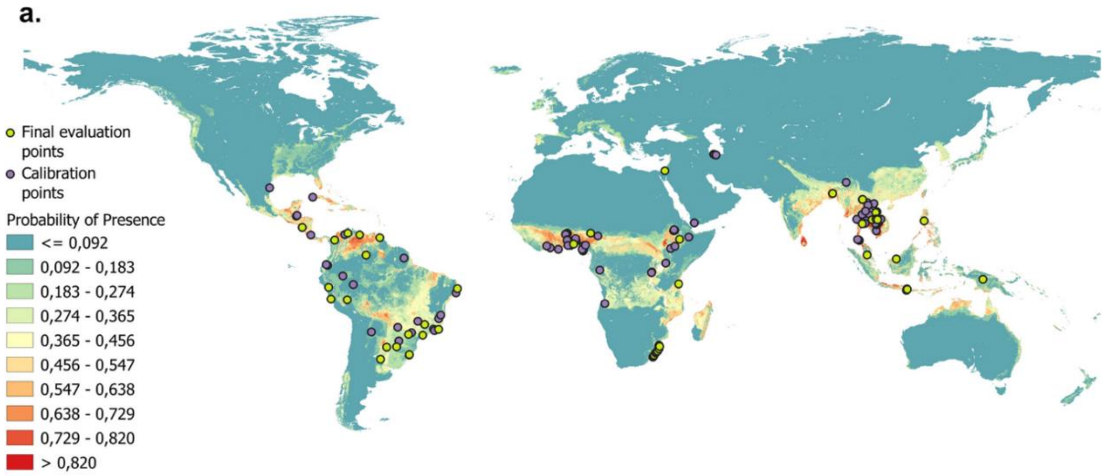

Figure 1.3 **Raster map of the global distribution of *S. stercoralis* infection** (Fleitas *et al.*, 2022)**.**

Nevertheless, based on the limited studies published in recent years, the median prevalence of strongyloidiasis is 25.7%. The recent prevalence studies are presented in **Table 1.2**. For instance, in Cambodia, the prevalence of *S. stercoralis* was 30.5% (Forrer *et al.*, 2019). In Bolivia and Argentina, particularly in the Gran Chaco and Yungas Rainforest regions, the prevalence was 20.9% (Cimino *et al.*, 2020). A survey in Ecuador indicated that the prevalence of strongyloidiasis was 20.7% among the rural population (Guevara *et al.*, 2020). Moreover, a systematic review and meta-analysis by Peru indicated that the prevalence of strongyloidiasis in the general population was 7.3% (Ortiz-Martínez *et al.*, 2021). The prevalence of strongyloidiasis is 55.7% in the rural regions of Ethiopia (Aramendia et al., 2020). In addition, the seroprevalence of *S. stercoralis* among South Indian adults is 33.0% (Munisankar *et al.*, 2022). Among the residents of Papua, Indonesia, the prevalence of strongyloidiasis is 32.0%, which is higher than that of other major soil-transmitted helminths (Kridaningsih *et al.*, 2020). In northern Vietnam, the seroprevalence of *S. stercoralis* is 20.0% (Van De *et al.*, 2019).

Table 1.2 Summary of recent studies about the prevalence of strongyloidiasis in various countries

Country/ Region	Study population	Prevalence (%)	Study type	Diagnostic method	Reference
Cambodia	General with children*	30.5	Cross-sectional	ELISA-*S. ratti* Ag	Forrer et al., 2019
Bolivia & Argentina	General with children*	20.9	Cross-sectional	ELISA-NIE	Cimino et al., 2020
Ecuador (rural)	General with children*	20.7	Cross-sectional	ELISA-*S. ratti* Ag	Guevara et al., 2020
Peru	General with children*	7.3	Systematic review	Heterogenous methods	Ortiz-Martínez et al., 2021
Ethiopia (rural)	General with children*	55.7	Cross-sectional	Baermann technique and qPCR	Aramendia et al., 2020
India (southern)	General	33.0	Cross-sectional	ELISA-NIE	Munisankar et al., 2022
Indonesia (Papua)	General with children*	32.0	Cross-sectional	qPCR	Kridaningsih et al., 2020
Vietnam (northern)	General	20.0	Cross-sectional	ELISA	Van De et al., 2019

