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Saarland University, Homburg/Saar, Germany

Innovative MALDI-TOF mass spectrometry techniques to improve the diagnosis of parasites and bacteria in clinical samples

A Dissertation

Submitted in Fulfillment of the Requirements for the Degree of

Doctor of Philosophy (PhD)/Doktor der Naturwissenschaften (Dr. rer. nat.)

Faculty of Medicine

Saarland University

2024

by

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Declaration

The work presented in this thesis was conducted in the period from July 2019 to October 2023 at the Institute of Medical Microbiology and Hygiene (IMMH) at Saarland University Medical Center.

This dissertation is written cumulatively, and the respective publications are presented in the results section of this thesis (section V), after having obtained permission from all participating authors. A list of all manuscripts this author has published and/or peer-reviewed is presented in the publications and conferences section. The author of this thesis is the main author and/or a co-author in the following peer-reviewed publications:

- **Article n° 1. Research article:** Identification of Adult *Fasciola* spp. Using Matrix-Assisted Laser/Desorption Ionization Time-of-flight (MALDI-TOF) Mass Spectrometry

  Sy I, Margardt L, Ngbede EO, Adah MI, Yusuf ST, Keiser J, Rehner J, Utzinger J, Poppert S, Becker SL. Identification of Adult *Fasciola* spp. Using Matrix-Assisted/Laser Desorption Ionization Time-of-flight (MALDI-TOF) Mass Spectrometry. *Microorganisms*. 2021; 9(1):82. https://doi.org/10.3390/microorganisms9010082

- **Article n° 2. Research article:** Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry for differential identification of adult *Schistosoma* worms

  Ebersbach JC, Sato MO, de Araújo MP, Sato M, Becker SL, **Sy I**. Matrix-Assisted laser desorption/ionization time-of-flight mass spectrometry for differential identification of adult *Schistosoma* worms. *Parasites Vectors* 16, 20(2023). https://doi.org/10.1186/s13071-022-05604-0

- **Article n° 3. Research article:** Evaluating Different Storage Media for Identification of *Taenia saginata* Proglottids Using MALDI-TOF Mass Spectrometry

  Wendel TP, Feucherolles M, Rehner J, Poppert S, Utzinger J, Becker SL, **Sy I**. Evaluating Different Storage Media for Identification of *Taenia saginata* Proglottids Using MALDI-TOF Mass Spectrometry. *Microorganisms*. 2021; 9(10):2006. https://doi.org/10.3390/microorganisms9102006

- **Article n° 4. Review article:** Recent Advances and Potential Future Applications of MALDI-TOF Mass Spectrometry for Identification of Helminths

  Sy I, Conrad L, Becker SL. Recent Advances and Potential Future Applications of MALDI-TOF Mass Spectrometry for Identification of Helminths. *Diagnostics*. 2022; 12(12):3035. https://doi.org/10.3390/diagnostics12123035

- **Article n° 5. Research article:** Research article: Evaluation of the Qvella FAST™ System and the FAST-PBC Cartridge for Rapid Species Identification and Antimicrobial Resistance Testing Directly from Positive Blood Cultures

  Sy I, Bühler N, Becker SL, Jung P. Evaluation of the Qvella FAST™ System and the FAST-PBC cartridge for rapid species identification and antimicrobial resistance testing directly from positive blood cultures. *J Clin Microbiol*. 2023; 61(10):e00569-23. https://doi.org/10.1128/jcm.00569-23

Table of Contents

Declaration 1

List of figures 4

Abbreviations and acronyms 5

Summary 7

Zusammenfassung 9

1. Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) Mass spectrometry (MS) 12

1.1. Mass spectrometry and proteomics 12

1.2. Principle of MALDI-TOF MS 12

1.3. MALDI-TOF MS in clinical microbiology 13

2. Parasitic pathogens: helminth and neglected tropical diseases (NTDs) 16

2.1. Background 16

2.2. Taxonomy and classification 16

2.3. Pathogen entry pathways 17

2.4. Helminthic life cycles in the infected host 17

2.5. Taxonomic groups and key pathogens 18

3. Bacterial and fungal pathogens: bloodstream infections (BSI) 53

3.1. Global burden and incidence of BSI 53

3.2. Sepsis and septic shock 54

3.3. Gram-positive bloodstream infections (GPBSI) 55

3.4. Gram-negative bloodstream infections (GNBSI) 55

3.5. Yeast/fungal bloodstream infections 56

3.6. Current methods for diagnosis of BSI 56

3.7. Antimicrobial susceptibility testing (AST) 60

3.8. FAST™ System 62

4. Goal and objectives of the present PhD thesis 64

5. Helminth identification and pathogen detection in blood cultures using MALDI-TOF MS 66

5.1. Article n° 1. Research article: Identification of adult *Fasciola* spp. using matrix-assisted laser/desorption ionization time-of-flight (MALDI-TOF) mass spectrometry 66

5.2. Article n° 2. Research article: Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry for differential identification of adult *Schistosoma* worms 78

5.3. Article n° 3. Research article: Evaluating different storage media for identification of *Taenia saginata* proglottids using MALDI-TOF mass spectrometry 92

5.4. Article n° 4. Review article: Recent advances and potential future applications of MALDI-TOF mass spectrometry for identification of helminths 104

5.5. Article n° 5. Research article: Research article: Evaluation of the Qvella FAST™ system and the FAST-PBC cartridge for rapid species identification and antimicrobial resistance testing directly from positive blood cultures 117

6. Discussion ... 132
6.1. Enhancing helminthic parasite detection by MALDI-TOF MS ... 132
6.2. Clinical application of new tools for detection of BSI-causing pathogens ... 135
6.3. Future perspectives ... 137
References ... 139
Publications and conferences ... 175
Curriculum Vitae ... 177
Acknowledgments ... 178

List of figures

Figure 1. Principle of MALDI-TOF MS....13
Figure 2. MALDI-TOF MS workflows in clinical microbiology laboratory. 14
Figure 3. Helminth classification and taxonomy. 17
Figure 4. Life cycle of *T. saginata*....21
Figure 5. *Taenia solium* 23
Figure 6. Life cycle of *Taenia solium*....23
Figure 7. Parenchymal (**A**, cysticercosis encephalitis) and extraparenchymal (**B**, intraventricular cyst) neurocysticercosis....25
Figure 8. Close human-pig contact is a major risk factor for *T. solium* infections....27
Figure 9. (**A**) Morphology and development stages of *E. granulosus s.l.* and *E. multilocularis*. (**B**) Global distribution of *E. granulosus* s.l. and *E. multilocularis*....28
Figure 10. Life cycle of *Echinococcus* spp 29
Figure 11. Rural slaughterhouse in Peru: dogs are occasionally fed with infected viscera containing hydatid cysts....31
Figure 12. Different stages of *A. lumbricoides* eggs: (**A**) unfertilized and (**B**) fertilized eggs. (**C**) Life cycle of *Ascaris lumbricoides*....33
Figure 13. Adult *Ascaris lumbricoides* extracted from the jejunum of a 7-year-old boy 34
Figure 14. Number of pre-school aged children and school-aged children treated and progress in preventive chemotherapy (PC) coverage, 2003-2017....36
Figure 15. *Trichuris trichiura*: (A) female adult, (B) male adult, (C) egg 36
Figure 16. The life cycle of *Trichuris trichiura*....37
Figure 17. Different stages of hookworm species....40
Figure 18. The life cycle of hookworms (*N. americanus* and *A. duodenale*) 41
Figure 19. Multi-intervention integrated deworming strategy for sustainable control of STH in Jiangsu Province, China from 1989 to 2019....45
Figure 20. Life cycle of *Fasciola* spp. 47
Figure 21. Life cycle of *Schistosoma mansoni*, *Schistosoma japonicum* and *S. haematobium* 49
Figure 22. Interaction between bacteria (gut microbiome), bacterial translocation and sepsis 55
Figure 23. FAST™ System and Fast PBC cartridge producing LC after processing PBC 62

Abbreviations and acronyms

A

alveolar echinococcosis
(AE) 25
Ancylostoma secreted proteins
(ASPs)...40
antimicrobial resistance
(AMR)...61
antimicrobial stewardship
(AMS) 62
Antimicrobial susceptibility testing
(AST) 61

B

Blood Culture Identification
(BCID) 59
blood culture sample preparation
(BCSP)..58
bloodstream infections
(BSI)...53

C

central nervous system
(CNS)...16
coagulase negative Staphylococci
(CoNS)..59
computed tomography
(CT)...28
cystic echinococcosis
(CE)...25

D

Desoxyribonucleic acid
DNA..23

E

eggs per gram
(EPG) 33
electrospray ionization mass spectrometry
(ESI-MS)..57
enzyme-linked immunoelectrotransfer blot
(EITB)...23
enzyme-linked immunosorbent assay
(ELISA) 20
European Committee on Antimicrobial Susceptibility Testing
(EUCAST) 62
excretory/secretory products
(ESPs) 41
Extracellular vesicles
(EVs)..47

F

fecal egg-counting
(FEC) 41
fluorescence in-situ hybridization
(FISH) 57
formol-ether concentration
(FEC) 33

G

gastrointestinal
(GI) 56
germinal layer
(GL) 26
Gram-Negative Blood Culture
(BC-GN) 60
Gram-Positive Blood Culture
(BC-GP)...60

H

hepatobiliary and pancreatic ascariasis
(HPA)...32
high income countries
(HICs) 53
human immunodeficiency virus/acquired immunodeficiency syndrome
(HIV/AIDS) 14

I

intensive care unit
(ICU)..55
internal medicine wards
(IMW)..57
iron deficiency anemia
(IDA) 36

K

Kato-Katz
(KK)...32

L

laminated layer

(LL)...26
Liquid Colony
(LC)...63
loop-mediated isothermal amplification
(LAMP)...20
low and middle-income countries
(LMICs)...14
lymphatic filariasis
(LF)...14

M

magnetic resonance imaging
(MRI)...23
mass drug administration
(MDA)...33
Mass spectrometry
(MS)...10
mass-to-charge
(m/z)...10
matrix-assisted laser desorption/ionization
(MALDI)...10
Minimun concentration inhibition
MIC...62
modified catheterization technique
MoCAT...28
molecular rapid diagnostic tests
(mRDT)...57
Mueller
Hinton (MH)...62
multi-resistant organisms
(MRO)...61

N

neglected tropical diseases
(NTDs)...14
neurocysticercosis
(NCC)...21
Nucleic acid amplification test
NAAT...23

P

polymerase chain reaction
(PCR)...18
praziquantel
(PQZ)...20
preventive chemotherapy
(PC)...33
puncture, aspiration, injection, and re-aspiration
(PAIR)...28

Q

quadrupole time of flight
(Q-Q-ToF)...10

S

sodium dodecyl sulphate
(SDS)...58
soil-transmitted helminths
(STHs)...14
standard operating procedure
(SOP)...12

T

T helper cell type 2
(Th2)...27
tandem time of flight
(ToF-ToF)...10
time of flight
(TOF)...10
Trichuris dysentery syndrome
(TDS)...36

U

ultrasound
(US)...28
urinary tract
(UT)...56

W

water, sanitation, and hygiene
(WASH)...34
World Health Organization
(WHO)...14

A

α-cyano-4-hydroxycinnamic acid
(HCCA)...10

Summary

Introduction: Parasitic helminth infections and bacterial bloodstream infections (BSI) are major health problems affecting millions of people worldwide. Helminthic parasites are mainly found in tropical and subtropical areas, especially in low and middle-income countries (LMICs), where poor sanitation and hygiene conditions can increase disease transmission and the risk of infection. Most helminth infections (e.g. taeniasis, schistosomiasis, ascariasis, trichuriasis, and hookworm infection) are part of the so-called neglected tropical diseases (NTDs) and mainly lead to chronic morbidity such as persistent diarrhea, stunting or anemia. In contrast, BSI are acute, life-threatening events and are characterized by the presence of bacterial or fungal pathogens in the blood. The routine microbiological diagnosis of BSI and helminths relies mainly on blood culture and microscopic examination of parasite eggs, respectively. Both techniques have limitations, e.g. with regard to diagnostic accuracy, and there is a need to improve laboratory detection methods. Many efforts have been made through the development of new tools such as polymerase chain reaction (PCR), or matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS). MALDI-TOF MS has become the gold standard method for bacterial diagnosis in many laboratories, particularly in high-income settings. This thesis aims to investigate and evaluate the capacity of innovative MALDI-TOF MS approaches to improve the diagnosis of helminthic parasites and BSI in different scenarios.

Methods: MALDI-TOF MS was employed for identification of different helminths by creating an in-house database containing protein spectra profiles (reference spectra, also known as main spectra profiles; MSP) that were generated from protein extracts obtained from a portion of the worm or the entire worm, depending on the size. Adults trematodes stemming from animals (*Fasciola gigantica*, and *F. hepatica* isolated from slaughtered cattle in Nigeria and Switzerland, respectively; *Schistosoma mansoni* and *S. japonicum* isolated from experimental mice in Japan), and cestodes (*Taenia saginata* proglottids isolated from stool samples of infected patients in Switzerland) were analyzed by MALDI-TOF MS for species identification. Subsequently, the effect of different sample storage media (e.g., ethanol, sodium chloride, formalin, or RNA*later*) on spectra acquisition and the diagnostic accuracy was also investigated. In a second part of the thesis, a validation of the commercially available FAST™ System for improved and more rapid MALDI TOF-based BSI identification was performed. The system allows the purification of microorganisms in patient blood samples without an otherwise necessary subculture step by generating a so-called liquid colony (LC). LC-based MALDI-TOF MS was compared to the reference method based on culture-grown colonies by prospectively analyzing 261 positive blood culture (PBC) samples collected on different wards at Saarland University Medical Center in Homburg. Additionally, antimicrobial susceptibility testing (AST) patterns following LC processing were compared to the reference standard (i.e., broth microdilution by MicroScan WalkAway performed on grown colonies, and directly inoculated disk diffusion).

Results: The helminth studies revealed that adult trematodes (*Fasciola* spp., and *Schistosoma* spp.) and cestodes (*T. saginata*) can reliably be identified by MALDI-TOF MS with identification rates ranging between 98.7%-100% for *Fasciola* spp., 81% (species level) and 100% (genus level) for *Schistosoma* spp., and 97.2%-99.7% for *T. saginata*. In addition, it was shown that prolonged storage up to 24 weeks in ethanol, water, sodium chloride and RNA*later* did not affect the subsequent MALDI-TOF-based identification of *Taenia* parasites, whereas formalin storage impeded later identification due to protein degradation. With regard to BSI diagnostics, it was shown that MALDI-TOF MS combined with the FAST™ System device enabled a correct and reliable identification of PBC samples. Indeed, in comparison to the reference technique, diagnostic agreement rates of 97.4% (150/154) and 95.7% (67/70) were observed for Gram-positive and Gram-negative pathogens, respectively. Subsequently, when compared to the routine workflow, the FAST™ System LC indicated AST results with a categorical agreement (CA) of 96.1% and 98.7% for MicroScan and disk diffusion, respectively. The turnaround time for correct identification and AST was significantly reduced by one day as compared to the standard methods.

Conclusion: MALDI-TOF MS can successfully be employed as a rapid and accurate diagnostic tool for identification of helminth parasites such as *Fasciola* spp., *Schistosoma* spp. or *T. saginata*. However, the creation and validation of an in-house library containing species-specific MSPs is necessary as no commercial database for parasite identification is available thus far. With regard to bacterial BSI, the herein presented results show that the FAST™ LC System allows to speed up the diagnostic process of PBC by reducing significantly the time to correct identification and AST results as compared to the reference method.

Zusammenfassung

Einleitung: Parasitäre Helminthen-Infektionen und bakterielle Blutstrominfektionen (BSI) sind große Gesundheitsprobleme, von denen Millionen von Menschen weltweit betroffen sind. Helminthische Parasiten kommen hauptsächlich in tropischen und subtropischen Gebieten vor, insbesondere in Ländern mit niedrigem und mittlerem Einkommen, wo schlechte sanitäre und hygienische Bedingungen die Krankheitsübertragung und das Infektionsrisiko erhöhen können. Die meisten Helmintheninfektionen (z. B. Taeniasis, Schistosomiasis, Ascariasis, Trichuriasis und Hakenwurminfektionen) gehören zu den so genannten vernachlässigten Tropenkrankheiten („neglected tropical diseases"; NTD) und führen hauptsächlich zu einer chronischen Krankheitslast mit z.B. chronischem Durchfall, Wachstumsstörungen oder Anämie. Im Gegensatz dazu sind BSI akute, lebensbedrohliche Ereignisse und zeichnen sich durch das Vorhandensein von bakteriellen Erregern oder Pilzen im Blut aus. Die mikrobiologische Routinediagnostik von BSI und Helminthen stützt sich hauptsächlich auf Blutkulturen bzw. die mikroskopische Untersuchung von Parasiteneiern. Beide Techniken haben Einschränkungen, z. B. in Bezug auf die diagnostische Genauigkeit, und es besteht die Notwendigkeit, die Labornachweisverfahren zu verbessern. Durch die Entwicklung neuerer Techniken wie der Polymerase-Kettenreaktion (PCR) oder der Matrix-assistierten Laser-Desorption/Ionisations- Flugzeit- (MALDI-TOF)-Massenspektrometrie (MS) stehen bereits zahlreiche neue Ansätze zur Verfügung. Die MALDI-TOF-MS hat sich in vielen Laboren, vor allem in Ländern mit hohem Einkommen, zur Hauptidentifikationsmethode für die Bakteriendiagnostik entwickelt. Ziel dieser Arbeit ist es, die Anwendbarkeit innovativer MALDI-TOF MS-Ansätze zur Verbesserung der Diagnostik von Helminthen-Infektionen und BSI in verschiedenen Szenarien zu untersuchen und zu bewerten.

Methoden: MALDI-TOF MS wurde zur Identifizierung verschiedener Helminthen eingesetzt, indem eine interne Datenbank mit Proteinspektrumsprofilen (Referenzspektren, auch bekannt als „main spectra profile"; MSP) erstellt wurde, welche aus Proteinextrakten gewonnen wurden, die je nach Größe aus einem Teil des Wurms oder dem gesamten Wurm stammen. Adulte Trematoden wurden gewonnen (*Fasciola gigantica* und *F. hepatica*, isoliert von geschlachteten Rindern in Nigeria bzw. der Schweiz; *Schistosoma mansoni* und *S. japonicum*, isoliert von Versuchsmäusen in Japan) und ebenso wie Zestoden (*Taenia saginata* Proglottiden, isoliert von Stuhlproben infizierter Patienten in der Schweiz) mittels MALDI-TOF MS zur Speziesbestimmung analysiert. Anschließend wurde die Auswirkung verschiedener Probenlagerungsmedien (z.B. Ethanol, Natriumchlorid, Formalin oder RNA*later*) auf die Spektrenqualität und die diagnostische Genauigkeit untersucht. In einem zweiten Abschnitt der vorliegenden Promotionsarbeit wurde eine Validierung des kommerziell erhältlichen FAST™-Systems für eine verbesserte und schnellere MALDI TOF-basierte BSI-Identifizierung durchgeführt. Das System ermöglicht die Aufreinigung von Mikroorganismen in Patientenblutproben ohne einen ansonsten notwendigen Schritt einer Subkultur auf Agarmedien, indem eine sogenannte Flüssigkolonie

gewonnen wird („Liquid Colony“; LC). Die LC-basierte MALDI-TOF MS wurde mit der Referenzmethode, die auf Agar-gewachsenen Kolonien basiert, verglichen, indem 261 positive Blutkulturproben von verschiedenen Stationen des Universitätsklinikums des Saarlandes in Homburg prospektiv analysiert wurden. Darüber hinaus wurden die Ergebnisse einer Antibiotika-Resistenztestung aus der LC mit dem Referenzstandard verglichen (d. h. Mikrodilution mittels MicroScan WalkAway und direkt inokulierte Agarplättchen-Diffusionstestung).

Ergebnisse: Die Helminthen-Untersuchungen ergaben, dass adulte Trematoden (*Fasciola* spp. und *Schistosoma* spp.) und Cestoden (*T. saginata*) mit MALDI-TOF MS zuverlässig identifiziert werden können, wobei die Identifizierungsraten zwischen 98,7% und 100% für *Fasciola* spp., 81% (Speziesidentifikation) und 100 % (Gattungsidentifikation) für *Schistosoma* spp., und zwischen 97,2% und 99,7% für *T. saginata* lagen. Darüber hinaus wurde gezeigt, dass eine längere Lagerung von bis zu 24 Wochen in Ethanol, Wasser, Natriumchlorid und RNA*later* die anschließende MALDI-TOF-basierte Identifizierung von *Taenia*-Parasiten nicht beeinträchtigt, während die Lagerung in Formalin eine spätere Diagnostik aufgrund des Proteindenaturierung unmöglich macht. Im Hinblick auf die BSI-Diagnostik konnte gezeigt werden, dass MALDI-TOF MS in Kombination mit dem FAST™ System eine korrekte und zuverlässige Identifizierung von positiven Blutkultur-Proben ermöglicht. So wurden im Vergleich zur Referenztechnik diagnostische Übereinstimmungsraten von 97,4% (150/154) und 95,7% (67/70) für grampositive bzw. gramnegative Erreger festgestellt. Im Vergleich zum Routine-Workflow zeigte das FAST™ System LC Antibiotika-Resistenztestungsergebnisse mit einer kategorischen Übereinstimmung („categorical agreement; CA“) von 96,1% bzw. 98,7% für MicroScan und Agardiffusion. Die benötigte Zeit bis zur korrekten Identifizierung wurde im Vergleich zu den Standardmethoden deutlich um etwa einen Tag verkürzt.

Schlussfolgerung: MALDI-TOF MS kann erfolgreich als schnelles und genaues Diagnoseinstrument zur Identifizierung von Helminthen wie *Fasciola* spp., *Schistosoma* spp. oder *T. saginata* eingesetzt werden. Allerdings ist die Erstellung und Validierung einer laboreigenen Datenbank mit speziespezifischen MSP erforderlich, da bisher keine kommerzielle Datenbank zur Parasitenidentifizierung zur Verfügung steht. In Bezug auf bakterielle BSI zeigen die hier vorgestellten Ergebnisse, dass das FAST™ LC-System die Diagnostik bei positiven Blutkulturen verbessern kann, indem es die Zeit bis zur korrekten Identifizierung und die Resistenztestungsergebnisse im Vergleich zur Referenzmethode erheblich verkürzt.

I

MALDI-TOF mass spectrometry in clinical microbiology

1. Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) Mass spectrometry (MS)

1.1. Mass spectrometry and proteomics

Mass spectrometry (MS) is a technique for studying and analyzing biomolecules such as proteins, peptides, and lipids. With the development of genomic analyses and advances in sequencing, MS has become a key technique for identifying protein structures and sequences (Watt *et al.*, 2003). Proteins or peptides can be easily and rapidly identified by correlating sequence data with MS measurements (Domon and Aebersold, 2006). Mass spectrometers consist of three principal components which are the ion source, the analyzer, and the ion detector (Guerrera and Kleiner, 2005). The development of soft ionization techniques such as electrospray ionization (ESI) (Fenn *et al.*, 1989) or matrix-assisted laser desorption/ionization (MALDI) (Karas and Hillenkamp, 1988), which allow ionized molecules to be transferred to a gas phase without fragmentation, has stimulated the emergence of new instruments [e.g., time of flight (TOF), hybrid quadrupole time of flight (Q-Q-ToF), tandem time of flight (ToF-ToF)], making it possible to analyze polypeptides, proteins, and the proteome by MS (Aebersold and Mann, 2003). Depending on the specific features of each instrument (i.e., whether it is a single-stage or multi-stage MS, the mode of operation, etc.), MS analysis can be performed to measure only the molecular mass of peptides/polypeptides (e.g., single-stage MS: MALDI-TOF) or to determine their structural characteristics such as posttranslational modifications (e.g., tandem MS/MS) (Domon and Aebersold, 2006).

MALDI-TOF MS is a relatively simple technique that performs well in terms of accuracy, sensitivity, and resolution. The analysis of proteins from mixtures by peptide mass fingerprinting is based on the separation of ions according to their time of flight, which is directly related to their mass-to-charge (m/z) values (Guerrera and Kleiner, 2005). The main proteins detected by MALDI-TOF MS analysis are ribosomal proteins, structural proteins, and putative proteins (Ryzhov and Fenselau, 2001).

1.2. Principle of MALDI-TOF MS

MALDI-TOF MS is an ionization technique in which a co-crystalized mixture of analyte and photosensitive matrix (e.g., α-cyano-4-hydroxycinnamic acid (HCCA)) is exposed to ultraviolet lasers that form ions, which will then migrate through the electric field of the TOF analyzer to the detector. Ions are separated according to their mass-to-charge (m/z) ratios determined by TOF estimation. Hence, small molecules will reach the detector first (Hou, Chiang-Ni and Teng, 2019). Mass spectra profiles are presented m/z displayed on the x-axis and the intensities on the y-axis (**Figure 1**). The identification method is based on a comparison of the spectra profiles with a commercial database developed by the device-manufacturing companies. Currently, most commercially available instruments [e.g., MALDI

Biotyper® (Bruker Daltonics, Germany), VITEK® MS (bioMerieux, France)] contain databases of reference spectra specific to the different species of bacteria, mycobacteria, and fungi.

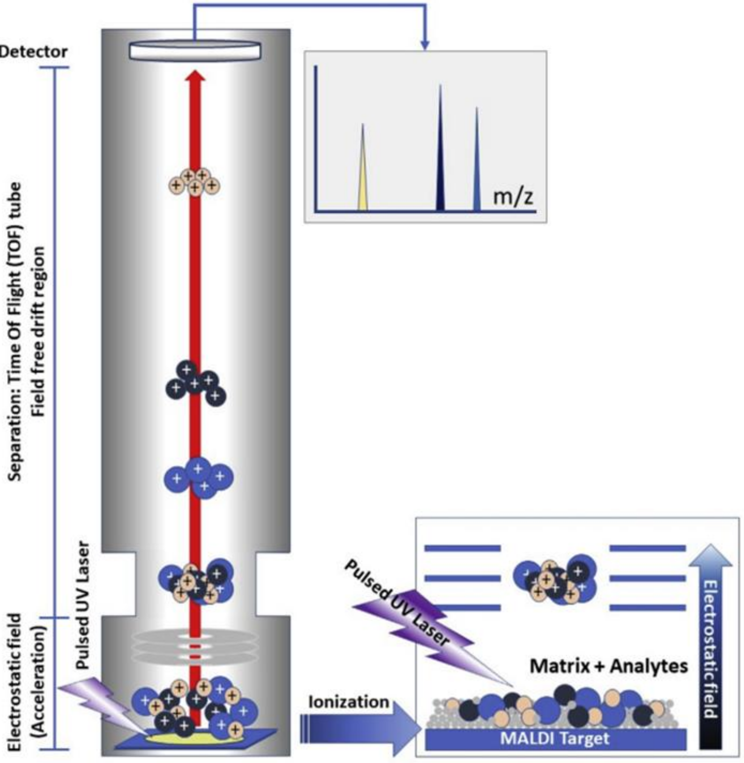

***Figure 1**. Principle of MALDI-TOF MS (adapted from (Hou, Chiang-Ni and Teng, 2019)).*

1.3. MALDI-TOF MS in clinical microbiology

In clinical microbiology, one of the most important tasks is to search, identify and characterize pathogenic microorganisms in biological samples. Traditional identification methods are mainly based on culture combined with phenotypical and/or biochemical tests using specific characteristics such as morphology, color, motility, oxygen tolerance, presence of enzymatic activities, fermentative activity, etc. However, these methods have limited discriminating power and the required time to obtain successful identification is relatively long (two to three days). To expedite the diagnosis process, efforts have been made to reduce the turnaround time. Hence, automated systems for identification of microorganisms have been developed (e.g., Vitek Biomérieux, Phoenix BD (Becton Dickinson), etc.). In the same vein, MALDI-TOF MS was introduced into clinical microbiology laboratories in the late 2000s and has become the standard method for pathogen identification (Seng *et al.*, 2009a, 2010). It is a rapid, powerful, accurate, and cost-effective tool for identifying human pathogens (bacteria, mycobacteria, fungi, archaea, viruses, etc.) based on analysis of their protein profiles (Seng *et al.*, 2010). Depending on the workflow or standard operating procedure (SOP), MALDI-TOF MS analysis in clinical microbiology laboratories can be carried out on culture-grown colonies (direct deposition of the colony or prior proteins extraction using formic acid and acetonitrile) or directly from biological

samples (e.g., blood, urine) (La Scola and Raoult, 2009; Hou, Chiang-Ni and Teng, 2019) (**Figure 2**). In addition to species identification, the application of MALDI-TOF MS has recently been extended to the subtyping of single strains (Kudirkiene *et al.*, 2015; Jadhav, Shah and Palombo, 2021) and the detection of antibiotic/antifungal resistance (Sparbier *et al.*, 2012; Neonakis and Spandidos, 2019).

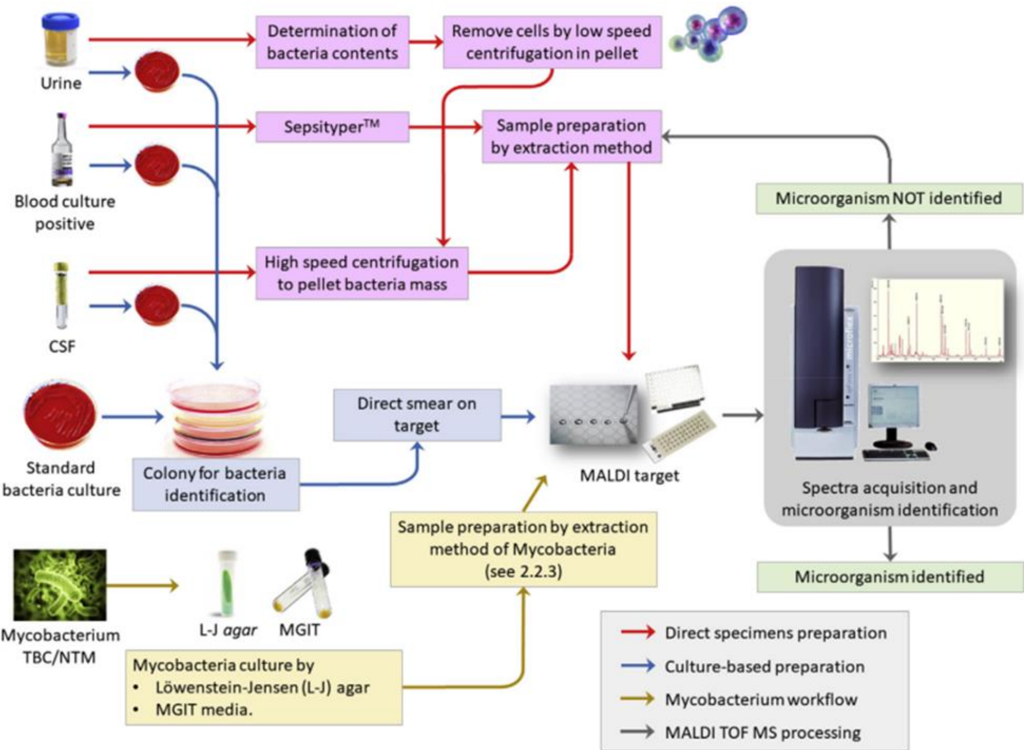

Figure 2. *MALDI-TOF MS workflows in clinical microbiology laboratory (adapted from (Hou, Chiang-Ni and Teng, 2019)).*

II

Parasitic pathogens: helminths and neglected tropical diseases (NTD)

2. Parasitic pathogens: helminth and neglected tropical diseases (NTDs)

2.1. Background

Helminthiasis is a term that comprises a host of parasitic diseases caused by parasitic worms called helminths. These parasites are mainly found in tropical and subtropical areas, especially in low and middle-income countries (LMICs) where poor sanitation and hygiene conditions increase the transmission of disease and the risk of infection (Crompton and Nesheim, 2002). A series of landmark papers published in 2005 (Molyneux, Hotez and Fenwick, 2005) and 2006 (Hotez *et al.*, 2006) established a list of parasitic diseases categorized as neglected tropical diseases (NTDs), including nine helminthic infections [i.e., cysticercosis/taeniasis, dracunculiasis (guinea-worm disease) food-borne trematodiasis, lymphatic filariasis, onchocerciasis, schistosomiasis, and the three main soil-transmitted helminthiases (ascariasis, trichuriasis, and hookworm infection)] (Molyneux, Hotez and Fenwick, 2005; Utzinger *et al.*, 2012). According to the World Health Organization (WHO), NTDs are defined as a diverse group of diseases and/or conditions principally affecting poor populations living in tropical areas and sharing common features. They are of minor importance in high-income countries, which is why they were neglected by research efforts over many years. However, taken together, their global disease burden is comparable to that of the "big three" (malaria, tuberculosis, human immunodeficiency virus/acquired immunodeficiency syndrome (HIV/AIDS)) (Hotez *et al.*, 2007). Several helminth species can infect both humans and animals, thus resulting in zoonotic diseases (e.g., cysticercosis, fascioliasis, etc.). They generally have complex life cycles that include intermediate hosts. School-aged children (including adolescents) and pre-school-aged children are the most affected people. Helminth infections can lead to mild or severe health problems, and the clinical features are manifold, ranging from subtle long-term morbidity to fatal courses (Hotez *et al.*, 2008).

2.2. Taxonomy and classification

Helminths are classified into different groups based on their morphological features such as their body shape. There are two major phyla known to be endoparasites of humans: the nematodes or Nematoda (also called roundworms) including the major intestinal worms also known as soil-transmitted helminths (STHs) and the filarial worms that cause lymphatic filariasis (LF) and onchocerciasis; and the platyhelminths (flatworms) including the flukes (trematodes) and the tapeworms (cestodes) (Hotez *et al.*, 2008; Else *et al.*, 2020) (**Figure 3**). Of note, there is also a third phylum of parasitic worms: the Acanthocephala phylum. These worms very rarely infect humans, who are accidental hosts.

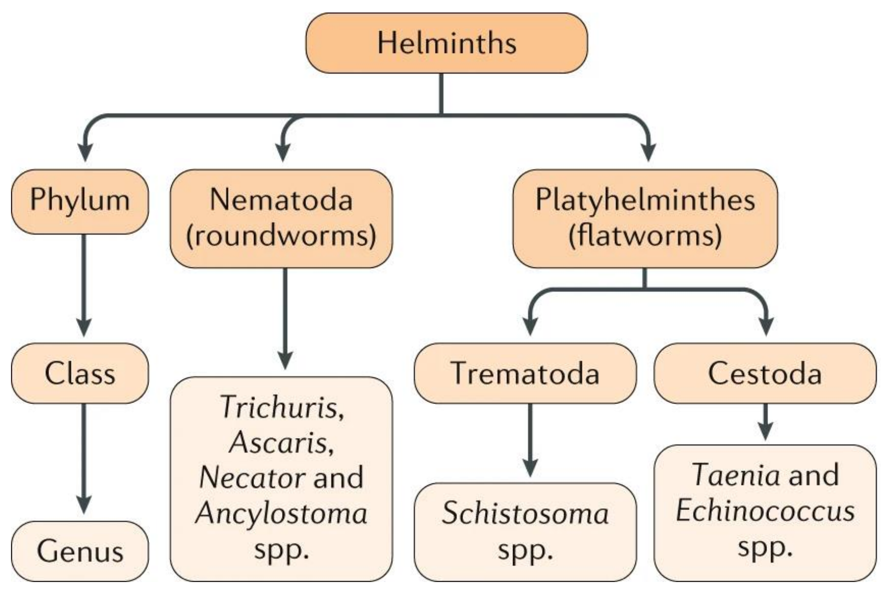

Figure 3. *Helminth classification and taxonomy (adapted from (Else* et al.*, 2020)).*

2.3. Pathogen entry pathways

Helminths use diverse and complex strategies for penetrating the host and establishing infection. For intestinal nematodes such as *Ascaris lumbricoides* or *Trichuris trichiura*, infection occurs after acquisition of embryonated eggs present in the soil or after consumption of contaminated food (e.g., fruits and vegetables). For other nematodes (e.g., hookworm, *Strongyloides* spp.), infection occurs when infective larval stages (e.g., L_3 larvae) hatched from eggs and present in soil enter the human host through the intact skin (Knopp *et al.*, 2012). For certain trematode species, free-living larvae (called cercariae) released in freshwater by intermediate snail hosts can infect humans through skin penetration (e.g., *Schistosoma* spp.). For other trematodes known as plant-borne trematode species (e.g., *Fasciola* spp.), infection begins after ingestion of contaminated food harboring infective agents (i.e., metacercaria) attached to aquatic vegetation (Mas-Coma, Bargues and Valero, 2005). Cestodes are mainly transmitted orally. Most human infections result from the consumption of raw or undercooked infected food (e.g., fish, beef, or pork) containing cysticercoid larvae. Moreover, accidental ingestion of cestode eggs can lead to human cysticercosis and human echinococcosis (Garcia, 2016).

2.4. Helminthic life cycles in the infected host

Parasitic helminths occupy diverse niches (liver, lungs, intestines, bloodstream, etc.) inside the human host. For intestinal parasites such as hookworms or *Strongyloides* (nematodes), after penetration of the host, the infective larva spends time in the bloodstream and the lungs before migrating to the gut via the pharynx and the esophagus (Else *et al.*, 2020). In some cases, helminth parasites (i.e., *T. solium* causing cysticercosis) may be located in the central nervous system (CNS) (Garcia, Gonzalez and Gilman, 2020). The reproduction process of adult helminths depends essentially on the group of

helminths and/or the location of the adult worm. For intestinal nematodes, eggs are produced by adults and released with the feces outside the host where they can continue their development and restart a new life cycle (Else *et al.*, 2020). For cestodes (tapeworms), as the body of adults is segmented and with each segment (proglottid) being hermaphroditic, self-fertilization occurs. Adult tapeworms shed segments into the small intestine, and they can exit the host actively. While exiting through feces, some segments disintegrate and release eggs which can then continue their development (Webb and Cabada, 2017). Regarding trematodes (flukes), most species are hermaphroditic except for the schistosomes. Therefore, self-fertilization, as well as cross-fertilization between two trematodes can occur during reproduction. Intestinal trematodes produce eggs that are released through feces, while eggs of the lung trematodes exit the host when they are coughed up in sputum or with the feces if they are swallowed (Keiser and Utzinger, 2009). For schistosomes, an adaptation mechanism assures that produced eggs exit the human host. Eggs are deposited against the wall of the blood vessel. Equipped with sharp spines and lytic enzymes, *Schistosoma* eggs are able to pass through the vessel endothelium and gut wall to cross the surface of either the intestine (e.g., for *S. mansoni*, *S. japonicum*) or the bladder (e.g., for *S. haematobium*) before entering the lumen to be finally expelled through feces or urine (Colley *et al.*, 2014). Helminths are also known to inhabit complex environments (e.g., CNS, blood vessels, etc.). For example, adult Schistosomes live in blood vessels, where one would expect a constant action from the immune system. However, the worms can still live there for several years (Gryseels *et al.*, 2006). Immune evasion mechanisms such as integument hiding the adults or granulomas surrounding eggs have been described. In addition, helminths can produce and release substances (e.g., L_3-hydrolytic enzymes) into the host for their survival (McManus *et al.*, 2018). With the capacity to produce peptides that block host coagulation, platelet aggregation, and host inflammation, hookworms can freely ingest blood in the intestinal mucosa (Loukas *et al.*, 2016).

2.5. Taxonomic groups and key pathogens

In this section, the most important helminths in the medical setting, belonging to the three main groups (cestodes, nematodes, and trematodes) will be discussed through detailed examples. For each species chosen as an example of key pathogen, the biology will be reviewed, focusing on aspects such as life cycles, pathogenesis, and clinical manifestations, as well as methods of diagnosis, treatment, prevention and control.

2.5.1. Cestodes

Also known as tapeworms, cestodes are parasitic worms capable of infecting humans and various types of animals (Craig and Ito, 2007). They belong to the class Cestoda in the phylum Platyhelminthes and are characterized by their flat shape and segmented body composed of a scolex, a neck, and a strobila (Garcia, 2016). The scolex represents the head and attachment point between host and parasite. It may be equipped with suckers, hooks, or elongated grooves facilitating the attachment. The neck is undifferentiated and is prolonged by the strobila which is composed of a series of segments called

proglottids (Schantz, 1996). Proglottids are composed of muscle layers (circular, longitudinal, and transversal) enabling movements (Craig and Ito, 2007). A nervous system originating in the central ganglia located in the scolex links the different segments that are anatomically independent (Schantz, 1996). Tapeworms have no digestive tract. All individual segments have a specific structure and function that allows nutrients to pass through actively (Schantz, 1996; Craig and Ito, 2007). Waste excretion and osmoregulation take place via a lateral pair of excretory tubules. Proglottids are classified as immature, mature, and gravid. They are hermaphroditic (*i.e*., both male and female sex organs are present), but self-reproduction within the same segment is rare (Despommier *et al.*, 2020).

Gravid proglottids containing hundreds or thousands of embryonated eggs detach from the rest of the tapeworm body (i.e., apolysis) and are released along with feces. Depending on the species, tapeworm segments are released in their entire form (*e.g*., *Taenia* spp.), or disintegrate (e.g., *H. nana*) before exiting the host (Schantz, 1996). Upon deposition in the environment, embryonated eggs (i.e., containing oncosphere larvae) can remain viable in the soil for several weeks or months. Their life cycle is complex and involves both intermediate and definitive hosts. Humans usually serve as definitive hosts, but in some cases, they also serve as intermediate hosts (Garcia, 2016).

Adult tapeworms in the human intestine do not usually cause severe pathology, but sometimes chronic symptoms like diarrhea, constipation, abdominal pain, etc. can occur (Webb and Cabada, 2017). However, when a human accidentally becomes an intermediate host (*e.g*., by carrying larvae of the pig tapeworm *Taenia solium*, responsible for neurocysticercosis), this results in a morbidity associated with clinical pathologies such as new-onset seizures (Garcia, 2016).

The four major species distributed worldwide and known to be responsible for human intestinal infections are *Taenia saginata*, *Taenia solium*, *Diphyllobothrium latum, Echinococcus* spp.*, and Hymenolepis nana*. There are other zoonotic species (e.g., *Taenia asiatica*, *Diphyllobothrium pacificum*, *D. nihonkaiense*, etc.) that can also infect humans (Craig and Ito, 2007). The way of transmission depends on the species. Generally, eggs are ingested through contaminated food (vegetables), soil, or water. Larvae, on the other hand, are transmitted through consumption of contaminated raw or undercooked meat.

For the microbiological diagnosis, tapeworm eggs or proglottids can be identified microscopically in fecal samples. However, not all pathologies lead to the release of eggs in human feces, and the eggs of some species are morphologically indistinguishable – despite significant clinical differences (e.g. *T. solium* and *T. saginata*), which would then require further molecular subtyping (e.g. polymerase chain reaction (PCR) of specific genes) (*e.g*., PCR) (Schantz, 1996; Webb and Cabada, 2017).