Abbreviations: ELISA, enzyme-linked immunosorbent assay; Ag, antigen; qPCR, real-time polymerase chain reaction.
*Children are aged 12 years and below

1.2.2(b) Local epidemiology in Malaysia

Malaysia was not spared of this disease. Among Southeast Asian countries, Malaysia has one of the highest prevalence rates of strongyloidiasis (Schär *et al.*, 2016). However, the same applies here, where there is paucity in the reporting of the disease distribution and prevalence of strongyloidiasis. In 2019, a study found that the prevalence of *S. stercoralis* infection among Orang Asli schoolchildren was 15.2% (Al-Mekhlafi *et al.*, 2019), whereas among migrant workers, the overall seroprevalence was 35.8% using an ELISA commercial kit (Sahimin *et al.*, 2019). In addition, the reported seropositivity rate for Orang Asli communities in Selangor, Peninsular Malaysia was 31.5% (Ahmad *et al.*, 2013), and 11% in East Malaysia

(Sabah and Sarawak) (Ngui *et al.*, 2016). In summary, the median prevalence rate in Malaysia is 11%, with the Orang Asli and migrant workers being the population with the highest risk of having the disease (Rahmah *et al.*, 1997; Basuni *et al.*, 2011; Ahmad *et al.*, 2013; Ngui *et al.*, 2016; Al-Mekhlafi *et al.*, 2019; Sahimin *et al.*, 2019).

1.2.3 Transmission of strongyloidiasis

S. stercoralis infection is most commonly acquired via the transcutaneous route via contact with contaminated soil, thereby earning a spot within the category of soil-transmitted helminths (World Health Organization, 2023). Therefore, the major risk factors for infection by *S. stercoralis* are strongly linked to the exposure of soil contaminated with faeces discharged by *Strongyloides*-positive individuals, coupled with environmental conditions that favour the survival of the helminth's infective larvae (Krolewiecki and Nutman, 2019). The first discovery of the skin-penetrating ability of *S. stercoralis* larvae was credited to a parasitologist named Looss when he infected himself transcutaneously with hundreds of infective larvae and later found larvae in his faeces 64 days post-infection (Looss, 1905). Similarly, because the soil harbours infective *Strongyloides* larvae, strongyloidiasis can be acquired through the gastrointestinal route via contact or ingestion of food grown on contaminated soil and contaminated, unsterilised water. The discovery of the gastrointestinal infection route was made by Wilms when he observed the presence of larvae in faeces 17 days after ingestion of the larva by a human volunteer (Wilms, 1897). Closer to modern times, infective larvae were discovered in vegetables within *Strongyloides*-prevalent regions such as Egypt and Malaysia, causing vegetable farmers, vendors, and workers that prepared vegetables for human consumption to have a high risk of contracting strongyloidiasis when handling infected produce (Zeehaida *et al.*, 2011; El-Badry, Hamdy and Abd El Wahab, 2018). It is easy to see why the tropics and sub-tropics have the highest prevalence of strongyloidiasis because these regions have high humidity, and the people living there regularly walk barefooted and are highly involved in the farming industry. High-risk occupations include gardeners, farmers, miners, and healthcare workers (Puthiyakunnon *et al.*, 2014).

Nonetheless, besides transcutaneous and gastrointestinal routes, there are conjectures about alternative transmission routes, particularly after the observance of a high number of institutionalised *S. stercoralis*-infected patients. Direct person-to-person transmission is rare, but not impossible. One case reported spousal transmission of strongyloidiasis from a husband suffering from hyperinfection to his wife via pulmonary secretions (Czachor and Patrick Jonas, 2000). This observation raises concerns regarding nosocomial transmission. Fortunately, it was proven that adherence to standard precautions by healthcare workers who are in contact with strongyloidiasis patients is sufficient to prevent hospital-acquired infection with this pathogen (Maraha *et al.*, 2001; Sugiyama *et al.*, 2006). Furthermore, in terms of sexual transmission, one study showed that the risk of transmission of strongyloidiasis in heterosexual couples is small but not entirely impossible (Grove, 1982). However, the same cannot be said for homosexual men, as there are several studies denoting a high risk of transmission of strongyloidiasis from one infected partner, especially the passive or receiving counterpart giving it to the other, due to anal penetration, which results in penile contact with the perianal area that is possibly soiled with larvae-infested faeces (Sorvillo *et al.*, 1983; Ross *et al.*, 2020). Apart from that, there are some concerns regarding the possibility of transmammary transmission of strongyloidiasis, but so far, there are no reports of such cases in humans for *S.*

stercoralis. Human transmammary transmission has only been reported for *S. fuelleborni kellyi* (Ashford, Barnish and Viney, 1992), whereas for *S. stercoralis,* it has only been observed in dogs (Shoop *et al.*, 2002; De Liberato *et al.*, 2022).

1.2.4 Diagnosis of strongyloidiasis

Due to the immunological complexity and diagnostic difficulty of this disease, research and development on the diagnosis of strongyloidiasis is very extensive compared to other research fields of this disease. However, to date, there is no gold standard for the diagnosis of strongyloidiasis (Mendes *et al.*, 2017). A graphical summary of common and popular diagnostic tests for strongyloidiasis mentioned in this thesis is presented in **Figure 1.4**. Strongyloidiasis is detected via two categories of methods: direct and indirect. As the name implies, direct diagnostic methods directly detect the presence of *S. stercoralis* eggs, larvae, or adult worms through parasitological or molecular techniques. On the other hand, indirect methods do not detect any components of the parasites, but rather indicators of immune defense against the parasite, such as haematological and serological diagnosis, to determine the presence of parasitic infection, and thus indirectly. Each detection method varies in terms of diagnostic specificity, accuracy, sensitivity, and technological requirements (Siddiqui and Berk, 2001; Kearns *et al.*, 2017).