2.5.1.1. Key pathogen #1: *Taenia saginata*

a) Background

T. saginata is also known as the ‘beef tapeworm’ and was first correctly described in 1782 by Johann Goeze (Goeze, 1782). It is one of the common human cestode pathogens living in the small intestine of

an infected person. An adult *T. saginata* measures about 8-10 m and has a scolex with four suckers, a short neck (3-7 mm), and a long segmented strobila. Gravid proglottids located at the posterior end and containing eggs are often excreted along with feces (Swastika *et al.*, 2017). Eggs are small, slightly oval, have a thick and ridged shell, inhibit the six-hooked embryo (oncospheres), and measure around 31-43 m (Garcia, 2016). Human infection occurs when raw or undercooked beef containing larvae is consumed. During human taeniasis, only a few symptoms are usually present (Tembo and Craig, 2015), but in cattle, bovine cysticercosis has a significant economic impact, particularly in industries (*i.e.*, insurance costs, regular inspections, etc.), and a high burden can lead to economic losses (Dorny and Praet, 2007; Jansen *et al.*, 2018; Laranjo-González *et al.*, 2018). Human taeniasis caused by *T. saginata* has a worldwide distribution (Okello and Thomas, 2017). However, the most endemic areas are Africa, particularly the sub-Saharan African region (Dermauw *et al.*, 2018; Hendrickx *et al.*, 2019; Saratsis *et al.*, 2019), parts of Asia (Eichenberger *et al.*, 2020) and South America (Braae *et al.*, 2018) and, to a lesser extent, Eastern Europe (Trevisan *et al.*, 2018). A few cases have also been reported in North America (Braae *et al.*, 2018) and Western European countries (Laranjo-González *et al.*, 2017).

b) Life cycle

Human infection begins with the consumption of contaminated raw or undercooked beef. The larvae (cysticerci) mature into adult *T. saginata* in the small intestine after 5 to 12 weeks (Garcia, 2016). Using their suckers, they attach themselves to the intestinal mucosa where they feed by actively transporting nutrients (e.g., amino acids, sugars etc.) through their tegument. Gravid proglottids containing eggs (around 100,000) are released with human feces into the environment. When these latter are ingested by an intermediate host (bovine), eggs hatch and release oncosphere larvae in the duodenum which then migrate through the intestinal wall before being transported via lymphatics or bloodstream to tissues and muscles where they mature into cysticerci, which measure approximately 7.5-10 mm wide and 4-6 mm long (Garcia, 2016; CDC, 2019c) (**Figure 4**).

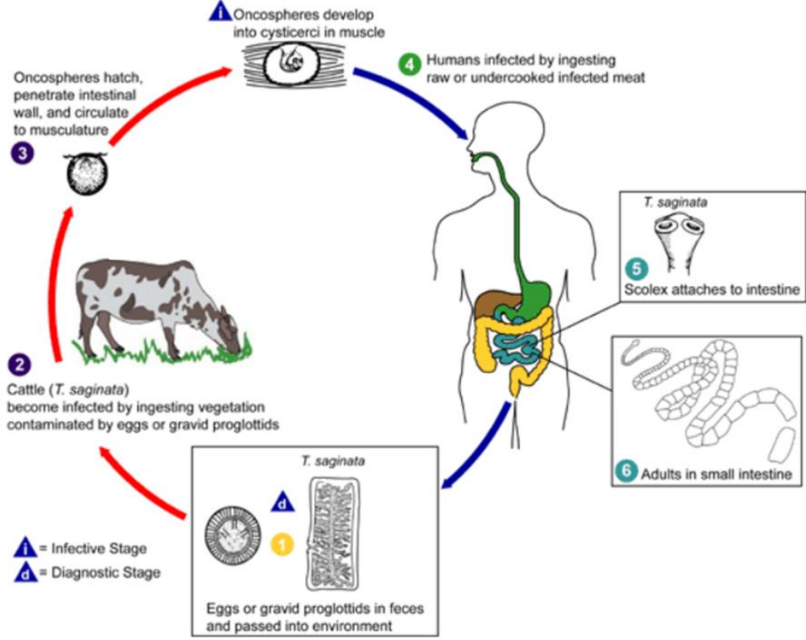

Figure 4. *Life cycle of T. saginata (adapted from (CDC, 2019c)).*

c) Pathogenesis

The presence of adult *T. saginata* in humans induces the production of specific serum antibodies, but only low gut inflammatory responses occur. Moderate eosinophilia associated with increased IgE levels may be observed (Schantz, 1996).

d) Symptoms

In the majority of cases, *T. saginata* infections are asymptomatic. However, symptoms such as epigastric pain, vomiting, nausea, or bowel perforation may also occur (Jongwutiwes *et al.*, 2004; Bhandari *et al.*, 2022; Farhat *et al.*, 2022). Another clinical manifestation is the discharge of gravid proglottids from the anus or mouth (*i.e.*, during vomiting) of the infected person (Raja, 2021).

e) Diagnosis

Morphology-based methods are the most frequently used techniques for the diagnosis of *T. saginata*. Once expelled, intact gravid proglottids can be examined and distinguished from other *Taenia* species by formaldehyde fixation and India ink staining, or hematoxylin-eosin staining. Indeed, they present a typical number of branches (15-20 branches) on either side of the uterus (Mayta *et al.*, 2000; Garcia, 2016). Eggs present in stool sample cannot be distinguished from other species and are generally reported as *Taenia* spp. (Gemmell *et al.*, 1983). In addition, the scolex can occasionally pass into feces, enabling species-specific identification on the basis of scolex characteristics (Schantz, 1996). More recent methods based on molecular techniques (*e.g.*, PCR, loop-mediated isothermal amplification (LAMP), and multiplex PCR) (González *et al.*, 2002; Nunes *et al.*, 2003; Sako *et al.*, 2013; Ng-Nguyen *et al.*, 2017), or serology (*e.g.*, enzyme-linked immunosorbent assay (ELISA)) (Jansen *et al.*, 2016) can

also be employed for species identification. However, these methods are utilized more in research than in clinical routine diagnosis.

f) Treatment

The effective anthelmintic drugs commonly used for the treatment of *T. saginata* infections are praziquantel (PQZ) (5-10 mg/kg) (46) and niclosamide (2 g orally in a single dose, or on three consecutive days if persistence) (Vermund, MacLeod and Goldstein, 1986). These treatments allow the discharge of the worms which are subsequently expelled through feces (Lightowlers *et al.*, 2021). PQZ is highly effective against both mature and immature stages. It induces neuromuscular effects which increase motility, leading to paralysis, scolex detachment and disintegration in the intestine (Frayha *et al.*, 1997). Niclosamide is a multifunctional drug that acts by inhibiting glucose uptake, electron transport in the mitochondrion (*i.e*., uncouple oxidative phosphorylation), and modulating the signaling pathways of Wnt/ß-catenin, mTORC1, STAT3, NF-κB, and Notch (Frayha *et al.*, 1997; Chen *et al.*, 2018).

g) Prevention and Control

Tapeworm infections by *T. saginata* can be prevented by combining good hygiene practices (i.e., proper disposal of human waste, ensure that eggs are killed before using the human wastes as fertilizer, cooking beef properly etc.) and meat inspection. The latter is not routinely implemented in many endemic areas, particularly in resource-constrained countries (Okello and Thomas, 2017).

2.5.1.2. Key pathogen #2: *Taenia solium*

a) Background

T. solium, also called the 'pig tapeworm' is a large tapeworm capable of infecting humans. The adult worm measures about 2-7 m and lives in the host's small intestine (Delobel *et al.*, 2004; Garcia, 2016). It consists of a scolex with four suckers (**Figure 5-C**) and a double ring of hooks, a short neck (3-7 mm), and a long segmented strobila (**Figure 5-D**) (Schantz, 1996). Like *T. saginata*, gravid proglottids contain eggs with round, radiated shell measuring about 30-35 µm in diameter (**Figure 5-A**). Infection occurs when infected pork is eaten raw or undercooked. Unlike *T. saginata*, if *T. solium* eggs are accidentally ingested by human host, they can develop into larvae and lead to human cysticercosis (Garcia, Gonzalez and Gilman, 2020). The larvae can reach the CNS, thus causing neurocysticercosis (NCC). Endemic regions are Latin America (Mexico, Brazil, etc.), sub-Saharan Africa (Burundi, Tanzania, South Africa, etc.), and the Eastern Europe (Garcia, Gonzalez and Gilman, 2020) (**Figure 5-E**).

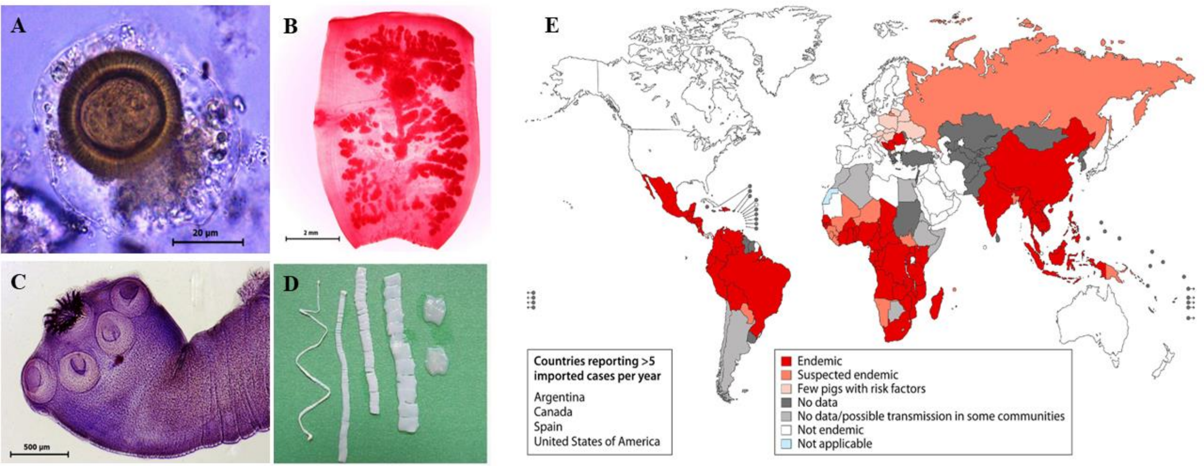

Figure 5. *Taenia solium. (**A**) egg, (**B**) gravid proglottid, (**C**) scolex, (**D**) strobila, and (**E**) Global distribution (adapted from (García* et al.*, 2003; Garcia, Gonzalez and Gilman, 2020))*

b) Life cycle

Infection starts with the consumption of raw or undercooked pork meat containing encysted larvae of *T. solium* which, after being digested out of the meat, attach themselves in the small intestine by using their scolex equipped with suckers and hooks (Flisser, 1994; Mendlovic *et al.*, 2014) to become adults after 5-12 weeks (Garcia, 2016). Eggs produced by adult *T. solium* are released along with gravid proglottids in human feces. Upon reaching the environment (vegetation, grass, etc.), encysted eggs can be ingested by intermediate hosts (pigs). In the intestine of the pigs, eggs hatch and release oncosphere larvae which, with the help of various proteases, cross the intestinal mucosa and migrate (via the bloodstream) to muscles and develop into cysticerci (Zimic *et al.*, 2012; Garcia, Gonzalez and Gilman, 2020). (**Figure 6**).

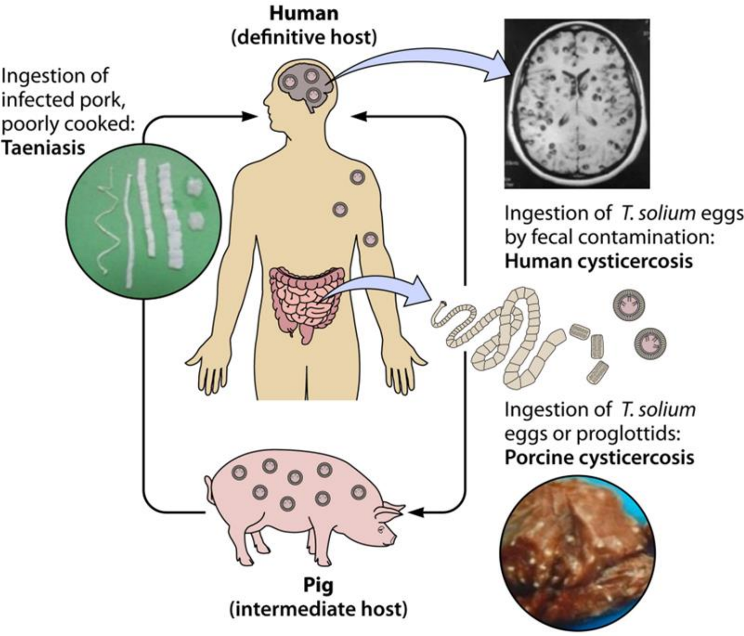

Figure 6. *Life cycle of Taenia solium (adapted from (Garcia, Gonzalez and Gilman, 2020)).*

c) Pathogenesis

In the host gut, although adults of *T. solium* do not provoke significant inflammatory responses, they do induce antibody production (DeGiorgio *et al.*, 2005; Al-Awadhi, Iqbal and Ahmad, 2020). In the case of *T. solium* NCC, which is the most serious complication, oncosphere larvae in the CNS develop with a weak immune response. Indeed, modulation of immune responses is favored by the expression of immune evasion molecules (*e.g*., taeniastatin, somatostatin, protease inhibitor, paramyosin, immunoglobulin-cleaving proteases, etc.) (Leid, Grant and Suquet, 1987; Laclette *et al.*, 1992; Khumbatta *et al.*, 2014), and the activation of immune evasion mechanisms (e.g., covering itself with host immunoglobulins) (Damian, 1987; Garcia *et al.*, 2014). However, an intense immune response is triggered when the parasite begins to die (White, Robinson and Kuhn, 1997). This is due, on the one hand, to the suppression of immune response modulation, and on the other hand, to the presence of cellular reactions (*i.e*., interleukin signaling from T cells, presence of IgM and killer cells) (Restrepo *et al.*, 1998; Garcia *et al.*, 2014).

d) Symptoms

Human taeniasis caused by adult *T. solium* in the host intestine is generally asymptomatic. Rare symptoms like abdominal pain or diarrhea may occur during infection, but these may be due to other causes, and the link is not yet clearly established (Song, Kim and Lee, 2004; Gonzales *et al.*, 2016). The main clinical manifestations in humans are associated with the presence of larvae resulting from the direct ingestion of *T. solium* eggs: cysticercosis. They are of two types: (i) extraneural cysticercosis corresponding to cysticercosis outside the CNS (*e.g*., ocular, intramuscular, or subcutaneous), which is most frequently asymptomatic (Dixon *et al.*, 2021), and (ii) NCC which corresponds to cysticercosis within the CNS (*e.g*., intraparenchymal, or extraparenchymal) (**Figure 7**) with severe manifestations such as myriad lesions, chronic headache, seizures, hydrocephalus, encephalitis, cognitive deficits, epilepsy, etc. (Rangel *et al.*, 1987; Rajshekhar, 1991; Elliott and Landaker, 2017; Ramirez-Bermudez *et al.*, 2017; Duque *et al.*, 2018; Nau *et al.*, 2018).

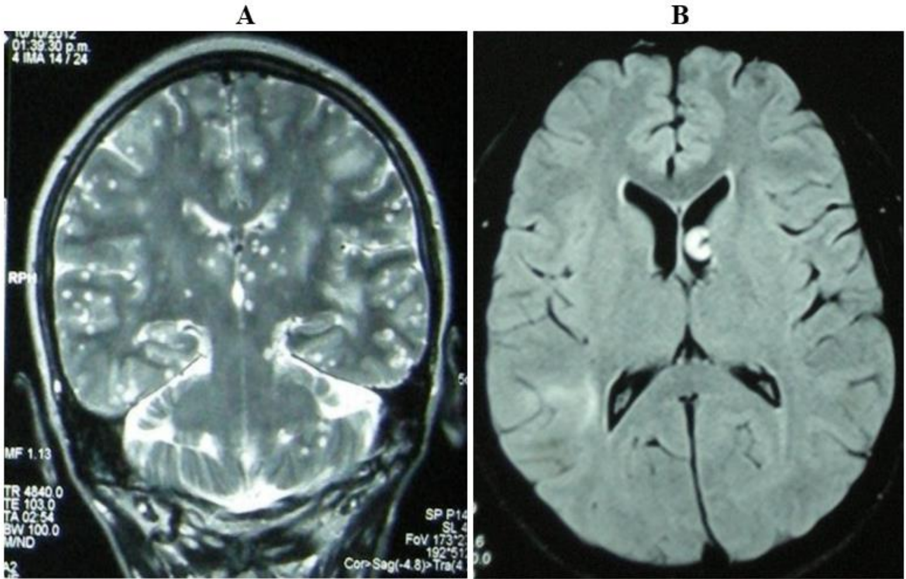

Figure 7. *Parenchymal (**A**, cysticercosis encephalitis) and extraparenchymal (**B**, intraventricular cyst) neurocysticercosis (adapted from (Garcia, Gonzalez and Gilman, 2020)).*

e) Diagnosis

For human taeniasis, diagnosis can be performed by microscopic examination of materials expelled with the feces (*e.g*., gravid proglottids, eggs, or scolex). Indeed, specific characteristics of *T. solium* such as gravid proglottid that has less than 12 uterine branches (see **Figure 7**-**B**), or the scolex having hooks represent key elements for identification (see **Figure 7-C**). These morphological-based methods are widely used, although they have some limitations. Eggs cannot be distinguished from other species, and an intact scolex is rarely expelled. Alternative methods such as DNA dot blot test (Yamasaki *et al.*, 2004; Mayta *et al.*, 2008), NAAT, and coproantigen detection tests by ELISA (Allan *et al.*, 1990; Praet *et al.*, 2013) are also available for the differentiation of *T. solium* from other *Taenia* species.

For human cysticercosis and NCC, diagnosis is generally based on imaging tests like computed tomography (CT), or magnetic resonance imaging (MRI) (see **Figure 7**) (Lerner *et al.*, 2012; Hernández, Durán and Lujambio, 2014). Based on the capacities and limitations of these two techniques, the utilization of a combination of both techniques improves the diagnosis, as recommended by the current U.S guidelines (White *et al.*, 2018). Subsequent serological tests such as the enzyme-linked immunoelectrotransfer blot (EITB) (Tsang, Brand and Boyer, 1989), or ELISA (Carod *et al.*, 2012; Garcia *et al.*, 2018) could provide additional information to confirm the imaging-based diagnosis (Rodriguez, Wilkins and Dorny, 2012). Furthermore, molecular methods (*e.g*., PCR, or sequencing) have been recently introduced but the sensitivity level is not optimal (Liu *et al.*, 2018; Goyal *et al.*, 2020; O'Connell *et al.*, 2020).

f) Treatment

Like *T. saginata* taeniasis, the treatment of choice for *T. solium* taeniasis are PQZ (5-10 mg/kg, single oral dose) and niclosamide (2g, single oral dose) (Garcia, Gonzalez and Gilman, 2020). For NCC, especially in case of symptomatic NCC, treatment requires medical therapy with symptomatic medication (*i.e*., analgesics, steroids to reduce intracerebral edema, antiepileptics, etc.) (Bustos, García and Del Brutto, 2016). Additionally, antiparasitic treatment may be used to destroy viable or degenerating cysticerci (Otte *et al.*, 2013; Romo *et al.*, 2015; Zhao *et al.*, 2016). For single parenchymal NCC, albendazole 15mg/kg/day for 7 to 15 days is the treatment of choice. In case of multiple viable cysts, a combination of albendazole (same regimen) and PQZ 50 mg/kg/day for 10 days demonstrated a better efficacy (Garcia, Lescano, *et al.*, 2016). However, due to the enhanced inflammatory responses caused by cysticercal death, steroids would be required to reduce swelling. Occasionally, surgery may be necessary in patients with NCC. In the situation where all aforementioned diagnosis tests and treatments are not always available (e.g., in resource-limited countries), patient management should at least include symptomatic treatment (Millogo, Kongnyu Njamnshi and Kabwa-PierreLuabeya, 2019).

g) Prevention and control

T. solium infections constitute a serious public health problem even in non-endemic countries due to the increase of cysticercosis. In endemic areas, the phenomenon of "free roaming pigs" in close contact to humans, especially children, is an important factor leading to human infections and transmission of cysticercosis (Millogo, Kongnyu Njamnshi and Kabwa-PierreLuabeya, 2019) (**Figure 8**). To control taeniasis and cysticercosis/NCC, especially in endemic areas, prevention measures should be considered seriously. This would necessarily need a large integrated approach, as proven in Peru, where *T. solium* transmission was eliminated in 105/107 villages of about 80'000 people using a bundle of human and porcine mass chemotherapy (i.e. application of anthelmintic drugs), pig vaccination, and coproantigen detection tests (Garcia, Gonzalez, *et al.*, 2016). Additionally, one should also consider an extended approach integrating socio-economic aspects such as better hygiene and sanitation conditions, improved health systems, access to clean water, and educating populations in the general aspects of hygiene behavior.

***Figure 8**. Close human-pig contact is a major risk factor for T. solium infections (adapted from (Millogo, Kongnyu Njamnshi and Kabwa-PierreLuabeya, 2019) and (WHO, 2022a)).*

2.5.1.3. Key pathogen #3: *Echinococcus granulosus senso lato* (*s.l.*) and *Echinococcus multilocularis*

a) Background

E. granulosus s.l. and *E. multilocularis* are cestode parasites causing diseases known respectively as cystic echinococcosis (CE) and alveolar echinococcosis (AE). They represent the two most important zoonoses in terms of health and economics (Deplazes *et al.*, 2017). Most of the human infections are caused by *E. granulosus* (Griffin, Donaghy and Edwards, 2014). *E. granulosus* was described in 1782 by Johann Goeze who described juvenile forms (*i.e.*, protoscoleces) (Goeze, 1782), and in 1853 by Carl von Siebold who described adult forms (Despommier *et al.*, 2020). The larval stage of *E. granulosus s.l.* is a fluid-filled vesicle (*i.e*., hydatid cyst) measuring about 1-15 cm in diameter (Bhutani and Kajal, 2018). While for *E. multilocularis*, the larvae are smaller and have external buds (Gottstein *et al.*, 2017). Adult worms of both species measure about 3-7 mm long, consisting of a scolex and a strobila segmented in three parts (**Figure 9-A**), and live in the small intestine of definitive hosts (mainly dogs for *E. granulosus*, and dog/fox for *E. multilocularis*) (Wen *et al.*, 2019). *E. granulosus* is globally distributed (Algeria, Kenya, Sudan, USA, Chine, Russia, Japan, Syria, Argentina, Brazil, Peru, Australia, etc.) with the highest prevalences found in South America, North Africa, East Africa, Central Asia, and Mongolia (Jenkins *et al.*, 2014; Deplazes *et al.*, 2017; Pavletic *et al.*, 2017). *E. multilocularis* is mainly found in Asia, Europe, and North America and is also endemic in Germany (McManus *et al.*, 2003; Deplazes *et al.*, 2017) (**Figure 9-B**). The average global burden estimated in disability-adjusted -life years (DALYs) was 285,500 for CE (Torgerson *et al.*, 2010; Cm *et al.*, 2013) and 666,434 for AE (Torgerson *et al.*, 2010). Both pathogens and their respective diseases are highly endemic in resource-limited communities in China (Torgerson *et al.*, 2018).

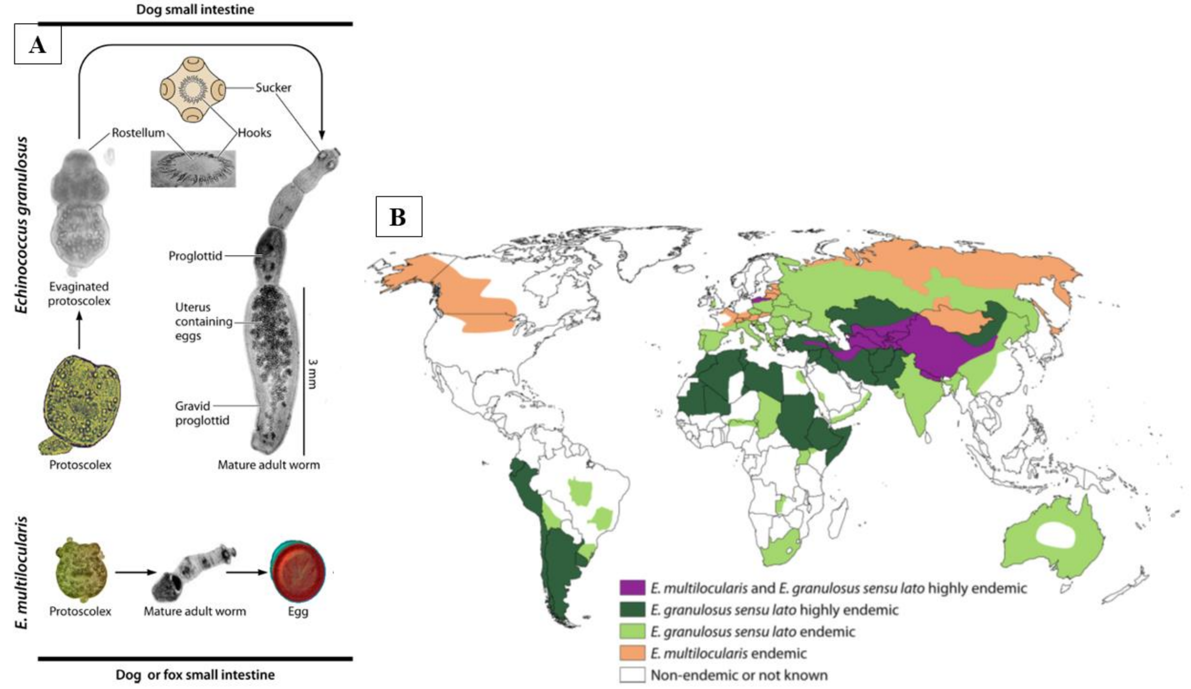

***Figure 9**. (**A**) Morphology and development stages of E. granulosus s.l. and E. multilocularis. (**B**) Global distribution of E. granulosus s.l. and E. multilocularis (adapted from (Wen* et al.*, 2019)).*

b) Life cycle

Eggs produced by adult worms and located in the gravid proglottid are expelled in the environment (grass, vegetation, etc.) through carnivore's feces. When ingested by the intermediate host (sheep or ruminants for *E. granulosus*, and rodents for *E. multilocularis*), embryonated eggs hatch and release juvenile oncosphere larvae, which then cross the intestinal barrier and are carried by the bloodstream to the liver where they develop into metacestodes (*i.e.*, hydatid cysts structured as small vesicle with an inner germinal layer (GL) and an outer laminated layer (LL)). Of note, organs other than liver may also be affected (*e.g.*, lungs, kidney, heart, brain etc.) (Romig *et al.*, 2017). Inside each cyst, the GL produces brood capsules that lead to protoscoleces (fertile form of the parasite) (Gottstein *et al.*, 2017). When protoscoleces contained in the cysts are ingested by the definitive host, scoleces emerge and attach to the intestinal wall using their suckers and hooks (Wen *et al.*, 2019). Most importantly, humans can be accidental intermediate hosts, thus the development of *Echinococcus* spp. cysts inside the human body lead to CE or AE (**Figure 10**) (Wen *et al.*, 2019).

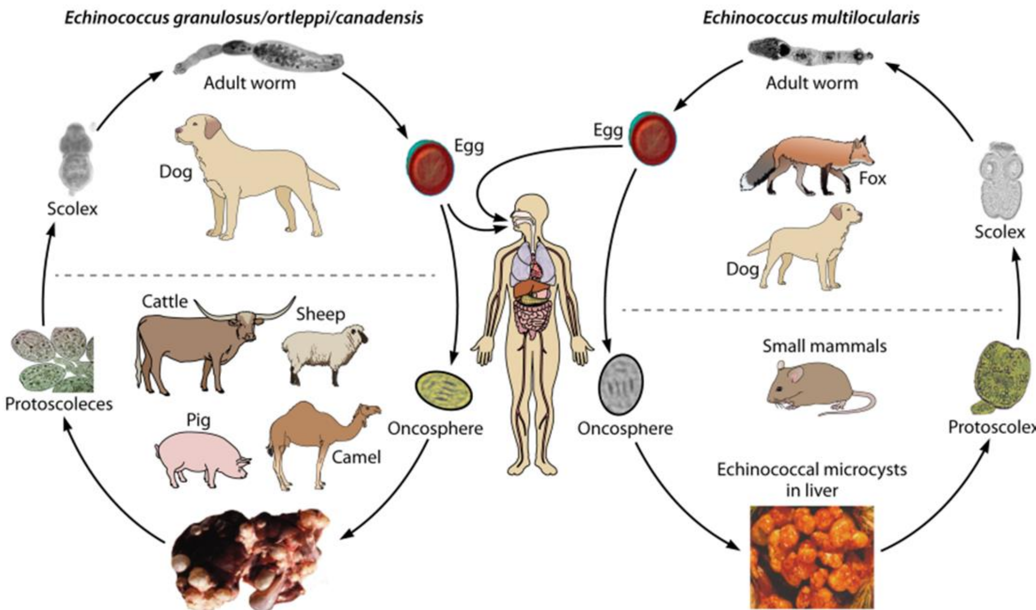

Figure 10. *Life cycle of Echinococcus spp. (adapted from (Wen* et al.*, 2019)).*

c) Pathogenesis

In human CE, *E. granulosus* can passively escape the immune response by growing within the cyst and using the LL as a barrier (Siracusano, Teggi and Ortona, 2009). The second strategy consists of immunomodulation through active interaction with the host immune system. During AE, in addition to its role in maintaining tolerance, TGF-ß present around parasitic lesions may participate in the production of collagen and other extracellular components, and thus induce the formation of hepatic fibrosis which is a characteristic of AE (Bartram and Speer, 2004; Higashiyama, Kinoshita and Asano, 2007).

d) Symptoms

Most cysts are asymptomatic. However, clinical signs associated with CE are mainly related to space occupied by the cyst which can grow quite big in size. A cyst rupture can lead to allergic reactions like urticaria, angioedema or anaphylactic shock (Gottstein *et al.*, 2017). For symptomatic AE, the most frequent symptoms are tumor-like liver lesions with an infiltrative, malignancy-like growth pattern, hepatomegaly and cholestatic jaundice, liver abscess, portal hypertension, and Budd-Chiari syndrome. At the early stage of infection, unspecific clinical manifestations can occur (*e.g*., epigastric pain, or signs of anicteric cholestasis) (Gottstein *et al.*, 2017). *E. multilocularis* infections can also be asymptomatic for a long period (5-15 years) (Eckert and Deplazes, 2004).

e) Diagnosis

The diagnosis of CE and AE is mainly based on clinical examination and imaging with a typical morphology pattern which is suggestive of the diseases (ultrasound (US), MRI, or computed tomography (CT)) (Bresson-Hadni *et al.*, 2006; Bhutani and Kajal, 2018; Solomon *et al.*, 2018). The use of portable US devices, especially in endemic areas, represents a suitable option for screening and

detection of asymptomatic patients (Wen *et al.*, 2019). In addition, the recent introduction of LAMP as a first-line method for screening allows the detection of *Echinococcus* spp. DNA (Wassermann, Mackenstedt and Romig, 2014). In non-endemic areas, the history of the patient (i.e., travel to endemic areas, dog owner, etc.) should be considered (Bhutani and Kajal, 2018). Other methods including serology (*e.g*., ELISA, or detection of antigens) (Zhang, Li and McManus, 2003; Pagnozzi *et al.*, 2016), qPCR (Boufana *et al.*, 2013; Maas *et al.*, 2016), and microscopy (i.e., detection of hooklets on sputum sample) (Antoniou and Tselentis, 1993) can be employed to confirm imaging results.

f) Treatment

Depending on the diagnostic findings, different approaches may be used for the treatment of CE and AE, including anthelmintic drugs (albendazole or mebendazole) (Ammann *et al.*, 1988; Vuitton *et al.*, 2016; Siles-Lucas *et al.*, 2018), "watch-and-wait" strategy (Junghanss *et al.*, 2008), non-surgical interventions (*e.g*., PAIR technique = puncture, aspiration, injection, and re-aspiration, or MoCAT = modified catheterization technique) (Chen and Xusheng, 2007; Gupta *et al.*, 2011), and surgical interventions (Chen and Xusheng, 2007; Soni *et al.*, 2011). Overall, the use of multidisciplinary and coordinated approaches leads to better therapeutic conclusions and an improved patient outcome (Piarroux *et al.*, 2011).

g) Prevention and control

Prevention and control of CE and AE consists, primarily, of an implementation and improvement of slaughtering conditions associated with good management of dogs (avoiding feeding dogs with contaminated viscera (**Figure 11**), periodic treatment of dogs with PQZ, or niclosamide etc.) (Zhang *et al.*, 2009). Subsequently, the implementation of multidisciplinary approaches in a one-health perspective (US screening, dog dosing, optimized management of patients, etc.) during control programs would be necessary (Merino *et al.*, 2017). Dog and sheep vaccinations would be an effective alternative to eliminate echinococcosis transmission (Zhang and McManus, 2008).

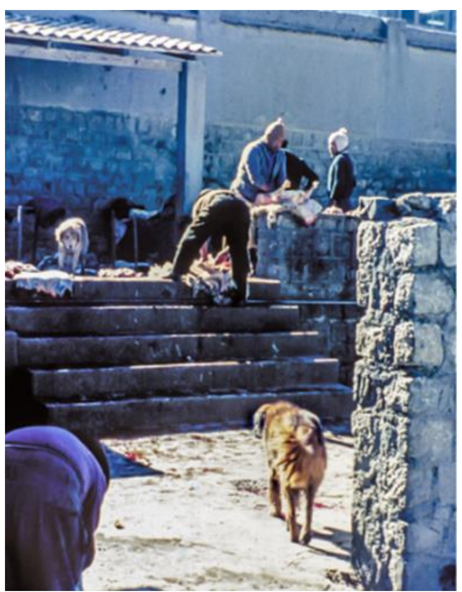

***Figure 11**. Rural slaughterhouse in Peru: dogs are occasionally fed with infected viscera containing hydatid cysts (adapted from (Despommier* et al.*, 2020)).*

2.5.2. Nematodes

Nematodes or roundworms are non-segmented worms belonging to the phylum Nematoda. Most nematodes are free-living organisms inhabiting soil, fresh or saltwater. Some of them are parasitic and capable of infecting human hosts (Hotez *et al.*, 2008). The most common human nematodes are soil-transmitted helminths (STHs): the roundworm *Ascaris lumbricoides*, the whipworm *Trichuris trichiura*, and the two hookworm species *Necator americanus* and *Ancylostoma duodenale* (CDC, 2022). These infections occur mainly in LMICs, areas where sanitation and hygiene conditions are poor. Children are the most affected population group and suffer from diverse consequences (e.g., abdominal pain, diarrhea, malnutrition, physical growth retardation, and deficits in cognitive and intellectual development) when they are heavily infected, while light infections usually do not give rise no symptoms. These STHs, also called intestinal parasites, live in the intestine of their host and produce thousands of eggs that are released with the feces (Else *et al.*, 2020).

2.5.2.1. Key pathogen #4 : *Ascaris lumbricoides*

a) Background

First described as *Lumbricus teres* by Edward Tyson in 1683 (Tyson, 1683), *A. lumbricoides*, according to F.E.G Cox in "*History of human parasitology*" (Cox, 2002), owes its name to Carl Linnaeus who named it for its resemblance to the earthworm *Lumbricus terrestrias*. Adult worms measure around 15-25 cm (males) or 20-35 cm (females) and can live up to 1-2 years. The body cavity contains an alimentary canal, an excretory system, a nervous system, and a reproductive system with genital organs well-developed in both sexes (Khuroo *et al.*, 2016). The life cycle was correctly described in 1917 by

the American zoologists/parasitologists Brayton Howard Ransom and W. D. Foster (Ransom and Foster, 1917).

b) Life cycle

Adult worms live in the lumen of the small intestine. The adult *Ascaris* female can produce up to 200,000 eggs per day, which exit the host with feces. Eggs mature and become fertile and embryonated within 2-8 weeks depending on the environmental condition (humidity, moisture, temperature, shaded soil, tropical soil, etc.). Of note, *Ascaris* eggs can survive in the environment (soil) for up to 6 (Stephenson and Holland, 1987) 10 (Khuroo, 1996), or even 14 years (Stephenson and Holland, 1987). It was reported that in tropical soils, *Ascaris* eggs can be depleted within 2 months if there is no further contamination. Eggs that do not reach the soil directly (e.g., sewage) can survive up to two months (Bryan, 1977). This resistance ability is due to the presence of a lipoprotein called ascaroside, which occupies a portion of the inner layer of the egg. Once infective eggs are ingested by the host (i.e., human), the egg-protease is activated by alkaline conditions in the small intestine. In addition, bile salts help the solubilization of certain outer layers of the eggshell. These conditions stimulate the hatching and release of L_3 larvae, which invade the intestinal mucosa. They are then transported via the portal blood vessels to the liver, then to the heart, and finally to the lungs, where they continue their maturation (10 to 14 days). In the lungs, they penetrate the alveolar spaces, and travel through the bronchial tree to the pharynx, where they are coughed up and swallowed. Upon reaching the small intestine, they develop into L_4, then L_5 larvae, and finally become adults. Two to three months are required from ingestion of the infective eggs to the adult stage. Adults can live for one to two years (**Figure 12**) (Else *et al.*, 2020; CDC, 2022).

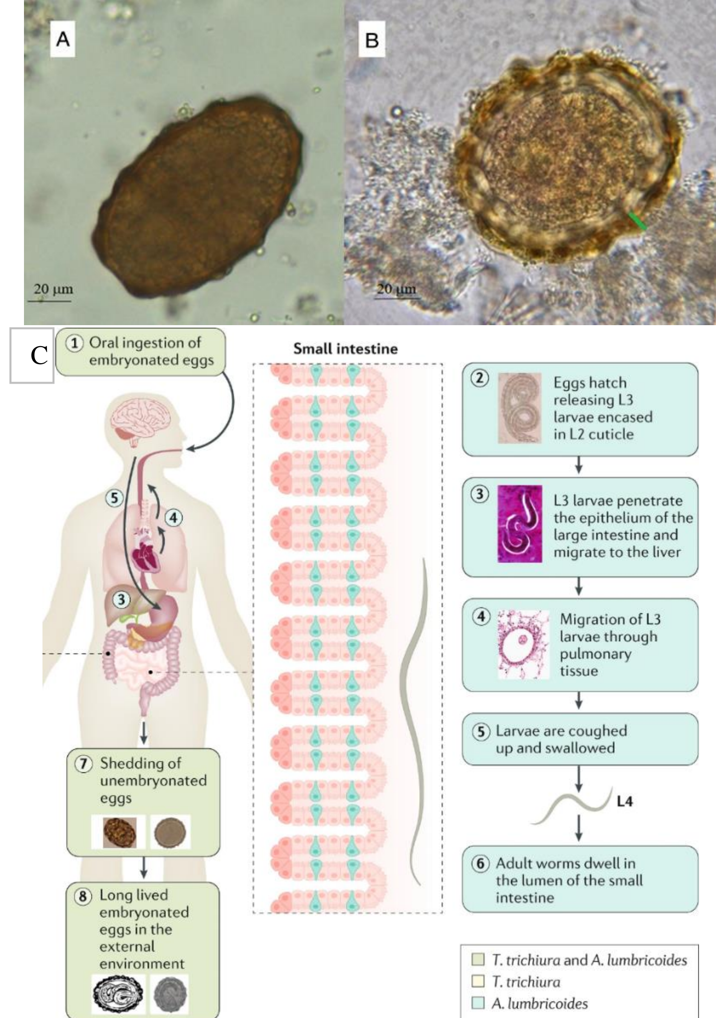

***Figure 12**. Different stages of A. lumbricoides eggs: (**A**) unfertilized and (**B**) fertilized eggs. (**C**) Life cycle of Ascaris lumbricoides (adapted from (Else* et al.*, 2020; Maurelli* et al.*, 2021)).*

c) Pathogenesis

The pathogenesis of *A. lumbricoides* is primarily due to the mechanical damage caused by the hepato-tracheal migration of the larvae. Antigens released by the parasite during this phase have allergenic properties, causing inflammation and damage to the tissues, but also antibody responses inducing an increase of IgE levels in serum (Else *et al.*, 2020). In the small intestine, adult worms, due to their large size, can cause a range of pathological changes, including mechanical obstruction of the intestinal lumen, inflammation of the intestinal wall, malabsorption of nutrients, or even intestinal perforation leading to life-threatening complications (Else *et al.*, 2020).

d) Symptoms

The onset and severity of ascariasis symptoms are related to the worm burden. During the larval migration phase, in the case of light infection (only a few parasites penetrate the host's tissues), the host's response remains insignificant, and the infected person is asymptomatic. On the other hand, in the case of heavy infection (ingestion of hundreds or thousands of eggs), severe symptoms are observed

in early infection phases, such as an eosinophilic pneumonia-like disease (Löffler syndrome), liver hypertrophy, etc. (Else *et al.*, 2020). During the intestinal phase of a severe infection, adult worms, because of their big size, can cause intestinal obstruction (**Figure 13**) (Yetim *et al.*, 2009; López *et al.*, 2010; Ali, Mohamed Abdi and Mambet, 2023). In endemic countries, airway obstruction may also occur (Bailey and Warner, 2010). Symptoms typically include abdominal pain, anemia, and vomiting. Adult worms can also migrate to the biliary tree, resulting in hepatobiliary and pancreatic ascariasis (HPA) (Khuroo *et al.*, 2016; Lynser and Marbaniang, 2016; Temesgen, Abebe and Abera, 2022) characterized by cholecystitis, hepatic abscess, and pancreatitis.

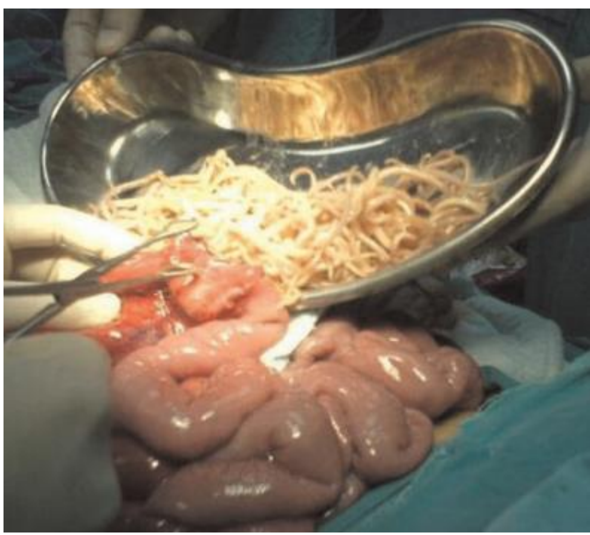

***Figure 13**. Adult Ascaris lumbricoides extracted from the jejunum of a 7-year-old boy (adapted from (Yetim* et al.*, 2009)).*

e) Diagnosis

Ascariasis cannot be diagnosed specifically based on clinical manifestations. In addition to the clinical suspicion of helminth infection, stool microscopy on three consecutive samples should be ordered. In diagnostic laboratories, there are various techniques for diagnosing ascariasis (Lamberton and Jourdan, 2015). A Kato-Katz (KK) smear based on duplicate slides is a widely used method recommended by the WHO in endemic areas (WHO, 2011). It is relatively inexpensive, easy to perform, can detect other co-endemic parasites species (e.g., *Trichuris trichiura*), and correlates well with the intensity of infection expressed as number of eggs per gram (EPG) of feces (Speich *et al.*, 2015). Other stool examination methods are also available: direct smear, formol-ether concentration (FEC), McMaster, FLOTAC, and Mini-FLOTAC (Nikolay, Brooker and Pullan, 2014; Lamberton and Jourdan, 2015). However, the KK technique has some limitations such as a high variance of EPG and limited sensitivity in light-intensity infection (156–158).