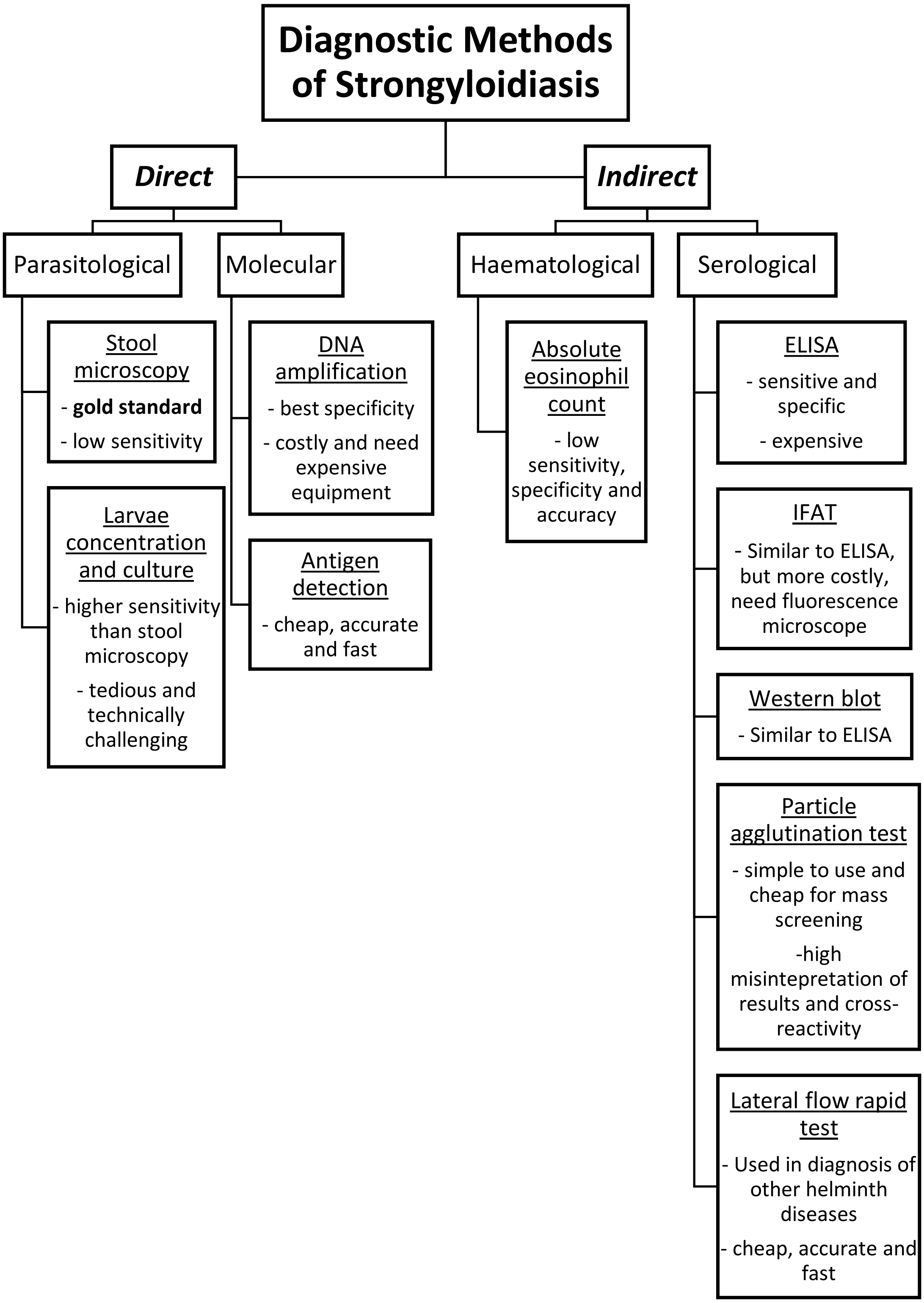

Figure 1.4 **Graphical summary of common and popular diagnostic methods for strongyloidiasis.**

1.2.4(a) Direct diagnostic methods

1.2.4(a)(i) Stool microscopy, and larvae concentration and culture methods

Of all the direct methods, the most common is the microscopic examination of faecal smear, which is also known as stool microscopy. This method is generally considered the gold standard for parasitological detection of many gastrointestinal helminthic diseases, including strongyloidiasis, as it is cheap and does not require sophisticated diagnostic equipment. In such diseases, the eggs and larvae of the parasites are released by adult worms in the lumen of the small intestine or colon and subsequently excreted via faeces. This is the basic operating principle of stool microscopy (Siddiqui and Berk, 2001). However, in strongyloidiasis, eggs and larvae of the parasite are rarely found in the stool of infected patients, especially in acute and asymptomatic infections. Because of this, stool microscopy diagnosis frequently yielded a false negative result, especially at the early stage of infection, where the success rate was only up to 30% for single stool microscopy examinations of infected patients (Montes, Sawhney and Barros, 2010). Therefore, a wise decision is to increase the number of stool microscopy replicates. However, previous studies have shown that the diagnostic sensitivity only increased to 50% with three subsequent examinations, and seven consecutive tests were needed to achieve a sensitivity of nearly 100 %. In typical scenarios, many replicates are impractical. Hence, relying solely on stool microscopy for diagnosis is not rational. as the infection can be overlooked in infected patients (Siddiqui and Berk, 2001; Requena-Méndez *et al.*, 2013). Because of this issue of low diagnostic sensitivity, various concentration and culture methods have been devised to increase parasite detection in stool samples. Examples of concentration methods include the formalin-ether concentration (Ritchie, 1948; Ridley and Hawgood, 1956; Allen and Ridley, 1970) and the Baermann concentration techniques (Baermann, 1917; Graeff-

Teixeira *et al.*, 1997; Hernández-Chavarría and Avendaño, 2001), whereas examples of culture techniques are Harada-Mori (Harada and Mori, 1955; Martín-Rabadán *et al.*, 1999) and Koga agar plate culture techniques (Koga *et al.*, 1991; Kaewrat *et al.*, 2020).

1.2.4(a)(ii) Deoxyribonucleic acid (DNA) amplification assays

There are two subcategories of direct molecular detection techniques, namely deoxyribonucleic acid (DNA) amplification and antigen detection. Molecular detection via DNA amplification has many variations, including conventional polymerase chain reaction (PCR), PCR-restriction fragment length polymorphism (RFLP), multiplex PCR, real-time PCR (qPCR), nested PCR, loop-mediated isothermal amplification (LAMP), and high-resolution melting (HRM)-PCR (Zarlenga and Higgins, 2001; Watts *et al.*, 2014, 2019; Llewellyn *et al.*, 2016; Barda *et al.*, 2018; Fakhrieh-Kashan *et al.*, 2019; Kumar *et al.*, 2021). Although there are many variations in DNA amplification techniques, as listed above, it relies on the basic principle of PCR, which is to use a set of primers that will recognise a designated sequence within the genome of the targeted parasite, subsequently amplifying the sequence and detecting the product of amplification. Because of this characteristic, these methods are preferred by many people, as they generally have a higher diagnostic sensitivity and specificity than other methods (Kadri, 2020). For instance, PCR-RFLP can easily differentiate different isolates, including *S. stercoralis* from dogs or humans, *S. ratti* from rodents, and *S. fuelleborni* from monkeys, by specifically amplifying sequences within nuclear DNA, mitochondrial DNA (mtDNA), 18s and 28s ribosomal DNA (rDNA), and internal transcribed spacer (ITS) sequences (Ramachandran, Gam and Neva, 1997; Nagayasu *et al.*, 2017).

Given the aforementioned facts, it is prudent to use DNA amplification methods as the gold standard for strongyloidiasis diagnosis. Conversely, as much as DNA amplification techniques sound like a perfect replacement for the gold standard, this was not possible in reality. As expected, DNA amplification requires sophisticated instruments and expensive molecular reagents, and therefore, a well-equipped laboratory, which is unfortunately not available to many underdeveloped countries where strongyloidiasis is highly prevalent (Levenhagen and Costa-Cruz, 2014). In addition, in the case of strongyloidiasis, especially in chronic cases, DNA amplification assays may not be reliable enough for a consistent and accurate diagnosis. This is due to the characteristics of chronic strongyloidiasis, where larval output is variable and sporadic, thereby affecting the diagnostic sensitivity of PCR-based tests. This insight was supported by a meta-analysis and systematic review of the accuracy of molecular diagnostic assays in detecting strongyloidiasis compared with parasitological methodologies. Their study revealed that PCR and qPCR tests had diagnostic sensitivities of 71.8% and 64.4%, respectively, in comparison to parasitological methods; these values dropped further to 61.8% and 56.5%, respectively, when serological assays were included within the reference tests. The review concluded that molecular detection methods were insufficient for screening purposes but were highly suitable for diagnostic confirmation (Buonfrate *et al.*, 2018). Another study also discovered a similar trend, where the sensitivity of molecular methods was only 67% when compared with parasitological methods (Javanian, Gorgani-Firouzjaee and Kalantrai, 2019). Hence, these drawbacks reinforce the need for a more cost-efficient, rapid, and simple diagnostic test without sacrificing diagnostic sensitivity and accuracy.

1.2.4(a)(iii) Antigen detection assays

A major solution to this problem is the next subcategory of molecular detection tests, antigen detection tests. The operating principle behind this test is the use of antibodies in the solid phase to detect specific antigens of the parasite in the liquid phase, that is, a diagnosis sample (Wu *et al.*, 2000). Two types of antibodies can be used for this purpose: (1) polyclonal antibodies that can bind to one or multiple epitopes of an antigen and (2) monoclonal antibodies that bind exclusively to one specific epitope of an antigen (Lipman *et al.*, 2005). Because of the simplistic nature of antigen detection compared with DNA amplification-based methods, it is much cheaper and faster in detecting the targeted disease, albeit compromising a little accuracy and sensitivity (Levenhagen and Costa-Cruz, 2014).