Aiming to improve sensitivity and specificity, alternative diagnostic methods such as serological or molecular methods were developed. Using *Ascaris*-antibody (Ab) ELISA, certain immunoglobulins (e.g., IgG4) can be utilized as specific markers for detecting chronic *Ascaris* infection (Chatterjee *et al.*,

1996; Bhattacharyya *et al.*, 2001; Santra *et al.*, 2001; Dana *et al.*, 2022). However, it is common to find cross-reactivity with epitopes from other helminth species (Zaman *et al.*, 2018). This method does not distinguish active and past infection, and therefore may overestimate the true prevalence. Molecular methods, which are mainly based on DNA amplification allow better sensitivity (Easton *et al.*, 2016; Mationg *et al.*, 2017; Zendejas-Heredia *et al.*, 2021). They are more expensive and mainly utilized in research context (Else *et al.*, 2020).

f) Treatment

Current treatments of choice for ascariasis are albendazole, mebendazole, levamisole, and pyrantel pamoate (**Table 1**) (WHO, 2011; Else *et al.*, 2020). Usually, a single dose of albendazole 400 mg or mebendazole 500 mg is effective. They have been used widely for decades against *A. lumbricoides* in the treatment of individual patients and in mass drug administration (MDA) programs (Else *et al.*, 2020). Other alternative drugs such as ivermectin, moxidectin, and tribendimidine are also effective against *A. lumbricoides* (Moser, Schindler and Keiser, 2017). Sometimes, if a heavy infection leads to intestinal obstruction, surgical intervention is necessary (Yetim *et al.*, 2009; Gupta *et al.*, 2012).

Table 1. Recommended preventive chemotherapy (PC) regimens and efficacy of anthelminthic drugs against *A. lumbricoides* (*adapted from* (Else *et al.*, 2020)).

Treatment	**Mechanism of action**	***A. lumbricoides* infection**		
		Preventive chemotherapy	**Cure rate (%)**	**Egg reduction rate (%)[a]**
Albendazole	β-Tubulin binding	Once	96.5	99.7
Mebendazole	β-Tubulin binding	Once	96.8	99.5
Albendazole-ivermectin	NA	Once	96.7	99.9
Levamisole	L-subtype nAChR agonist	Once	93.0	97.0
Pyrantel pamoate	L-subtype nAChR	Once	97.5	91.7

NA: not applicable; nAChR: nicotinic acetylcholine receptor; [a]After single dose administration

g) Prevention and control

The prevention of ascariasis depends on the combination of several approaches attempting to reduce the prevalence. In addition to the preventive chemotherapy by MDA, which is the cornerstone of the current WHO-recommended strategy in endemic areas to reduce the mortality and morbidity in school-aged children (**Figure 14**) (Marocco *et al.*, 2017; Bah *et al.*, 2019; WHO, 2020), the water, sanitation, and hygiene (WASH) program encourages improvements in access to clean water as well as awareness in hygiene procedures (*i.e.*, hand washing before eating and after defecation using soap) (Fewtrell *et al.*, 2005; Vaz Nery *et al.*, 2019).

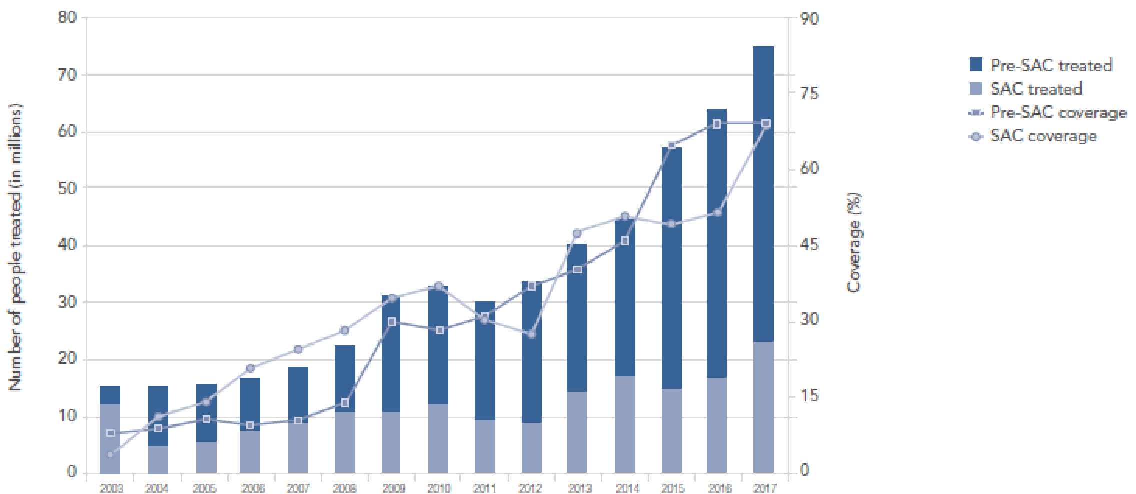

***Figure 14**. Number of pre-school aged children and school-aged children treated and progress in preventive chemotherapy (PC) coverage, 2003-2017 (adapted from (WHO, 2020)).*

2.5.2.2. Key pathogen #5 : *Trichuris trichiura*

a) Background

T. trichiura, also called human whipworm, was first described in 1740 by Giovanni Morgagni. In 1761, Johannes Roederer depicted the morphological aspect (Despommier *et al.*, 2020). The adult female measure between 30 and 50 mm, while the adult male size is 30-45 mm (Viswanath, Yarrarapu and Williams, 2023). They can live inside the host for up to 1.5-2 years. Eggs released by adults have specific characteristics ("lemon-shaped") and measure around 50-55 µm in length, and 20-25 µm in width (Despommier *et al.*, 2020) (**Figure 15**).

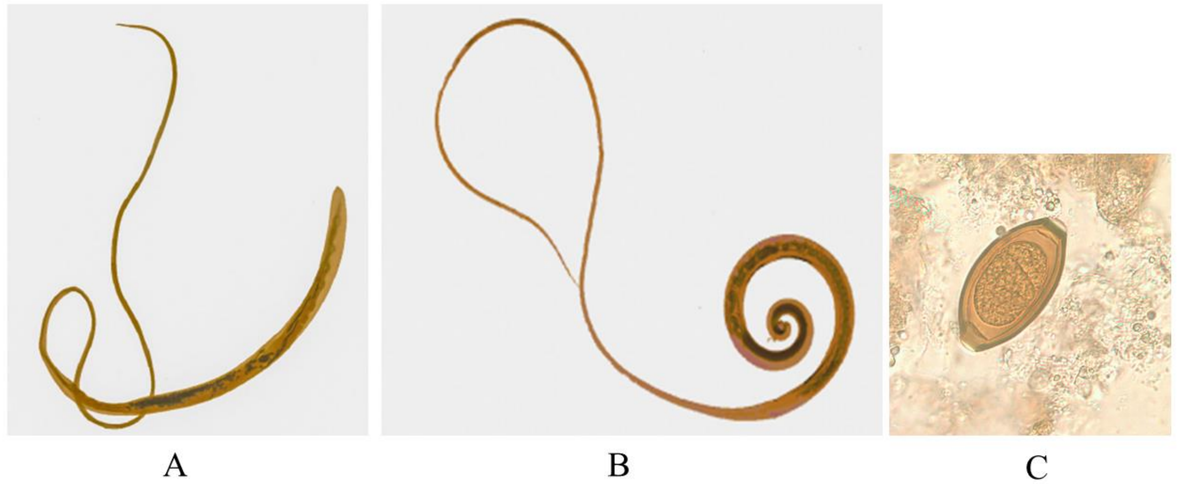

***Figure 15**. Trichuris trichiura: (A) female adult, (B) male adult, (C) egg (adapted from (CDC, 2019b; Despommier* et al.*, 2020))*

b) Life cycle

Like other *Trichuris* species, *T. trichiura* infection begins with the ingestion of an embryonated egg via contaminated food or water. Upon arrival in the small intestine, the egg hatches and releases an L_1 larva, which penetrates the gut epithelium cells. After several molts (L_2, L_3, and L_4) in the intracellular niche, the immature adult arises in the large intestine. However, from the L_3 larval stage, the parasite is no longer totally intracellular; only the thin anterior end is embedded within a syncytial tunnel of host epithelial cells without compromising the gut barrier. The posterior end protrudes into the gut lumen (Else *et al.*, 2020). The pre-patent period (i.e., time from infection to egg production) in *T. muris* is estimated to be 33-35 days in mice with 2000 to 8000 eggs per day released by the adult female (Pike, 1969). In humans, a study suggested a pre-patent period of more than 60-70 days (Vavricka, Manz and Burri, 2009). Unembryonated eggs (non-infective) are expelled with host feces, and depending on the environmental conditions (humidity, temperature, etc.), embryonation of *T. trichiura* eggs takes 2 to 4 weeks (**Figure 16**) (Beer, 1976).

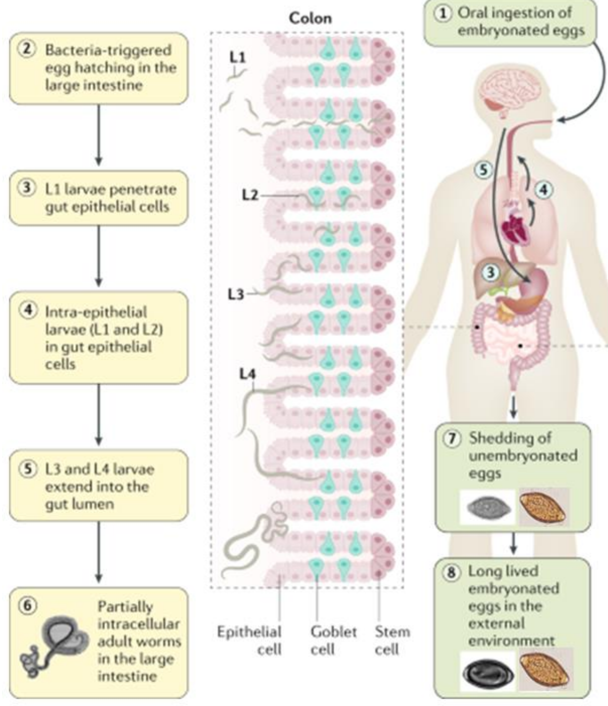

***Figure 16**. The life cycle of Trichuris trichiura (adapted from (Else et al., 2020)).*

c) Pathogenesis

Heavy infection of *T. trichiura*, particularly in children, depends on the incapacity of the host immune system to establish a strong Th2 response (Jackson *et al.*, 2004). During the process of colonic mucosa invasion, pore formation occurs in the epithelial cell membrane, facilitates the invasion, and allows the parasite to maintain in the cecal epithelium. Pore formation occurs and is induced by a channel-forming protein released by the adult worm (Drake *et al.*, 1998; Eichenberger *et al.*, 2018).

d) Symptoms

Clinical manifestations caused by *T. trichiura* infection are linked to intensity of infection. Light infections induce mild symptoms (Else *et al.*, 2020). In contrast, heavy infections with several hundreds of worms (*i.e.*, ≥800 worms (Layrisse *et al.*, 1967), or >5,000 eggs per gram of stool (EPG) (Ramdath *et al.*, 1995) cause chronic colitis, or chronic iron deficiency anemia IDA) in adults (Khuroo, Khuroo and Khuroo, 2010). Children may present with bloody diarrhea, malnutrition, failure to thrive, or the so-called *Trichuris* dysentery syndrome (TDS), which is also known as massive infantile trichuriasis (i.e., a severe illness associated with a prolapsed rectum (MacDonald *et al.*, 1991; Kaminsky, Castillo and Flores, 2015).

e) Diagnosis

Trichuriasis diagnosis is mainly based on stool examination for egg detection using microscopy (Ghai *et al.*, 2014). Adult *T. trichiura* worms can also be identified by direct visualization on colonoscopy, especially when there are no symptoms and stool examination is negative (Ok *et al.*, 2009). Like ascariasis, WHO also recommends the use of the KK method (WHO, 2011). *T. trichiura* eggs have specific features (*i.e.*, "lemon-shape") and are relatively easy to identify. However, in case of light infections, egg concentration (e.g., centrifugation) is necessary prior performing microscopy. Other methods such as McMaster or Mini-FLOTAC can also be used (Barda *et al.*, 2014). Egg examination only can sometimes be difficult, as different species of *Trichuris* can be very similar (Ghai *et al.*, 2014). Molecular methods (*e.g.*, qPCR) have been developed for alternative diagnostic tools allowing a more precise identification at the species level (Phosuk *et al.*, 2018) with a better sensitivity and specificity (Mationg *et al.*, 2017). However, the application of such methods in endemic regions is limited as they require specific and more expensive equipment.

f) Treatment

The treatment of choice for trichuriasis is mebendazole or albendazole (Moser, Schindler and Keiser, 2017). In deworming programs, a single dose (or combined with ivermectin or oxantel) is usually effective, but several doses can be required for a complete cure (Sirivichayakul *et al.*, 2003; Benjamin Speich *et al.*, 2014). These drugs act by inhibiting microtubule polymerization by binding the unique beta-tubulin of invertebrates (**Table 2**). Of note, these drugs are less effective against *T. trichiura* infection as compared to other STH and there is a pressing need for better therapeutic tools to treat trichuriasis.

Table 2. Recommended treatment regimens and efficacy of anthelminthic drugs against *T. trichiura* (*adapted from* (Else *et al.*, 2020)).

Treatment	Mechanism of action	*T. trichiura* infection		
		Dosage	Cure rate (%)	Egg reduction rate (%)[a]
Albendazole	β-Tubulin binding	Once	32.1	64.3
Mebendazole	β-Tubulin binding	Once	44.4	80.7
Albendazole-ivermectin	NA	Once	60.0	95.5
Levamisole	L-subtype nAChR agonist	Once	23.4	41.8
Pyrantel pamoate	L-subtype nAChR	Once	28.5	62.3

NA: not applicable; nAChR: nicotinic acetylcholine receptor; [a]After single dose administration.

g) Prevention and Control

As with other diseases caused by STHs, the prevention and control of trichuriasis require a strategic approach aiming to reduce mortality, morbidity, and prevalence in endemic areas by combining anthelmintic treatment, environmental sanitation and hygiene, and health education (Else *et al.*, 2020). This would consist of conducting preventive chemotherapy (i.e., MDA) and raising awareness among people living in endemic areas about hygiene procedures, especially with regard to the WASH program (Strunz *et al.*, 2014).

2.5.2.3. Key pathogen #6: Hookworm (*Necator americanus* and *Ancylostoma duodenale*)

a) Background

Hookworms are nematode parasites belonging to the family Ancylostomatidae. The two main hookworm species affecting humans are *Ancylostoma duodenale* and *Necator americanus* (Hotez *et al.*, 2004). A third hookworm species, *A. ceylanicaum* is mainly found in cats and dogs, and was also described as a common human pathogen in Southeast Asia and the Pacific (Ngui *et al.*, 2012; Stracke, Jex and Traub, 2020). Human hookworm infection with *A. duodenale* was first reported by Angelo Dubini in 1843, while *N. americanus* was reported by Stiles in 1902 (Despommier *et al.*, 2020). Previous reports estimated around 500 million people to be infected in developing countries (Loukas *et al.*, 2016). The adult worms inhabit the small intestine of the host and measure between 7-11 mm (male) and 9-13 mm (female) (**Figure 17**). The percutaneous transmission of hookworms through the invasion of a third-stage (L_3) larva into the human host as well as the life cycle was first demonstrated and clarified by the German scientist named Loos (Despommier *et al.*, 2020).

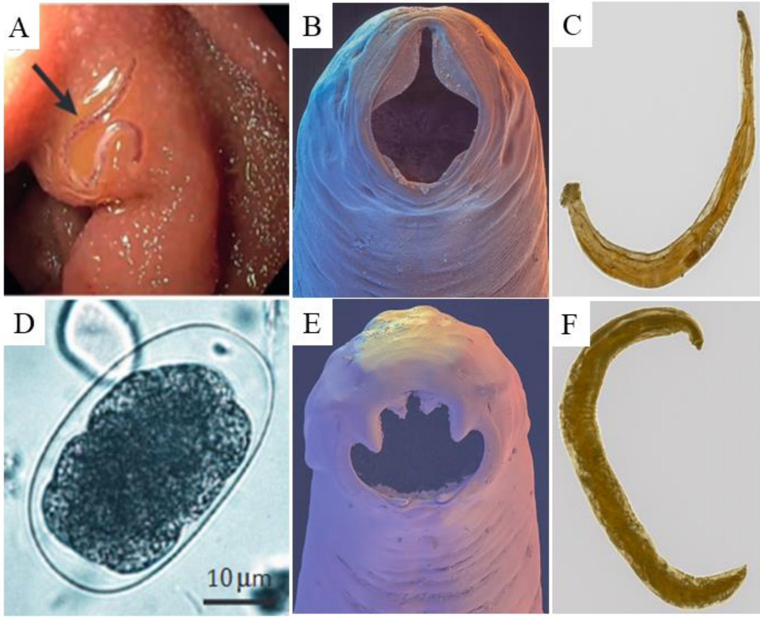

***Figure 17**. Different stages of hookworm species. (A) A copula (male and female) of N. americanus in the small bowel of a human. (B) A scanning electron micrograph of the head of N. americanus showing the "cutting plates". (C) Adult male A. duodenale. (C) Hookworm egg in human feces. (E) A scanning electron micrograph of the head of A. duodenale showing the "cutting teeth". (F) Adult female A. duodenale (adapted from (Loukas* et al.*, 2016; Despommier* et al.*, 2020)).*

b) Life Cycle

Hookworm eggs expelled through the feces of an infected person hatch after 1-2 days in soil and release first-stage (L_1) rhabditiform larvae, which, after two molts in 5-10 days become infective L_3 filariform larvae which measure approximately 0.5-0.6 mm in length. When the environmental conditions are optimal (temperature, humidity, etc.), the L_3 larvae can survive for 3-4 weeks on soil, grass, or vegetation awaiting exposure to human skin. On contact with the human skin (*e.g*., bare foot), the L_3 larvae penetrate the human host through the skin and enter the bloodstream. They migrate to the heart and then the lungs. In the lungs, they penetrate the bronchial tree via the pulmonary alveoli before reaching the pharynx, from which they enter the gastrointestinal tract to finally reach the small intestine where they mature and become adults after two molts (**Figure 18**). With the help of their "teeth" (in *Ancylostoma* spp.) or their "cutting plates" (in *Necator* spp.), adult hookworms cling to the small intestinal mucosa to avoid being ejected by the gut peristalsis, but also to be able to feed on the host's blood and tissues. Mature female hookworms produce about 10'000 eggs (*N. americanus*) or 28'000 eggs (*A. duodenale*) per day. The patency period (*i.e*., the time between infection and egg production) is estimated between 6 and 8 weeks (Loukas *et al.*, 2016).

Of note, apart from the percutaneous transmission, *A. duodenale* L_3 larvae can also infect the host through oral ingestion (Wang *et al.*, 2011). The larvae then become adults after two molts without leaving the intestine (Brooker, Bethony and Hotez, 2004; Loukas *et al.*, 2016) and might cause a so-

called Wakana disease. In this case, the host must ingest a high number of larvae to become sick (Wang *et al.*, 2011).

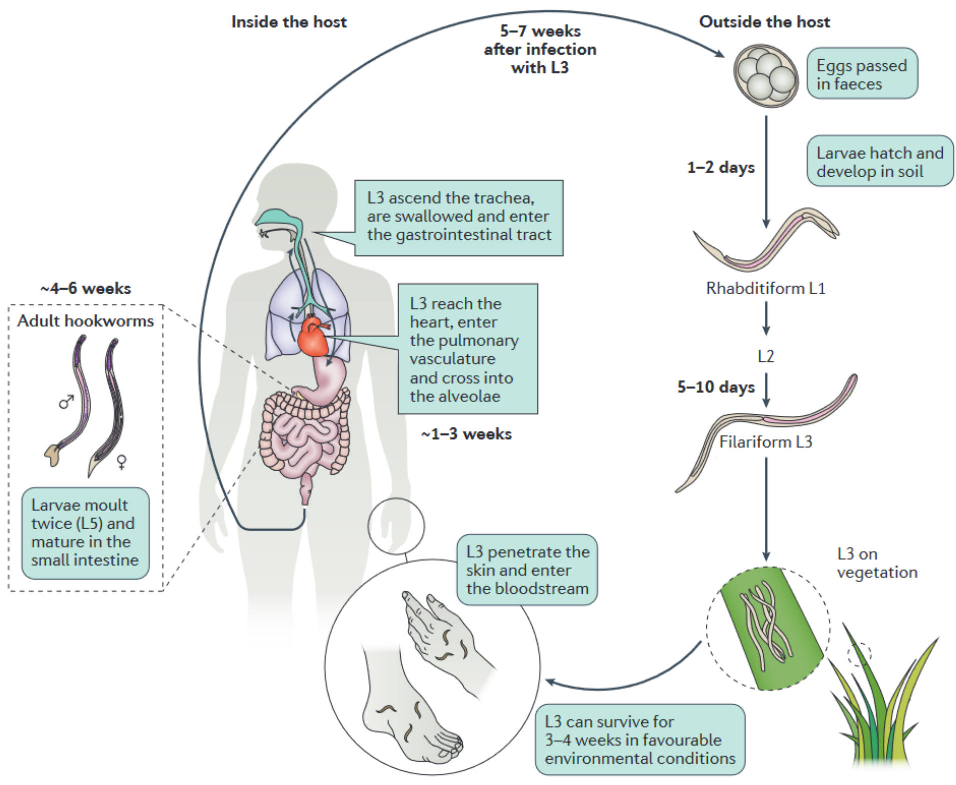

Figure 18*. The life cycle of hookworms (N. americanus and A. duodenale) (adapted from (Loukas* et al.*, 2016)).*

c) Pathogenesis

During hookworm infections, L_3 larvae secrete macromolecules (*e.g.*, hydrolytic enzymes) that facilitate the entry and invasion process (Yoshida *et al.*, 1974). These enzymes include metalloproteases and a family of cysteine-rich secretory proteins known as *Ancylostoma* secreted proteins (ASPs) (Hawdon *et al.*, 1996; Asojo *et al.*, 2005). ASPs play a fundamental role in the development and maturation of the parasite and represent one-third of its proteome (Mulvenna *et al.*, 2009). One of these ASPs (*e.g.*, ASP-2) is known to be allergenic (Diemert *et al.*, 2012). Thus, repeated infections lead to hypersensitivity and other inflammatory responses such as hookworm dermatitis called "ground itch" (Loukas *et al.*, 2016).

d) Symptoms

The most common clinical manifestations associated with hookworm disease are related to the development stage (larva or adult), the way of infection (oral or percutaneous), and the worm burden (intensity of infection). Dermatitis (i.e., ground itch) is due to repeated skin penetration of L_3 larvae (Loukas *et al.*, 2016), pneumonia and eosinophilia are caused by L_3 migration to the lungs (Maxwell *et al.*, 1987; Cheepsattayakorn and Cheepsattayakorn, 2014). Oral ingestion of many L_3 *Ancylostoma*

larvae causes a syndrome known as Wakana disease characterized by nausea, vomiting, pharyngeal irritation, cough, and breathing difficulties (Harada, 1962; Kojima, 1999). The intestinal phase is often asymptomatic, but abdominal pain may appear in case of heavy infection (Daveson *et al.*, 2011). Gastrointestinal bleeding associated with chronic IDA (Nair *et al.*, 2016) and the accompanying hypoalbuminemia and hypoproteinemia caused by blood loss are related to different factors such as the intensity of the infection, the daily iron intake or the host's iron reserves, and the hookworm species (Basta *et al.*, 1979; Stoltzfus *et al.*, 1996, 1997; Albonico *et al.*, 1998). Blood loss caused by *A. duodenale* is higher than the blood loss caused by *N. americanus* (Albonico *et al.*, 1998). In children, severe and chronic IDA could lead to physical growth retardation as well as deficits in cognition (Hotez *et al.*, 2005). During pregnancy, hookworm infections may impact maternal and child health, hence chronic hookworm anemia can lead to prematurity or low birth weight (Bundy, Chan and Savioli, 1995; Lee and Okam, 2011). Vertical transmission (*i.e*., from mother to fetus) may occur when larvae are present in breast milk, and this can cause syndromes like infantile ancylostomiasis associated with severe anemia, melena, abdominal distension, and growth retardation (Bhatia *et al.*, 2010).

e) Diagnosis

Hookworm diagnostics are essentially based on microscopic examination of stool samples using KK thick smear method, as recommended by the WHO (WHO, 2011). Specific characteristics can be used to identify hookworm eggs. It is also possible to estimate the worm burden, by quantifying the number of EPG of feces (*i.e*., fecal egg-counting (FEC)) (Zendejas-Heredia *et al.*, 2021). Other copro-based methods using flotation techniques are also available (*e.g.*, FLOTAC, mini-FLOTAC, McMaster, etc.) (Inpankaew *et al.*, 2014; Cools *et al.*, 2019). Eggs of the two hookworm species (i.e., *N americanus* and *A. duodenale*) are indistinguishable due to their resemblance. Moreover, the exclusive use of microscopy-based methods for the identification of hookworm eggs can lead to misidentification with other "hookworm-like eggs" of other free-living nematodes (*e.g*., *Caenorhabditis elegans*) (Irisarri-Gutiérrez *et al.*, 2016). Species specification can be achieved by alternative methods like molecular methods (*i.e*., qPCR) (Hughes *et al.*, 2023). Such methods are often restricted to research laboratories and are rarely available in endemic areas for routine diagnostics (van Mens *et al.*, 2013; Phuphisut *et al.*, 2014). Adult worms can also be identified by *in vivo* endoscopic imaging followed by samples collection from the intestine using biopsy forceps (Barakat, Ibrahim and Nasr, 2012). **Table 3** below lists the different techniques available for hookworm diagnostic with their specificities, advantages, and disadvantages.

Table 3. Microscopy-based and molecular methods for diagnosis of hookworm infection (*adapted from* (Loukas *et al.*, 2016))

Test	Mechanism	Detects ova?	Detects larvae?	Sample requirements	Benefits	Limitations
Microscopic-based examinations						
FECT	Concentration	Yes	Yes	10 g of fresh feces	Sensitive, low technology and can detect other STHs	Only qualitative
Diethyl-acetate	Concentration	Yes	Yes	20 g of fresh feces	Sensitive, low technology and can detect other STHs	Only qualitative
Harada-Mori	L_3 migration	No	Yes	2 g of fresh feces	Specific by morphological examination	L_3 collection only
MacMaster	Flotation	Yes	No	2 g of fresh feces	Sensitive, low technology and can detect other STHs	Trematodes
FLOTAC	Flotation	Yes	No	2 g of fresh feces	Sensitive, low technology and can detect other STHs	Availability of apparatus
Kato-Katz	Glycerol	Yes	No	10, 20, or 50 mg of fresh feces	Standardized (templates) and can detect other STHs	Desiccates eggs after 60 min
Molecular-based examinations						
NGS	Genetic sequencing	Yes	Yes	Fresh, fixed, or frozen feces	Sensitive and can use frozen material	Availability of apparatus
PCR	Primers	Yes	Yes	Fresh, fixed, or frozen feces	Sensitive and can use frozen material	Availability of apparatus
LAMP	DNA	Yes	Yes	Fixed, or frozen feces	Sensitive and can use frozen material	Availability of apparatus

FECT, formalin-ether concentration technique; L3, third-stage larvae; LAMP, loop-mediated isothermal amplification; STH, soil-transmitted helminth.

f) Treatment

The most used anthelmintic drugs for the treatment of human hookworm infections are currently the benzimidazoles (*i.e*., albendazole, and mebendazole) (Dayan, 2003). A single dose of 400 mg albendazole is more effective than a single dose of 500 mg of mebendazole (Keiser and Utzinger, 2008). Single dose usage is widely applied for PC in MDA programs, but for high cure rates, repeated rounds of drug administration (*i.e*., double-dose or triple-dose regimens) are necessary (Steinmann *et al.*, 2011; Soukhathammavong *et al.*, 2012; Ejigu, Hailu and Alemu, 2021). Due to their embryotoxicity and their teratogenicity, the benzimidazoles are not recommended in children under the age of 1 year, and in pregnant women during the first trimester of pregnancy (Loukas *et al.*, 2016). From the second trimester of pregnancy, and in children ≥ 1 year, albendazole and mebendazole can be used without adverse effects, as recommended by the WHO (Montresor, Awasthi and Crompton, 2003). Alternative anthelmintic drugs such as Pyrantel pamoate, levamisole (Köhler, 2001), tribendimidine (Xiao *et al.*,

2013), and ivermectin are also available. **Table 4** below highlights the recommended anthelmintic drugs for the treatment of hookworm infections.

Table 4. Recommended drugs for the treatment of hookworm infections (*adapted from* (Loukas *et al.*, 2016)).

Patient population	**Intestinal hookworm infection (*N. americanus* and *A. duodenale*)**	**Mechanism of action**	**Cutaneous larva migrans (zoonotic hookworms)**
Non-pregnant adults	**First line**		**First line**
	Albendazole	Inhibition of microtubule polymerization	Ivermectin
	Mebendazole		
	Alternative		
	Pyrantel pamoate	Muscle cell depolarization and spastic paralysis	
Pregnant women	**First trimester**		**Symptomatic relief**
	Treatment not recommended	NA	Topical corticosteroids
	Second and third trimester		- Antihistamines
	Regimens as for non-pregnant adults	Inhibition of microtubule polymerization	
Children	Age : 12-24 months		Age : 12-24 months
	- Albendazole	Inhibition of microtubule polymerization	Ivermectin
	- Mebendazole		
	Age : >24 months		Age : >24 months
	Regimens as for non-pregnant adults	Inhibition of microtubule polymerization	Albendazole

g) Prevention and control

Current methods employed to control hookworm infections include strategies such as MDA associated with improvement of sanitary conditions (*e.g*., WASH program) (WHO, 2011). These approaches tend to first reduce the prevalence rates, morbidity, and mortality, and then to control hookworm infections (Ziegelbauer *et al.*, 2012; Strunz *et al.*, 2014). The effectiveness of these strategies has proved to be successful in high-income countries like China (**Figure 19**), the Republic of Korea, the USA, or Japan (Hong *et al.*, 2006; Mao *et al.*, 2021). On the other hand, in low-income countries, MDA and WASH interventions have so far failed to achieve a remarkable long-term reduction of hookworm prevalence due to ongoing transmission, particularly among adults who are not targeted by MDA programs and could therefore constitute potential reservoirs (Zacharia *et al.*, 2023). In fact, even with a reduction in prevalence, the level of infection remains so high that it is still considered a public health problem (Okoyo *et al.*, 2020). Sustainable control of the public health problem caused by hookworm infections as well as other STHs in resource-limited endemic countries would require integrated management strategies (*i.e*., MDA, health education, environmental sanitation improvements (*e.g*., WASH

program)), but also simultaneous and substantial economic development (*i.e.*, general improvement in socio-economic conditions) (Hong *et al.*, 2006; Loukas *et al.*, 2016; Mao *et al.*, 2021).

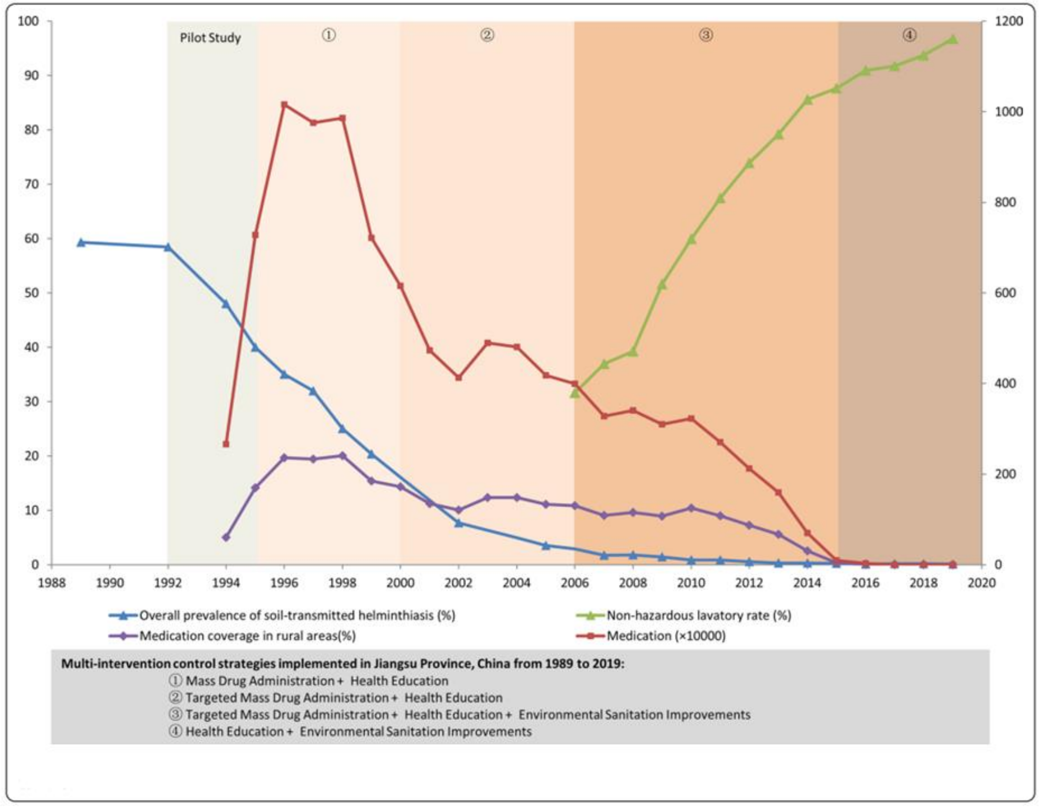

***Figure 19**. Multi-intervention integrated deworming strategy for sustainable control of STH in Jiangsu Province, China from 1989 to 2019 (adapted from (Mao* et al.*, 2021)).*

2.5.3. Trematodes

Trematodes, also known as flukes, belong to the Phylum of Plathelminths and are obligate parasites living in the blood (blood flukes), intestine (intestinal flukes), and tissues (tissue flukes, as liver flukes or lung flukes) of the host, thus causing trematodiasis (Keiser and Utzinger, 2009). Human infection occurs after ingestion of raw or undercooked aquatic food (fish, snails, snakes, water plants (e.g., watercress), etc.) (Chai and Jung, 2019), or also by penetration of the larvae through an intact skin in contact with contaminated freshwater (Gryseels *et al.*, 2006). Other mammals (e.g., livestock) are susceptible to infection (Fürst *et al.*, 2012). Adults are equipped with two suckers (a ventral sucker and an anterior oral sucker) they use to attach themselves within the host. The anterior sucker also serves as oral cavity for food ingestion. Ingested food migrated through the intestinal tract to be digested. Due to the lack of posterior cavity, wastes and non-digested materials are regurgitated into the host. They have a tegument covered with a membrane-bound microvilli that allows absorption of nutrients and other molecules (Halton, 2004). Below the tegument, there are several layers of muscles that serve for motility. The nervous system includes a pair of ganglia prolonged by lateral nerves to the muscle layers and other organs (gut, reproductive organs, etc.). Generally, most trematodes are hermaphroditic

(Despommier *et al.*, 2020). Reproduction methods differ according to species: self-fertilization where both reproductive organs are present in the same adult worm (e.g., *Fasciola hepatica*), cross-fertilization where two adult worms possess both reproductive organs (*Paragonimus westermani*), and fertilization between two adult worms with opposite sex (*Schistosoma* spp.). The life cycle is complex including an intermediate host with certain characteristics specific to the species. The most important species infecting humans are *Opisthorchis viverrini*, *Opisthorchis felineus, Clonorchis sinensis*, *Fasciola gigantica*, *Fasciola hepatica*, *Schistosoma mansoni*, *Schistosoma japonicum*, *Schistosoma haematobium*, etc. (Despommier *et al.*, 2020). A few detailed examples of medical and veterinary importance are presented below.

2.5.3.1. Key pathogen #7: *Fasciola hepatica* and *Fasciola gigantica*

a) Background

F. hepatica and *F. gigantica* are so-called liver flukes that are foodborne (i.e. plant-born or waterborne) pathogens giving rise to a disease called fascioliasis (Toledo, Esteban and Fried, 2012). Fascioliasis is a zoonotic disease affecting both human and animals. Adult worms measure about 35 mm long and 15 mm width for *F. hepatica*, 75 mm long and 15 mm width for *F. gigantica*, and live in the bile ducts of the definitive host. *F. hepatica* was first described in 1684 by Francesco Redi (Redi, 1684) but named as *F. hepatica* in 1758 by Carl Linnaeus (Lima *et al.*, 2009). *F. gigantica* was described in 1856 by Thomas Spencer Cobbold (Kendall and Parfitt, 1953). Human fascioliasis (*F. hepatica* and *F. gigantica*) was reported in many countries around the world (*F. hepatica* has a worldwide distribution, while *F. gigantica* is mainly present in Africa and South-East Asia) with about 2.4 million people affected annually (Nyindo and Lukambagire, 2015; Chai and Jung, 2022). Highest prevalences were found in Egypt, China, Vietnam, and South America (e.g., Peru, Bolivia) (Cwiklinski *et al.*, 2015; Nyindo and Lukambagire, 2015). Animal infections were estimated of being 300 million in cattle and 250 million in sheep leading to a productivity loss of $3 billion (Mas-Coma, Bargues and Valero, 2005).

b) Life Cycle

Adult worms produce eggs that are released in the bile ducts and carried to the intestine before being expelled with the feces. Upon reaching freshwater, eggs embryonate after 9-15 days, then hatch and release miracidia larvae. Free-swimming miracidia penetrate the snail intermediate host (*Galba truncatula* for *F. hepatica*, *Lymnea natalensis* and *L. rubiginosa* for *F. gigantica*) and develop into sporocysts, rediae, and cercaria which are then released in water where they move freely until attachment on vegetation and encyst as metacercaria (Nyindo and Lukambagire, 2015). When the metacercaria are ingested by the definitive host (human or animal (sheep, cattle)), they excyst in the small intestine and the juvenile worms migrate across the intestinal wall, via the peritoneal cavity to the

liver and bile ducts where they mature (**Figure 20**) (Robinson and Dalton, 2009; Toledo, Esteban and Fried, 2012).

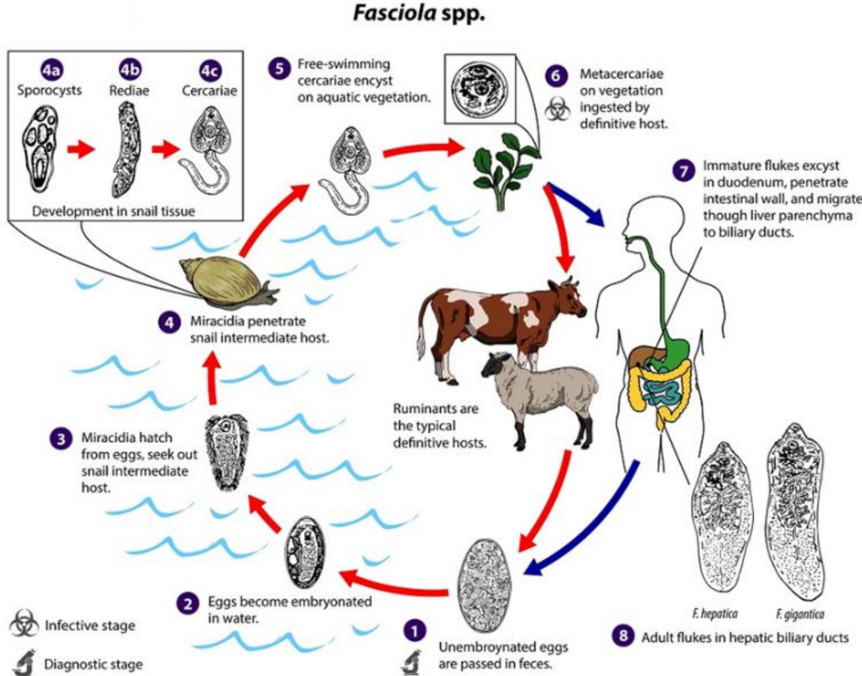

Figure 20*. Life cycle of Fasciola spp. (adapted from (CDC, 2019a)).*

c) Pathogenesis

Extracellular vesicles (EVs) secreted by the parasite play an important role in infection sustainability, e.g., by maintaining the Th2/regulatory environment tolerant for fluke survival and reproduction, or by modulating host immune cell function (Cwiklinski *et al.*, 2015). They facilitate the parasite migration through tissues (e.g., *Fasciola* cathepsin Ls degrade macromolecules like collagen) and escape the host immune response (Cwiklinski *et al.*, 2015).

d) Symptoms

The acute form of fascioliasis is accompanied by symptoms like fever, anorexia, urticaria, respiratory troubles, eosinophilia, and jaundice (Fica *et al.*, 2012; Leerapun *et al.*, 2021). During the chronic stage, the patient may develop epigastric pain, nausea, fatty food intolerance, acute pancreatitis, etc. (González-Miguel *et al.*, 2019).

e) Diagnosis

Diagnosis of fascioliasis can be achieved by microscopic examination of stool samples for eggs detection. They appear brownish-yellow and measure 130-150 µm in length and 60-90 µm in width (Valero *et al.*, 2009). However, eggs are seldom excreted in hepatic infections, and thus, a combination of clinical suspicion, pathological imaging studies (e.g. US, CT) and a positive serology may point toward fascioliasis (Cevikol *et al.*, 2003; Zali *et al.*, 2004; Rokni *et al.*, 2018; Tran *et al.*, 2019). This is

particularly common in the early phase of infection, as stool examination may give negative results because the prepatent period is around 4 months (Gonzales Santana *et al.*, 2013).

f) Treatment

The treatment of choice is triclabendazole (single dose of 10 mg/kg; possibility to repeat after 12-24 hours in case of persistent heavy infection) (Saba *et al.*, 2004). For MDA programs, a single dose of 40 mg/kg is used (Keiser and Utzinger, 2009). If triclabendazole is not available, the alternative could be nitazoxanide (Favennec *et al.*, 2003), or even bithionol, an antibacterial agent, but long treatment (10 to 15 days) would be required (Keiser and Utzinger, 2009).

g) Prevention and control

Fascioliasis can be controlled by protecting freshwater and educating farmers and people in regular contact with freshwater to apply sanitary rules (*e.g.*, not eating raw watercress or other plants growing in freshwater, and avoiding open defecation, as contaminated feces could reach the water, thus perpetuating the parasite's life cycle and facilitating transmission).

2.5.3.2. Key pathogen #8: *Schistosoma* spp.

a) Background

Human infections caused by *Schistosoma* species give rise to a disease known as schistosomiasis or 'bilharzia' (this term is frequently used in southern Africa). At present, human schistosomiasis is known to be caused by six different species: *S. mansoni*, *S. japonicum*, *S. intercalatum*, *S. guineensis* and *S. mekongi*, responsible for intestinal schistosomiasis, and *S. haematobium* causing urogenital schistosomiasis (WHO, 2022b). Hybrids of schistosome species of human and veterinary importance (e.g. *S. bovis*) have also been described. However, the clinically by far most important species affecting humans are *S. mansoni*, *S. japonicum*, and *S. haematobium* (Colley *et al.*, 2014), on which the subsequent sections will focus.

Schistosomes were first described in 1851 by Theodor Bilharz (Jordan, 2000). Unlike other flukes mentioned in **section 2.5.3.1** (*Fasciola* spp.) which are hermaphroditic, *Schistosoma* species are dioecious (separate sexes). Adult intestinal schistosomes live in copula in mesenteric veins of the bowel with a thin female (7-17 mm in length) residing in the gynecophoral canal within the thick male that measures 6-12 mm in length. *S. mansoni* and *S. haematobium* are mainly found in sub-Saharan African countries and in South America (e.g., Brazil, Venezuela, Puerto Rico), while *S. japonicum* is endemic in Asia (e.g., China, Indonesia, Philippines) (McManus *et al.*, 2018).

b) Life cycle

Schistosoma eggs produced by adult females are expelled with host feces. Upon reaching the freshwater, they hatch and release miracidium larvae which penetrate the snail intermediate host (*Biomphalaria* spp. for *S. mansoni*, and *Oncomelania* spp. for *S. japonicum, and Bulinus* spp. for *S. haematobium*) (Steinmann *et al.*, 2006). The intermediate hosts can only survive in freshwater, and hence, schistosomiasis cannot be acquired in seawater. Within the snail, miracidia develop into sporocysts (two generation by asexual reproduction), which, after ~ 4-6 weeks under sunny conditions, produce free-swimming cercaria that are then released into the water (Colley *et al.*, 2014). In contact with human hosts, cercaria penetrate through the skin, lose their tails, and become schistosomula, which are then carried by the bloodstream (via venous circulation) to the lungs, then to the heart, and finally to the liver where they mature before moving to the mesenteric veins surrounding the intestine (intestinal schistosomes) or the venous plexus (*S. haematobium*) in copula. The prepatent period is ~5 weeks (McManus *et al.*, 2018) (**Figure 21**).

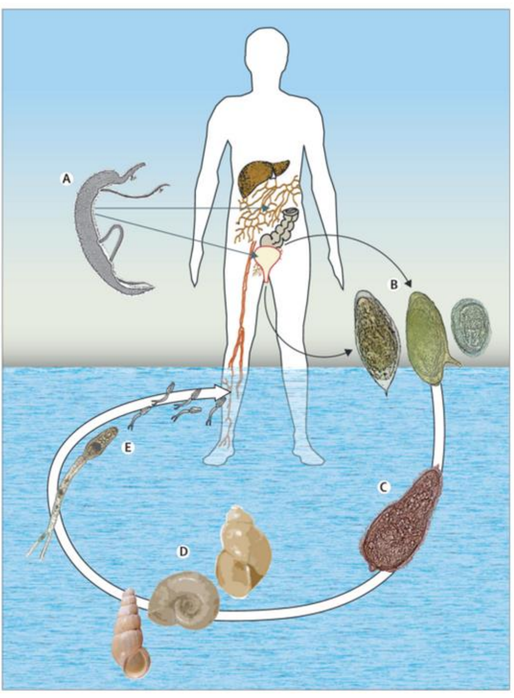

Figure 21. *Life cycle of Schistosoma mansoni, Schistosoma japonicum and S. haematobium. (A) paired adults, (B) eggs, (C) miracidium, (D) intermediate snail hosts, (E) cercariae (adapted from (Gryseels* et al.*, 2006)).*

c) Pathogenesis

Active skin penetration induces hypersensitive reactions leading to dermatitis (McManus *et al.*, 2018). The presence of adult worms in blood vessels does not cause any trouble due to their somatic stem cells allowing them to regenerate their surface tegument that act as protective, thus hiding them from the host antigens and immune responses (Colley *et al.*, 2014)). Produced eggs secrete glycoproteins facilitating

their migration to the intestine, the liver, or the urinary bladder, and the formation of granulomas (groups of eosinophils, neutrophils, lymphocytes, macrophages) surrounding the eggs (McManus *et al.*, 2018).

d) Symptoms

Clinical manifestations can be classified based on the infection stage and/or the involved pathogen. Skin penetration by cercariae is often asymptomatic, however, mild symptoms like dermatitis, or pulmonary lesions may be observed (WHO, 2022b). In the case of acute schistosomiasis, immune-naïve individuals may develop an allergy-like reaction called 'Katayama syndrome', which is characterized by eosinophilia, intestinal pain, diarrhea, fatigue, fever, myalgia, aches, weight loss, lymphadenopathy (Ross *et al.*, 2007). If established active and late chronic infection occurs, inflammatory reactions (i.e., granuloma formation) as well as obstruction due to immune responses against the eggs trapped in host tissues are the main factors giving rise to specific clinical symptoms (WHO, 2022b). Symptoms related to intestinal schistosomes (*S. mansoni*, and *S. japonicum*) are abdominal discomfort, diarrhea, and rectal bleeding. In contrast, *S. haematobium* infection typically causes haematuria, urinary and suprapubic discomfort, and may give rise to bladder dysplasia, which can develop into squamous cell carcinoma of the bladder (Colley *et al.*, 2014).

e) Diagnosis

Standard methods for diagnosing schistosomiasis are based on microscopic examinations: KK stool examination (Katz, Chaves and Pellegrino, 1972) in the case of *S. mansoni* and *S. japonicum* infections; polycarbonate filters or urine dipstick assays in the case *of S. haematobium* infection (Colley *et al.*, 2014). These parasitological methods are suitable for active and chronic infections, but their sensitivity remains limited for detecting acute infections (McManus *et al.*, 2018). Alternative methods such as molecular (e.g., real-time PCR) (Obeng *et al.*, 2008; Cnops *et al.*, 2012), serological (e.g., ELISA) (Tsang and Wilkins, 1997), or imaging (e.g., ultrasonography) (Akpata *et al.*, 2015) techniques are also available.

f) Treatment

Praziquantel (40 mg/kg) is the treatment of choice for schistosomiasis and has been used for over 30 years (McManus *et al.*, 2018). In infected patients, the treatment can be repeated until elimination (no eggs in stool or urine). However, in MDA programs, a single dose is administered following WHO recommendations (WHO, 2022b). This drug is effective against adult worms of all *Schistosoma* species, but not against larvae (schistosomula) (McManus *et al.*, 2018). Artemisinin and derivates (e.g., artesunate, artemether) are known to be effective against the immature stage (schistosomula) and can be used in combination with praziquantel to improve treatment as well as chemoprophylaxis (McManus *et al.*, 2018). However, this is not regularly done in clinical practice, where artemisinin is exclusively used for treatment of malaria.

g) Prevention and control

In endemic regions, people living in rural areas have difficulties accessing clean water. Thus, regular contact with freshwater constitutes a permanent risk of *Schistosoma* infection. Facilitating access to clean water and improving sanitary and hygiene conditions, as well as promoting chemical snail control (e.g., molluscicides) (ref: WHO 2017. Field use of molluscicides in schistosomiasis control programmes: an operational manual for programme manager) are important factors for prevention (Grimes *et al.*, 2015). In addition, the socio-economic aspect must be considered. Indeed, in such regions, most people practice fishing in rivers or lakes for or as a source of income (Olsen, Kinung'hi and Magnussen, 2015).

III

Bacterial and fungal pathogens: bloodstream infections (BSI)

3. Bacterial and fungal pathogens: bloodstream infections (BSI)

3.1. Global burden and incidence of BSI

Bloodstream infections (BSI) are severe, potentially life-threatening infections during which the causative agent can be detected in the peripheral blood of the infected patients through microbiological techniques such as blood culture. They encompass a wide variety of pathogens representing a major cause of global mortality and morbidity. Regardless of the level of socio-economic development, the incidence of BSI has been described in LMICs (Marks *et al.*, 2017; Gezmu *et al.*, 2021) as well as in high income countries (HICs) (Laupland *et al.*, 2016; Mehl *et al.*, 2017).

Previous estimates from Europe and North America suggest 1.775-1.877 million BSI cases and 236,000-251,000 deaths per year, with over 1.2 million occurrences and 157,000 deaths per year in Europe (England: 96,000 occurrences and 12,000-19,000 deaths; Denmark: 91,000 BSI occurrences and 1900 deaths; Finland: 87,000 occurrences and 1100 deaths), and around 575,000-677,000 occurrences and 79,000-94,000 deaths in North-America (USA: 536,000-628,000 occurrences and 72,000-85,000 deaths; Canada: 40,000-49-000 occurrences and 7,000-9,000 deaths) (Goto and Al-Hasan, 2013).

In Africa, despite the limited resources of clinical microbiology laboratories (infrastructure, equipment/materials, highly qualified personel, etc.) to diagnose non-malaria BSI, it has been reported that BSI is frequently observed in febrile patients and associated with high mortality rates. Prevalence rates of bacterial and/or fungal BSI were previously estimated at 13.5% (2,051/15,166) in adults, and 8.2% (3,527/43,130) in children. The mortality rates were estimated at 18%. Most pathogens isolated in adults and children were *Salmonella enterica* and *Streptococcus pneumoniae*. Other pathogens such as *Staphylococcus aureus*, *Escherichia coli*, *Hemophilus influenzae*, *Mycobacterium tuberculosis* complex, and yeast were also encountered (Reddy, Shaw and Crump, 2010).

In South and Southeast Asia, occurrences of bacterial BSI have also been reported. A review article published in 2012 summarized all studies published between 1990 and 2010 including 40,644 patients. Prevalence rates of 12% (1784/14386) and 7% (1722/26258) were reported in adults and children, respectively. *S. enterica* serotype Typhi was the most isolated pathogen, but other species (e.g., *S. aureus*, *E. coli*, *S. pneumoniae*, *H. influenzae*, etc.) were also detected. The overall mortality rate among patients was 9% (Deen *et al.*, 2012).

A more recent systematic review published in 2019 has reported data on community-acquired BSI (CO-BSI) collected in 19 African and Asian countries including a total of 29,022 patients (children and adults) with severe febrile illness, of whom 3,146 (10.8%) had BSI (Marchello *et al.*, 2019). In addition, another study based on the analysis of risk(water)-adjusted typhoid disease burden in LIMCs estimated 11.9 million cases and 128,775 deaths (Mogasale *et al.*, 2014).

The incidence of short-term mortality (e.g., 30-day mortality) as well as a long-term mortality (3-year mortality) following BSI, which represents a major risk of death compared to other comorbidities, have been demonstrated (Lillie *et al.*, 2013; McNamara *et al.*, 2018). However, other sequelae can lead to more complicated consequences such as sepsis or septic shock, which also contribute to increased mortality and morbidity rates (Huerta and Rice, 2019).

3.2. Sepsis and septic shock

Sepsis is a life-threatening condition and a major cause of health loss associated with organ dysfunction caused by a dysregulated host response to infection (Singer *et al.*, 2016). It represents a global health problem in terms of loss of life and economic costs. In 2017, global sepsis cases were estimated at 48.9 million (20.3 million cases in children under 5 years old, 4.9 million cases in 5-19 years old, and 23.7 million cases in ≥20 years old), with an incidence of 677.5 cases per 100,000 and 11 million sepsis-related deaths (Rudd *et al.*, 2020). This incidence was more observed in women (716.5 cases per 100,000) than in men (642.8 cases per 100,000). Globally, the most attributable cause of sepsis was diarrheal diseases with 9.21 million cases, and respiratory infections were leading cause of sepsis-related deaths (1.8 million) (Rudd *et al.*, 2020). Of note, not all sepsis cases derived from BSI or other infections. In fact, BSI causes around 21-30% of sepsis cases, and the microorganisms most frequently found are bacteria and fungi (Tulloch *et al.*, 2017; Guo *et al.*, 2023). It was reported that the type of pathogens (i.e., Gram-positive bacteria, Gram-negative bacteria, or fungi) isolated from a patient with sepsis influences the patient's prognosis (i.e., 30-day mortality rate, duration of hospital stay, intensive care unit (ICU) stay , ventilation, etc.) (Cohen *et al.*, 2004; Guo *et al.*, 2023). For example, Gram-negative bacteria are more likely to cause sepsis than Gram-positive bacteria (Guo *et al.*, 2023), probably due to their more intense inflammatory response (Abe *et al.*, 2010). Nevertheless, other non-communicable diseases or injuries can lead to sepsis. In LMICs, most sepsis-related deaths are due to infections (BSI, respiratory infections, etc.), but in HICs they are mainly attributed to non-communicable diseases, followed by infection diseases (Rudd *et al.*, 2020).

Septic shock is a subset of sepsis (i.e., a severe form of sepsis) in which particularly profound circulatory, cellular, and metabolic abnormalities are associated with an in-hospital risk of mortality greater than with sepsis alone (Singer *et al.*, 2016). Indeed, increased serum lactate levels (i.e., hyperlactatemia: >2 mmol/L [18 mg/dL]) indicate cellular dysfunction (Singer *et al.*, 2016) and are associated with higher mortality (Cecconi *et al.*, 2014). The combination of hyperlactatemia with two other variables (hypotension and sustained need for vasopressor therapy), also defined as indicators of severity, shows higher mortality rates than each considered alone (Singer *et al.*, 2016).

The interaction between bacterial flora and the human host plays an important role in the pathogenesis and/or development of certain diseases. Indeed, the human microbiome comprises a wide diversity of bacteria including commensal, beneficial bacteria, and opportunistic pathogens. In case of physical and/or physiological disorders (e.g., dysbiosis), opportunistic pathogens can become infectious and

reach normally sterile locations such as the bloodstream (i.e., bacteremia) leading to BSI and sepsis (**Figure 22**) (MacFie *et al.*, 2006; Vaishnavi, 2013; Kang and Thomas, 2021). In addition, the presence of bacteria in the blood of ICU-patients receiving probiotic treatment (i.e., capsules) has also been reported. However, the mechanism of transmission remains unclear whether it is a translocation from the gut or not (Kunz, Noel and Fairchok, 2004; Honeycutt *et al.*, 2007; Yelin *et al.*, 2019). Other source of infections, especially in hospitalized patients are catheter-related infections colonized by patient's skin flora, and/or soft tissue infections (Gahlot *et al.*, 2014).

Apart from blood parasites (e.g., *Plasmodium*) or viral blood infections (e.g., hepatitis, human immunodeficiency virus (HIV)), microorganisms isolated from blood in clinical microbiology laboratories include Gram-negative bacteria, Gram-positive bacteria, and yeasts. More details about these microorganisms are discussed in the following sections.

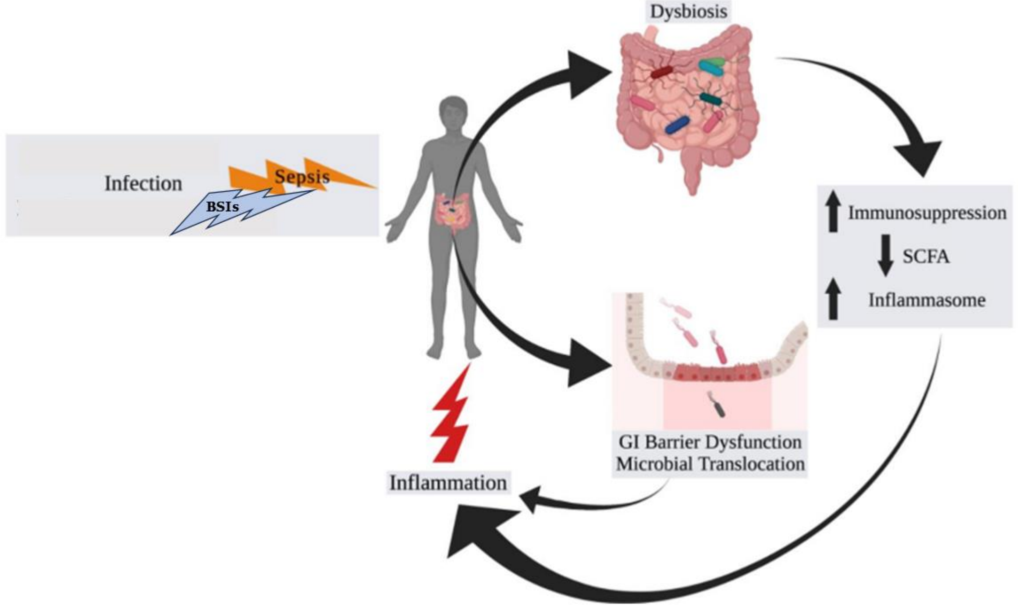

***Figure 22**. Interaction between bacteria (gut microbiome), bacterial translocation and sepsis (adapted from (Kang and Thomas, 2021)).*

3.3. Gram-positive bloodstream infections (GPBSI)

GPBSI is defined as the detection of Gram-positive pathogens in the bloodstream. In the healthcare facilities and in the community in the United States of America (USA) and Europe, *S. aureus* (MRSA and MSSA), and *Enterococcus* spp., followed by coagulase-negative *Staphylococcus* spp. (CoNS) and *Streptococcus* spp. are the most frequently encountered pathogens (Thwaites *et al.*, 2011; Dantes *et al.*, 2013; Mendes *et al.*, 2018). These pathogens contribute significantly to the burden associated with GPBSIs with mortality rates of >20% for *S. aureus*. (Caniff, Rebold and Rybak, 2023).

3.4. Gram-negative bloodstream infections (GNBSI)

GNBSI are caused by the presence of Gram-negative pathogens (e.g., *E. coli*) in the bloodstream from diverse sources, including nosocomial and community-acquired infections. They account for up to half

of all BSI, with a mortality rate of 20-40% (Cogliati Dezza *et al.*, 2020). The most frequently isolated Gram-negative pathogens are *K. pneumoniae*, *E. coli*, and *P. aeruginosa* (Schamroth Pravda *et al.*, 2024). GNBSIs are more likely to derive from the urinary tract (Shimon *et al.*, 2018). In addition, bacterial translocation (i.e., passage of bacteria from the gastrointestinal (GI) tract, known to be the main reservoir for *E. coli,* into the blood circulation) can also occur (Vollmerhausen *et al.*, 2014). For example, due to certain clinical conditions such as bacterial overgrowth, immunodeficiency, or physical alteration of epithelium membrane integrity, intestinal and/or urinary tract (UT) bacteria can migrate through the epithelial layer or via the kidney to the bloodstream, resulting in BSI (McNally *et al.*, 2013; Sato *et al.*, 2014; Nagpal and Yadav, 2017; Czajkowski *et al.*, 2023).

3.5. Yeast/fungal bloodstream infections

Yeasts and/or fungal BSI are mainly caused by *Candida* species, with *C. albicans*, *C. glabrata, C. parapsilosis, C. tropicalis,* and *C. krusei* being the main species isolated (Eliakim-Raz *et al.*, 2016; Bac *et al.*, 2019). Mortality rates associated with candidaemia reach around 35-75% (Jiménez-Aguilar, López-Cortés and Rodríguez-Baño, 2019). Most patients with candidaemia detected in clinical settings present with several comorbidities. Parenteral nutrition, urinary catheters, central line catheters, mechanical ventilation, etc., are the most frequent clinical characteristics reported in candidemic patients admitted to internal medicine wards (IMW) patients, in ICU or in surgery departments (Eliakim-Raz *et al.*, 2016).

3.6. Current methods for diagnosis of BSI

3.6.1. Standard culture-based method

Blood culture (BC) is the standard method for diagnosing BSIs (Rutanga and Nyirahabimana, 2016; Dubourg, Lamy and Ruimy, 2018). This method involves adding a blood sample to blood culture bottles [e.g., Plus Aerobic, Plus Anaerobic, etc. (Becton Dickson)] and incubating them in an automated system (e.g., BD BACTEC FX System) for 12 to 24 hours to allow for bacterial/fungal growth. These blood culture media have unique formulations. For example, *Plus Aerobic culture vials* contain resin beads having porous surface structure, which neutralize a wide range of antimicrobial agents to enable enhanced growth conditions for microorganisms present in the sample. *Lytic anaerobic culture vials* contain saponins, which promote lysis of white blood cells, releasing intracellular microorganisms that can further replicate. Using sensing technologies that measure CO_2 concentration and detection algorithms through fluorescence, the BD BACTEC instrument can identify samples as being either positive (detection of viable microorganisms) or negative (absence of viable microorganisms). After being flagged positive, pathogens can be identified by Gram staining, followed by culture-grown colonies on agar plates, which also requires 12 to 24 hours for most culturable pathogens (Dubourg, Lamy and Ruimy, 2018). The resulting colonies can be identified either by conventional

phenotypic/biochemical methods (i.e., Gram staining, catalase, coagulase, lactose, etc.) (Murray *et al.*, 2006) or by more rapid methods such as MALDI-TOF MS (Seng *et al.*, 2009b).
However, due to the prolonged time to results of the standard bacterial culture-based method, efforts have been made to develop rapid alternatives to blood cultures such as molecular-based approaches [i.e., molecular rapid diagnostic tests (mRDT)] for BSIs identification directly from positive blood cultures (PBCs) [e.g., MALDI-TOF MS with the Sepsityper® kit (Kok *et al.*, 2011), fluorescence in-situ hybridization (FISH) (Abdelhamed *et al.*, 2015), FilmArray (Agnetti *et al.*, 2023), and Verigene systems (Suzuki *et al.*, 2015), etc.].

3.6.2. Direct identification approaches from PBC

a) MALDI-TOF MS

MALDI-TOF MS, a protein profiling-based technique has become a widely used method in clinical microbiology laboratories due to its rapidity, reliability, and cost-effectiveness (Patel, 2015). Primarily applied on cultured colonies for bacteria/fungi identification, subsequent developments allowed a more direct and rapid approach for identification of BSIs (Opota *et al.*, 2015). MALDI-TOF MS can also be employed for rapid identification of BSIs directly from purified pellets (i.e., bacterial/fungal biomass) derived from PBCs using commercial kits (e.g., Bruker Sepsityper®) or in-house lysis and purification methods (e.g., sodium dodecyl sulphate (SDS), saponin, etc.) (Meex *et al.*, 2012; Tanner *et al.*, 2017; Ponderand *et al.*, 2020). Furthermore, a time saving fully automated sample preparation/extraction methods have also been developed [i.e., Bruker Galaxy® for automated addition of formic acid and HCCA matrix, and the blood culture sample preparation (BCSP) associated with VITEK® MS] (P *et al.*, 2018).

b) Molecular amplification methods: Polymerase chain reaction (PCR)

Molecular detection of pathogens causing BSIs using real-time PCR (qPCR) represents a valuable approach in clinical diagnostic microbiology laboratories due to its high sensitivity (Fujita *et al.*, 2011). With the further development of multiplex PCR assays, simultaneous detection of a broad range of species in one reaction is now possible (Quiles *et al.*, 2015; Carlesse *et al.*, 2016; Wu *et al.*, 2022). The greatest advantage of this technique is its flexibility. Indeed, molecular patterns (e.g., partial, or whole genes) can be specifically targeted through primers designing. However, many commercial kits based on molecular techniques, such as Accelerate Pheno™ system (Ullberg and Özenci, 2020), Verigene™ Blood Culture panels (Nanosphere) (Bhatti *et al.*, 2014), Biofire FilmArray panels (Altun *et al.*, 2013) etc., are now available, which are further explained below. Of note, despite their impact in clinical microbiology laboratories, these techniques mentioned below still require a preliminary blood culture incubation step (i.e., they are performed on PBC).

i. Fluorescence in situ hybridization (FISH)

Fluorescence *in situ* hybridization (FISH) technique is a multicolor, qualitative nucleic acid hybridization assay based on the complementary fit between a fluorescent probe and the targeted molecule within the sample (AdvanDx, 2020d, 2020c, 2020a, 2020b). Depending on Gram staining results, different kits can be used to perform the assay (Enroth *et al.*, 2019). The following kits commercialized by *AdvanDx* Inc., are available for in vitro diagnostic (IVD) use so far: Gram-Negative *Quick*FISH® BC for identification of *E. coli*, *K. pneumoniae*, and *P. aeruginosa* within 20 minutes (AdvanDx, 2020b); *Enterococcus Quick*FISH® BC for identification of *E. faecalis* and other selected Enterococci within 20 minutes (Deck *et al.*, 2014; AdvanDx, 2020a); *Staphylococcus Quick*FISH® BC identification of *S. aureus* and coagulase negative Staphylococci (CoNS) within 20 minutes (Deck *et al.*, 2012; Koncelik and Hernandez, 2016; AdvanDx, 2020c); Yeast Traffic Light® PNA FISH® for identification of *C. albicans* and/or *C. parapsilosis*, *C. tropicalis*, *C. glabrata* and/or *C. krusei* within 90 minutes (Abdelhamed *et al.*, 2015; AdvanDx, 2020d). These techniques have high sensitivity and specificity (>95%) and can identify the most prominent pathogens encountered in clinical settings (e.g., *S. aureus*, CoNS, Enterococci, *E. coli*, *K. pneumoniae*, *P. aeruginosa,* etc.) (Martinez *et al.*, 2014; Dubourg, Raoult and Fenollar, 2019). However, in some cases, limitations occur, and bacterial/fungal isolation by subculture is needed due to the lack of ability to distinguish polymicrobial PBCs or certain closely related species (e.g., these following species cannot be distinguished one from another: *E. faecalis*, *E. caccae* and *E. haemoperoxidus*; *Escherichia. coli*, *Shigella* spp., *E. albertii*, and *E. fergusonii*; *C. albicans* and *C. parapsilosis*; *C. glabrata* and *C. krusei*) (AdvanDx, 2020a, 2020b, 2020d). However, a few number of pathogens (e.g., *Staphylococcus simulans*, *Staphylococcus felis*, *Enterococcus dispar*, *Enterococcus saccharolyticus*) still cannot be identified .

ii. Biofire® FilmArray® Blood Culture Identification (BCID) Panel

The Biofire® FilmArray® Blood Culture Identification (BCID) panel (BioFire Diagnostics, LLC, Salt Lake City, USA) is a closed and fully automated PCR-based system harboring all necessary chemistry to isolate and detect DNA pathogens from PBC samples. The technique is based on nucleic acid purification, and amplification by nested multiplex PCR. A single PBC sample is tested for twenty-four organisms and/or organism groups included in the Biofire® FilmArray® BCID Panel (**Table 5**) (bioMérieux, 2024). The setup time is around 5 minutes and time to results around 1 hour, with sensitivity and specificity of 96-98.5% and 98.4-99%, respectively (Dubourg, Raoult and Fenollar, 2019; Agnetti *et al.*, 2023). Nevertheless, false positive results resulting from the detection of nucleic acids of non-viable microorganisms were reported, especially for *P. aeruginosa* and *Enterococci* (Dubourg, Raoult and Fenollar, 2019). Also, clinically relevant microorganisms identified by subculture (21/386; 5.4%) and not covered by the Biofire® FilmArray® BCID Panel were reported (Agnetti *et al.*, 2023).

More recently in 2022, an updated version called Biofire® FilmArray® Blood Culture Identification 2 (BCID2) Panel (BioFire Diagnostics, LLC, Salt Lake City, USA) including additional organisms, has been released (bioMérieux, 2024), hence allowing the detection and identification of other clinically relevant groups/species (**Table 5**).

Table 5. Biofire® FilmArray® BCID Panels (adapted from (bioMérieux, 2024)).

Version	Gam-Positive Bacteria	Gam-Negative Bacteria	Yeast
BCID	*Enterococcus* spp.	*Acinetobacter baumannii*	*Candida albicans*
	Listeria monocytogenes	*Haemophilus influenzae*	*Candida glabrata*
	***Staphylococcus* spp.**	*Neisseria meningitidis* (encapsulated)	*Candida krusei*
	Staphylococcus aureus	*Pseudomonas aeruginosa*	*Candida parapsilosis*
	Streptococcus	***Enterobacteriaceae***	*Candida tropicalis*
	Streptococcus agalactiae	*Enterobacter cloacae complex*	***AMR genes***
	Streptococcus pneumoniae	*Escherichia coli*	*mecA*-methicillin resistance
	Streptococcus pyogenes	*Klebsiella oxytoca*	*vanA/B*-vancomycin resistance
		Klebsiella pneumoniae	bla_{KPC}-carbapenem resistance
		Proteus spp.	
		Serratia marcescens	
Version	**Additional Gram-Positive**	**Additional Gram-Negative**	**Additional Yeast**
BCID2	*Enterococcus faecalis*	*Acinetobacter calcoaceticus-baumannii complex*	*Candida auris*
	Enterococcus faecium	*Bacteroides fragilis*	*Cryptococcus neoformans/gattii*
	Staphylococcus epidermidis	*Klebsiella aerogenes*	**Additional *AMR genes***
	Staphylococcus lugdunensis	*Salmonella* spp.	*mecA/C* and MREJ (MRSA)
		Stenotrophomonas maltophilia	$bla_{CTX\text{-}M/IMP/NDM/OXA48\text{-}like/VIM}$
			mcr-1

iii. Verigene® Blood Culture Tests

Verigene® Gram-Positive Blood Culture (BC-GP) and Verigene® Gram-Negative Blood Culture (BC-GN) tests (Luminex Corporation, Northbrook, IL, USA) are multiplexed, automated nucleic acid tests based on hybridization of specific bacterial DNA targets employing a gold nanoparticle probe-based technology in a microarray format enabling pathogens identification (Nanosphere Inc., 2023b, 2023a). The Verigene® System includes panels that can detect both Gram-positive and Gram-negative pathogens (~21 bacterial species) as well as resistance gene markers (**Table 6**) within 2 hours with specificity and sensitivity of 98% and 90%, respectively (Dubourg, Raoult and Fenollar, 2019). Nevertheless, false negative results occur, especially in polymicrobial BSIs and the covered

microorganisms comprised in the panel are low compared to MALDI-TOF MS (Arroyo and Denys, 2017).

Table 6. The Verigene® (BC-GP and BC-GN tests) panels (adapted from (Nanosphere Inc., 2023a, 2023b) (Nanosphere Inc., 2023b, 2023a))

Verigene® Gram-Positive Blood Culture (BC-GP): Gam-Positive pathogens	**Verigene® Gram-Positive Blood Culture (BC-GP): Gam-Negative pathogens**
Micrococcus spp.	*Acinetobacter* spp.
Listeria spp.	*Citrobacter* spp.
***Staphylococcus* spp.**	*Enterobacter* spp.
Staphylococcus aureus	*Proteus* spp.
Staphylococcus epidermidis	*Escherichia coli*
Staphylococcus lugdunensis	*Klebsiella oxytoca*
***Streptococcus* spp.**	*Klebsiella pneumoniae*
Streptococcus agalactiae	*Pseudomonas aeruginosa*
Streptococcus pneumoniae	*Serratia marcescens*
Streptococcus pyogenes	***AMR genes***
Streptococcus anginosus (Group)	$bla_{KPC/CTX\text{-}M/IMP/NDM/OXA/VIM}$ (carbapenems)
Enterococcus faecalis	*mecA* (methicillin)
Enterococcus faecium	*vanA/B* (vancomycin)

3.7. Antimicrobial susceptibility testing (AST)

Antimicrobial resistance (AMR), and multi-drug resistant organisms (MDRO) have emerged and became a global public health problem. The emergence of AMR represents a significant challenge in the management of infectious diseases, especially bloodstream infection (Salam *et al.*, 2023). It is one of the major factors causing increased mortality rates and healthcare costs (Brink, 2019). Detection of antimicrobial resistance in BSIs is crucial for the general management of patients, especially for the optimization of the therapy, but also for limiting the misuse of antibiotics and the spread of resistant pathogens (Giacobbe *et al.*, 2020; Pfaller *et al.*, 2020). Many techniques (phenotypic and genotypic) and devices (manual or automated) have been developed to perform AST (Salam *et al.*, 2023). In this section, conventional standard methods as well as a few innovative/recent methods utilized in clinical microbiology laboratories will be detailed.

3.7.1. Standard culture-based methods

Culture-based methods mostly based on phenotypic tests remain the gold standard method widely used for AST (Baquer *et al.*, 2021). Subsequently to bacterial isolation from PBC and species identification, manual phenotypic AST is mainly performed using agar disk diffusion known as Kirby-Bauer disk

diffusion (i.e., tests consisting of inoculating bacterial suspension of 0.5 McFarland turbidity ~ 1.5×10^8 colony forming units/mL of the isolated colonies onto Mueller Hinton (MH) agar plates, then spotting onto the bacterial lawn, antimicrobial filter paper disks containing a defined antibiotic concentration) (Salam *et al.*, 2023). Other phenotypic AST methods such as gradient diffusion strip [minimum inhibition concentration (MIC) test strips (Liofilchem, Italy)], or broth microdilution kits (Balouiri, Sadiki and Ibnsouda, 2016; Humphries *et al.*, 2018) are also available. These culture-based methods require 24 to 72 hours to get results, hence limiting their impact in decision making regarding the optimal antimicrobial treatment, especially for urgent clinical cases (e.g., ICU sepsis patients). In addition, a direct application method consisting of MH-agar inoculation of PBC aliquot without subculture, known as rapid antimicrobial susceptibility testing (RAST) have been developed by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) with specific breakpoints to be read after 4, 6 and/or 8, or 16-20 hours of incubation. However, this method can be performed only for a limited number of species, and cannot be applied to polymicrobial blood cultures bottles with mixed species (Åkerlund *et al.*, 2020).

3.7.2. Automated systems

During the past decades, automated systems enabling enhancing the rapidity of AST in a matter of hours rather than days and less hands-on-time have been developed (Doern *et al.*, 1994). These new rapid techniques may add an important benefit in the clinical outcome of the hospitalized patients, especially when associated with antimicrobial stewardship (AMS) with an AST profile reported within a few hours allowing timely initiation of appropriated antimicrobial treatment, and hence, reducing misuse of antimicrobials and development of antimicrobial resistance, which may cause increase of mortality rates and hospitalization costs, (Doern *et al.*, 1994; Lamy *et al.*, 2020). Automated systems based on genotypic tests are gaining interest in many clinical microbiology laboratories. For example, the Biofire® FilmArray® (BioFire Diagnostics, LLC, Salt Lake City, USA), allows the detection of genetic markers of resistance to methicillin (*mecA*), vancomycin (*vanA* and *vanB*), ß-lactams including penicillins, cephalosporins, monobactams, carbapenems ($bla_{\text{CTX-M}}$, bla_{IMP}, bla_{KPC}, bla_{NDM}, $bla_{\text{OXA48-like}}$, bla_{VIM}) (**Table 5**); the Verigene® system (Luminex Corporation, Northbrook, IL, USA) allows the detection of the following resistance markers: CTX-M (ESBL), bla_{IMP}, bla_{KPC}, bla_{NDM}, bla_{OXA}, bla_{VIM} (carbapenems), *mecA* (methicillin), and *vanA/B* (vancomycin) (**Table 6**).

Moreover, other automated commercial systems such as the VITEK 2 (BioMérieux, Durham, NC) or the MicroScan WalkAway (Beckman Coulter, Germany) also afford to test, simultaneously, multiple antibiotics using microtiter 96-well plates. However, these techniques do not allow direct assay from PBC, but require the preparation of a broth microdilution from bacterial suspension (Beckman, 2024). Of note, negative results of such PCR-based methods do not exclude phenotypic resistance due to the existence of other resistance mechanisms than those included in the test panels (Idelevich and Becker, 2016; Bard and Lee, 2018).

3.8. FAST™ System

The FAST™ System (Qvella Corporation, Canada) is an automated device allowing bacteria purification from PBC. The principle of this recent innovative technology consists of isolating and concentrating bacterial cells directly from PBC using commercial, single-use FAST PBC Prep cartridges (Qvella Corporation, Canada). Briefly, for sample preparation, 2-mL aliquots are drawn from a PBC bottle and processed in a single-use cartridge to purify and isolate the bacterial suspension within 24 (run with one cartridge) or 38 minutes (run with two cartridges). The so-called Liquid Colony ("LC") can then be used for species ID and AST (**Figure 23**) (Qvella Corporation, Richmond Hill and Canada, 2024).

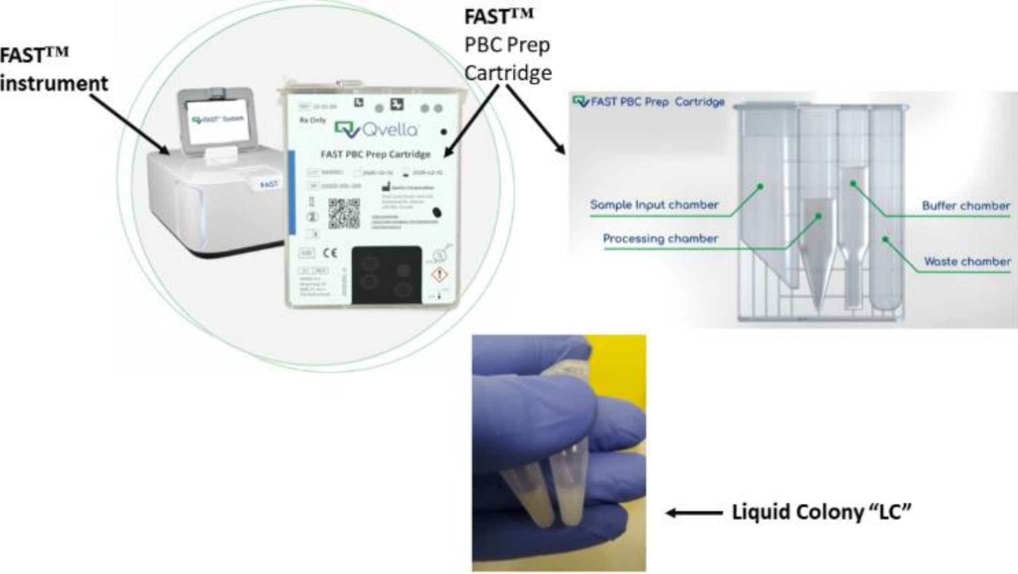

***Figure 23**. FAST™ System and Fast PBC cartridge producing LC after processing PBC (adapted from (Qvella Corporation, Richmond Hill and Canada, 2024)).*

IV

Goal and objectives of the present PhD thesis

4. Goal and objectives of the present PhD thesis

Helminthic parasite infections and BSI are major health issues affecting millions of people each year and causing thousands of deaths and significant morbidity, as is e.g. expressed in DALYs (Khubchandani and Bub, 2019; Kern and Rieg, 2020). Rapid and accurate diagnosis of microorganisms responsible for these infections is crucial for effective treatment and management of patients (Fabre, Carroll and Cosgrove, 2022). Despite significant technological advances and progress, many challenges remain, e.g. with regard to the fast differentiation of pathogens present in PBCs and pertaining to an accurate species identification of helminths (Sy, Conrad and Becker, 2022; Sy *et al.*, 2023).

Nowadays, MALDI-TOF is being used as a standard method in routine microbiology laboratories, especially in HICs, for the identification of clinically relevant bacterial and fungal species using commercially available databases (e.g., Bruker Daltonics, BioMérieux, etc.). In addition, special commercial kits (e.g., Sepsityper) have also been introduced to allow pathogen identification directly from PBC. Regarding parasites, no commercial database for MALDI-TOF identification has been commercialized thus far. However, recent publications have shown that the development of in-house databases or libraries allow for reliable identification of parasites using MALDI-TOF MS, even though very few have focused on helminths (305). Hence, the purpose of this thesis was to investigate innovative diagnostic tools for (1) helminth identification and (2) the microbiological identification of pathogens giving rise to BSI.
The overall goal of this thesis is to investigate the potential of MALDI-TOF MS for identification of helminthic pathogens of medical and veterinary importance in different scenarios, and to improve the diagnosis of bacterial BSI..

The following specific objectives are related to this goal:

a) To assess the ability of MALDI-TOF MS to identify and distinguish adult *Fasciola* spp.
b) To investigate the capacity of MALDI-TOF MS to identify and differentiate adult *Schistosoma* spp.
c) To assess the potential of MALDI-TOF MS to identify other important helminths (e.g. *Taenia* spp.) and to evaluate the effect of different storage media on the MALDI-based identification.
d) To perform a literature review to summarize and outline current and future strategies pertaining to the application of mass spectrometry techniques for parasites.
e) To evaluate the performance of the FAST™ System-generated liquid colony for rapid pathogen identification of PBC by MALDI-TOF MS and subsequent AST using manual (i.e., disk diffusion) and automated (i.e., MicroScan Walk*Away plus*) systems.

V
RESULTS

5. Helminth identification and pathogen detection in blood cultures using MALDI-TOF MS

In this chapter, key findings from this thesis will be presented in the form of manuscripts that have been published in the international peer-reviewed literature. The first two articles are research articles (see **article n°1&2**) pertaining to the application of MALDI-TOF MS for trematode identification and differentiation [i.e., *Fasciola* spp., (see **article n°1**), and *Schistosoma* spp. (see **article n°2**)], followed by a third article in which the performance of MALDI-TOF MS was tested for identifying *T. saginata* proglottids stored in different conservation media (sodium chloride, ethanol, formalin, and water) (see **article n°3**). In the fourth article, a comprehensive review highlights the recent advances and potential future applications of MALDI-TOF MS for helminth diagnostics (see **article n°4**). Finally, the fifth article is a research article evaluating the FAST™ System-generated liquid colony (Qvella Corporation) for the diagnosis of bacterial and fungal pathogens giving rise to BSI, facilitated by the use of MALDI-TOF MS and the subsequent detection of antimicrobial resistance using manual (disks diffusion) and automated (MicroScan Walk*Away*) systems (see **article n°5**).

5.1. Article n° 1. Research article: Identification of adult *Fasciola* spp. using matrix-assisted laser/desorption ionization time-of-flight (MALDI-TOF) mass spectrometry

Sy I, Margardt L, Ngbede EO, Adah MI, Yusuf ST, Keiser J, Rehner J, Utzinger J, Poppert S, Becker SL. Identification of Adult *Fasciola* spp. Using Matrix-Assisted/Laser Desorption Ionization Time-of-flight (MALDI-TOF) Mass Spectrometry. *Microorganisms*. 2021; 9(1):82. https://doi.org/10.3390/microorganisms9010082

Article

Identification of Adult *Fasciola* spp. Using Matrix-Assisted Laser/Desorption Ionization Time-of-Flight (MALDI-TOF) Mass Spectrometry

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Abstract: Fascioliasis is a neglected trematode infection caused by *Fasciola gigantica* and *Fasciola hepatica*. Routine diagnosis of fascioliasis relies on macroscopic identification of adult worms in liver tissue of slaughtered animals, and microscopic detection of eggs in fecal samples of animals and humans. However, the diagnostic accuracy of morphological techniques and stool microscopy is low. Molecular diagnostics (e.g., polymerase chain reaction (PCR)) are more reliable, but these techniques are not routinely available in clinical microbiology laboratories. Matrix-assisted laser/desorption ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) is a widely-used technique for identification of bacteria and fungi; yet, standardized protocols and databases for parasite detection need to be developed. The purpose of this study was to develop and validate an in-house database for *Fasciola* species-specific identification. To achieve this goal, the posterior parts of seven adult *F. gigantica* and one adult *F. hepatica* were processed and subjected to MALDI-TOF MS to create main spectra profiles (MSPs). Repeatability and reproducibility tests were performed to develop the database. A principal component analysis revealed significant differences between the spectra of *F. gigantica* and *F. hepatica*. Subsequently, 78 *Fasciola* samples were analyzed by MALDI-TOF MS using the previously developed database, out of which 98.7% (n = 74) and 100% (n = 3) were correctly identified as *F. gigantica* and *F. hepatica*, respectively. Log score values ranged between 1.73 and 2.23, thus indicating a reliable identification. We conclude that MALDI-TOF MS can provide species-specific identification of medically relevant liver flukes.

Keywords: diagnosis; *Fasciola gigantica*; *Fasciola hepatica*; fascioliasis; helminth; matrix-assisted laser/desorption ionization time-of-flight (MALDI-TOF) mass spectrometry; trematode

Citation: Sy, I.; Margardt, L.; Ngbede, E.O.; Adah, M.I.; Yusuf, S.T.; Keiser, J.; Rehner, J.; Utzinger, J.; Poppert, S.; Becker, S.L. Identification of Adult *Fasciola* spp. Using Matrix-Assisted Laser/Desorption Ionization Time-of-Flight (MALDI-TOF) Mass Spectrometry. *Microorganisms* **2021**, *9*, 82. https://doi.org/10.3390/microorganisms9010082

Received: 18 November 2020
Accepted: 25 December 2020
Published: 31 December 2020

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1. Introduction

Fascioliasis is a food-borne parasitic disease in animals and humans, caused by the digenetic trematodes *Fasciola gigantica* and *Fasciola hepatica* [1]. While the occurrence of *F. gigantica* is restricted to areas of Asia and Africa [2,3], *F. hepatica* is widely distributed throughout Africa, Asia, Europe, Oceania and the Americas [4–7]. Fascioliasis and other food-borne trematode infections are classified as neglected tropical diseases. An estimated 2.4–17 million people are infected with *Fasciola* spp. [8]. However, these numbers may be considerable underestimations of the true number of infections, and hence, the global

disease burden. In veterinary surveys, high prevalences of fascioliasis have been reported. For example, recent studies carried out in Lake Chad area and in a coastal region of Vietnam reported a prevalence in cattle of 68% and 23%, respectively; additionally, a significant risk for spillover of infections from animals to humans was noted [9,10].