Currently, most antigen detection tests use antibodies that are produced via the hyper-immunisation of rabbits, phage display, or hybridoma cell technologies with the help of recombinant proteins of *S. stercoralis* that display a high degree of sensitivity for detection by either monoclonal or polyclonal antibodies. Well-known examples of such recombinant proteins include Ss-NIE, Ss-IR, and Ss-1a. In addition, antibodies raised against antigens from species within the *Strongyloides* genus, such as *S. ratti* and *S. venezuelensis*, have also been used in antigen detection assays. For example, monoclonal antibodies against *S. ratti* antigens that were produced via both mouse immunisation and hybridoma cell technology (Sykes and McCarthy, 2011) and polyclonal antibodies against *S. ratti* excretory/secretory (E/S) antigens produced via rabbit hyper-immunisation (Mahmuda *et al.*, 2018) successfully detected the parasite antigens in animal and human sera, respectively. For *S. venezuelensis*, Avian IgY antibodies against its parthenogenetic female (pF) and filariform larvae (L3) detected strongyloidiasis immune complexes in patient sera. The corresponding diagnostic specificity and sensitivity for both types of antibodies were 91.11% and 88.89%, respectively, for anti-pF IgY, while the values were 95.56% for both parameters for anti-L3 IgY (de Faria *et al.*, 2019).

1.2.4(b) Indirect detection methods

1.2.4(b)(i) Absolute eosinophil count

The absolute eosinophil count, also known as the peripheral blood eosinophil count, is a blood test that measures the levels of eosinophils in a subject's peripheral blood. In general, when an individual is infected with a helminth such as *S. stercoralis*, eosinophilia is often manifested, which is defined as an unusual elevation in the quantity of eosinophils in the peripheral blood circulation. Eosinophilia is caused not only by a helminth infection, but also a myriad of factors, including but not limited to, dermatological diseases, hypersensitivity reactions, asthma, and neoplasm. Above the normal range of 500 cells/mm^3, eosinophilia can be categorised as mild (500 – 1500 cells/mm^3), moderate (1500 – 5000 cells/mm^3), or severe (more than 5000 cells/mm^3) (Park *et al.*, 2018; Klion, 2019; Kuang, 2020). Peripheral eosinophilia has been reported frequently in up to 80% of immunocompetent patients with chronic strongyloidiasis and was even more evident in individuals who had travelled to parasite-endemic regions (Herrick *et al.*, 2015; Klion, 2019). As a result, the British Infection Society published a stark recommendation for testing for eosinophilia for all individuals hailing from tropical countries to detect any *S. stercoralis* infection (Checkley *et al.*, 2010).

Eosinophilia is a key symptom of strongyloidiasis. In fact, 38% of eosinophilia cases were caused by *S. stercoralis* infection when investigated among migrants and travellers (Nutman *et al.*, 1987; Libman, MacLean and Gyorkos, 1993; Nutman, 2017). Therefore, refugees and immigrants from strongyloidiasis endemic regions should be screened for the disease. This statement is supported by evidence in the literature. For instance, a study found that 12% of refugees who came to the United States from 1998 to 2002 had eosinophilia, and 58% of the eosinophilic individuals were from areas with strongyloidiasis (Seybolt, Christiansen and Barnett, 2006). In addition, refugees from Southeast Asia with eosinophilia were highly associated with *S. stercoralis* infections (Mitchell *et al.*, 2018). Because of this, many places check for eosinophilia when suspecting a person to be infected with *S. stercoralis*.

However, although absolute eosinophil count seems to be a good test to diagnose strongyloidiasis, this detection method still lacks diagnostic sensitivity, accuracy, and specificity. This is because of the inherent nature of the disease to be asymptomatic, including eosinophilia, in the majority of cases for both immunocompetent and immunocompromised patients (Beknazarova, Whiley and Ross, 2016; Levenhagen, Conte and Costa-Cruz, 2016). Studies have revealed that 33% of African refugees and 27% of Southeast Asian refugees who test positive for strongyloidiasis do not manifest eosinophilia (Naidu, Yanow and Kowalewska-Grochowska, 2013; Mitchell *et al.*, 2018). In addition to that, eosinophilia was often found to be absent in immunocompromised individuals that have hyperinfection and disseminated strongyloidiasis (Newberry *et al.*, 2005). Hence, although the absolute eosinophil count is a good indicator test for strongyloidiasis, the absence of eosinophilia does not guarantee that the individual is not infected with *S. stercoralis*; further confirmatory tests are needed to ensure a true negative result (Ligas et al., 2003; Seybolt, Christiansen and Barnett, 2006; Naidu, Yanow and Kowalewska-Grochowska, 2013).