The two *Fasciola* species share similar life cycles [1,11]. In brief, the life cycles are complex and require a snail intermediate host of the family Lymnaeidae (e.g., *Galba truncatula* for *F. hepatica*, *Lymnaea natalensis* for *F. gigantica* [12,13], and *Pseudosuccinea columella* for both species [14]). Adult worms are located in the biliary ducts of the definitive host (e.g., ruminants and humans). They release unembryonated eggs that are passed with the feces. These eggs become embryonated by contact with unprotected surface water. Subsequently, a miracidium hatches, which in turn infects the intermediate snail host. Free-swimming cercariae are released from the snail that form metacercariae and can attach to aquatic vegetation. Human infection occurs most frequently by the unintended ingestion of freshwater or vegetables (e.g., watercress) that are contaminated with infective metacercariae [7]. Following ingestion by humans or animals, immature worms excyst in the duodenum and penetrate the intestinal wall from where they migrate through the liver parenchyma [15].

Current methods used for the diagnosis of human and veterinary fascioliasis, in particular for the identification of adult worms, rely on morphological analyses of the trematodes [16], molecular methods (e.g., polymerase chain reaction (PCR)) and sequencing [7,11,17]. The latter two approaches have several limitations, including a lack of rigorous standardization of the morphological identification in different settings, relatively high costs and unavailability of PCR-based testing using specific primers for e.g., trematodes outside highly specialized research laboratories. While the morphological identification is a rapid and less costly procedure, *Fasciola* spp. are rarely detected outside endemic settings and the waning of parasitological experience among laboratory technicians in clinical laboratories is a particular challenge. Hence, the development of an accurate, rapid, less expensive and more accessible diagnostic technique for parasite identification would be desirable. During the past decade, matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) has become a widely employed tool for the diagnosis of bacteria and fungi in clinical samples [18–20]. More recently, a variety of studies have reported the use of MALDI-TOF MS as a potentially promising tool for the identification of mosquitoes [21,22], ticks [23], and, to a lesser extent, parasites (protozoa and helminths) [24]. In contrast to PCR-based techniques, MALDI-TOF MS allows for a broad, "untargeted" detection of microorganisms if these are present in the database used for pathogen identification. The goal of this study was to validate MALDI-TOF MS for the identification and differentiation of adult *Fasciola*.

2. Materials and Methods

2.1. Ethics Statement

For adult *Fasciola* samples originating from Nigeria, written permission was obtained from the management board of the Kubwa abattoir (Abuja, Nigeria) for collection and subsequent analyses of the fluke samples from slaughtered cattle (reference no.: FCT/ARD/TRN/004, 17 October 2019). Adult *F. hepatica* specimens were obtained from livers from a slaughterhouse in central Switzerland. Note that such specimens are utilized for routine diagnostic work and research purposes at the Swiss Tropical and Public Health Institute (Swiss TPH; Basel, Switzerland), adhering to local laws and regulations.

2.2. Sample Collection

For the current investigation, adult *Fasciola* worms were collected by experienced veterinarians from the liver tissue of slaughtered cattle at the Kubwa abattoir in Abuja, the Federal Capital Territory of Nigeria in December 2019 and January 2020. Samples were stored in 70% (*v*/*v*) ethanol at room temperature and were transferred to the Institute of Medical Microbiology and Hygiene in Homburg, Germany.

Additional samples were collected in early 2020 from the livers of cattle from a slaughterhouse in Oensingen, Switzerland. Livers were routinely screened by the slaughterhouse veterinarians. Infected livers were put aside and, on the same day, transferred and examined by experienced laboratory technicians at Swiss TPH. *Fasciola* specimens were stored in 0.7% (v/v) sodium chloride solution (NaCl) and transferred on ice to the Institute of Medical Microbiology and Hygiene in Homburg. Upon receipt in Homburg, samples were kept at −20 °C pending further examination.

2.3. Sample Preparation

Adult worms were removed from the storage solution and dried at room temperature to allow for evaporation of organic solvents. Two small pieces (each weighing approximately 15 mg) of the posterior part of each fluke were cut with a sterile scalpel to be used for subsequent molecular analyses and MALDI-TOF MS.

2.4. Molecular Analysis

2.4.1. DNA Extraction, PCR and Sequencing

The DNeasy Blood and Tissue Kit (Qiagen GmbH; Hilden, Germany) was utilized for DNA extraction, adhering to the manufacturer's instructions. Briefly, pieces measuring approximately 5 mm of each fluke were placed into a 1.5 mL Eppendorf tube, adding 280 µL lysis buffer and 20 µL proteinase K. The flukes were gently crushed in this mixture. Next, the mixture was incubated using a thermomixer (Eppendorf; Hamburg, Germany) at 56 °C and 800× g for 1 h. After digestion, a washing step was performed using AW1 and AW2 buffers from the Qiagen kits and a column with a silica membrane. The extracted DNA was eluted in 200 µL of AE buffer (Qiagen; Hilden, Germany) and stored at −20 °C pending molecular analyses.

PCR amplification of the partial mitochondrial cytochrome oxidase 1 gene (COX1) of all *Fasciola* specimens was carried out in Homburg, using a previously described protocol with the forward primer 5′-TTGGTTTTTTGGGCATCCT-3′ and the reverse primer 5′-AGGCCACCACCAAATAAAAGA-3′ [6]. The amplicons generated were sequenced using the Capillary Electrophoretic GenomeLab genetic analysis system (Beckman Coulter; Brea, CA, USA).

2.4.2. Sequence Analysis and Species Identification

The forward and reverse sequences obtained were edited and combined to generate a consensus sequence for each specimen, using the software BioEdit© version 7.2.5 (Tom Hall; Carlsbad, CA, USA) [25]. The consensus sequences were then queried against the National Center for Biotechnology Information (NCBI) GenBank database for identification, using the Basic Local Alignment Search Tool (BLASTn) [26]. Sequences obtained from the eight isolates that were used for MALDI-TOF MS database development during this study were submitted to GenBank, comprising the consecutive accession numbers MW258701 to MW258708.

2.5. MALDI-TOF Analysis

2.5.1. Protein Extraction

A small piece corresponding to approximately 15 mg from the posterior part of each adult fluke was cut thinly with a sterile scalpel in order to facilitate the release of molecules from other locations than the fluke's surface area, and was subsequently put into a 1.5 mL tube (Figure 1). Of note, no eggs can be found in the posterior part of *Fasciola*, as uterine structures are localized in the anterior part of this helminth. The "complete extraction protocol", recommended by the manufacturer (Bruker Daltonics; Bremen, Germany) for protein extraction from bacteria for subsequent MALDI-TOF analysis, was readily adapted to the fluke samples. In brief, 300 µL of LC-MS grade water (Merck KG; Darmstadt, Germany) and 900 µL of 100% (v/v) ethanol (Merck KG) were added to the samples and mixed by vortexing. The mixture was centrifuged at 18,312× g for 2 min and the

supernatant discarded. The pellet was resuspended in 50 µL of 70% formic acid and 50 µL of acetonitrile and mixed by vortexing. Of note, we also employed additional steps, including the use of Zirconium beads, during the development of our helminth extraction protocol, but did not observe differences in the obtained spectral profile. Hence, we decided not to use beads to reduce the working steps and the hands-on time per sample.

Figure 1. Morphology of an adult *Fasciola gigantica* fluke. The red line shows the area where the incision of the helminth was performed, and the red arrow indicates the posterior body part that was used for molecular analysis and MALDI-TOF MS.

2.5.2. Target Plate Preparation and Measurements

The protein extracts obtained with the mix of formic acid and acetonitrile above was centrifuged at 18,312× *g* for 2 min and 1 µL of the clear supernatant spotted onto the MALDI-TOF target plate, followed by overlaying with 1 µL of α-cyano-4-hydroxycinnamic acid (CHCA) matrix solution (Bruker Daltonics), composed of saturated CHCA 50% (v/v) of acetonitrile, 2.5% (v/v) of trifluoroacetic acid and 47.5% (v/v) of LC-MS grade water. Bacterial test standard (BTS) (Bruker Daltonics), which is an extract of *Escherichia coli* that is spiked with two high molecular weight proteins, was used to calibrate the machine. After drying at room temperature, the plate was placed into the Microflex LT Mass Spectrometer (Bruker Daltonics) for MALDI-TOF MS.

2.5.3. MALDI-TOF MS Parameters

Measurements were performed using the AutoXecute algorithm in the FlexControl© software version 3.4 (Bruker Daltonics). For each spot, 240 laser shots in six random positions were carried out automatically to generate protein mass profiles in linear positive ion mode with a laser frequency of 60 Hz, a high voltage of 20 kV and a pulsed ion extraction of 180 ns. Mass charge ratios range (m/z) were measured between 2 k and 20 k Da.

2.5.4. Spectral Analysis and Database Creation

For the creation of species-specific main spectra profiles (MSPs), protein extracts of one *F. hepatica* and seven *F. gigantica* specimens were spotted onto the MALDI-TOF target plate eight times per sample. Next, each spot was measured four times to generate 32 raw spectra per sample. For each specimen, this procedure was carried out on two replicates on the same day (to demonstrate repeatability) and on one replicate on a different day (to demonstrate reproducibility). Hence, a total of 96 raw spectra were acquired for each sample, using the FlexControl® software version 3.4 (Bruker Daltonics). These raw spectra

were analyzed and curated using the FlexAnalysis® software version 3.4 (Bruker Daltonics) for a "cleaning step", i.e., to withdraw all flatlines and outlier peaks and to smoothen intensities and edit peak shifts within spectra whenever these exceeded 500 ppm. Following this editing step, replicates containing at least 22 remaining spectra were randomly chosen for the creation of species-specific MSPs. These MSPs were created using the automatic function of the MALDI Biotyper Compass Explorer© software version 3.0 (Bruker Daltonics). Finally, the newly created MSPs of both *Fasciola* species were included in a previously developed in-house MALDI-TOF database for helminth identification, which already contained several nematodes (e.g., *Ascaris lumbricoides*), cestodes (e.g., *Taenia saginata*) and trematodes (e.g., *Schistosoma mansoni*).

MSP dendrogram analysis was carried out with the MALDI Biotyper Compass Explorer and employed the following parameters: distance correlation, linkage by average and score threshold values of 300 and 0 (arbitrary unit) for a single and a related organism, respectively. For a principal component analysis and a discriminatory analysis of the species-specific MSPs, we used the software BioNumerics® version 7.5 (Applied Maths N.V.; Sint-Martens-Latem, Belgium).

2.5.5. Validation Test

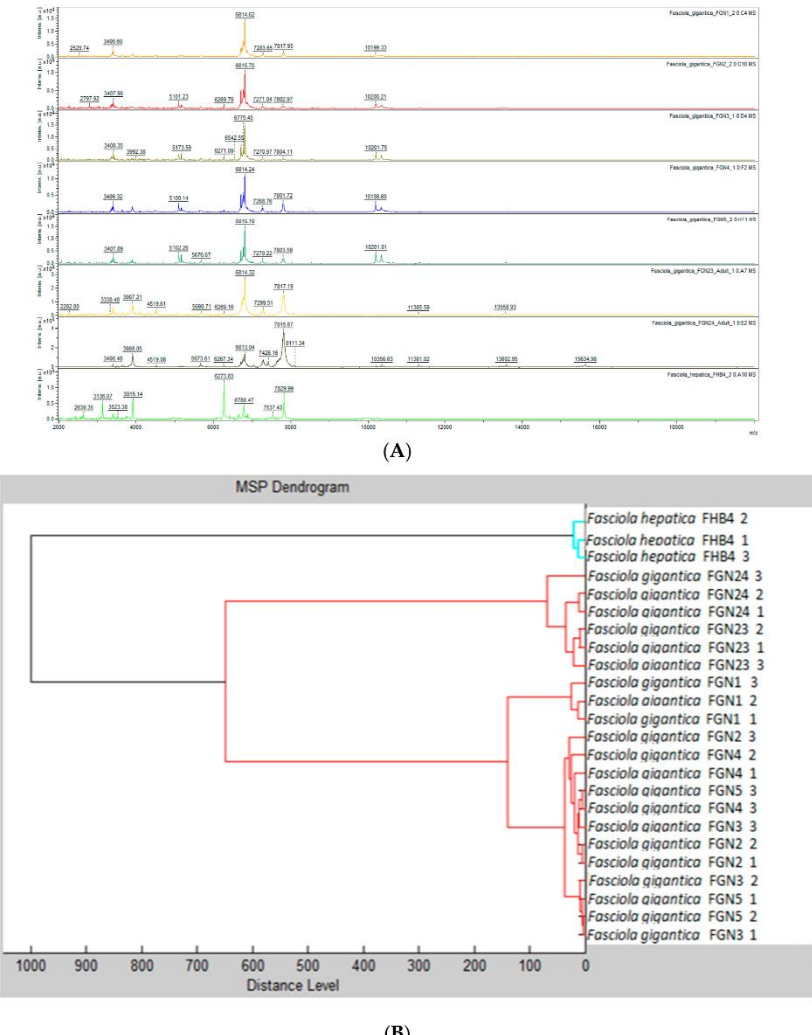
The newly developed in-house database was subjected to two different validation procedures. First, an internal validation, during which all raw spectra of *Fasciola* spp. obtained during the process of MSP creation were analyzed. Second, an external validation, during which raw spectra from new, unidentified *Fasciola* specimens were measured by MALDI-TOF MS to assess the database's ability to reliably identify these samples.

All spectra were tested against the commercially available official database released by Bruker Daltonics for identification of bacteria and fungi (Bruker Taxonomy, Maldi Biotyper Compass Explorer version 3.0). Next, the spectra were subjected to a combination of the official database and our in-house helminth database. The reliability of identification was assessed by log score values (LSVs), which are generated for each result. We adhered to the "official" grading system put forth by the manufacturer for bacteria (i.e., LSVs may range between 0 and 3; LSV of $\geq$1.70 is considered as a threshold for identification; LSVs ranging between 1.70 and 1.99 indicate a reliable identification at the genus level; and LSVs equal to or higher than 2.0 are interpreted as reliable species identification).

3. Results

3.1. Comparative Analysis of Samples Used for MSP Database Creation

For the creation of MSPs to be included in the in-house database, we included seven randomly selected adult flukes obtained from cattle in Nigeria, which had previously been identified as *F. gigantica* based on morphological characteristics, and one adult *F. hepatica* provided from Switzerland. Partial sequencing of the COX1 confirmed the species diagnosis in the eight reference samples. In comparison to previously deposited sequences, the BLAST analysis revealed sequence homologies between 98.5% and 99.8% for *F. gigantica* (reference accession numbers: MN586868.1, MN586869.1, MN913872.1 and MN913873.1) and 99.4% for *F. hepatica* (reference accession number: GQ231551.1). The visualization of spectra obtained by MALDI-TOF MS is displayed in Figure 1. Considerable differences between *F. gigantica* and *F. hepatica* were observed (Figure 2A), which were confirmed by a dendrogram analysis (Figure 2B). Of note, there was some heterogeneity with regard to the spectra within the *F. gigantica* cluster (e.g., samples FGN23 and FGN24), which was further substantiated by a principal component analysis and discriminant analysis (Figure 3). The COX1 sequences of these two samples (FGN23 and FGN24) showed sequence homologies of 99.5% and 98.6%, respectively, to the GenBank reference sequences of *F. gigantica*.



(**B**)

Figure 2. (**A**) Spectra obtained by matrix-assisted laser/desorption ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) for seven adult *Fasciola gigantica* (FG) specimens and one adult *Fasciola hepatica* (FH) specimen. The peak intensities of ionised molecules are shown on the y-axis and the corresponding mass on the x-axis. Numbers indicate the resulting mass-to-charge-ratios. (**B**) Dendrogram analysis displaying the relatedness of the different samples. Of note, each specimen was measured thrice to assure reproducibility.

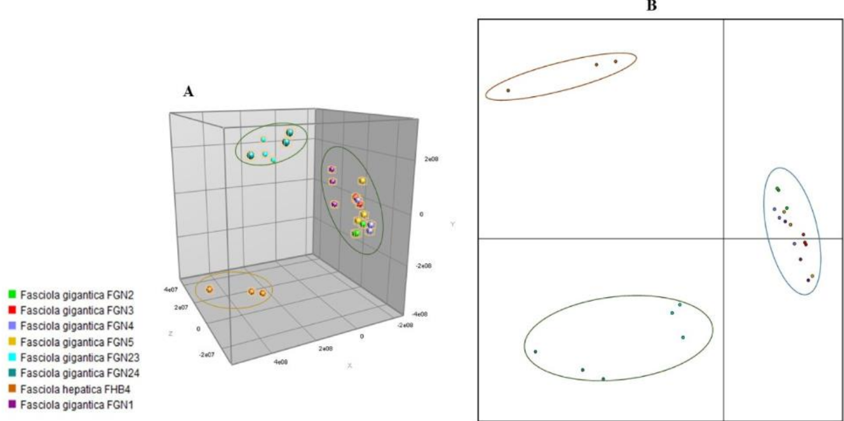


Figure 3. Statistical analysis of main spectra profiles (MSPs) obtained during the creation of an in-house database for identification of adult *Fasciola gigantica* (FG) and *Fasciola hepatica* (FH) using MALDI-TOF MS. (**A**) Three-dimensional view of a principal component analysis, displaying three distinct clusters, i.e., (i) *F. hepatica* FHB4; (ii) *F. gigantica* FGN23 and FGN24; and (iii) the remaining *F. gigantica* samples. (**B**) Two-dimensional view of a discriminant analysis, using the same samples. The analyses were carried out using the software BioNumerics.

3.2. Internal Database Validation

When the reference spectra were analyzed with the commercially available database for bacteria and fungi identification, no reliable identification was achieved, and all LSVs were below 1.7. In the subsequent analysis of the raw spectra obtained during MSP creation, which used a combination of the official and the in-house database, a reliable species identification as either *F. gigantica* or *F. hepatica* was achieved in all eight samples, with LSVs ranging from 2.17 to 2.86 (average LSV = 2.5).

3.3. Analysis of Samples for External Database Validation

A total of 78 *Fasciola* samples were subjected to MALDI-TOF analysis, using the newly developed database. There were 75 samples from Nigeria that had previously been identified as *F. gigantica*, based on morphological characteristics. The remaining three specimens stemmed from Switzerland and were reported as *F. hepatica*. PCR was performed on all samples and confirmed the species identification in all cases.

When using the previously developed in-house database and considering an LSV threshold of $\geq$1.70, MALDI-TOF MS correctly identified 74/75 (98.7%) of the *F. gigantica* and 3/3 (100%) of the *F. hepatica* samples. In one sample, MALDI-TOF MS did not identify sufficient protein spectra to provide an identification. When an LSV threshold of $\geq$2.0 was used, the identification rate was 31/75 (41.3%) for *F. gigantica*, while it remained unchanged for *F. hepatica* (Table 1). Of note, no *F. gigantica* sample was misidentified as *F. hepatica* and vice versa.

Table 1. Identification of 78 adult *Fasciola* spp. samples obtained from Nigeria and Switzerland by MALDI-TOF MS, using a newly developed in-house database. LSV, log-score value.

Species	Number of Samples	Identification		LSV Range
		LSV $\geq$ 1.70	LSV $\geq$ 2.00	
Fasciola gigantica	75	74 (98.7%)	31 (41.3%)	1.73–2.13
Fasciola hepatica	3	3 (100%)	3 (100%)	2.15–2.23

4. Discussion

The purpose of this study was to determine whether MALDI-TOF MS can be utilized as a diagnostic tool for the identification and differentiation of adult *Fasciola* species. Our results show that MSPs created from repetitive measurements of eight adult *Fasciola* specimens, utilized as part of a helminth-specific in-house database, allowed for an unambiguous identification of 78 additional *Fasciola* samples. Indeed, 77 of 78 specimens were correctly identified, and there was no misidentification at the species level. A deeper investigation using principal component analysis and discriminant analysis for comparative spectra visualization confirmed species-specific differences that enable accurate diagnosis. In the case of the single specimen that was not correctly identified, we had already noted a slightly different morphological aspect on macroscopic inspection, and no meaningful protein spectra of sufficient quality were obtained by MALDI-TOF MS, which probably indicates previous degradation of the proteins.

MALDI-TOF MS has become a standard diagnostic tool for bacteria [27,28] in the microbiological laboratory and is now increasingly being utilized for identification of yeasts and filamentous fungi as well as mycobacteria. In research settings, entomological studies have also employed MALDI-TOF for identification of ticks [23], mosquitoes [21,29], fleas [30], and lice [31]. Yet, data on application of MALDI-TOF MS for parasites, particularly helminths, are scarce. A recent systematic review identified only five published studies pertaining to diagnostic helminthology [24], using MALDI-TOF MS specifically for *Ascaris* spp., cyathostomin helminths, *Dirofilaria* spp. and *Trichinella* spp. Recently, two studies described the application of MALDI-TOF MS for identification of helminths; one focusing on *Trichinella* spp. in France [32] and the other on *Anisakis* spp. in Italy [33]. Using different cestode, nematode and trematode samples, our group has also generated species-specific MSPs and developed an in-house database for helminth identification, which will prospectively be validated on well characterized clinical samples [34]. It is important to note that the protocols used for protein extraction and MALDI-TOF spectra acquisition varied slightly between studies, and the development of one standardized approach will be an important feature to generate (i) accurate databases; and (ii) specific and reproducible results across different laboratories in future studies.

Interestingly, while creating the MSPs for the in-house database, it was observed that two out of seven *F. gigantica* samples used (i.e., isolates no. FGN23 and FGN24) clustered together in a group with slightly different spectral patterns than the other five *F. gigantica* samples. Such observed intra-species differences in the mass spectra profiles could be explained by a minor genetic variation (e.g., a non-synonymous mutation, which could affect the protein profile). In this context, it is important to mention that several studies have reported the existence of an intermediate "hybrid" species of *Fasciola*, which can only be discriminated by specific molecular methods [34]. Liu and colleagues [35] studied the sequence data of protein-encoding genes and showed that the intermediate form of *Fasciola* is more closely related to *F. gigantica* than *F. hepatica*. Similar studies from sub-Saharan Africa have suggested that the epidemiology of fascioliasis in this part of the world may be more complex than previously thought, and that *F. gigantica* is not the only species occurring [36]. "Hybrid" *Fasciola* spp. have also been reported from Chad [37], and these might also occur in Nigeria, where the *F. gigantica* samples for the current MALDI-TOF MS were obtained. Since "hybrid" species cannot be accurately identified by amplification of the COX1 as performed here, further molecular investigations pertaining to the genomics (e.g., sequencing of the internal transcribed spacer (ITS) region as reported by Evack and colleagues [37]) of these samples will be interesting. Indeed, while we aligned and comparatively analyzed the obtained COX1 sequences, we were unable to identify specific variable sites, which would have allowed to accurately identify "hybrid" isolates.

Our study is limited by the small sample size for *F. hepatica* (i.e., only four adult worms), analysis of samples stemming from only two different geographical areas (i.e., Nigeria and Switzerland) and the use of samples preserved in different media (70% ethanol and 0.7% NaCl for *F. gigantica* and *F. hepatica*, respectively). It is important to mention that

potential effects of different preservation media used were not thoroughly investigated in this proof-of-concept study. Another limitation relates to the hosts, as we only analyzed *Fasciola* spp. stemming from animals and further research is needed to confirm that our developed in-house database would also reliably identify flukes from other hosts, e.g., specimens extracted from human bile ducts. It should also be noted that more than half of all *F. gigantica* samples were identified with LSVs < 2.0, which would only relate to a genus-specific, but not to a species-specific identification if thresholds for bacteria were used. However, MALDI-TOF MS did not misidentify any of the two *Fasciola* species. For the further development and improvement of a helminth-specific MALDI-TOF database, a more comprehensive investigation with specimens from other geographical settings in Africa, Asia, Europe and the Americas and samples originating from different hosts would be desirable. Additionally, while we only analyzed adult flukes, future studies should also consider *Fasciola* eggs and different larval stages to obtain a more accurate taxonomic typing and to further improve the species identification via MALDI-TOF MS. Indeed, a direct MALDI-TOF-based identification of *Fasciola* eggs in stool samples of human and veterinary origin would represent a major achievement to improve the diagnosis of fascioliasis, including "hybrid" species, in many laboratories. Pending further research on the effect of different storage solutions on the resulting protein spectra, this method might also be employed as a suitable alternative to PCR for a diagnostic identification of helminths after storage for many years (e.g., historical parasite collections in research laboratories).

5. Conclusions

We conclude that MALDI-TOF MS is a promising tool for rapid and reliable identification of adult *Fasciola* and potentially other food-borne trematode species. It is important to note that the creation and validation of a specific in-house database is necessary for identification of taxonomic groups (e.g., food-borne trematodes) that are not covered by commercial databases. To our knowledge, this study is the first to employ MALDI-TOF for differentiation of the causative agents of fascioliasis.

Author Contributions: Conceptualization, I.S., E.O.N., S.P. and S.L.B.; methodology, I.S., L.M., E.O.N. and S.L.B.; contribution of samples, E.O.N., M.I.A., S.T.Y., J.K. and S.P.; investigation and formal analysis, I.S., L.M. and E.O.N.; writing—original draft preparation, I.S.; writing—review and editing, I.S., E.O.N., J.R., J.U., S.P. and S.L.B. All authors have read and agreed to the published version of the manuscript.

Funding: E.O.N. was supported by the Cooperation Visits Programme of The World Academy of Sciences and Deutsche Forschungsgemeinschaft (TWAS-DFG). We acknowledge support by the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) and Saarland University within the funding programme Open Access Publishing.

Institutional Review Board Statement: Written permission was obtained from the management board of the Kubwa abattoir (Abuja, Nigeria) for collection and subsequent analyses of the fluke samples from slaughtered cattle (reference no.: FCT/ARD/TRN/004, 17 October 2019). No approval was required for analysis of the Swiss samples.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Acknowledgments: We are grateful to Maureen Feucherolles from the Luxembourg Institute of Science and Technology (LIST) for assistance during statistical analysis. We are grateful to Anna Stuermann from the Swiss Tropical and Public Health Institute for isolating the *F. hepatica* samples from animal livers in the Swiss component of the study.

Conflicts of Interest: The authors declare no conflict of interest.

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5.2. Article n° 2. Research article: Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry for differential identification of adult *Schistosoma* worms

Ebersbach JC, Sato MO, de Araújo MP, Sato M, Becker SL, **Sy I**. Matrix-Assisted laser desorption/ionization time-of-flight mass spectrometry for differential identification of adult *Schistosoma* worms. *Parasites Vectors* 16, 20(2023). https://doi.org/10.1186/s13071-022-05604-0

Ebersbach *et al. Parasites & Vectors* (2023) 16:20
https://doi.org/10.1186/s13071-022-05604-0

Parasites & Vectors

RESEARCH **Open Access**

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry for differential identification of adult *Schistosoma* worms

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Abstract

Background Schistosomiasis is a major neglected tropical disease that affects up to 250 million individuals worldwide. The diagnosis of human schistosomiasis is mainly based on the microscopic detection of the parasite's eggs in the feces (i.e., for *Schistosoma mansoni* or *Schistosoma japonicum*) or urine (i.e., for *Schistosoma haematobium*) samples. However, these techniques have limited sensitivity, and microscopic expertise is waning outside endemic areas. Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) has become the gold standard diagnostic method for the identification of bacteria and fungi in many microbiological laboratories. Preliminary studies have recently shown promising results for parasite identification using this method. The aims of this study were to develop and validate a species-specific database for adult *Schistosoma* identification, and to evaluate the effects of different storage solutions (ethanol and RNA*later*) on spectra profiles.

Methods Adult worms (males and females) of *S. mansoni* and *S. japonicum* were obtained from experimentally infected mice. Species identification was carried out morphologically and by cytochrome oxidase 1 gene sequencing. Reference protein spectra for the creation of an in-house MALDI-TOF MS database were generated, and the database evaluated using new samples. We employed unsupervised (principal component analysis) and supervised (support vector machine, *k*-nearest neighbor, Random Forest, and partial least squares discriminant analysis) machine learning algorithms for the identification and differentiation of the *Schistosoma* species.

Results All the spectra were correctly identified by internal validation. For external validation, 58 new *Schistosoma* samples were analyzed, of which 100% (58/58) were correctly identified to genus level (log score values ≥ 1.7) and 81% (47/58) were reliably identified to species level (log score values ≥ 2). The spectra profiles showed some differences depending on the storage solution used. All the machine learning algorithms classified the samples correctly.

Conclusions MALDI-TOF MS can reliably distinguish adult *S. mansoni* from *S. japonicum*.

Keywords Identification, *Schistosoma mansoni*, *Schistosoma japonicum*, Helminth, Matrix-assisted laser desorption/ionization-time of flight mass spectrometry, Trematode, Storage media, Machine learning

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Background

In tropical and subtropical areas, schistosomiasis is a major cause of morbidity; it is one of the clinically most relevant water-borne parasitic diseases worldwide [1, 2], and an estimated 250 million people are infected with it in a total of 78 countries [3, 4]. Schistosomiasis can cause severe allergy-like reactions in initial disease stages (e.g., Katayama syndrome), while it can later lead to significant long-term morbidity, e.g., diarrhea, hematuria, depending on the site of infection, and considerable complications (e.g., hepatic fibrosis, bladder cancer) [5]. *Schistosoma* belong to the family Schistosomatidae, with three main species infecting humans: *Schistosoma mansoni*, occurring mainly in the sub-Saharan region and South America; *Schistosoma japonicum*, mainly found in China, the Philippines, and Sulawesi (Indonesia); and *Schistosoma haematobium*, found in Africa and parts of the Middle East. Active infections are most frequently found in schoolchildren and young adults [4].

Schistosoma has a complex life cycle that requires an intermediate host (freshwater snail). When they come into contact with water, *Schistosoma* eggs hatch and release miracidia. The miracidia penetrate snail tissues and develop into cercariae, which are released into the water and constitute the infective stage. Humans and other mammals (definitive hosts) can become infected through contact with contaminated freshwater. The schistosomulae successively enter the lungs, the heart, and the liver via the venous circulation, and leave the liver via the portal system once the maturation process is complete. Male and female adult worms live in the mesenteric veins, where they copulate, and lay their eggs in the small venules of the portal and perivesical systems [3]. The transmission cycle continues when hosts infected with schistosomiasis contaminate freshwater sources with their excreta, which contain the parasite's eggs [6, 7].

Morphological identification of eggs from stool or urine samples using microscopy is the most widely employed standard technique for the diagnosis of schistosomiasis [3]. However, this method is laborious and has limited sensitivity, especially for infections of light intensity [8]. Hence, several other tests have been developed, e.g., using polymerase chain reaction (PCR) [9], and serology [8]. However, these have shortcomings with regard to their field applicability, diagnostic accuracy and/or accessibility—for example, no commercially available PCR test kits are available for *Schistosoma* diagnostics [10]. The serological method is very sensitive, but its specificity is poor, as it cannot distinguish active from past infections [2, 7].

Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) is the standard diagnostic method for the identification of culture-grown bacteria, mycobacteria, and fungi in clinical laboratories. Recently, this technique has also been investigated in clinical research as a new tool for the identification of mosquitoes [11, 12], ticks [13] and other arthropods [14] and, to a lesser extent, intestinal parasites (e.g., helminths) [8]. Species identification through MALDI-TOF MS is based on the comparison of protein spectra of unknown, independent samples to reference spectra of well-characterized species that are held in a MALDI database. Compared to the other identification techniques, the advantages of this method are its reliability and rapidity, cost-effectiveness due to the reagents used, its availability in many microbiological laboratories, particularly in high-income countries, where expertise in the identification and differentiation of parasitic elements is waning, and its ease of use once a database (which includes the reference spectra of the targeted microorganisms) is available. However, there are scant data on trematode identification from MALDI-TOF MS. Against this background, the aims of this study were (i) to employ MALDI-TOF MS for the identification of adult *Schistosoma* species, even though adult worms are not used in human diagnosis, as this allowed us to evaluate the potential of this technology for future applications in clinical diagnostics using available materials (i.e., egg samples from stool or urine); (ii) to assess the accuracy of identification for the differentiation of adult *S. mansoni* from *S. japonicum* (interspecies classification); and (iii) to evaluate the effect of different storage media [RNA*later*, and 70% (*v*/*v*) ethanol] on samples by using MALDI-TOF MS coupled with machine learning (ML) classification algorithms.

Methods

Ethics statement

All the experimental procedures involving animals were conducted in strict accordance with the Institutional Animal Care Guidelines and approved by the Ethical Committee for Animal Experimentation of Dokkyo Medical University under number 1307.

Origin of the parasitic material

Adult worms of *S. mansoni* (Puerto Rican strain) and *S. japonicum* (Japanese Yamanashi strain) used in this study were obtained from experimentally infected BALBc mice. The infected animals were maintained at the animal facility of the Laboratory of Tropical Medicine and Parasitology of Dokkyo Medical University. For the current investigation, a total of 62 adults of *Schistosoma* spp. were obtained from either the mesenteric or portal veins of the experimental animals and washed with PBS several times, before being morphologically identified and stored in two different media:

70% (*v*/*v*) ethanol; RNA*later* (Invitrogen, USA), which is an aqueous, nontoxic, tissue and cell collection reagent that stabilizes and protects RNA and proteins in intact, unfrozen tissue and cell samples. Sample identification was further confirmed by DNA sequencing (see "Molecular identification of *Schistosoma* spp. samples" section). Forty samples were identified as *S. mansoni*, while the remaining 22 corresponded to *S. japonicum*. Of the 40 *S. mansoni* isolates, 19 (seven mixed males/females, six males, six females) were placed in 70% (*v*/*v*) ethanol, and 21 (11 mixed males/females, five males, five females) in RNA*later*. For the 22 *S. japonicum* isolates, 11 (five mixed males/females, three males, three females) were stored in 70% (*v*/*v*) ethanol and 11 (five mixed males/females, three males, three females) in RNA*later*. All the samples were stored at −40 °C before being transferred to the Institute of Medical Microbiology and Hygiene (Homburg, Germany), where they were stored at −20 °C pending further examination.

Molecular identification of *Schistosoma* spp. samples

For molecular confirmation of the individual *Schistosoma* species obtained from the mice, genomic DNA of two adult worms from each experimental infection was extracted using a commercially available DNA extraction kit (DNeasy Blood & Tissue Kit; QIAGEN, USA) according to the manufacturer's instructions. Genomic DNA from morphologically identified *S. mansoni* or *S. japonicum* was used for the amplification of cytochrome oxidase 1 (*COX1*) by PCR using primer pairs specific for *S. mansoni* (TCCTTTATCAATTTGAGAGG/CR: CCAACCATAAACATATGATG) and *S. japonicum* (CCGTTTTTTTTTGAGTATGAG/CR: CCAACCATA AACATATGATG), with an expected length of 479 and 614 base pairs, respectively [15]. The reactions were carried out in a final volume of 50 µL, using KOD One PCR Master Mix (Toyobo, Japan) with 10 µmol each of the forward and reverse primers and 1.0 µL (approximately 10 ng/µL) genomic DNA. Cycling conditions for the PCR consisted of a 2-min denaturation step at 94 °C, followed by 35 cycles of denaturation at 98 °C for 10 s, annealing at 58 °C for 30 s, and extension at 68 °C for 30 s, and final extension at 72 °C for 7 min. PCR products were detected in 2% agarose gel stained with 1% ethidium bromide using Tris–borate–ethylenediaminetetraacetic acid buffer. The PCR products were purified using a commercial DNA purification kit (QIAquick Gel Extraction Kit; QIAGEN, Hilden, Germany) following the manufacturer's protocol. Purified PCR products were sequenced in a 3130xl Genetic Analyzer (Applied Biosystems, USA). Sequences were assembled using Molecular Evolutionary Genetics Analysis version 10 [16] and a Nucleotide Basic Local Alignment Search Tool (BLASTn) search (https://blast.ncbi.nlm.nih.gov) was performed for the confirmation of sequence identity of the generated consensus sequences. A phylogenetic tree based on the analysis of *COX1* gene sequences was constructed after 1000 bootstrap replications, using the maximum likelihood method and the Hasegawa-Kishono-Yano model. The outgroup sequence *S. haematobium* (accession ID: ON237718), as well as other *COX1* sequences of *S. mansoni* (accession IDs: MK171834, MF919418, and MG562513) and *S. japonicum* (accession IDs: KU196387, KU196397, and KU196417) were retrieved from GenBank and added to the analysis.

MALDI-TOF MS analysis

Sample preparation

Adult worm samples were removed from the storage solution and dried at room temperature in a biosafety cabinet for about 5 min, to allow for the evaporation of organic solvents prior to the subsequent analyses.

Protein extraction

A previously employed protocol was adapted and applied for protein extraction of the adult *Schistosoma* samples [10]. In brief, adult worms were manually crushed in 300 µL liquid chromatography-mass spectrometry (LC–MS) grade water (Merck, Darmstadt, Germany). Then, 900 µL of 100% (*v*/*v*) absolute ethanol (Merck, Darmstadt, Germany) was added before mixing by vortexing. The mixture was centrifuged at 18,312 × *g* for 2 min, and the supernatant was discarded. After having completely dried the pellet, it was resuspended in 20 µL of 70% (*v*/*v*) formic acid and mixed by vortexing. Finally, 20 µL acetonitrile was added before mixing again.

MALDI target plate preparation and measurements

The protein extracts (see the "Protein extraction" section) mixed with formic acid and acetonitrile were centrifuged at 18,312 × *g* for 2 min. One microliter of the clear supernatant was spotted onto the MALDI-TOF MS target plate (Bruker Daltonics, Bremen, Germany) then allowed to dry completely before covering it with 1 µL of α-cyano-4-hydroxycinnamic acid matrix solution (Bruker Daltonics) composed of saturated α-cyano-4-hydroxycinnamic acid, 50% (*v*/*v*) acetonitrile, 2.5% (*v*/*v*) trifluoroacetic acid and 47.5% (*v*/*v*) LC–MS grade water. The protein extracts of each sample were spotted onto the MALDI-TOF MS target plate at eight different spots, and each spot was measured four times to assure reproducibility. Hence, a total of 32 raw spectra per sample were generated using FlexControl® software version 3.4 (Bruker Daltonics). The bacterial test standard (Bruker Daltonics), which is an extract of *Escherichia coli* spiked with two high molecular weight proteins, was used to

calibrate the mass spectrometer. After drying at room temperature, the MALDI target plate was placed into a Microflex LT Mass Spectrometer (Bruker Daltonics) for the measurements.

MALDI-TOF MS parameters

Measurements were performed using the AutoXecute algorithm implemented in FlexControl software version 3.4. For each spot, a total of 240 laser shots (40 shots each, six random positions) were carried out automatically to generate protein mass profiles in linear positive ion mode with a laser frequency of 60 Hz, a voltage of 20 kV, and a pulsed ion extraction of 180 ns. Mass charge ratios range (m/z) were measured between 2 and 20 kDa.

Spectra inspection and creation of reference spectra

Raw spectra were visualized using FlexAnalysis software version 3.4 (Bruker Daltonics). The spectra were edited, i.e., all flatlines and outlier peaks were removed, intensities were smoothed, and peak shifts within replicated spectra were set at 300 p.p.m. After this editing step, spectra of four mixed (males/females) adult worm samples (two *S. mansoni*, two *S. japonicum*) from both of the storage media (RNA*later*, ethanol), comprising at least 27 remaining spectra each, were randomly selected for the creation of reference spectra (main spectra profiles; MSPs). These MSPs were created using the automatic function of MALDI Biotyper Compass Explorer® software version 3 (Bruker Daltonics). The newly created MSPs of both *Schistosoma* species were included in a previously developed in-house MALDI-TOF MS database for helminth identification, which already contained MSPs from different helminths such as cestodes (e.g., *Taenia saginata*) and trematodes (e.g., *Fasciola* spp.) [10, 17].

Database validation

To verify the purity and check if any of the spectra matched bacterial spectra, all the acquired spectra were tested against the commercially available, official BDAL database released by Bruker Daltonics for the identification of bacteria and fungi. The newly expanded in-house helminth database was subjected to two different validation procedures. First, to an internal validation procedure, in which all raw spectra of *Schistosoma* spp. obtained during the MSP process were tested to verify whether it was possible to identify them from existing spectra in the database. Second, to an external validation procedure, where spectra from the 58 remaining, independent, adult *Schistosoma* specimens were investigated to assess whether they could be reliably identified from the database. For this purpose, spectra were examined using a combination of the official BDAL database (Bruker Daltonics) and our in-house helminth database. The reliability of the identification was interpreted by log score values (LSVs) generated for each identification result. We used the scoring system recommended by the manufacturer for bacteria identification (i.e., an LSVs of 1.70 was considered the threshold for reliable identification; LSVs between 1.70 and 1.99 indicated reliable identification at the genus level, and LSVs equal to or higher than 2.0 were interpreted as indicating reliable species identification) [10].

Classification and comparisons analysis

Pre-processing parameters

A total of 1657 edited spectra were exported into the free online software Clover MS Data Analysis (https://platform.clovermsdataanalysis.com/, Clover BioSoft, Granada, Spain) (last accessed May 2022) for further investigation. Default parameters were used during pre-processing [18]. A Savitzky–Golay filter (window length, 11; order 3 polynomial) was applied to smooth the spectra, and the baseline was removed using the top-hat filter method (factor 0.02). To obtain one average spectrum per sample for use in the classification and comparisons analysis, replicated spectra were aligned using the following parameters: allowed shift, medium; constant tolerance, 0.2 Da; linear tolerance, 2000 p.p.m.

Classification using ML algorithms

Peak matching was performed to generate a peak matrix from pre-processed spectra that were used for comparison analysis. Total ion current normalization was applied, followed by a threshold method (factor 0.01), where peaks with an intensity below 1% of the maximum intensity were not considered; the constant tolerance was 0.5 Da and the linear tolerance 500 p.p.m. [18].

Classification analysis was carried out at two levels. First, at the interspecies level, where all isolates were investigated to distinguish *S. mansoni* from *S. japonicum.* Second, at the intraspecies level, where samples of the same species were compared to assess the discrimination related to the effect of the storage solutions [70% (*v*/*v*) ethanol, and RNA*later*]. Unsupervised [principal component analysis (PCA), hierarchical clustering], and supervised ML algorithms were used to assess the classification. A PCA is a dimensionality reduction algorithm (it reduces a high-dimensional dataset to a set of coordinates to allow for better visualization of different clusters and relationships among specimens for the identification of subgroups) and provides information about the "true"

nature of a dataset [19]. The hierarchical clustering was performed using the Chebyshev method for distance calculation and the complete method for the metric. For the supervised ML methods, four widely used algorithms for MALDI-TOF mass spectra analysis [linear support vector machine (SVM), partial least squares-discriminant analysis (PLS-DA), Random Forest (RF), and *k*-nearest neighbors (KNN)] were evaluated [20]. The *k*-fold cross-validation method ($k=10$) was used for the internal validation. A confusion matrix (generating values such as accuracy, specificity, sensitivity, F1 score, positive prediction value or precision, and negative prediction value), as well as the area under the receiver operating characteristic curve, and the area under the precision recall curve, were used as performance metrics of the supervised ML algorithms.

Results

Molecular analysis

The morphological identification of *S. mansoni* and *S. japonicum* was confirmed from the sequences obtained from the four isolates. Partial sequences of the *COX1* gene were deposited in the GenBank database with the consecutive accession IDs LC733206–LC733209. The phylogenetic tree based on the *COX1* sequences revealed two main groups corresponding to the two species. Bootstrap values obtained for both clades (100%) indicated high similarities with the sequences recovered from GenBank (Fig. 1).

Spectra analysis and purity control

Spectra with high intensities were generated for *Schistosoma* samples stored in ethanol, as well for those stored in RNA*later*. The spectra profiles obtained by MALDI-TOF MS of different samples of *S. mansoni* and *S. japonicum* were unique for each of the two species. However, a few differences related to the preservation media were also observed (Fig. 2). None of the spectra matched those of bacterial species included in the database, which indicated that none of the samples were contaminated with any of these bacteria (Additional file 1: Table S1).