1.2.4(b)(ii) Enzyme-linked immunosorbent assay (ELISA)

Serological diagnosis is primarily dependent on antibodies or immunoglobulins (Ig) which result from the body's immune defense response, particularly B-cells, towards an infection of any type. The enzyme-linked immunosorbent assay (ELISA) is a classic test that uses this basis. In conventional ELISA, an antigen is coated on a solid phase, followed by incubation with samples or serum potentially containing the antibody of interest, a detection antibody that is usually conjugated with an enzyme, and a chromogenic substrate of that particular enzyme. The concentration of antibodies within the analyte was interpreted by the intensity of the colour after the addition of the chromogenic substrate, which can be measured in terms of optical density (OD) using a spectrophotometer. As a result, ELISA has a high degree of versatility and diversity in diagnostic applications, simply by swapping the necessary components of an ELISA test to detect the analyte of interest and assay improvisation (Crowther, 1995; Aydin, 2015).

For the diagnosis of *S. stercoralis*, various classes of antibodies in the analyte have been targeted for detection, including IgA, IgE, and IgG with its subclasses IgG1 and IgG4 (Requena-Méndez *et al.*, 2013; Levenhagen and Costa-Cruz, 2014; Bosqui *et al.*, 2015; De Souza *et al.*, 2020). Patient serum is often used as an analyte, but there is evidence that urine can also be used (Ruantip *et al.*, 2018). In terms of the coating antigen, different types and sources have been utilised, such as *Strongyloides* spp. crude lysates or recombinant proteins such as 14-3-3, Ss-IR, Ss-1a, and NIE (Ravi *et al.*, 2002; Krolewiecki *et al.*, 2010; Rascoe *et al.*, 2015; Arifin *et al.*, 2018; Masoori *et al.*, 2019). Owing to the popularity of antibody ELISA, an assortment of antibody ELISA test kits is available for the diagnosis of strongyloidiasis, such as the IVD *Strongyloides* Serum Antibody Detection Microwell ELISA test kit, SciMedx

Strongy-96 test kit, Bordier *Strongyloides ratti* IgG ELISA kit, and InBios Strongy Detect^{TM} IgG ELISA (Anderson *et al.*, 2014; Bisoffi *et al.*, 2014; Buonfrate *et al.*, 2015; Ruantip *et al.*, 2018). Overall, the diagnostic sensitivity for antibody ELISA in detecting strongyloidiasis ranges from 37% to 100%, whereas its specificity ranges between 60% and 100% (Levenhagen and Costa-Cruz, 2014; Kalantari *et al.*, 2020).

1.2.4(b)(iii) Immunofluorescence antibody test (IFAT)

Analogously, the immunofluorescence antibody test (IFAT) uses an underlying principle similar to ELISA, with the major modification being the use of fluorochrome-conjugated antibodies as the secondary antibody (Odell and Cook, 2013). For the diagnosis of strongyloidiasis using IFAT, whole larvae of *S. stercoralis* are frequently used as antigens, followed by the addition of serum and fluorochrome-conjugated antibodies to detect antigen-antibody interactions. The larvae are often killed and fixed using chemicals such as acetone or paraformaldehyde. Fluorescence was visualised using a fluorescence microscope (Boscolo *et al.*, 2007). The diagnostic sensitivity and specificity for IFAT were 97% and 98%, respectively, when compared to parasitological reference tests, and 93.9% and 92.2%, respectively, when compared to serological tests.

Nonetheless, from a technical point of view, the skills needed to perform this test are challenging, especially for the preparation of larvae and interpretation of fluorescence results. Therefore, trained and highly skilled technicians and expensive fluorescence microscope are needed if IFAT is to be used widely to diagnose *S. stercoralis* infections (Rigo *et al.*, 2008; Requena-Méndez *et al.*, 2013). In addition, intact *S. stercoralis* larvae are notoriously difficult to obtain from confirmed infected