In-house database validation

All the spectra were identified correctly through the internal validation of our database, i.e., 100% correct identification (Table 1). For the external validation, 58 "new" *Schistosoma* samples were analyzed; correct identification at the genus level (LSVs ≥ 1.7) was achieved for all samples (100%), while 81% (47/58) were correctly identified at the species level (LSVs ≥ 2.0). Of note, more *S. japonicum* (85%) than *S. mansoni* (79%) samples were correctly identified at the species level (Table 1). There was no species mismatch for any of the samples, even within an LSV range of 1.7–2.0.

Interspecies classification

Peak matching analysis using parameters described in the "Classification using ML algorithms" section generated a peak matrix which was used as input for classification. A PCA based on detected peaks showed clear separation between *S. mansoni* and *S. japonicum* (Fig. 3a). Likewise, all the supervised classification algorithms (SVM, RF, PLS-DA, KNN) showed good discrimination between *S. mansoni* and *S. japonicum* (Fig. 3b). The tenfold cross-validation results indicated accuracy values ranging from 96.8 to 100%, and F1 scores (the harmonic mean of precision and sensitivity) between 95.5% and 100% (Table 2), and thus indicated a species-specific spectra profile. However, the RF algorithm showed better results, with no misclassification, i.e., an accuracy of 100% and an F1 score of 100%. Moreover, area under receiver operating characteristic curve values (0.955 for SVM, 0.995 for PLS-DA, 0.995 for RF, and 0.983 for KNN) and area under the precision-recall curve values (0.98 for SVM, 1.00 for PLS-DA, 1.00 for RF, and 0.97 for KNN) confirmed these observations (Additional file 2: Fig. S1).

Intraspecies classification

To further investigate the intraspecies variability of the spectra based on the different storage solutions, the dataset was split into two subsets: one subset with all isolates of *S. mansoni*, and one with *S. japonicum* isolates. Peak matrices generated for each dataset were then used as input for intraspecies classification. For both species, the PCA revealed clear discrimination between isolates stored in 70% (*v*/*v*) and those stored in RNA*later* (Fig. 4). In addition, the dendrogram analysis showed similar results, with distinct groups and with a clear separation between the two species. Moreover, we also observed distinct groups where samples kept in the same storage medium clustered together, except for one sample (*S. mansoni* female 3, in ethanol) that was misclassified (Fig. 5). These observations were confirmed by all the supervised classification algorithms tested, i.e., SVM, PLS-DA, RF, and KNN (Fig. 6a, b). The *k*-fold cross-validation of both datasets showed an accuracy of 100% and an F1 score of 100% for all the tested algorithms, except for SVM tested with the *S. mansoni* dataset (accuracy 97.5%, F1 score 97.6%) (Table 2).

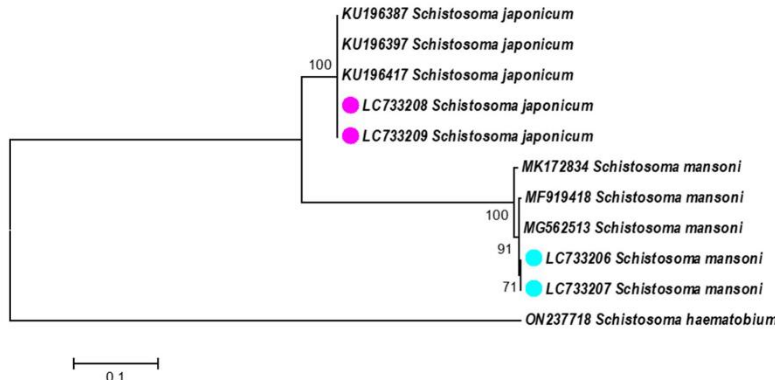

Fig. 1 Phylogenetic tree based on cytochrome oxidase 1 gene (*COX1*) sequences by the maximum likelihood method. The values on the nodes represent bootstrap values. The colored circles (light blue for *Schistosoma mansoni*, violet for *Schistosoma japonicum*) indicate isolates analyzed in this study

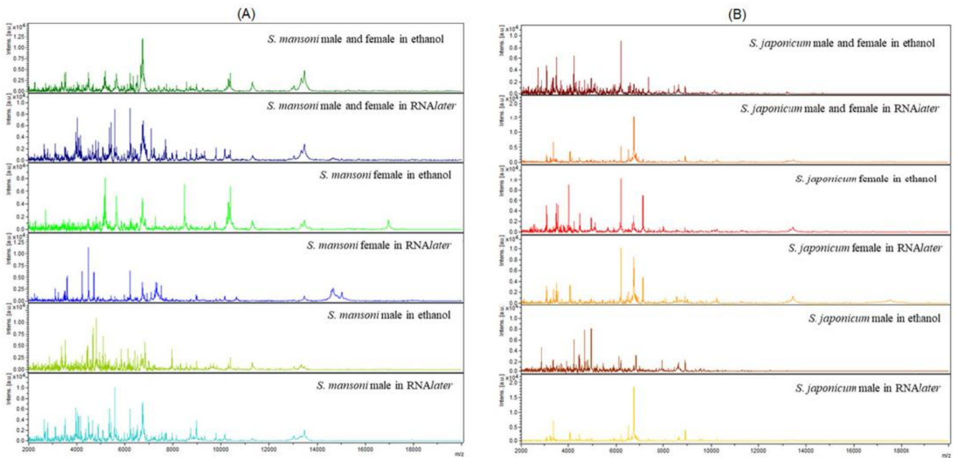

Fig. 2 Representative matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) spectra profiles of adult **a** *Schistosoma mansoni* and **b** *Schistosoma japonicum* stored in 70% (*v*/*v*) ethanol and RNA*later*. *x*-axis Mass-to-charge (m/z) ratio; *y*-axis intensity values in arbitrary units

Discussion

Species-specific spectra generated by MALDI-TOF MS allowed for the identification and specific differentiation of adult *Schistosoma* worms. Indeed, all the isolates were correctly identified, and an LSV of ≥ 2, which is the reference for species-specific identification for bacteria, was achieved for 81% of all the isolates. Of note, no misidentification between the two species was observed. Hence, these results confirm previous reports on the applicability of MALDI-TOF MS for the identification of trematode species [10, 21]. The application of MALDI-TOF MS for parasite identification has attracted considerable attention, and the technique has been employed for other helminth species such as nematodes, i.e., *Trichuris* spp. [22] and *Anisakis* spp. [23], and the cestode *Taenia saginata* [17].

Table 1 Internal and external validation of the identification of *Schistosoma* specimens stored in different media [70% (*v/v*) ethanol, RNA*later*], using a newly expanded in-house database, implemented in MBT Compass Explorer software (Bruker Daltonics, Bremen, Germany)

Internal validation

Species	Preservation media	Number of samples	Identification results		
			Number of spectra	LSV ≥ 1.7	LSV ≥ 2.0
				Mixed adults (males/females)	Mixed adults (males/females)
Schistosoma mansoni	Ethanol	1	31	31/31 (100%)	31/31 (100%)
	RNA*later*	1	27	27/27 (100%)	27/27 (100%)
Schistosoma japonicum	Ethanol	1	29	29/29 (100%)	29/29 (100%)
	RNA*later*	1	32	32/32 (100%)	32/32 (100%)
	Total	4	119	119/119 (100%)	119/119 (100%)

External validation

Species	Preservation media	Number of samples	Identification results							
			LSV ≥ 1.7				LSV ≥ 2.0			
			Mixed adults (males/females)	Females	Males	% Correct	Mixed adults (males/females)	Females	Males	% Correct
S. mansoni	Ethanol	18	6/6 (100%)	6/6 (100%)	6/6 (100%)	18/18 (100%)	6/6 (100%)	5/6 (83.3%)	6/6 (100%)	11/18 (61.1%)
	RNA*later*	20	10/10 (100%)	5/5 (100%)	5/5 (100%)	20/20 (100%)	10/10 (100%)	4/5 (80%)	5/5 (100%)	19/20 (95%)
S. japonicum	Ethanol	10	4/4 (100%)	3/3 (100%)	3/3 (100%)	10/10 (100%)	4/4 (100%)	0/3 (0%)	3/3 (100%)	7/10 (70%)
	RNA*later*	10	4/4 (100%)	3/3 (100%)	3/3 (100%)	10/10 (100%)	4/4 (100%)	3/3 (100%)	3/3 (100%)	10/10 (100%)
Total		58	26/26 (100%)	17/17 (100%)	17/17 (100%)	58/58 (100%)	24/24 (100%)	12/17 (70.6%)	11/17 (64.7%)	47/58 (81%)

LSV Log score value

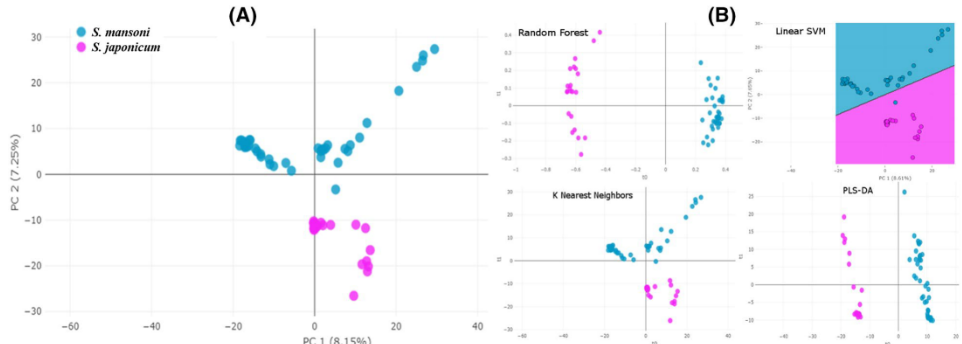

Fig. 3 **a**, **b** Interspecies classification of adult worms of *Schistosoma* species examined using unsupervised and supervised algorithms. **a** Two-dimensional view of a principal component (*PC*) analysis (PCA) based on a peak matrix generated with a threshold of 1% and total ion current (TIC) normalization (TICp). **b** Two-dimensional view showing the distance plot of four classification algorithms [Random Forest (RF), linear support vector machine (*SVM*), *k*-nearest neighbors (KNN), and partial least square-discriminant analysis (*PLS-DA*)] using Clover MS Data Analysis software

Table 2 Tenfold cross-validation results showing scores (%) obtained with four different classification algorithms [linear support vector machine (*SVM*), partial least squares-discriminant analysis (*PLS-DA*), Random Forest (*RF*), and *k*-nearest neighbors (*KNN*)]

Schistosoma mansoni vs *Schistosoma japonicum*[a]			
Actual group	Predicted group		% Correct
	S. japonicum	*S. mansoni*	
SVM			
S. japonicum	22 (TP)	0 (FN)	100 (Sensitivity)
S. mansoni	2 (FP)	38 (TN)	95 (Specificity)
	91.7% (PPV)	100% (NPV)	96.8 (Accuracy)
			95.7 (F1 score)
PLS-DA			
S. japonicum	21 (TP)	1 (FN)	95.5 (Sensitivity)
S. mansoni	1 (FP)	39 (TN)	97.5 (Specificity)
	95.5% (PPV)	97.5% (NPV)	96.8 (Accuracy)
			95.5 (F1 score)
RF			
S. japonicum	22 (TP)	0 (FN)	100 (Sensitivity)
S. mansoni	0 (FP)	40 (TN)	100 (Specificity)
	100% (PPV)	100% (NPV)	100 (Accuracy)
			100 (F1 score)
KNN			
S. japonicum	21 (TP)	1 (FN)	95.5 (Sensitivity)
S. mansoni	1 (FP)	39 (TN)	97.5 (Specificity)
	95.5% (PPV)	97.5% (NPV)	96.8 (Accuracy)
			95.5 (F1 score)
S. mansoni (ethanol vs RNA*later*)[b]			
Actual group	Predicted group		% Correct
	RNA*later*	Ethanol	
SVM			
RNA*later*	20 (TP)	1 (FN)	95.24 (Sensitivity)
Ethanol	0 (FP)	19 (TN)	100 (Specificity)
	100% (PPV)	95% (NPV)	97.5 (Accuracy)
			97.6 (F1 score)
PLS-DA			
RNA*later*	21 (TP)	0 (FN)	100 (Sensitivity)
Ethanol	0 (FP)	19 (TN)	100 (Specificity)
	100% (PPV)	100% (NPV)	100 (Accuracy)
			100 (F1 score)
RF			
RNA*later*	21 (TP)	0 (FN)	100 (Sensitivity)
Ethanol	0 (FP)	19 (TN)	100 (Specificity)
	100% (PPV)	100% (NPV)	100 (Accuracy)
			100 (F1 score)
KNN			
RNA*later*	21 (TP)	0 (FN)	100 (Sensitivity)
Ethanol	0 (FP)	19 (TN)	100 (Specificity)
	100% (PPV)	100% (NPV)	100 (Accuracy)
			100 (F1 score)

Table 2 (continued)

S. japonicum (ethanol vs RNA*later*)[c]			
Actual group	Predicted group		% Correct
	RNA*later*	Ethanol	
SVM			
RNA*later*	11 (TP)	0 (FN)	100 (Sensitivity)
Ethanol	0 (FP)	11 (TN)	100 (Specificity)
	100% (PPV)	100% (NPV)	100 (Accuracy)
			100 (F1 score)
PLS-DA			
RNA*later*	11 (TP)	0 (FN)	100 (Sensitivity)
Ethanol	0 (FP)	11 (TN)	100 (Specificity)
	100% (PPV)	100% (NPV)	100 (Accuracy)
			100 (F1 score)
RF			
RNA*later*	11 (TP)	0 (FN)	100 (Sensitivity)
Ethanol	0 (FP)	11 (TN)	100 (Specificity)
	100% (PPV)	100% (NPV)	100 (Accuracy)
			100 (F1 score)
KNN			
RNA*later*	11 (TP)	0 (FN)	100 (Sensitivity)
Ethanol	0 (FP)	11 (TN)	100 (Specificity)
	100% (PPV)	100% (NPV)	100 (Accuracy)
			100 (F1 score)

TP True positive, *TN* true negative, *FP* false positive, *FN* false negative, *PPV* positive predictive values, *NPV* negative predictive values

[a] Discrimination of adult *S. japonicum* from adult *S. mansoni*; *S. japonicum* considered a positive category

[b] Discrimination of *S. mansoni* isolates based on the effect of the storage medium; RNA*later* considered a positive category

[c] Discrimination of *S. japonicum* isolates based on the effect of the storage medium; RNA*later* considered a positive category

We were able to confirm the robustness of this approach for the reliable differentiation of adult *S. mansoni* from adult *S. japonicum* through classification of the protein spectra using unsupervised (PCA) and supervised (SVM, PLS-DA, RF, and KNN) ML algorithms. The successful application of ML algorithm classification has been repeatedly demonstrated for microbes [19], e.g., for the subtyping of bacterial species [24], and for the detection of antimicrobial resistance [18, 25]. More recently, studies have also been published on the application of ML algorithms for parasite identification. Kalafi et al. [26] reported the automated identification of monogeneans (flatworms present on the gills and skin of fish), with an overall classification accuracy of 90% using KNN, and an accuracy of 91.25% using the leave-one-out cross-validation method. An ML-based study that used nucleotide sequence analysis for the taxonomic evaluation of *Strongyloides fuelleborni* and *Strongyloides stercoralis* also indicated the utility, and another possible application, of this approach [27].

An effect of storage solution on spectra profiles was observed in the present study. Indeed, differences between the spectra profiles of samples stored in ethanol or RNA*later* were noted during a preliminary visual inspection. Furthermore, both unsupervised and supervised algorithms showed that isolates of the same species clustered differently according to the storage solution used. Of note, PCA, SVM, and KNN highlighted sub-clustering based on sex (or type) for samples stored in ethanol, with three subgroups corresponding to males, females, and mixed (male, and female) samples, respectively.

In applied and diagnostic parasitology, a host of different protocols for sample collection and storage are commonly employed. Alcohol or formalin are most frequently utilized as storage solutions, especially for long-term storage outside of a fridge or freezer [8]. Most published studies on the identification of parasites via MALDI-TOF MS used either 70% (*v*/*v*) ethanol [10, 21, 28] or sodium chloride [29] for sample preservation. Wendel et al. [17] recently evaluated the effect of four different storage media [ethanol 70% (*v*/*v*), sodium chloride 0.45% (v/), LC–MS grade water, and formalin 37% (*v*/*v*)] on the identification of *T. saginata* proglottids,

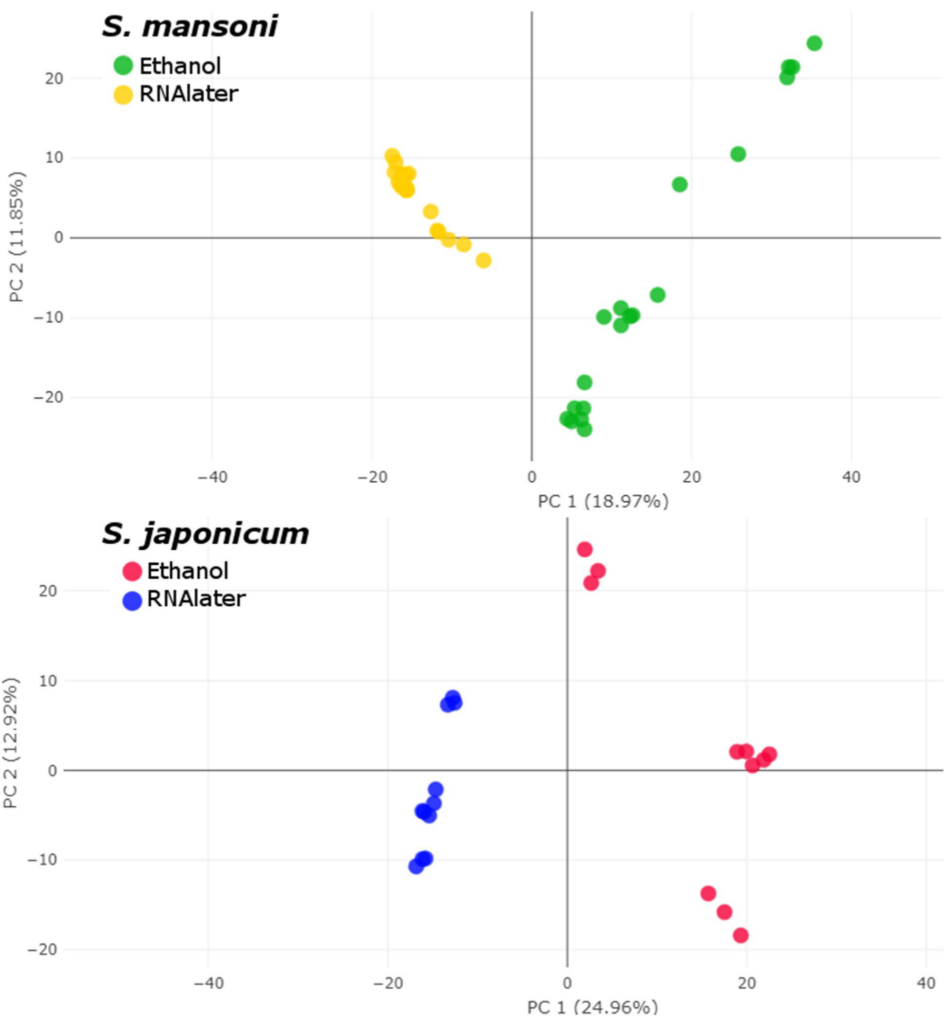

Fig. 4 Intraspecies classification of adult worms of *Schistosoma* species by an unsupervised algorithm. Two-dimensional view of a PCA based on a peak matrix generated with a threshold of 1% and TICp. For abbreviations, see Fig. 3

and reported similar results in terms of spectra profiles and identification score values for all media for up to 24 weeks of storage, except for formalin 37% (*v*/*v*), for which no spectrum was obtained [17]. Mayer-Scholl et al. [30] evaluated the impact of different conservation conditions (sample freezing, preservation in ethanol 70%) and reported only minor, non-significant, variations in the generated spectra, which did not significantly affect identification. However, in the present study we observed different peaks and different peak intensities depending on the storage solution used. ML algorithms hold promise for the differentiation of samples stored in these different storage media. Furthermore, the accuracy of identification for new, independent samples using the helminth

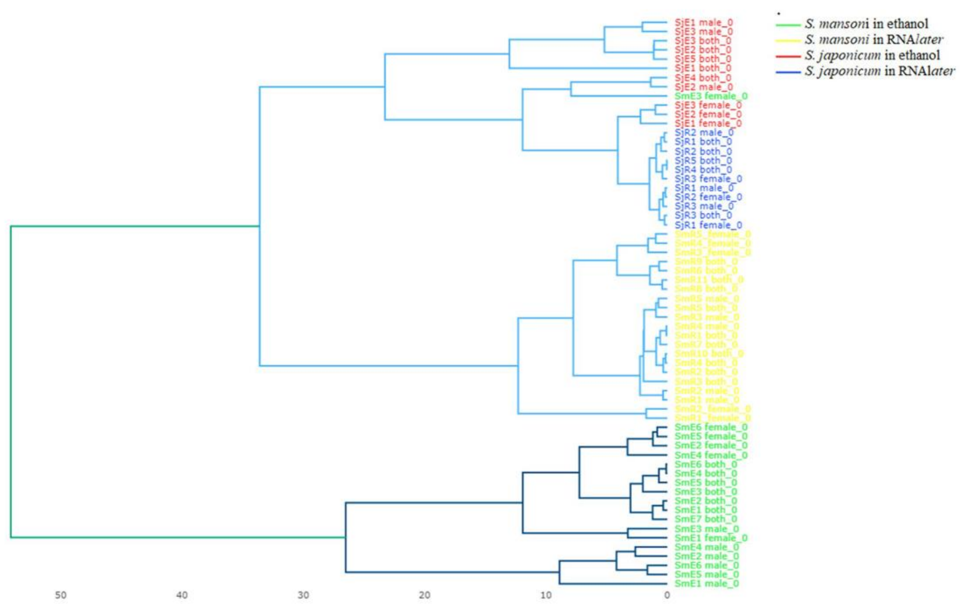

Fig. 5 Dendrogram analysis. Hierarchical clustering based on spectral data showing the relatedness of adult *Schistosoma mansoni* and *Schistosoma japonicum* stored in RNA*later* and in ethanol

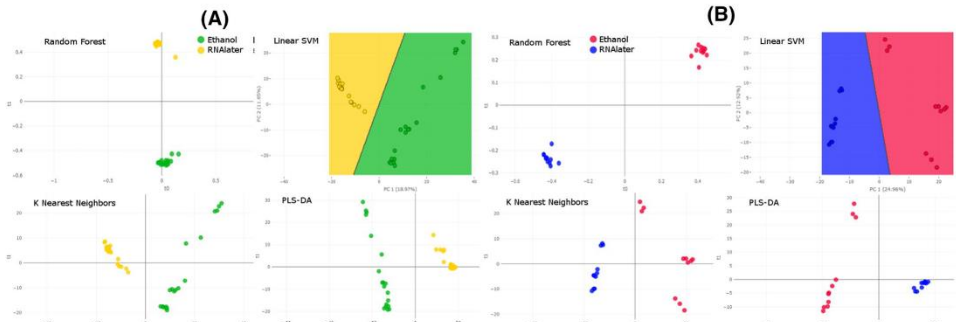

Fig. 6 **a**, **b** Intraspecies classification of adult *Schistosoma* by supervised algorithms. Two-dimensional view of the results of four classification algorithms (RF, linear SVM, KNN, and PLS-DA) based on a peak matrix generated with a threshold of 1% and TICp, using Clover MS Data Analysis software. **a** Classification of *Schistosoma mansoni* isolates stored in 70% ethanol versus those stored in RNA*later*. **b** Classification of *Schistosoma japonicum* isolates in 70% ethanol versus those stored in RNA*later*. For abbreviations, see Fig. 3

database was better for samples stored in RNA*later* (95% and 100% correct identification at the species level for *S. mansoni* and S. *japonicum*, respectively) than for samples stored in ethanol (correct species identification of 61% for *S. mansoni* and 70% and *S. japonicum*). These results may indicate that preservation in RNA*late*r offers better stability and better integrity of the proteins (Table 2, External validation). Saito et al. [31] demonstrated that

RNA*later* was more effective than ethanol in preventing the degradation of proteins from *Synechococcus* WH8102 (a marine cyanobacterium), which were preserved in a total of five different solutions.

This study had several limitations. First, the number of samples was relatively small and originated from animals—experimentally infected mice—kept in just a few different settings. Indeed, before this method is used in laboratories that carry out routine diagnostics, additional research is warranted to validate our approach with a larger number of samples stemming from different locations and various hosts. Second, another limitation was that we did not examine samples of the parasites at different development stages, i.e., we only investigated adult worms, not eggs or larvae. As infected human beings expel eggs through their feces or urine, future studies should try to apply a similar approach to the one used here for eggs originating from stool specimens (in the case of *S. mansoni* and *S. japonicum*) and/or urine samples (in the case of *S. haematobium*). Third, due to a lack of new, independent samples, not all of the ML models used for classification could be subjected to external validation. External validation (a blind test) using new samples is desirable to further validate the performance of the ML models.

Conclusions

To our knowledge, this is the first study to employ MALDI-TOF MS for the identification of adult *Schistosoma* worms. Our results provide evidence that MALDI-TOF MS is an efficient, rapid, and promising tool for the reliable identification and differentiation of adult *Schistosoma* worms. The creation, and validation, of species-specific reference spectra is necessary in the absence of a commercially available database for parasite identification. Thus, ML-based classification algorithms could also be used as predictive models for parasite species discrimination, as well as for the detection of possible variations in spectra profiles caused by the storage media employed.

Abbreviations

COX1	Cytochrome oxidase 1 gene
KNN	*k*-nearest neighbor
LC–MS	Liquid chromatography-mass spectrometry
LSV	Log score value
MALDI-TOF MS	Matrix-assisted laser desorption ionization time-of-flight mass spectrometry
ML	Machine learning
MSP	Main spectra profile
PCA	Principal component analysis
PLS-DA	Partial least square-discriminant analysis
RF	Random Forest
SVM	Support vector machine

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13071-022-05604-0.

Additional file 1: Table S1. MALDI identification using the commercial database released by Bruker Daltonics for bacterial identification to check the purity of the spectra and possible contamination with spectra from bacteria.

Additional file 2: Figure S1. Receiver operating characteristic (ROC) and precision-recall (PR) curves and their related areas under the curves [area under receiver operating characteristic curve (AUROC) and area under the precision-recall curve (AUPR)] for *Schistosoma* species classification using supervised machine learning (ML) algorithms. **a** Support vector machine, **b** partial least square–discriminant analysis (PLS-DA), **c** Random Forest (RF), and **d** *k*-nearest neighbor (KNN).

Acknowledgements
We would like to thank Prof. Masashi Kirinoki, Dokkyo Medical University, for the maintenance of *S. mansoni* and *S. japonicum* in the laboratory. We thank Jesús Jiménez for his valuable help in using the Clover BioSoft software.

Author contributions
JCE, SLB, and IS designed the study. MOS and MPA provided the samples and performed the morphological and molecular identification. JCE and IS performed the MALDI-TOF MS analysis. JCE and IS wrote the original draft. All authors read and approved the final manuscript.

Funding
Open Access funding enabled and organized by Projekt DEAL. We acknowledge support by the Deutsche Forschungsgemeinschaft (German Research Foundation) and Saarland University within the funding program Open Access Publishing.

Availability of data and materials
The data presented in this study are available on reasonable request from the corresponding author.

Declarations

Ethics approval and consent to participate
Approved by the Ethics Committee for Animal Experimentation of Dokkyo Medical University under number 1307.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

Received: 23 September 2022 Accepted: 30 November 2022
Published online: 19 January 2023

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5.3. **Article n° 3. Research article:** Evaluating different storage media for identification of *Taenia saginata* proglottids using MALDI-TOF mass spectrometry

Wendel TP, Feucherolles M, Rehner J, Poppert S, Utzinger J, Becker SL, **Sy I**. Evaluating Different Storage Media for Identification of *Taenia saginata* Proglottids Using MALDI-TOF Mass Spectrometry. *Microorganisms*. 2021; 9(10):2006. https://doi.org/10.3390/microorganisms9102006

Article

Evaluating Different Storage Media for Identification of *Taenia saginata* Proglottids Using MALDI-TOF Mass Spectrometry

Tabea P. Wendel [1], Maureen Feucherolles [2], Jacqueline Rehner [1], Sven Poppert [3,4], Jürg Utzinger [3,4], Sören L. Becker [1,3,4,*] and Issa Sy [1]

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Abstract: *Taenia saginata* is a helminth that can cause taeniasis in humans and cysticercosis in cattle. A species-specific diagnosis and differentiation from related species (e.g., *Taenia solium*) is crucial for individual patient management and disease control programs. Diagnostic stool microscopy is limited by low sensitivity and does not allow discrimination between *T. saginata* and *T. solium*. Molecular diagnostic approaches are not routinely available outside research laboratories. Recently, matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) was proposed as a potentially suitable technique for species-specific helminth diagnosis. However, standardized protocols and commercial databases for parasite identification are currently unavailable, and pre-analytical factors have not yet been assessed. The purpose of this study was to employ MALDI-TOF MS for the identification of *T. saginata* proglottids obtained from a human patient, and to assess the effects of different sample storage media on the technique's diagnostic accuracy. We generated *T. saginata*-specific main spectral profiles and added them to an in-house database for MALDI-TOF MS-based diagnosis of different helminths. Based on protein spectra, *T. saginata* proglottids could be successfully differentiated from other helminths, as well as bacteria and fungi. Additionally, we analyzed *T. saginata* proglottids stored in (i) LC–MS grade water; (ii) 0.45% sodium chloride; (iii) 70% ethanol; and (iv) 37% formalin after 2, 4, 6, 8, 12, and 24 weeks of storage. MALDI-TOF MS correctly identified 97.2–99.7% of samples stored in water, sodium chloride, and ethanol, with log-score values $\geq$2.5, thus indicating reliable species identification. In contrast, no protein spectra were obtained for samples stored in formalin. We conclude that MALDI-TOF-MS can be successfully employed for the identification of *T. saginata*, and that water, sodium chloride, and ethanol are equally effective storage solutions for prolonged periods of at least 24 weeks.

Keywords: cestodes; diagnosis; helminth infections; matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry; neglected tropical diseases; taeniasis

Citation: Wendel, T.P.; Feucherolles, M.; Rehner, J.; Poppert, S.; Utzinger, J.; Becker, S.L.; Sy, I. Evaluating Different Storage Media for Identification of *Taenia saginata* Proglottids Using MALDI-TOF Mass Spectrometry. *Microorganisms* **2021**, *9*, 2006. https://doi.org/10.3390/microorganisms9102006

Academic Editor: Adriana Calderaro

Received: 8 August 2021
Accepted: 14 September 2021
Published: 22 September 2021

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1. Introduction

The beef tapeworm, *Taenia saginata*, is a zoonotic cestode that can cause taeniasis, an intestinal infection in humans, and cysticercosis in bovines [1]. It is the most common and most widely distributed *Taenia* species. While humans are the definitive host, cattle serve as intermediate hosts for *T. saginata*. *Taenia solium* and *Taenia asiatica* are less frequently occurring species, with *T. solium* being of particular clinical relevance, as it gives rise to intestinal disease and the potentially fatal human (neuro-)cysticercosis [2]. Humans acquire intestinal *Taenia* infection through the consumption of raw or undercooked meat of infected animals. Intestinal taeniasis mainly causes mild and unspecific symptoms, such as weight

loss and general malaise. More pronounced symptoms (e.g., diarrhea, abdominal pain, and nausea) are less frequent [3]. Severe complications, such as appendicitis or gall bladder perforation, have rarely been reported [4].

After the ingestion of infected bovine muscle tissue, a *Taenia* cysticercus develops within the human host's intestine into an adult worm during a prepatency period of approximately 2 months, and produces eggs and gravid proglottids, which are shed with the feces. In settings with poor sanitation, eggs can spread through water, wind, or simply attach to vegetation. Cattle become infected by ingesting contaminated plants [5].

Taeniasis is considered a neglected tropical disease (NTD) [6]. In recent years, several studies carried out by the European CystiNet network and others investigated the global occurrence of taeniasis. It was found that *Taenia* tapeworms occur worldwide, and that *T. saginata* is particularly frequent in East, Southeast, and South Asia [7]. In Europe, taeniasis cases are reported in 12 out of 18 surveyed countries, with an estimated prevalence ranging from 0.02 to 0.67% [1]. As taeniasis is associated with poor sanitation, low-income settings, and understaffed meat inspectorates, the disease is also frequently reported from parts of the Middle East, Africa [8], and Central and South America [9]. However, prevalence estimates lack accuracy, as taeniasis is a non-notifiable disease in most countries, and as for many NTDs, public health campaigns pay little attention to this disease [10]. In 2007, it was estimated that at least 60 million people were infected with *T. saginata* [11]. However, the global burden of taeniasis, as expressed in disability-adjusted life years (DALYs), has yet to be determined [12].

The diagnosis of human taeniasis mainly relies on the direct visualization of proglottids, or the microscopic detection of eggs in stool samples [4]. In research settings, other methods are also used, such as stool-based polymerase chain reaction (PCR) assays or copro-antigen enzyme-linked immunosorbent assay (ELISA) tests, which detect specific secretory antigens in fecal samples [3]. However, these techniques have several limitations. While the commonly employed microscopy can be rapidly performed and does not require well-equipped laboratories, its sensitivity is low [4], and a species differentiation between *T. saginata* and *T. solium* is only possible if proglottids are shed in the feces, because the eggs of both species are indistinguishable [13]. The copro-antigen ELISA is characterized by a relatively low specificity, as studies carried out on samples stemming from cattle reported relatively high rates of cross-reactivity with related species of veterinary importance, such as *Taenia hydatigena* and *Taenia multiceps* [14]. PCR-based assays allow highly sensitive species identification, but are costly, rarely available outside research laboratories, and require specific technical expertise. Hence, there is a need for simple-to-use, accurate diagnostic methods for taeniasis, as the correct identification of *Taenia* infections at the species level is an important requirement for clinical management and contact screening, particularly in case of *T. solium* infections that pose the risk of human neurocysticercosis [15].

Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) is an extensively validated diagnostic technique, which is nowadays routinely used in clinical microbiology laboratories for the species-specific diagnosis of bacteria and fungi in high-income countries [16]. Recently, several studies also reported MALDI-TOF MS, which analyzes pathogen-specific protein spectra to reach a specific diagnosis, as a suitable method for the identification of parasites [13], including helminths (e.g., *Fasciola* spp. [17], *Trichinella* spp. [18], and *Anisakis* spp. [19]). Besides high accuracy, the low cost of reagents needed for MALDI-TOF analysis in comparison to reagents required for PCR assays is a competitive advantage. However, there is uncertainty regarding the standardization of MALDI-TOF analytical protocols, and the effects of pre-analytical factors need to be elucidated. In this study, we utilized *T. saginata* proglottids to systematically assess whether the use of different sample storage media or the duration of storage affect the composition of the resulting protein spectra, and hence, the ability of MALDI-TOF MS to reach species-specific identification.

2. Materials and Methods

2.1. Ethics Statement

The *T. saginata* sample used in this study was obtained from an infected patient who sought routine diagnostic work-up for suspected parasite infection at the Swiss Tropical and Public Health Institute (Swiss TPH) in Basel, Switzerland. All procedures adhered to local laws and regulations.

2.2. Sample Collection

T. saginata proglottids were collected by an experienced medical laboratory technician from the stool sample of an infected patient at Swiss TPH in Basel. The specimen was stored in a freezer at −20 °C in 0.45% (v/v) sodium chloride solution. In October 2018, the sample was transferred to the Institute of Medical Microbiology and Hygiene in Homburg, Germany for further examination.

2.3. Study Design and Experimental Set-Up

Upon receipt at the Institute of Medical Microbiology and Hygiene in Homburg, the *Taenia* specimen was subjected to nucleic acid extraction, PCR, and partial sequencing for species-specific identification as *T. saginata*. Next, MALDI-TOF MS was carried out to generate protein spectral profiles, which were then transferred into an in-house database for MS-based identification of helminths. Subsequently, proglottids were put into different storage media and re-analyzed by MALDI-TOF MS after 2, 4, 6, 8, 12, and 24 weeks. At each time, the obtained spectra were compared to the initially measured spectra.

2.4. Molecular Diagnosis Using PCR and Partial Sequencing

For confirmatory molecular species identification, one proglottid of the *Taenia* specimen was thawed and subjected to DNA extraction using the DNeasy Blood and Tissue Kit (Qiagen GmbH; Hilden, Germany). In brief, a sample measuring approximately 1 cm was pounded into small pieces. Next, 180 µL of ATL buffer was added, the sample was vortexed, and 20 µL of proteinase K was added. The mix was vortexed and incubated at 56 °C in a thermomixer (Eppendorf; Hamburg, Germany) for 1 h. After incubation, the mix was vortexed again, and both 200 µL of AL buffer and 200 µL of 100% (v/v) ethanol were added. Subsequently, the DNeasy Mini column system (Qiagen; Hilden, Germany) was used for nucleic acid extraction, adhering to the manufacturer's protocol.

For gene amplification, the partial mitochondrial cytochrome oxidase 1 gene (COX-1) was used to perform a PCR as previously described [20]. Specific forward (5′-CATCATATGTTTACGGTTGG-3′) and reverse (5′-GACCCTAATGACATAACATAAT-3′) primers were used to amplify a gene of around 350 base pairs (bp), utilizing a peqSTAR thermocycler (VWR; Radnor, PA, USA). In brief, the assay consists of 12.5 µL Hotstart Mix (Qiagen; Hilden, Germany), 0.5 µL of forward primer, 0.5 µL of reverse primer, 9.5 µL of water, and 2 µL of *Taenia* DNA. The cycling conditions comprised an initial denaturation step at 95 °C for 5 min, followed by 56 °C for 1 min, and 72 °C for 2 min. Then, 45 amplification cycles were performed, each consisting of a denaturation step at 95 °C for 30 s, annealing at 56 °C for 30 s, and elongation at 72 °C for 30 s. Afterwards, a final elongation step at 72 °C for 4 min was performed.

For sequencing of the generated amplicons, the Capillary Electophoretic GenomeLab genetic analysis system (Beckman Coulter; Brea, CA, USA) was used. Consensus sequences were created by editing and merging raw forward and reverse sequences, using the BioEdit© software version 7.2.5 (Tom Hall; Carlsbad, CA, USA). The consensus sequence was aligned with sequences deposited in the National Center for Biotechnology Information (NCBI) GenBank database for final identification.

2.5. Differential Sample Storage Conditions

Taenia proglottids were removed from the original storage medium (sodium chloride 0.45% (v/v)) and placed on a Petri dish. Using a sterile scalpel, individual proglottids were

cut into small pieces of approximately 1 cm. Next, each specimen was placed into a 1.5 mL Eppendorf tube, and 1 mL of one of the following four different storage solutions was added: (i) sodium chloride 0.45% (*v*/*v*) (Merck KG; Darmstadt, Germany); (ii) ethanol 70% (*v*/*v*) (Merck KG); (iii) liquid chromatography (LC) MS grade water (Merck KG); and (iv) formalin 37% (*v*/*v*) (Merck KG). All samples were then stored at −20 °C in these media, before being consecutively subjected to MALDI-TOF MS after the aforementioned exposure periods. The experiment was carried out with 6 specimens for each storage medium, i.e., 24 proglottids in total.

2.6. MALDI-TOF Analysis

2.6.1. Protein Extraction

Prior to analysis, each proglottid sample was thawed and cut into three equal parts. Each part was then transferred to a new tube for subsequent MALDI-TOF MS measurements. For protein extraction, we employed a previously developed protocol [17].

2.6.2. MALDI-TOF Target Plate Preparation and Measurements

Using the protein extract, 1 µL of the supernatant was spotted onto the MALDI target plate. For each sample, eight specific spots on the target plate were used, as recommended by the manufacturer (MSP creation protocol V1.1; Bruker Daltonics; Bremen, Germany). After drying, 1 µL of α-cyano-4-hydroxycinamic acid (CHCA) matrix solution (Bruker Daltonics), composed of saturated CHCA, 50% (*v*/*v*) of acetonitrile, 2.5% (*v*/*v*) of trifluoracetic, and 47.5% (*v*/*v*) of LC–MS grade water, was added to each spot. A commercially available Bacterial Test Standard (BTS; i.e., *Escherichia coli* extract connected with two high molecular weight proteins) was used to calibrate the mass spectrometer. After drying at room temperature, the MALDI target plate was placed into the Microflex LT Mass Spectrometer (Bruker Daltonics; Bremen, Germany) for MALDI-TOF MS analysis. Each sample spot was measured four times to generate a total of 32 raw spectra (8 spots × 4). This procedure was carried out on two replicates on the same day (repeatability analysis), and on one additional replicate on a subsequent day (reproducibility analysis). Hence, a total of 96 raw spectra were acquired for each sample.

2.6.3. MALDI-TOF MS Parameters

All measurements were performed using the AutoXecute algorithm in the FlexControl® software version 3.4. (Bruker Daltonics; Bremen, Germany). For each spot, 240 laser shots (40 laser shots each using six random positions) were used to generate protein spectral profiles in linear positive ion mode. The laser frequency was 60 Hz, and a high voltage of 20 kV and pulsed ion extraction of 180 ns were employed. The mass charge ratio range (m/z) was measured between 2 and 20 k Da.

2.6.4. Spectral Analysis, MSP Creation, and Clustering Analysis

All raw spectra were analyzed with the FlexAnalysis® software version 3.4 (Bruker Daltonics; Bremen, Germany). To improve the spectral quality, raw spectra were edited by removing all flatlines and outlier peaks. The intensities were smoothened, and baseline subtraction was performed, as appropriate. Peak shifts within spectra were also edited when they exceeded 500 ppm. Following these steps, replicates containing at least 22 remaining spectra were maintained, and the measurement was repeated if these conditions were not reached.

The edited spectra of the initial *Taenia* sample were used to create a species-specific main spectral profile (MSP), utilizing the automated function of the MALDI Biotyper Compass Explorer® software version 4.1 (Bruker Daltonics; Bremen, Germany). The newly created *Taenia* MSP was added to a previously developed in-house database with several species, including cestodes (e.g., *Diphyllobothrium* spp.), nematodes (e.g., *Ascaris* spp.), and trematodes (e.g., *Fasciola* spp.), for helminth identification, and served as a reference spectrum for comparative analysis under different storage conditions. Subsequently, a

clustering analysis was performed on the edited spectra obtained after 2, 12, and 24 weeks using the BioNumerics® software version 7.6 (Applied Maths N.V.; Sint-Martens-Latem, Belgium). A dendrogram was generated using an unweighted pair group method with the arithmetic mean (UPGMA), and a curve-based similarity matrix was calculated using Pearson correlation. A principal components analysis (PCA) and a discriminant analysis were carried out using quantitative values.

2.6.5. MALDI-TOF Identification Parameters

All measured spectra were initially analyzed using the official Bruker Taxonomy Database designed for bacteria and fungi, containing 8936 MSPs, which is routinely used in clinical microbiology laboratories, to detect possible contamination with bacterial or fungal organisms. Next, protein spectra were analyzed by a combination of this official Bruker database (Bruker Taxonomy) and the previously developed in-house helminth database with around 98 MSPs, including the MSP of the initially analyzed *Taenia* proglottid. The reliability of identification was evaluated by log score values (LSVs), which were generated by MALDI-TOF MS. We followed the LSV thresholds used in routine microbiology for the identification of bacteria and fungi, i.e., LSVs $\leq$1.69, indicating an unreliable identification; LSVs ranging between 1.70 and 1.99, indicating an accurate genus and probable species identification; and LSVs $\geq$2.0, suggesting a reliable species identification.

3. Results

3.1. Molecular Identification of Taenia Proglottids

PCR and sequencing of the initial *Taenia* proglottid sample using primers of the COX1-gene confirmed the species diagnosis. An analysis using NCBI GenBank showed 100% sequence homologies with a previously described *T. saginata* sequence (reference accession number: MT074048.1). The sequence of our *Taenia* sample was deposited in the GenBank database (accession number: MZ720823).

3.2. Comparative MALDI-TOF MS Analysis after Different Storage Periods

3.2.1. Protein Spectra and LSV Analysis

A representative protein spectral profile for each storage medium is displayed in Figure 1. High peak intensities were observed and reached up to 1.0×10^4 arbitrary units (a.u.). With regard to the position and the intensity of the measured peaks, *Taenia* samples stored in LC–MS grade water, ethanol, and sodium chloride showed a similar profile to the original sample, with no significant changes over time. For samples stored in formalin, no protein spectra were found at any time point.

For all samples, the commercially available MALDI-TOF database for the identification of bacteria and fungi did not yield a reliable identification, with an LSV of 1.37 for the bacterium *Arthrobacter monumenti* being the highest score. When submitting the spectra to a combination of the commercially available and in-house helminth databases, a correct identification was achieved in 97.2%, 99.7%, and 99.0%, for samples stored in sodium chloride, ethanol, and LC–MS grade water, respectively, with LSVs ranging between 2.53 and 2.57. No identification was achieved for spectra of *Taenia* proglottids stored in formalin (Table 1).

When analyzing identification patterns over time, a high LSV ($\geq$2.3) was constantly observed at all measurements for each storage solution, except formalin. Small fluctuations of LSVs were found for all storage solutions, with slightly more fluctuation in the sodium chloride medium (Figure 2).

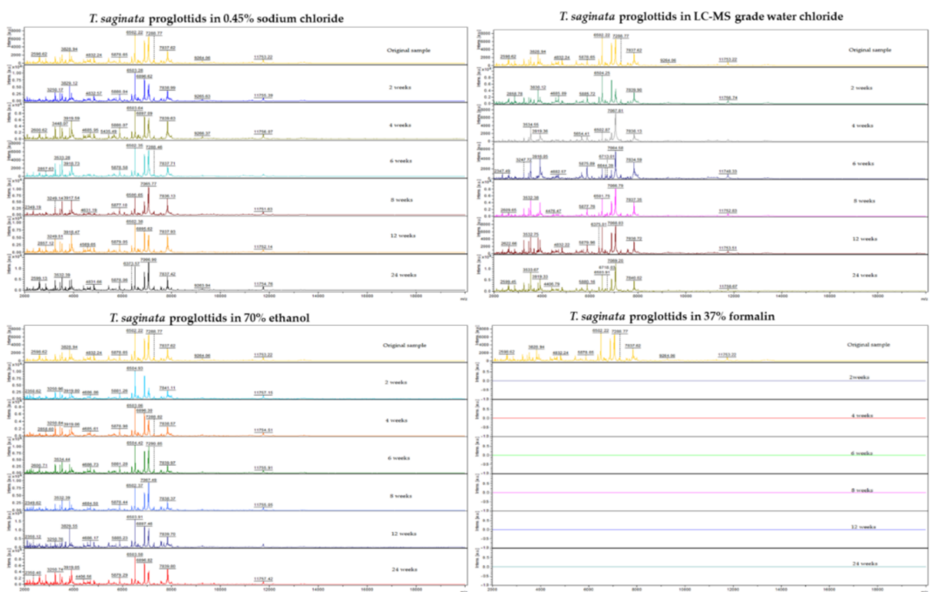

Figure 1. Protein spectral profiles of *Taenia saginata* proglottids. The peaks obtained when measuring the original sample, and protein profiles after prolonged storage in four different media, are displayed. *X*-axis, mass-to-charge ratio of (m/z); *Y*-axis, peak intensities of ionized molecules; a.u., arbitrary unit.

Table 1. Identification of *Taenia saginata* proglottids stored in different storage media (**A**) using Bruker Taxonomy, the commercially available database for bacteria and fungi, and (**B**) using a combination of Bruker Taxonomy and an in-house helminth database.

(**A**)

Sample Preservation Medium	Number of Samples	Number of Spectra	Bruker Taxonomy Database		
			Correct Identification	Average LSV	Most Frequently Suggested Result
0.45% sodium chloride	6	560	0%	1.38	*Arthrobacter monumenti*
70% ethanol	6	574	0%	1.39	*Arthrobacter monumenti*
LC–MS grade water	6	570	0%	1.38	*Arthrobacter monumenti*
37% formalin	6	0	0%	0	None

(**B**)

Sample Preservation Medium	Number of Samples	Number of Spectra	Combination of Bruker Taxonomy and In-House Helminth Database		
			Correct Identification	Average LSV	Most Frequently Suggested Result
0.45% sodium chloride	6	560	97.2% (560/576)	2.54	*T. saginata* proglottid
70% ethanol	6	574	99.7% (574/576)	2.53	*T. saginata* proglottid
LC–MS grade water	6	570	99.0% (570/576)	2.57	*T. saginata* proglottid
37% formalin	6	0	0%	0	-

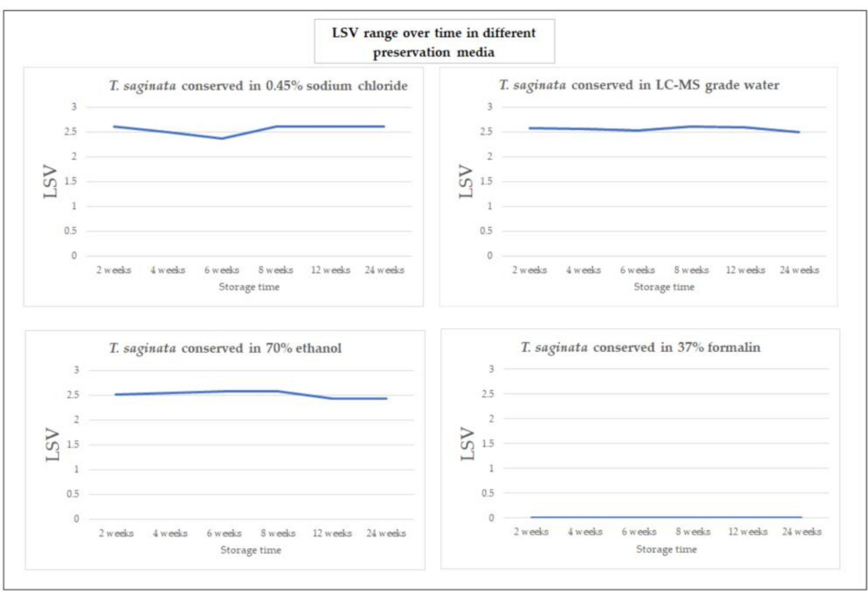

Figure 2. Average LSVs of protein spectra stemming from *Taenia* proglottids in different preservation media during a 24-week observation period. Spectra were identified using a combination of Bruker Taxonomy and an in-house helminth database.

3.2.2. Cluster Analysis

Cluster analysis to display the relatedness of the *Taenia* proglottids stored in sodium chloride, ethanol, and LC–MS grade water showed that all these proglottids clustered together and showed relatedness levels >85% (Figure 3). Subsequent statistical analyses (both PCA and discriminant analysis) performed on the summary spectra of *T. saginata* proglottids did not show specific differences pertaining to the different preservation media or the duration of storage (Figure 4), thus indicating an almost identical pattern of the protein spectra.

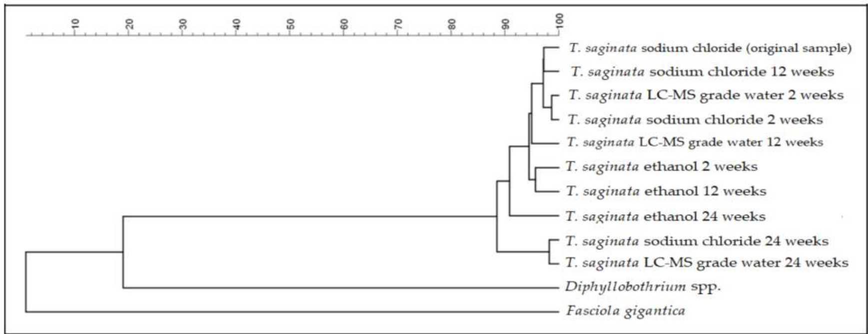

Figure 3. Dendrogram derived from a clustering analysis to assess and compare the different protein spectra of *Taenia saginata* proglottids stored in three storage media for different time periods. The cestode *Diphyllobothrium* spp. and the trematode *Fasciola gigantica* were added as outgroup samples.

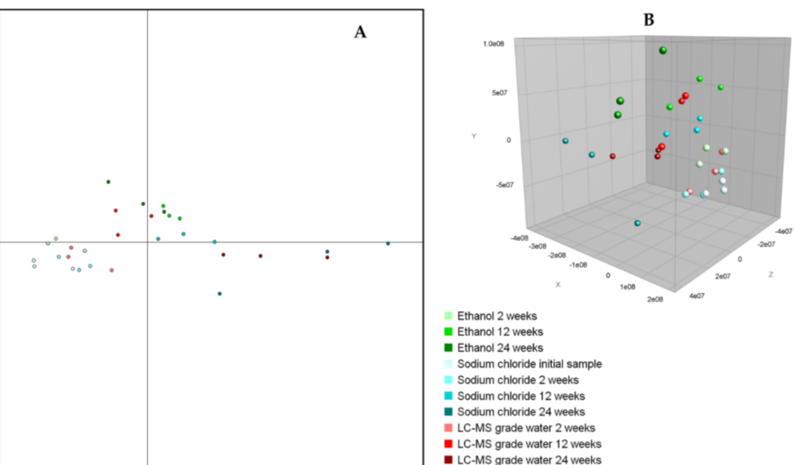

Figure 4. Discriminant analysis and principal components analysis (PCA) of *Taenia saginata* proglottids stored in different preservation media for different time periods. Each storage medium is depicted with a different color. Both statistical analyses indicate that the clusters are highly related and cannot be separated. (**A**) Two-dimensional view of the discriminant analysis. (**B**) Three-dimensional view of the PCA.

4. Discussion

The purpose of this study was to determine whether MALDI-TOF MS can be used as a diagnostic tool for the identification of *T. saginata* proglottids, and whether the use of different storage media may affect the technique's diagnostic accuracy. We found that *T. saginata* can be diagnosed by MALDI-TOF MS, and that its protein spectral analysis allows for reliable differentiation from other helminths, bacteria, and fungi. Indeed, *T. saginata* was consistently identified correctly in ≥97% of cases if LC–MS grade water, ethanol, or 0.45% sodium chloride was used as a storage solution, with no changes over time for storage periods of up to 24 weeks. Notably, preservation in 37% formalin did not allow for subsequent MALDI-TOF MS examinations.

Our findings might have important implications for future helminth diagnosis in epidemiologic studies. Indeed, MALDI-TOF MS is a widely used diagnostic tool in microbiologic routine diagnosis [21,22], which will also be increasingly available in laboratories of low- and middle-income countries [23]. Besides the identification of bacteria and fungi, this technique has also been successfully used for the differentiation of ticks and fleas [24], mosquitos [25], lice [26], and more recently, different helminths of medical and veterinary importance [13]. Hence, MALDI-TOF MS could also be employed for confirmatory testing of helminths in reference laboratories, for example, when no unambiguous identification is reached by conventional methods. However, prolonged transport periods of samples from peripheral healthcare centers to such reference laboratories are likely to be expected, and hence, information on the most appropriate sample storage media is key to ensure a reliable analysis by MALDI-TOF MS. In this context, it is important to note that different protocols were utilized in studies conducted thus far, as there is no consensus on the most suitable storage media. For the identification of *Fasciola* spp. [17], cyathostomins [27], and lice [26], 70% (v/v) ethanol was used as a storage solution, while studies on *Anisakis* spp. [19], *Dirofilaria* spp., and *Ascaris* spp. [28] employed a sodium chloride solution, which was sometimes even supplemented with antibiotics to prevent bacterial contamination. Nebbak et al. [24] analyzed the effects of different storage conditions on the identification of arthropods. The authors concluded that the immediate freezing of samples without the

addition of any fixative might be the best approach, closely followed by storage in 70% (v/v) ethanol at room temperature.

Only a few investigations have assessed the potential effects of different storage conditions on the subsequent MALDI-TOF MS-based identification of helminths. A study focusing on *Trichinella* spp. did not observe significant differences in identification rates when either freezing without any fixative or using 70% ethanol. Indeed, only minor alterations of measured peak intensities were reported, but no change in peak patterns or obtained LSVs [29]. In our study, LC–MS grade water, ethanol, and sodium chloride were equally effective in maintaining a high quality of protein spectra for up to 24 weeks, with correct identification rates ranging from 97.2% for sodium chloride to 99.7% for ethanol at −20 °C. In addition, a statistical analysis of the protein spectra did not reveal fixative-related clusters, thus confirming that all three media can be equally used as storage solutions for *T. saginata* proglottids until MALDI-TOF MS is carried out. Notably, preservation in formalin and subsequent protein extraction using formic acid and acetonitrile impeded any MALDI-TOF-based identification, and hence, should not be employed. This observation is not surprising, as formalin induces considerable molecular cross-linking that may change protein structures [30].

Several limitations restrict the generalizability of our findings. First, the proglottids used in this study were originally stored in sodium chloride for 12 months, before being assigned to the different storage media. Hence, future studies should employ fresh specimens. However, data from a study on suitable buffers for MALDI-based screening of biochemical targets suggest no concerns with regard to the use of sodium chloride [31]. The results obtained in this study may confirm this fact. Second, we only assessed potential effects on *T. saginata*; the in-house database is restricted as it does not contain other *Taenia* species, such as *T. solium*. While it is unlikely that other helminth species would react differently, a broader validation on similar cestodes—most importantly *T. solium*—as well as on nematodes and trematodes is desirable. Specifically, all developmental stages of helminths, including their eggs, should be subjected to MALDI-TOF-based examinations. Third, we compared the effects of different media stored at −20 °C, while future research should also assess the potential effects of storage at different temperatures.

5. Conclusions

We conclude that MALDI-TOF MS is a promising tool for the rapid and accurate identification of *T. saginata* proglottids. Samples can be reliably identified after prolonged storage in LC–MS grade water, sodium chloride solution, and ethanol, while formalin cannot be used as a fixative for later MALDI-TOF MS analysis.

Author Contributions: Conceptualization, T.P.W., M.F., S.L.B., and I.S.; methodology, T.P.W., M.F., S.L.B., and I.S.; contribution of samples, S.P.; investigation and formal analysis, T.P.W., M.F., and I.S.; writing—original draft preparation, T.P.W.; writing—review and editing, T.P.W., M.F., J.R., S.P., J.U., S.L.B., and I.S. All authors have read and agreed to the published version of the manuscript.

Funding: We acknowledge support from the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) and Saarland University within the funding program Open Access Publishing.

Institutional Review Board Statement: No approval was required for the analysis of Swiss TPH samples.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on reasonable request from the corresponding author.

Acknowledgments: We are grateful to Anna Stuermann from Swiss TPH for excellent technical support.

Conflicts of Interest: The authors declare no conflict of interest.

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5.4. Article n° 4. Review article: Recent advances and potential future applications of MALDI-TOF mass spectrometry for identification of helminths

Sy I, Conrad L, Becker SL. Recent Advances and Potential Future Applications of MALDI-TOF Mass Spectrometry for Identification of Helminths. *Diagnostics*. 2022; 12(12):3035. https://doi.org/10.3390/diagnostics12123035

Review

Recent Advances and Potential Future Applications of MALDI-TOF Mass Spectrometry for Identification of Helminths

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Abstract: Helminth infections caused by nematodes, trematodes, and cestodes are major neglected tropical diseases and of great medical and veterinary relevance. At present, diagnosis of helminthic diseases is mainly based on microscopic observation of different parasite stages, but microscopy is associated with limited diagnostic accuracy. Against this background, recent studies described matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry as a potential, innovative tool for helminth identification and differentiation. MALDI-TOF mass spectrometry is based on the analysis of spectra profiles generated from protein extracts of a given pathogen. It requires an available spectra database containing reference spectra, also called main spectra profiles (MSPs), which are generated from well characterized specimens. At present, however, there are no commercially available databases for helminth identification using this approach. In this narrative review, we summarize recent developments and published studies between January 2019 and September 2022 that report on the use of MALDI-TOF mass spectrometry for helminths. Current challenges and future research needs are identified and briefly discussed.

Keywords: diagnosis; helminths; cestodes; nematodes; trematodes; matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF); mass spectrometry (MS)

Citation: Sy, I.; Conrad, L.; Becker, S.L. Recent Advances and Potential Future Applications of MALDI-TOF Mass Spectrometry for Identification of Helminths. *Diagnostics* **2022**, *12*, 3035. https://doi.org/10.3390/diagnostics12123035

Academic Editor: Franco Falcone

Received: 4 November 2022
Accepted: 1 December 2022
Published: 3 December 2022

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1. Introduction

Helminths are parasitic worms that give rise to considerable disease burden in humans and animals. From a medical perspective, the major helminths are classified into three distinct groups, based on their external shape and the host organ they are located in: cestodes (tapeworms), nematodes (roundworms), and trematodes (flukes) [1]. They represent some of the most common infectious agents of humans, and helminthiasis is a major neglected tropical disease (NTD), which affects in particular individuals in the Global South [2,3]. A precise diagnosis of these infectious agents is crucial for control efforts and individual patient management. Currently used parasitological methods frequently rely on light microscopy and have limited diagnostic accuracy, especially in light-intensity infections. Moreover, some species such as hookworms [4] or *Taenia* spp. [5] have almost identical eggs and are thus indistinguishable on microscopic identification. While new methods have been proposed [6,7], different challenges remain. Molecular methods such as polymerase chain reaction (PCR)-based approaches can also be employed for helminth diagnosis [8]. These methods have a high sensitivity but are still expensive, require specific technical expertise, and are not really adapted for routine application, especially in resource-limited or developing countries, where helminths are endemic. Additionally, only a very limited set of PCR assays targeting helminths are commercially available thus far. In many parts of the world, where helminth infections are nowadays less common, expertise

in correct morphological identification is also waning. Hence, there is a need for innovative, examiner-independent techniques for helminth identification.

For more than one decade, matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) has become the widely accepted gold standard method in many clinical microbiology laboratories, and it is routinely used for the identification of bacteria [9,10] mycobacteria [11,12] and fungi [13]. This technology is based on the rapid and accurate identification of microorganisms using an analysis of pathogen-specific protein spectra profiles, which are measured and identified through comparison to an existing database [14]. Of note, MALDI-TOF MS analyses are not only limited to protein profile studies. Recent studies emphasized the possible use of MALDI-TOF MS for lipidological analysis and for prediction of antibiotic resistance pattern [15–18]. The principle of MALDI-TOF MS can be briefly described as follows: a sufficient amount of bacterial/fungal biomass (or their protein extracts; e.g., taken directly from an agar plate) is placed onto the MALDI plate and overlaid with a matrix (e.g., HCCA: alpha-cyano-4-hydroxycinnamic acid). The mixture (sample + matrix) is crystalized and then subjected to a laser beam. The heat energy of the laser ionizes the sample, and its molecules migrate through an electric vacuum field in the MALDI-TOF mass spectrometer. The ions are separated during the migration according to their mass-to-charge ratio (time-of-flight). Once they reach a detector, a specific spectra profile is generated and compared to a commercially available reference spectra database [19]. Within seconds, a result is generated as so-called log score value (LSV), which estimates the accuracy of identification. For bacteria, any LSV $\geq$ 1.70 is considered as genus-specific identification and LSVs $\geq$ 2.00 are considered as species-specific identification.

In contrast to bacteria and fungi, there is currently neither a commercial library for an MALDI TOF-based identification of parasites nor a standardized protocol for measurements of parasitic elements. However, the idea of employing MALDI-TOF MS for parasites and especially helminths is gaining more and more interest, and different research groups have worked on this topic [20]. As a first prerequisite, it is important to develop a spectral database for any subsequent identification. The MALDI-TOF MS apparatus released by the German company Bruker (Bruker Daltonics, Bremen, Germany) allows to create reference spectra (also called MSPs: main spectra profiles) using the Maldi Biotyper (MBT) compass explorer software (MSP creation protocol V1.1; Bruker Daltonics; Bremen, Germany). Figure 1 indicates a summary of the different steps that are required for MSP creation and database development and installation. Once the helminth-specific database is installed, new samples can be identified very rapidly using a simple procedure. Therefore, due to its accuracy, ease of use, and cost-effectiveness of reagents, the application of MALD-TOF MS for helminths identification offers a true advantage in comparison to microscopy or PCR-based methods.

In this article, we review the recent advances and highlight potential future applications of MALDI-TOF MS for the identification of helminths. Given the increasing availability of mass spectrometers in reference laboratories of low- and middle-income countries as well as the waning knowledge regarding identification of helminths among microbiologists in high-income settings, detection of helminths by MALDI-TOF MS might be advantageous in a host of different settings.

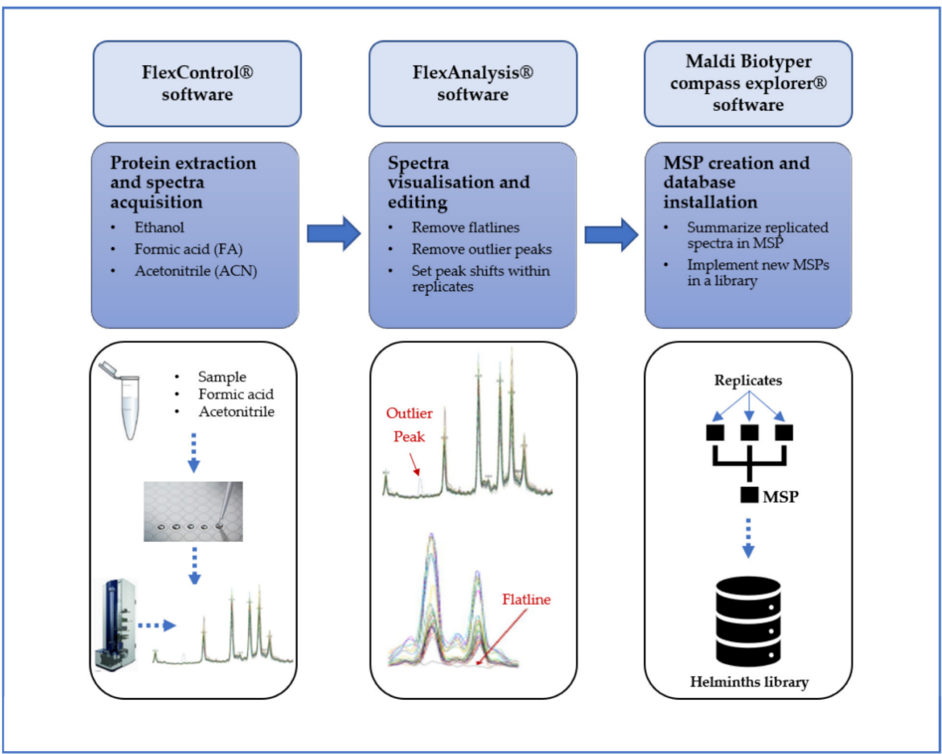

Figure 1. MSP creation and database installation using MBT compass explorer software version 3 (Bruker Daltonics, Bremen, Germany).

2. Materials and Methods

A literature review was conducted to assess the current state of MALDI-TOF MS as a diagnostic tool for helminths. As a previous review had included manuscripts published on this topic until late 2018 [20], we searched published articles from January 2019 to September 2022 in the following databases: PubMed/MEDLINE and Google Scholar. The search strategy included combinations of the following keywords: "matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS)", "helminth", "nematode", "cestode" and "trematode". The literature research was independently carried out by all three authors, and subsequently synthesized by the first two authors to generate this narrative review. No language restriction was used during the search.

3. Specific Application of MALDI-TOF MS for Different Helminths

3.1. Cestodes

We found only two studies that reported on the use of MALDI-TOF MS for cestodes. One study focused on the analysis of specific proteins from larval stages, while the second report investigated the identification of direct identification of adult worms (proglottids).

Diaz-Zaragoza et al. [21] examined the proteomic profiles of larval stages of the tapeworm *Taenia crassiceps* in a murine intraperitoneal model to assess the immunological T helper cell (Th) response during cysticercosis, in particular the differential Th1 and Th2 responses. After two-dimensional gel electrophoresis (2DE), proteins were selected and identified by MALDI-TOF MS. It was shown that *T. crassiceps* cysticerci are able to modify

their proteome depending on the activation of Th1 or Th2 response in the course of infection. Furthermore, the study assessed how cysticerci interact with their environment as well as which mechanisms allow for infection persistence in the host.

A more recent study carried out by Wendel and colleagues [22] showed that parts of adult cestodes (proglottids) can also be identified by MALDI-TOF MS. In this study, the authors showed that the beef tapeworm *Taenia saginata* can be reliably diagnosed by MALDI-TOF MS. For this purpose, *T. saginata* proglottides isolated from stool samples of infected humans were subjected to protein extraction and MALDI-TOF MS analysis. Protein spectra were generated under repeatability and reproducibility conditions and were analyzed in order to create species-specific MSPs to obtain an in-house database. Subsequently, samples were measured and analyzed by this database, leading to reliable LSVs in 97.2% to 99.7% of the analyzed spectra. Hence, this study showed the potential utility of such a diagnostic approach in humans but was limited by the low number of samples and the lack of external validation using new independent samples.

3.2. Nematodes

The utilization of MALDI-TOF MS for nematode identification has been reported by recent studies of either pathogen-associated proteins or the pathogen itself at different developmental stages (adults, larvae, or eggs) [23,24]. Bredtmann and colleagues [25] showed the possible differentiation of adult worms of two closely related cyathostomin species, i.e., *Cylicostephanus longibursatus* and *Cylicostephanus minutus*. In this study, the authors collected adult samples from horses (intestines, cecum, and feces) and performed MALDI-TOF analysis using an in-house MSP library. High-quality and repeatable spectra were acquired with fresh samples collected during necropsy, while samples recovered from feces after anthelminthic treatment gave spectra with low quality. The database validation test showed correct species identification of 89% (144/162) of all measured spectra. Similarly, Nagorny and colleagues [26] reported a MALDI-TOF MS-based identification of five adult female *Dirofilaria repens* originating from an infected human, and five *Dirofilaria immitis* isolated from dogs. They showed a concordance rate of 100% and 70% for *D. repens* and *D. immitis*, respectively. More recently, another study carried out by Rivero and colleagues [27] emphasized the identification of *Trichuris* spp. using MALDI-TOF MS. In this study, the authors analyzed different parts (esophagus and intestine) of five whipworms to create reference MSPs. Subsequently, they queried 20 new whipworms (18 esophagus and 20 intestines) to the reference database for external validation, resulting in nine esophagus samples (50%) and 16 intestines samples (80%) as being correctly identified (*Trichuris suis* with LSVs $\geq$ 2.00). However, a dendrogram analysis revealed that a differentiation between both parts (esophagus and intestine) was not possible by means of MALDI-TOF MS.

In addition to the identification of adult worms, other studies have also investigated the application of MALDI-TOF MS for the identification of larval stages. Karadjian and colleagues [28] analyzed larvae of *Trichinella* spp. that were isolated from different hosts (i.e., pigs, wild boars, red foxes, raccoon dogs, and horses) and different geographical areas (e.g., Italy, France, Romania, Serbia, Hungary, Serbia, Poland, Germany, Norway, Finland, Bulgaria, Tasmania, Russia, Japan, China, USA, Argentina). They reported a correct identification rate with LSVs $\geq$ 2.00 in 100% (70/70), 96.1% (49/51), and 83.3% (5/6) for *T. spiralis*, *T. britovi* and *T. nativa*, respectively. The authors also reported no close relationship between the *T. nativa* strains isolated from Germany, Norway, and Russia. Based on this observation, they suggest a possible difference in protein spectra associated with the geographical location. Dendrogram analysis also revealed varying distance levels of *T. britovi* and *T. nativa*, which may indicate intraspecific differences in both species. Taken together, this study underscored the possibility of correctly identifying *Trichinella* spp. based on protein spectra profiles. However, the study was limited by the use of rather old reference strains, and there is a need to also include additional spectra from more recent strains into the MSP database. Likewise, Marzano and colleagues [29] described the use of MALDI-TOF MS profiling as a new diagnostic tool for *Anisakis* spp. larvae

isolated from salmons. Clustering analysis performed on spectral data obtained from five *Anisakis* spp. larvae showed a phenotypic variability between the five larval samples (larvae 1 and 4 clustered together and formed a clade with larva 5; while larvae 2 and 3 formed a different cluster), possibly indicating the discriminatory power using protein fingerprints. In addition, the authors also identified 19 signals (peaks), which may be used as "fingerprinting classifier" specific to *Anisakis* spp. This study emphasized the potential use of this method, and this may lead in future to MALDI-TOF MS-based identification of *Anisakis* larvae obtained from e.g., the gastrointestinal tract of infected human patients.

Apart from using MALDI-TOF MS as a new tool for the identification of adult worms or larvae, other studies focused on the characterization and identification of specific nematode proteins. Khanmohammadi and colleagues [30] studied the characterization of immunogenic proteins of *D. immitis* using MALDI-TOF MS. Herein, authors collected adult worms from six infected dogs and cultivated them in cell culture flasks containing roswell park memorial institute (RPMI)-1640 medium and other substances. Afterwards, the medium was submitted to successive steps of centrifugation, filtration, homogenization, and sonication in order to retrieve the in vivo secretome (somatic and excretory/secretory (E/S) protein extracts). Immunoreactive protein bands detected by Western blots were then excised and analyzed by MALDI-TOF MS. The authors used UniProt and European molecular biology laboratory (EMBL) databases to characterize the identified proteins. Nine and eight proteins were identified in the somatic (polyprotein antigen, P22u, galectin, etc.) and E/S (pepsin inhibitor Dit33, superoxide dismutase, etc.) extracts, respectively, including six common proteins. Martini and colleagues [31] reported on the presence of N-glycans from *D. immitis* of up to 7000 Da using MALDI-TOF MS (both linear and reflector mode) in combination with other techniques (e.g., high-performance liquid chromatography (HPLC), enzymatic digestion, etc.). These N-glycans contain features that can be involved in immunomodulation or help to avoid the immune response. Similarly, Wang and colleagues [32] also studied the N-glycome and N-glycoproteome of the ruminant nematode *Haemonchus contortus*. They reported a total of 291 N-glycosylated proteins occurring primarily in the intestine and gonads. In the same direction, Petralia and colleagues [33] investigated the glycans expressed by the filarial nematode *Brugia malayi* at different stages of development (microfilariae, third-stage larvae (L3), and adult worms (males and females)) using MALDI-TOF MS combined with glycan sequencing, tandem mass spectrometry (MS/MS) and glycan microarrays. They identified several antigen motifs such as phosphorylcholine and terminal glucuronic acid. Antibody responses with a significant level of IgG were also detected during microarray screening.

3.3. Trematodes

In a French study by Huguenin and colleagues [34], MALDI-TOF MS was employed to identify cercarial stages of different trematodes. To this end, the authors collected different snails as they are the intermediate hosts of many trematodes. Cercariae from the genera Lymnaeidae and Planorbidae were released after specific hatching tests. Using molecular identification, the detected cercariae included the following species: *Trichobilharzia anseri*, *Diplostomum pseudospathaceum*, *Tylodelphys* sp., *Australapatemon* sp., *Cotylurus* sp., *Posthodiplostomum* sp., *Parastrigea* sp., *Alaria alata*, *Echinostoma revolutum*, *Petasiger phalacrocoracis*, *Echinoparyphium* sp. and *Plagiorchis* sp. MALDI-TOF MS analysis was performed for each species and the obtained spectra were processed. Based on this, reference spectra of all species except *Parastrigea* sp. and *Cotylurus* sp. were generated and integrated into an in-house database. Subsequently, the newly created database was evaluated by 'blind tests' with new samples. Of the analyzed spectra acquired from species in the database, 68.36% achieved an LSV of 1.7, and thus, reliable genus identification. The sensitivity was 81.7% and the specificity was 100%. After adding reference spectra of *Cotylurus* sp., 65.78% achieved an LSV of $\geq$1.7, and 100% were correctly identified. In the same way, Kästner et al. [35] conducted a study to identify *A. alata* mesocercariae isolated from meat by MALDI-TOF MS. When wild boar meat is examined for *Trichinella*, mesocercariae of *A. alata*

are frequently found. For this study, mesocercariae have been isolated in several countries from muscle samples from different hosts, including wild boars, lynxes, and amphibians. After developing an applicable protocol for protein extraction, MALDI-TOF analysis was performed. Spectra were analyzed and spectra of high quality were selected. A total of 10 mesocercariae were used to integrate species-specific MSPs into the database. Unknown spectra were then compared to the new database for validation. The protocol was tested in two different laboratories and proved to be reproducible. Thirty-six of 38 mesocercariae had LSVs of above 2.0 in all three individual spectra, which demonstrated the repeatability of the chosen approach. In addition, spectra from *Opisthioglyphe ranae* larvae were also analyzed by MALDI-TOF MS and queried to the new database. The obtained score values ($\leq$1.7) demonstrate the capacity to distinghuish between different trematodes species using their spectra profiles. The protein spectra of the mesocercariae in this study, however, did not correspond to those previously reported [34], which could be caused by the different larval stages as well as the host species or the slightly different protein extraction protocol. More recently, Huguenin and colleagues [36] also reported the identification of cercariae of schistosomes using MALDI-TOF. In the study, different *Schistosoma* species were included: *S. haematobium*, *S. mansoni*, *S. bovis*, *S. rodhaini*, and the hybrid *S. bovis* $\times$ *S. haematobium*. After spectra acquisition and analysis, MSPs were generated and added to the database. It was possible to distinguish between the individual species. Blind tests showed an accuracy of 94% and a specificity of 99.56%. The discrimination between *S. haematobium* and the hybrid species (*Corsican hybrids*), however, remained a challenge. Yet, the authors also used machine learning algorithms combined with MALDI-TOF MS spectra in order to increase the accuracy of the discrimination to 97%.

In addition to the use of cercariae, MALDI-TOF has also been employed for a rapid diagnosis and early screening of schistosomiasis in serum samples. Huang and colleagues [37] studied the identification of newly developed advanced schistosomiasis (NDAS), using 30 serum samples stemming from infected patients, as well as 30 sera from healthy people as control group. Protein extracts obtained from these two groups were subjected to MALDI-TOF MS after a weak cation exchange beads (MB-WCX) treatment, using the ClinProTool software. A comparison analysis revealed a total of 14 peaks (four upregulated peaks (increase of the intensity); and 11 downregulated peaks (decrease of the intensity)) as important for the discrimination of the two groups and were established as proteomic detection patterns (PDP). The authors also evaluated the specificity and sensitivity of the PDP by subjecting new mass fingerprints (spectra) of sera from 50 NDAS and 100 healthy controls for a blind test. They reported a sensitivity of 100% and a specificity of 92%. The PDP created in this study could potentially be used to identify NDAS patients, and the peaks might possibly serve as future biomarkers for diagnosis.

Beside using cercariae or serum as samples for the diagnosis of schistosomiasis by MALDI-TOF MS, two other studies employed different materials for identification of *Fasciola* spp., the causative agents of fascioliasis [38,39]. One study focused on the analysis of individual components of *Fasciola* spp., while the other one used adult *Fasciola* worms. Mohan et al. [39] conducted a study on the biomedical properties of haemoglobin from *Fasciola gigantica* using MALDI-TOF MS. Extracted peptides were applied to the MALDI target plate and analyzed. The resulting spectra, to which the authors referred as "peptide map" or "peptide mass fingerprints" (PMF), were then queried to a protein databases (e.g., Swiss-Prot) for identification. The authors found that 28.4% of the peptide sequences corresponded to *Fasciola hepatica* haemoglobin. In the second study Sy et al. [38] demonstrated that adult stages of *F. hepatica* and *F. gigantica* can be identified and differentiated with MALDI-TOF MS. For this purpose, the authors subjected the posterior part of the adult worm to molecular analysis and, after protein extraction, to MALDI-TOF MS analysis. Raw spectra of seven *Fasciola gigantica* and one *Fasciola hepatica* samples were analysed and processed to generate species-specific MSPs for both species, which were subsequently integrated into an in-house database for helminth identification. To validate this database, authors performed a blind test using spectra from new samples and obtained a correct

identification of 98.7% (74/75) for *F. gigantica* and 100% (3/3) for *F. hepatica* with LSVs above 1.7.

The following table summarizes the different studies published on MALDI-TOF MS for helminths since 2019, as well as the different helminth species and the type of material used (Table 1).

Table 1. Published studies pertaining to the application of MALDI-TOF MS on helminth samples between January 2019 to September 2022.

Pathogens	Analyzed Material				Correct Identification Rate in % [LSV]	References
	Serum of Infected Individuals	Specific Proteins	Larval Stage	Adult Helminth		
Cestodes						
Taenia crassiceps		√			NA	Diaz-Zaragoza et al., 2020
Taenia saginata				√	97.2–99.7% [≥2.5]	Wendel et al., 2021
Trematodes						
Alaria alata			√		94.73% [≥2]	Kästner et al., 2021
Cotylurus sp. *Diplostomum pseudospathaceum* *Echinostoma revolutum* *Echinoparyphium* sp.			√ √ √ √		65.78% [≥1.7]	Huguenin et al., 2019
***Fasciola* spp.:** *F. gigantica* *F. hepatica*				√ √	98.7–100% [1.73–2.23]	Sy et al., 2020
Parastrigea sp. *Petasiger phalacrocoracis* *Plagiorchis* sp. *Parastrigea* sp. *Posthodiplostomum* sp. *Petasiger phalacrocoracis*			√ √ √ √ √ √		65.78% [≥1.7]	Huguenin et al., 2019
***Schistosoma*:** *S. bovis* *S. haematobium* *S. mansoni* *S. rodhaini* *S. bovis* × *S. haematobium (hybrid)*			√ √ √ √ √		42.7% [≥1.7] ≥97% [NA]	Huguenin et al., 2022
Schistosoma japonicum	√				92–100% [NA]	Huang et al., 2019
Trichobilharrzia anseri *Tylodelphys* sp.			√ √		65.78% [≥1.7]	Huguenin et al., 2019
Nematodes						
Anisakis spp.			√		NA	Marzano et al., 2020
Brugia malayi		√			NA	Petralia et al., 2022

Table 1. *Cont.*

Pathogens	Analyzed Material				Correct Identification Rate in % [LSV]	References
	Serum of Infected Individuals	Specific Proteins	Larval Stage	Adult Helminth		
***Cyatosthomins*:**						
Cylicostephanus longibursatus				√	89% [1.13–2.44]	Bredtmann et al., 2019
Cylicostephanus minutus				√		
***Dirofilaria* spp.:**						Khanmohammadi et al., 2019 Martini et al., 2019 Nagorny et al., 2019
D. immitis	√	√		√	NA	
D. immitis		√			70–100% [NA]	
D. repens				√		
Haemonchus contortus		√		√	NA	Wang et al., 2021
***Trichinella* spp.:**						
T. britovi			√			
T. nativa			√			
T. patagoniensis			√		83.3–100% 100% [≥2]	Karadjian et al., 2020
T. pseudospiralis			√			
T. spiralis			√			
Trichinella sp. *T8*			√			
Trichinella sp. *T9*			√			
***Trichuris* spp.:**						
Trichuris sp.				√		
T. ovis				√		
T. suis				√	100% [1.84–2.36]	Rivero et al., 2022
T. trichiura				√		
T. vulpis				√		

NA: not applicable; LSV: log score value.

4. Effect of Storage Media and Duration

In the field, in research, or in diagnostic laboratories, the lack of common or standardized procedures for sample preservation prior to analysis implies that helminth samples are usually kept under different conditions (e.g., freezing, room temperature, etc.), and storage media (e.g., sodium chloride, ethanol, formalin, RNAlater, etc.) for a short or prolonged period of time before further sample processing [40–43]. For the purpose of using MALDI-TOF MS as a potential tool for the diagnostic of helminths, it is necessary to use suitable preservative media to avoid the degradation of proteins in order to generate high-quality spectra [20]. Thus far, we identified only two studies that have investigated the influence of different preservative media on MALDI-TOF MS analysis. Wendel and colleagues studied the influence of four storage media (70% ethanol, LC-MS grade water, formalin and 0.45% sodium chloride) on MALDI identification of *T. saginata* proglottids. They reported that all tested media except for formalin were suitable and equally effective for a prolonged storage period of up to 24 weeks. This study was limited by the fact that the comparatively analyzed *T. saginata* proglottids had previously been preserved in sodium chloride for 12 months [22]. Likewise, Mayer-Scholl and colleagues [44] compared two conservation methods (freezing of the native sample vs. storage in ethanol) and found that neither LSVs and nor protein peak patterns were affected after a period of storage of up to six months. However, slight differences in the intensities of the peaks were noticed. In this study, the samples were kept at −20 °C, but the authors did not specifically mention how long the samples were stored in alcohol. Further investigations using different preservation media and different conditions on different helminth species would be helpful for a better understanding and management of samples for MALDI-TOF MS analysis.

5. Challenges and Future Research Needs

For reference spectra to be included in databases for helminth identification, it is an essential prerequisite to be sure about the species identification, which is ideally achieved by molecular diagnostic methods in conjunction with microscopic or morphological observation. While MALDI-TOF MS holds promise to be employed in helminths [45,46], it has also been applied for identification of protozoa and different ectoparasites such as ticks [47], lice [48], fleas [49] or bed bugs [50]. As described above, MALDI-TOF-based differentiation seems to be challenging for closely related species, and it might thus be worthwhile to further investigate the use of machine learning (ML) algorithms to further enhance the identification process [36]. This might be particularly useful when analyzing biospecimens (e.g., serum) where pathogen and host proteins occur simultaneously.

Most helminth infections in humans are diagnosed by either urine or stool sample examinations, and the detectable stage are frequently the helminth eggs and sometimes the larvae (e.g., in the case of *Strongyloides stercoralis*), but very rarely parts of the adult worm (e.g., proglottids of *Taenia* spp.). Hence, in the perspective of future applications of MALDI-TOF MS for human helminth diagnosis, upcoming investigations should focus on the analysis of eggs and egg-containing samples (e.g., stool). However, MALDI-TOF MS analysis of stool samples would require a preliminary step of egg purification in order to generate egg-specific spectra that can be used as references. In addition, future studies should also consider the analysis of samples from different geographical locations. Finally, it would be valuable to develop a uniform protocol for sample preservation and for protein extraction, as well as the creation of a universal database for a wide range of parasites. Spectra reproducibility, especially inter-laboratory reproducibility could be solved by standardizing the procedures, from sample storage (by using suitable storage media and conditions allowing non-degradation of proteins), to sample preparation methods (by using a simple protocol). Drug susceptibility testing necessarily involves preliminary identification of the infectious agent in order to determine the appropriate drug for subsequent administration. Therefore, rapid and accurate identification by MALDI-TOF MS would allow for more effective treatment. However, measuring drug sensitivity or monitoring treatment efficacy on adult worms, larvae or eggs would require additional approaches such as real-time cell assay (RTCA) to evaluate changes in worm motility and/or egg hatching [51,52].

6. Conclusions

The narrative review highlights the recent advances in helminth diagnosis using MALDI-TOF MS as well as their current limitations. A series of research needs has been identified, which currently hinders the use of this technique as detection method in clinical practice. However, based on the promising available data, it seems likely that further progress will be made in the next years, and that MALDI-TOF MS might also find a potential niche for diagnostics of helminths in the medical and/or veterinary sector.

Author Contributions: Conceptualization, I.S. and S.L.B.; literature search, I.S., L.C. and S.L.B.; writing—original draft preparation, I.S. and L.C.; writing—review and editing, I.S., L.C. and S.L.B. All authors have read and agreed to the published version of the manuscript.

Funding: We acknowledge support by the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) and Saarland University within the funding programme Open Access Publishing.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

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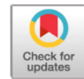
5.5. Article n° 5. Research article: Research article: Evaluation of the Qvella FAST™ system and the FAST-PBC cartridge for rapid species identification and antimicrobial resistance testing directly from positive blood cultures

Sy I, Bühler N, Becker SL, Jung P. Evaluation of the Qvella FAST™ System and the FAST-PBC cartridge for rapid species identification and antimicrobial resistance testing directly from positive blood cultures. *J Clin Microbiol*. 2023; 61(10):e00569-23. https://doi.org/10.1128/jcm.00569-23



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Evaluation of the Qvella FAST System and the FAST-PBC cartridge for rapid species identification and antimicrobial resistance testing directly from positive blood cultures

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ABSTRACT Blood culture diagnostics require rapid and accurate identification (ID) of pathogens and antimicrobial susceptibility testing (AST). Standard procedures, involving conventional cultivation on agar plates, may take up to 48 hours or more until AST completion. Recent approaches aim to shorten the processing time of positive blood cultures (PBC). The FAST System is a new technology, capable of purifying and concentrating bacterial/fungal pathogens from positive blood culture media and producing a bacterial suspension called "liquid colony" (LC), which can be further used in downstream analyses (e.g., ID and AST). Here, we evaluated the performance of the FAST System LC generated from PBC in comparison to our routine workflow including ID by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry using Sepsityper, AST by automatized MicroScan WalkAway *plus* and directly inoculated disk diffusion (DD), and MICRONAUT-AM for yeast/fungi. A total of 261 samples were analyzed, of which 86.6% (226/261) were eligible for the comparative ID and AST analyses. In comparison to the reference technique (culture-grown colonies), ID concordance of the FAST System LC and Sepsityper was 150/154 (97.4%) and 123/154 (79.9%), respectively, for Gram positive; 67/70 (95.7%) and 64/70 (91.4%), respectively, for Gram negative. For AST, categorical agreement (CA) of the FAST System LC in comparison to the routine workflow for Gram-positive bacteria was 96.1% and 98.7% for MicroScan and DD, respectively. Similar results were obtained for Gram-negative bacteria with 96.6% and 97.5% of CA for MicroScan and DD, respectively. Taken together, the FAST System LC allowed the laboratory to significantly reduce the time to obtain correct ID and AST (automated MicroScan) results 1 day earlier and represents a promising tool to expedite the processing of PBC.

KEYWORDS bloodstream infection, blood culture, diagnosis, antimicrobial susceptibility testing, Qvella FAST System, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), MicroScan WalkAway, disk diffusion

Bloodstream infections (BSIs) encompass a large variety of pathogens such as bacteria or fungi causing high rates of mortality and morbidity (1–3). Ineffective treatment of BSI due to delayed pathogen identification (ID) and late antimicrobial susceptibility testing (AST) can lead to adverse outcomes (4). Sepsis, defined as an organ dysfunction caused by a dysregulated host response to infection, is considered a major threat and one of the most important causes of health loss and deaths (4, 5). Previous studies estimated around 49 million cases and 11 million deaths per year, which would correspond to 20% of all deaths in the world (5–8). In this context, the rapid identification of pathogens followed by AST to enable appropriate antimicrobial therapy is crucial for effective management of septic patients. Since the standard method used in clinical laboratories is based on traditional culture methods and requires at least 24 hours for ID

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The authors declare no conflict of interest.

Received 4 May 2023
Accepted 28 July 2023
Published 28 September 2023

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and 48 hours for final AST results, the development of more rapid methods for pathogen ID from positive blood culture (PBC) and subsequent resistance testing is of great interest. To this end, several alternative methods have been developed, which employ molecular detection methods on PBC, such as Biofire FilmArray (3, 9, 10), LightCycler SeptiFast (11), Genmark ePlex (12), Verigene Blood Culture panels (13), Accelerate Pheno system (14), and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) with specific kits (3, 9, 15). In this study, we evaluated the performance of the recently released FAST System (Qvella Corporation, Richmond Hill, Canada)-generated liquid colony (LC) in comparison to our routine workflow, including Sepsityper for rapid MALDI-TOF-based pathogen ID, AST using MicroScan WalkAway *plus*, and directly inoculated disk diffusion (DD).

MATERIALS AND METHODS

Sample collection and study design

Between March and November 2022, a total of 261 prospective blood culture samples in different types of BD BACTEC blood culture bottles (Plus Aerobic, Plus Anaerobic, Lytic Anaerobic, Mycosis IC/F, and Peds Plus) (Becton Dickson, Heidelberg, Germany) were collected on different wards at Saarland University Medical Center in Homburg, southwest Germany. After being flagged positive in the BD BACTEC FX System (Becton Dickson), each sample was subjected to Gram staining and further processed using two procedures, which were performed in parallel: (i) the standard routine workflow and (ii) the FAST System workflow.

Inclusion/exclusion criteria

All positive blood culture samples obtained during routine diagnostics were included for the comparative assessment, except for the following exclusion criteria: post-mortem blood culture, polymicrobial blood culture (i.e., if different morphologies were seen on Gram staining, samples were not subjected to Qvella FAST System; if culture-grown colonies after 18–24 hours of incubation showed previously unexpected polymicrobial growth, these samples were retrospectively excluded), processing of the blood culture ≥16 hours after being flagged positive, incorrect labeling, and failed runs (cartridge processing errors or system failures).

Routine workflow

Each PBC sample was subjected, using the Sepsityper Kit accordingly to the manufacturer's recommendations, to the Bruker BDAL database using MALDI Biotyper version 3.0 for MALDI-TOF MS ID (Bruker Daltonics, Bremen, Germany). Next, directly inoculated DD was performed using the interpretative recommendations put forth by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) protocol version 12.0, 2022 (16), in order to obtain a preliminary AST result within 18 hours. In brief, 125 ± 5 µL aliquots of positive blood were directly inoculated on Mueller-Hinton or Mueller-Hinton fastidious agar plates (BD Biosciences, Franklin Lakes, NJ, USA), followed by the addition of appropriate antibiotic disks or epsilometry (E-test) gradient strips according to the manufacturer's protocol (Liofilchem, Italy) and incubated at 35 ± 1°C.

In parallel, the reference method using agar plate subculture was conducted: colonies grown overnight on trypticase soy agar plates supplemented with 5% sheep blood, McConkey agar plates, Columbia agar plates (for anaerobic bacteria), and Sabouraud glucose agar plates (for fungi) (all plates produced by BD Biosciences) were analyzed by MALDI-TOF MS for species ID. Then, agar plate colonies were subjected to automatized AST employing broth microdilution method using the MicroScan WalkAway *plus* System (Beckman Coulter, Germany), with POS MIC33 panels (for Gram-positive bacteria; except for streptococci) and NEG MIC1 panels (for Gram-negative bacteria), according to the manufacturer's guidelines. If fungal species had been identified, antifungal susceptibility

testing (AFST) using MICRONAUT-AM antifungal agent MIC plates (Bruker Daltonics, Germany) was performed.

FAST System workflow

The principle of the FAST System is based on the isolation and concentration of microbial cells directly from PBC using single-use FAST PBC Prep cartridges (Qvella Corporation, Canada). For sample preparation, 2-mL aliquots were drawn from PBC bottles. After a run of 24 (for one cartridge) or 38 (for two cartridges) minutes, the bacterial suspension called "liquid colony (LC)" was retrieved and further subjected to downstream analyses such as MALDI-TOF MS ID and AST. For the MALDI ID, 1 µL of the LC was spotted on the MALDI target plate in duplicate. After being dried, each spot was recovered with 1 µL of 70% (vol/vol) formic acid followed by the addition of 1 µL of saturated α-cyano-4-hydroxy-cinnamic acid matrix solution (Bruker Daltonics, Germany). For the AST, both manual (disk diffusion and/or E-tests) and automated ASTs were performed using a bacterial suspension of 0.5 McFarland prepared from the LC. Similar to the routine procedure, the automated AST method was performed using the MicroScan WalkAway *plus* System (Beckman Coulter, Germany), employing POS MIC33 panels (for Gram-positive bacteria; except for streptococci) and NEG MIC1 panels (for Gram-negative bacteria).

Comparative analysis

ID and AST (or AFST) results obtained with the two aforementioned workflows (routine workflow and FAST workflow) were compared for concordance. In addition, refer to the description of Inclusion/exclusion criteria, given above.

MALDI-TOF ID comparison

MALDI-ID obtained via Sepsityper or via the FAST System LC was compared to MALDI-ID obtained by culture-grown colonies as reference method. As two spots were measured for each sample, only the MALDI ID with the highest identification log score value was considered during the comparison (log score values ≥2 were considered as reliable species level ID, and score values ≥1.7, as probable species ID). Inconclusive IDs correspond to identification results with a score value below the threshold or "no peak found." Discrepant IDs correspond to identifications of different species for the same sample using two methods.

AST (or AFST) comparison

Results from DD (including *E*-tests) and automated AST (MicroScan WalkAway) (or AFST if fungal species were identified) obtained with the FAST System LC were compared to AST (or AFST) results obtained with the routine workflow. Categorical agreement (CA) was employed for the interpretation of the different AST results: "susceptible" (S), "resistant" (R), and "susceptible, increased exposure" (I) following the breakpoints put forth by EUCAST version 12.0 (16). Of note, AST results (i.e., MIC or diameter zone) in the area of technical uncertainty occurred in rare cases and were interpreted as given in Supplemental file S1-Table S1. Discrepancies were evaluated based on error types including minor errors (minE: results that are categorized increased [I] in one workflow but susceptible [S] or resistant [R] in the other), major errors (majE: results that are resistant [R] in the FAST System LC workflow but susceptible [S] in the routine workflow), and very major errors (VmajE: results that are susceptible [S] in the FAST System LC workflow, but resistant [R] in the routine workflow). Of note, as preliminary AST (i.e., directly inoculated DD) was compared with the DD results obtained from the FAST System workflow, isolates in which discrepancies (VmajE, majE, and minE) occurred were retested by performing standard DD from grown colonies obtained after reinoculation of cryo-cultures in order to clarify these discrepancies. The percentage of CA is calculated by dividing the number of categorical matches by the total number of antibiotics tested with the reference

method × 100. The rate of VmajE was calculated by dividing the number of VmajE by the total number of resistant bacteria tested with the reference method × 100. The rate of majE was calculated by dividing the number of majE by the total number of susceptible bacteria tested with the reference method × 100. The rate of minE was calculated by dividing the number of minE by the total number of antibiotics tested with the reference method × 100.

Time to results

To estimate the rapidity of the FAST System workflow compared to the routine workflow, the time to ID and AST results was calculated by measuring the necessary time to obtain conclusive ID and AST results for both workflows. For the FAST System, the time was measured with a run of two cartridges simultaneously. Statistical tests were performed using analysis of variance (ANOVA), and a P-value <0.05 was considered as significant.

RESULTS

Study sample characteristics

Thirty-five out of 261 (13.4%) samples were excluded from the analysis, including 28 (10.7%) polymicrobial PBCs that were not detected by the initial Gram staining and 7 (2.7%) failed runs corresponding to cartridge processing errors or software failures. The remaining 226 (86.6%) samples were eligible for comparison.

MALDI-TOF ID comparison

When being compared to the reference technique (culture-grown colonies), MALDI-TOF ID following the FAST workflow showed 96.9% (219/226) concordance, two discrepant IDs, and five inconclusive IDs (no peaks found or score value under the threshold). Sepsityper showed 82.74% (187/226) concordance, 1 discrepant ID, and 38 inconclusive IDs. The 219 samples correctly identified with the FAST System comprised 150 Gram-positive bacteria, i.e., 120/123 (97.6%) staphylococci, 19/20 (95%) enterococci, and 9/9 (100%) streptococci, as well as 1/1 (100%) *Micrococcus luteus* and 1/1 (100%) *Paenibacillus timonensis* (Table 1). The 69 remaining samples revealed 67 Gram-negative bacteria [59/61 (96.7%) Enterobacterales, with 33/33 (100%) *Escherichia coli* and 11/11 (100%) *Klebsiella pneumoniae* being the most represented species, 6/7 (85.7%) *Pseudomonas* spp., 1/1 (100%) *Bacteroides fragilis*, and 1/1 (100%) *Elizabethkingia anophelis*) (Table 2), and two yeasts both identified as *Candida glabrata*. Overall, the most common species were *Staphylococcus epidermidis* (n = 64) and *Escherichia coli* (n = 33). The two yeast species were successfully identified as *C. glabrata* following the FAST workflow (with MALDI score values of 1.86 and 1.93), while they were not successfully identified when the routine workflow (Sepsityper) was applied. Average MALDI scores of both workflows reached reliable values, comparable to those obtained for culture-grown colonies. For Gram-positive bacteria, we obtained scores of 2.00, 2.05, and 2.19, respectively, for FAST System, Sepsityper, and culture-grown colonies. While for Gram-negative bacteria, scores were 2.16, 2.12, and 2.24, respectively, for FAST System LC, Sepsityper, and culture-grown colonies. Likewise, log score values ≥2.00 were obtained for all groups (staphylococci, enterococci, Enterobacterales, etc.) with all three methods (Tables 1 and 2). Nevertheless, a few ID errors corresponding to inconclusive ID (i.e., no MALDI peaks found or MALDI score value below the threshold) and/or discrepant ID were observed. For Gram-positive bacteria, ID errors occurred in 4/154 (2.6%), 31/154 (20.1%), and 6/154 (3.9%) instances for FAST System, Sepsityper, and culture-grown colonies, respectively (Table 1). For Gram-negative bacteria, ID errors were observed in 3/70 (4.3%), 6/70 (8.6%), and 4/70 (5.7%) cases when using the FAST System, Sepsityper, and culture-grown colonies, respectively (Table 2). As compared to the reference (culture-grown colonies), MALDI ID using the LC from the FAST System revealed no discrepancies for Gram-positive bacteria. For Gram-negative bacteria, two discrepancies were noticed in the Enterobacterales group (*K. pneumoniae* were obtained instead of *K. variicola*) (Table 2).

TABLE 1 Identification of Gram-positive bacteria isolated from PBCs by MALDI-TOF MS using three different methods

Gram-positive bacteria			
	Culture-grown colonies (%)[c]	Sepsityper (%)	FAST System (%)
Staphylococci	119/123 (96.7%)	99/123 (80.5%)	120/123 (97.6%)
	2.17[b]	2.07[b]	2.01[b]
Staphylococcus epidermidis	63/65 (96.9%)	52/65 (80%)	64/65 (98.5%)
Staphylococcus aureus	28/29 (96.6%)	24/29 (82.8%)	29/29 (100%)
Staphylococcus hominis	11/11 (100%)	9/11 (81.8%)	10/11 (90.9%)
Staphylococcus haemolyticus	7/8 (87.5%)	5/8 (62.5%)	7/8 (87.5%)
Staphylococcus capitis	6/6 (100%)	6/6 (100%)	6/6 (100%)
Staphylococcus saccharolyticus	2/2 (100%)	1/2 (50%)	2/2 (100%)
Staphylococcus caprae	1/1 (100%)	1/1 (100%)	1/1 (100%)
Staphylococcus lugdunensis	1/1 (100%)	1/1 (100%)	1/1 (100%)
Inconclusive ID	4/123 (3.3%)	24/123 (19.5%)	3/123 (2.4%)
Discrepant ID	NA[a]	0/123 (0%)	0/123 (0%)
Enterococci	18/20 (90%)	16/20 (80%)	19/20 (95%)
	2.34[b]	2.00[b]	2.12[b]
Enterococcus faecium	10/11 (90.9%)	9/11 (81.8%)	10/11 (90.9%)
Enterococcus faecalis	7/8 (87.5%)	6/8 (75%)	8/8 (100%)
Enterococcus casseliflavus	1/1 (100%)	1/1 (100%)	1/1 (100%)
Inconclusive ID	2/20 (10%)	4/20 (20%)	1/20 (5%)
Discrepant ID	NA[a]	0/20 (0%)	0/20 (0%)
Streptococci	9/9 (100%)	7/9 (77.8%)	9/9 (100%)
	2.14[b]	2.02[b]	1.92[b]
Streptococcus mitis	3/3 (100%)	2/3 (66.7%)	3/3 (100%)
Streptococcus oralis	2/2 (100%)	2/2 (100%)	2/2 (100%)
Streptococcus dysgalactiae	1/1 (100%)	1/1 (100%)	1/1 (100%)
Streptococcus gallolyticus	1/1 (100%)	1/1 (100%)	1/1 (100%)
Streptococcus gordonii	1/1 (100%)	1/1 (100%)	1/1 (100%)
Streptococcus parasanguinis	1/1 (100%)	0/1 (0%)	1/1 (100%)
Inconclusive ID	0/9 (0%)	1/9 (11.1%)	0/9 (0%)
Discrepant ID	NA[a]	1/9 (11.1%)	0/9 (0%)
Other Gram positives	2/2 (100%)	1/2 (50%)	2/2 (100%)
	2.18[b]	2.26[b]	2.12[b]
Micrococcus luteus	1/1 (100%)	0/1 (0%)	1/1 (100%)
Paenibacillus timonensis	1/1 (100%)	1/1 (100%)	1/1 (100%)
Inconclusive ID	0/2 (0%)	1/2 (50%)	0/2 (0%)
Discrepant ID	NA[a]	0/2 (0%)	0/2 (0%)
Total Gram positive	148/154 (96.1%)	123/154 (79.9%)	150/154 (97.4%)2.00[b]
	2.19[b]	2.05[b]	

[a]NA, not applicable.
[b]Average MALDI score.
[c]Reference technique.

AST (or AFST) comparison

Manual and automated AST (or AFST) results obtained with the two workflows (routine and FAST) were compared using categorical agreement based on EUCAST guidelines version 12.

Automated AST results are presented in Table 3 (Gram positive) and Table 4 (Gram negative) with a total of 129 Gram-positive and 58 Gram-negative samples analyzed, comprising 115 staphylococci, 14 enterococci, 54 Enterobacterales, and 4 *Pseudomonas* spp. Of note, AST of 17 LC (five staphylococci and five enterococci, five Enterobacterales, and two *Pseudomonas* spp.) were not analyzed due to insufficient biomass to prepare a suspension of 0.5 McFarland or a failed run of the MicroScan. The rates of CA, VmajE, majE, and minE were, respectively, 1119/1165 (96.1%), 8/265 (3%), 16/824 (1.9%), and

TABLE 2 Identification of Gram-negative bacteria isolated from PBCs by MALDI-TOF MS using three different methods

	Gram-negative bacteria		
	Culture-grown colonies (%)[c]	Sepsityper (%)	FAST System (%)
Enterobacterales	57/61 (93.4%)	57/61 (93.4%)	59/61 (96.7%)
	2.25[b]	2.14[b]	2.19[b]
Escherichia coli	32/33 (97%)	32/33 (97%)	33/33 (100%)
Klebsiella pneumoniae	10/11 (90.9%)	11/11 (100%)	11/11 (100%)
Klebsiella variicola	4/5 (80%)	4/5 (80%)	3/5 (60%)
Enterobacter cloacae complex	4/4 (100%)	3/4 (75%)	4/4 (100%)
Citrobacter koseri	2/2 (100%)	2/2 (100%)	2/2 (100%)
Proteus mirabilis	2/2 (100%)	2/2 (100%)	2/2 100%)
Klebsiella oxytoca	1/2 (50%)	1/2 (50%)	2/2 100%)
Morganella morganii	1/1 (100%)	1/1 (100%)	1/1 100%)
Serratia marcescens	1/1 (100%)	1/1 (100%)	1/1 100%)
Inconclusive ID	4/61 (6.6%)	4/61 (6.6%)	0/61 (0%)
Discrepant ID	NA[a]	0/61 (0%)	2/61 (3.3%)
Pseudomonas spp.	7/7 (100%)	6/7 (85.7%)	6/7 (85.7%)
	2.24[b]	2.08[b]	2.14[b]
Pseudomonas aeruginosa	5/5 (100%)	4/5 (80%)	4/5 (80%)
Pseudomonas montelii	2/2 (100%)	2/2 (100%)	1/1
Pseudomonas sp.	NA[a]	NA[a]	1/1
Inconclusive ID	0/7 (3.3%)	1/7 (14.3%)	1/7 (14.3%)
Discrepant ID	NA[a]	0/7 (0%)	0/7 (0%)
Other Gram negatives	2/2 (100%)	1/2 (50%)	2/2 (100%)
	2.19[b]	2.03[b]	2.07[b]
Bacteroides fragilis	1/1 (100%)	0/1 (0%)	1/1 (100%)
Elizabethkingia anophelis	1/1 (100%)	1/1 (100%)	1/1 (100%)
Inconclusive ID	0/2 (0%)	1/2 (50%)	0/2 (0%)
Discrepant ID	NA[a]	0/2 (0%)	0/2 (0%)
Total Gram negative	66/70 (94.3%);	64/70 (91.4%);	67/70 (95.7%);
	2.24[b]	2.12[b]	2.16[b]

[a]NA, not applicable.
[b]Average MALDI score.
[c]Reference technique.

22/1165 (1.9%) for staphylococci and 110/114 (96.5%), 2/22 (9.1%), 2/83 (2.4%), and 0/114 (0%) for enterococci (Table 3). For Enterobacterales, rates of CA, VmajE, majE, and minE were 964/991 (97.3%), 16/152 (10.5%), 4/788 (0.5%), and 7/991 (0.7%), respectively, and 29/37 (78.4%), 0/1 (0%), 1/7 (14.3%), and 7/37 (18.9%) for *Pseudomonas* spp. (Table 4).

For DD, a total of 201 bacteria were analyzed, including 135 Gram-positive bacteria (113 staphylococci, 16 enterococci, 5 streptococci, and 1 *Micrococcus luteus*) and 66 Gram-negative bacteria (59 Enterobacterales, 5 *Pseudomonas* spp., 1 *B. fragilis*, and 1 *E. anophelis*). The percentages of CA, VmajE, majE, and minE were 434/436 (99.5%), 1/75 (1.3%), 1/361 (0.3%), and 0/436 (0%), respectively, for staphylococci; 85/89 (95.5%), 4/23 (17.4%), 0/59 (0%), and 0/89 (0%) for enterococci; 23/25 (92%), 2/3 (66.7%), 0/22 (0%), and 1/25 (4%) for streptococci; and 4/4 (100%), 0/1 (0%), 0/3 (0%), and 0/4 (0%) for *M. luteus* (Table 5). In Gram-negative bacteria, CA, VmajE, majE, and minE were 338/347 (97.4%), 1/26 (3.8%), 3/315 (0.9%), and 5/347 (1.4%), respectively, for Enterobacterales; 48/49 (98%), 0/2 (0%), 0/16 (0%), and 1/49 (2%), respectively, for *Pseudomonas* spp.; 6/6 (100%), 0/4 (0%), 0/2 (0), and 0/6 (0%), respectively, for other Gram-negative bacteria (i.e., *B. fragilis* and *E. anophelis*) (Table 6).

Furthermore, a complete overview of AST results is shown in Supplemental file S2-Table S4. Herein, for Gram-positive bacteria, the CA of the FAST LC in comparison to the routine procedure reached 96.1% and 98.4% for MicroScan and DD results,

TABLE 3 Automated AST results from MicroScan WalkAway of Gram-positive bacteria (staphylococci and enterococci) following the FAST System workflow as compared to our routine workflow[a]

Antibiotic	CA (%)	VmajE (%)	MajE (%)	MinE (%)
Staphylococci (*N* = 115)				
Clindamycin	105/115 (91.3)	2/40 (5)	3/70 (4.3)	5/115 (4.3)
Daptomycin	86/87 (98.9)	0/0 (NA)	1/87 (1.1)	0/87 (0)
Flucloxacillin	109/115 (94.8)	3/63 (4.8)	3/52 (5.8)	0/115 (0)
Fosfomycin	113/115 (98.3)	1/17 (5.9)	1/98 (1)	0/115 (0)
Gentamicin	108/114 ((94.7)	2/50 (4)	4/64 (6.3)	0/114 (0)
Levofloxacin	113/115 (98.3)	0/60 (0)	0/1 (0)	2/115 (1.7)
Linezolid	86/87 (98.9)	0/0 (NA)	1/87 (1.1)	0/87 (0)
Rifampicin	114/115 (99.1)	0/17 (0)	0/97 (0)	1/115 (0.9)
Teicoplanin	74/74 (100)	0/0 (NA)	0/74 (0)	0/74 (0)
Co-trimoxazole	99/115 (86.1)	0/18 (0)	2/81 (2.5)	14/115 (12.2)
Vancomycin	112/113 (99.1)	0/0 (NA)	1/113 (0.9)	0/113 (0)
Total	1119/1165 (96.1)	8/265 (3)	16/824 (1.9)	22/1165 (1.9)
Enterococci (*N* = 14)				
Ampicillin	14/14 (100)	0/5 (0)	0/9 (0)	0/14 (0)
Ciprofloxacin	14/14 (100)	0/6 (0)	0/8 (0)	0/14 (0)
Gentamicin	13/14 (92.9)	1/4 (25)	0/10 (0)	0/14 (0)
Imipenem	9/9 (100)	0/0 (NA)	0/0 (NA)	0/9 (0)
Levofloxacin	14/14 (100)	0/6 (0)	0/8 (0)	0/14 (0)
Linezolid	13/14 (92.9)	0/0 (NA)	1/14 (7.1)	0/14 (0)
Nitrofurantoin	8/8 (100)	0/0 (NA)	0/8 (0)	0/8 (0)
Teicoplanin	13/13 (100)	0/0 (NA)	0/13 (0)	0/13 (0)
Vancomycin	12/14 (85.7)	1/1 (100)	1/13 (7.7)	0/114 (0)
Total	110/114 (96.5)	2/22 (9.1)	2/83 (2.4)	0/114 (0)

[a]CA, categorical agreement; VmajE, number of very major errors encountered; majE, number of major errors encountered; minE, number of minor errors encountered; NA, not applicable.

respectively. Similar results were obtained for Gram-negative bacteria, with CA of 96.6% and 97.5% for MicroScan and DD, respectively (Supplemental file S2-Table S4).

However, a few discrepancies were also encountered. For MicroScan AST results of Gram-positive pathogens, a total of 50 errors were recorded. Most of the errors were minE (n = 22), detected mainly in *S. epidermidis* isolates with co-trimoxazole (13 discrepancies detected in 63 *S. epidermidis* tested) (Supplemental file S2-Table S1), followed by majE (n = 18) found mainly in *S. epidermidis* (three discrepancies with flucloxacillin, two with gentamicin, and two with co-trimoxazole detected in 63 *S. epidermidis* tested), and VmajE (n = 10) where gentamicin (n = 3) and flucloxacillin (n = 3) showed most discrepancies (Supplemental file S2-Table S1). When Gram-negative pathogens were tested, the majority of the 35 discrepancies were VmajE (n = 16) found mostly in coliform bacilli with fosfomycin (n = 5) being the most common discrepant substance, followed by minE (n = 14) and majE (n = 5) (Supplemental file S2-Table S1). Considering these results and to the best of our knowledge, our study is the first one combining the FAST LC with MicroScan WalkAway to assess automated AST.

For manual AST (DD), first, 53 discrepancies (21 for Gram positive and 32 for Gram negative) were found between the FAST System LC and our routine workflow based on direct inoculation DD of PBC, which was analyzed after 18 hours. Next, to clarify these errors, all isolates presenting these errors were re-analyzed by performing standard DD inoculated from grown colonies obtained after reinoculation of cryo-cultures on blood agar plates. It is important to highlight that 71.7% (38/53) of discrepant AST results were resolved, including 22 in Gram-negative bacteria and 16 in Gram-positive bacteria (Supplemental file S2-Table S2). Hence, the remaining discrepancies were for Gram-positive bacteria, seven VmajE mainly found in enterococci (n = 4) with gentamicin, tigecycline, and vancomycin, one majE in *S. epidermidis* with rifampicin, and one minE

TABLE 4 Automated AST results from MicroScan WalkAway of Gram-negative bacteria (Enterobacterales and *Pseudomonas* spp.) following the FAST System workflow as compared to our routine workflow[a]

Antibiotic	CA (%)	VmajE (%)	MajE (%)	MinE (%)
Enterobacterales (*N* = 54)				
Ampicillin	50/54 (92.6)	2/40 (5)	2/14 (14.3)	0/54 (0)
Amoxicillin-clavulanic acid	53/54 (98.1)	0/18 (0)	1/36 (2.8)	0/54 (0)
Piperacillin-tazobactam	52/54 (96.3)	2/7 (28.6)	0/42 (0)	0/54 (0)
Pivmecillinam	17/17 (100)	0/0 (NA)	0/17 (0)	0/17 (0)
Cefuroxime	48/53 (90.6)	2/12 (16.7)	0/0 (NA)	3/53 (5.7)
Cefotaxime	50/53 (94.3)	2/8 (25)	0/44 (0)	1/53 (1.9)
Ceftazidime	52/54 (96.3)	1/8 (12.5)	0/45 (0)	1/54 (1.9)
Cefepime	53/54 (98.1)	1/7 (14.3)	0/47 (0)	0/54 (0)
Ceftazidime-avibactam	6/6 (100)	0/0 (NA)	0/6 (0)	0/6 (0)
Ertapenem	54/54 (100)	0/1 (0)	0/53 (0)	0/54 (0)
Imipenem	51/51 (100)	0/1 (0)	0/50 (0)	0/51 (0)
Meropenem	54/54 (100)	0/1 (0)	0/53 (0)	0/54 (0)
Gentamicin	54/54 (100)	0/7 (0)	0/47 (0)	0/54 (0)
Tobramycin	53/54 (98.1)	1/8 (12.5)	0/46 (0)	0/54 (0)
Amikacin	54/54 (100)	0/0 (NA)	0/54 (0)	0/54 (0)
Ciprofloxacin	52/54 (96.3)	0/7 (0)	0/44 (0)	2/54 (3.7)
Levofloxacin	54/54 (100)	0/7 (0)	0/47 (0)	0/54 (0)
Co-trimoxazole	54/54 (100)	0/13 (0)	0/41 (0)	0/54 (0)
Fosfomycin	48/54 (88.9)	5/6 (83.3)	1/48 (2.1)	0/54 (0)
Nitrofurantoin	32/32 (100)	0/1 (0)	0/31 (0)	0/32 (0)
Trimethoprim	23/23 (100)	0/0 (NA)	0/23 (0)	0/23 (0)
Total	964/991 (97.3)	16/152 (10.5)	4/788 (0.5)	7/991 (0.7)
Pseudomonas spp. (*N* = 4)				
Piperacillin-tazobactam	4/4 (100)	0/0 (NA)	0/0 (NA)	0/4 (0)
Ceftazidime	4/4 (100)	0/0 (NA)	0/0 (NA)	0/4 (0)
Cefepime	4/4 (100)	0/0 (NA)	0/0 (NA)	0/4 (0)
Ertapenem	1/1 (100)	0/1 (0)	0/0 (NA)	0/1 (0)
Imipenem	3/4 (75)	0/0 (NA)	0/0 (NA)	1/4 (25)
Meropenem	4/4 (100)	0/0 (NA)	0/4 (0)	0/4 (0)
Tobramycin	3/4 (75)	0/0 (NA)	1/1 (100)	0/4 (0)
Amikacin	4/4 (100)	0/0 (NA)	0/0 (NA)	0/4 (0)
Ciprofloxacin	1/4 (25)	0/0 (NA)	0/1 (0)	3/4 (75)
Levofloxacin	1/4 (25)	0/0 (NA)	0/1 (0)	3/4 (75)
Total	29/37 (78.4)	0/1 (0)	1/7 (14.3)	7/37 (18.9)

[a]CA, categorical agreement; VmajE, number of very major errors encountered; majE, number of major errors encountered; minE, number of minor errors encountered; NA, not applicable.

in *S. mitis* with co-trimoxazole (Supplemental file S2-Table S3). While for Gram-negative bacteria, 10 discrepancies were found: one VmajE in *E. coli* with piperacillin-tazobactam; three majE in *E. coli* (*n* = 2) with piperacillin-tazobactam and ceftazidime-avibactam and in *K. variicola* (*n* = 1) with cefotaxime; and 6 minE in *E. coli*, *K. variicola*, *K. pneumoniae*, and *P. aeruginosa* with ceftazidime and ciprofloxacin (Supplemental file S2-Table S3).

In addition, the two yeasts species (*C. glabrata*), which were correctly identified using the FAST System LC, showed 14/16 (87.5%) CA and 1/16 (6.3%) minE found with itraconazole. The only resistant profile found with itraconazole using the reference method showed a discrepancy (1/1 VmajE) when compared to the AST result obtained via the FAST System (Table 7; Supplemental file S2-Table S4).

Time to results

The time to obtain ID and AST results using the FAST System LC was estimated in comparison to our routine workflow. Here, the time required to obtain correct MALDI

TABLE 5 Manual AST results from DD of Gram-positive bacteria (staphylococci, enterococci, streptococci, and *M. luteus*) following the FAST System workflow as compared to our routine workflow (DD)[a]

Antibiotic	CA (%)	VmajE (%)	MajE (%)	MinE (%)
Staphylococci (*N* = 113)				
Cefoxitin	108/108 (100)	0/59 (0)	0/49 (0)	0/108 (0)
Flucloxacillin	1/1 (100)	0/1 (0)	0/0 (NA)	0/1 (0)
Linezolid	112/112 (100)	0/2 (0)	0/110 (0)	0/112 (0)
Rifampicin	108/110 (98.2)	1/13 (7.7)	1/97 (1)	0/110 (0)
Vancomycin	105/105 (100)	0/0 (NA)	0/105 (0)	0/105 (0)
Total	434/436 (99.5)	1/75 (1.3)	1/361 (0.3)	0/436 (0)
Enterococci (*N* = 16)				
Ampicillin	15/15 (100)	0/8 (0)	0/7 (0)	0/15 (0)
Gentamicin	13/14 (92.9)	1/3 (33.3)	0/11 (0)	0/14 (0)
Imipenem	15/15 (100)	0/8 (0)	0/0 (NA)	0/15 (0)
Linezolid	14/14 (100)	0/0 (NA)	0/14 (0)	0/14 (0)
Tigecycline	14/15 (93.3)	1/1 (100)	0/14 (0)	0/15 (0)
Vancomycin	14/16 (87.5)	2/3 (66.7)	0/13 (0)	0/16 (0)
Total	85/89 (95.5)	4/23 (17.4)	0/59 (0)	0/89 (0)
Streptococci (*N* = 5)				
Clarithromycin	2/2 (100)	0/1 (0)	0/1 (0)	0/2 (0)
Clindamycin	4/4 (100)	0/0 (NA)	0/4 (0)	0/4 (0)
Erythromycin	3/5 (60)	2/2 (100)	0/3 (0)	0/5 (0)
Penicillin	5/5 (100)	0/0 (NA)	0/5 (0)	0/5 (0)
Co-trimoxazole	4/4 (100)	0/0 (NA)	0/4 (0)	1/4 (25)
Vancomycin	5/5 (100)	0/0 (NA)	0/5 (0)	0/5 (0)
Total	23/25 (92)	2/3 (66.7)	0/22 (0)	1/25 (4)
Micrococcus luteus (*N* = 1)				
Cefuroxime	1/1 (100)	0/0 (NA)	0/1 (0)	0/1 (0)
Ciprofloxacin	1/1 (100)	0/1 (0)	0/0 (NA)	0/1 (0)
Linezolid	1/1 (100)	0/0 (NA)	0/1 (0)	0/1 (0)
Meropenem	1/1 (100)	0/0 (NA)	0/1 (0)	0/1 (0)
Total	4/4 (100)	0/1 (0)	0/3 (0)	0/4 (0)

[a]CA, categorical agreement; VmajE, number of very major errors encountered; majE, number of major errors encountered; minE, number of minor errors encountered; NA, not applicable.

ID and MicroScan AST results by means of FAST System LC was significantly reduced by 1 day as compared to the routine workflow using culture-grown colonies. Hence, the average time for the FAST System LC to obtain ID results was 1.11 (±0.16) hours. While for the workflow of the reference method using overnight culture in our laboratory, the average time was 27.4 (±7.30) hours. This difference is statistically significant ($P < 0.05$). In contrast, the difference between FAST and Sepsityper is not significant. For MicroScan AST, the average times for Gram-positive pathogens were 19.3 (±2.55) and 45.1 (±5.14) hours for FAST System LC and culture-grown colonies, respectively. Similarly, for Gram-negative pathogens, 22.2 (±2.6) and 44.5 (±3.5) hours were necessary to obtain MicroScan AST results, respectively, for FAST System workflow and culture-grown colonies. Both differences were statistically significant ($P < 0.05$). For DD AST, the turnaround time for both Gram positive and Gram negative was comparable, and there was no significant difference between FAST System and direct inoculation (Fig. 1).

DISCUSSION

One of the most important objectives in clinical microbiology is to provide a rapid and accurate microbiological diagnosis of BSI. Thus, tools to accelerate PBCs processing are urgently needed. Many of those tools are based on syndromic nucleic acid amplification such as multiplex PCR, which provides good sensitivity but are usually limited to a preselected panel of pathogens. Furthermore, such molecular techniques are limited by

TABLE 6 Manual AST results from DD of Gram-negative bacteria (Enterobacterales, *Pseudomonas* spp., *B. fragilis*, and *E. anophelis*) following the FAST System workflow as compared to our routine workflow (DD)[a]

Antibiotic	CA (%)	VmajE (%)	MajE (%)	MinE (%)
Enterobacterales (*N* = 59)				
Piperacillin-tazobactam	56/58 (96.6)	1/8 (12.5)	1/50 (2)	0/59 (0)
Cefotaxime	57/58 (98.3)	0/7 (0)	1/51 (2)	0/58 (0)
Ceftazidime	56/59 (94.9)	0/4 (0)	0/52 (0)	3/59 (5.1)
Ceftazidime-avibactam	56/57 (98.2)	0/0 (NA)	1/57 (1.8)	0/57 (0)
Meropenem	58/58 (100)	0/0 (NA)	0/57 (0)	0/58 (0)
Ciprofloxacin	55/57 (96.5)	0/7 (0)	0/48 (0)	2/57 (3.5)
Total	338/347 (97.4)	1/26 (3.8)	3/315 (0.9)	5/347 (1.4)
Pseudomonas spp. (*N* = 5)				
Piperacillin-tazobactam	5/5 (100)	0/0 (NA)	0/0 (NA)	0/5 (0)
Cefiderocol	2/2 (100)	0/0 (NA)	0/2 (0)	0/2 (0)
Ceftazidime	5/5 (100)	0/0 (NA)	0/0 (NA)	0/5 (0)
Cefepime	5/5 (100)	0/0 (NA)	0/0 (NA)	0/5 (0)
Ceftazidime-avibactam	2/2 (100)	0/0 (NA)	0/2 (0)	0/2 (0)
Ceftolozane-tazobactam	2/2 (100)	0/0 (NA)	0/2 (0)	0/2 (0)
Imipenem	5/5 (100)	0/1 (0)	0/0 (NA)	0/5 (0)
Meropenem	5/5 (100)	0/1 (0)	0/2 (0)	0/5 (0)
Tobramycin	4/4 (100)	0/0 (NA)	0/4 (0)	0/4 (0)
Amikacin	4/4 (100)	0/0 (NA)	0/4 (0)	0/4 (0)
Ciprofloxacin	5/6 (83.3)	0/0 (NA)	0/0 (NA)	1/6 (16.7)
Levofloxacin	4/4 (100)	0/0 (NA)	0/0 (NA)	0/4 (0)
Total	48/49 (98)	0/2 (0)	0/16 (0)	1/49 (2)
Other Gram-negatives (B. fragilis [*n* = 1], and E. anophelis [*n* = 1])				
Penicillin	1/1 (100)	0/1 (0)	0/0 (NA)	0/1 (0)
Piperacillin-tazobactam	2/2 (100)	0/1 (0)	0/1 (0)	0/2 (0)
Clindamycin	1/1 (100)	0/0 (NA)	0/1 (0)	0/1 (0)
Meropenem	1/1 (100)	0/1 (0)	0/0 (NA)	0/1 (0)
Ciprofloxacin	1/1 (100)	0/1 (0)	0/0 (NA)	0/1 (0)
Total	6/6 (100)	0/4 (0)	0/2 (0)	0/6 (0)

[a]CA, categorical agreement; VmajE, number of very major errors encountered; majE, number of major errors encountered; minE, number of minor errors encountered; NA, not applicable.

the types of organisms and resistance mechanisms included in the panel. In this study, we examined an alternative approach to accelerating culture-based processing. The FAST System generates a LC after an approximative run of 24 minutes by purifying the pathogens directly from the positive blood culture. Here, IDs (by MALDI-TOF MS) and AST (by MicroScan WalkAway *plus* and disk diffusion) results were analyzed and compared to

TABLE 7 Micronaut AST results of *C. glabrata* following the FAST System workflow as compared to our routine workflow[a]

Antibiotic	CA (%)	VmajE (%)	MajE (%)	MinE (%)
C. glabrata (*N* = 2)				
Amphotericin	2/2 (100)	0/0 (NA)	0/2 (0)	0/2 (0)
5-Fluorocytosin	2/2 (100)	0/0 (NA)	0/2 (0)	0/2 (0)
Fluconazole	2/2 (100)	0/0 (NA)	0/2 (0)	0/2 (0)
Voriconazole	2/2 (100)	0/0 (NA)	0/2 (0)	0/2 (0)
Micafungin	2/2 (100)	0/0 (NA)	0/2 (0)	0/2 (0)
Anidulafungin	2/2 (100)	0/0 (NA)	0/2 (0)	0/2 (0)
Caspofungin	2/2 (100)	0/0 (NA)	0/2 (0)	0/2 (0)
Itraconazole	0/2 (0)	1/1 (100)	0/0 (NA)	1/2 (50)
Total	14/16 (87.5)	1/1 (100)	0/14 (0)	1/16 (6.3)

[a]CA, categorical agreement; VmajE, number of very major errors encountered; majE, number of major errors encountered; minE, number of minor errors encountered; NA, not applicable.

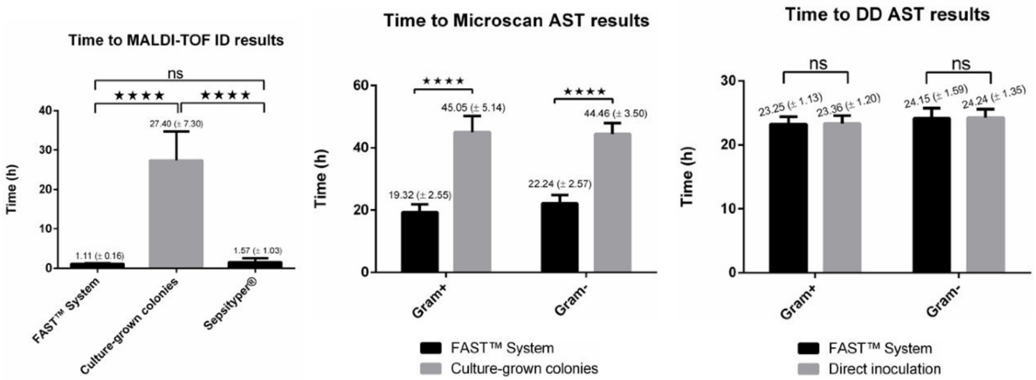

FIG 1 Average time required to obtain ID and AST results (by MicroScan WalkAway *plus* and direct-inoculation DD) using different methods: FAST System, Sepsityper, and culture-grown colonies.

those obtained via a culture-based routine workflow. By using the FAST System LC, correct and accurate ID results were achieved with a concordance of 96.9% as compared to the reference technique (culture-grown colonies). A concordance rate of 82.74% was achieved with Sepsityper. Similar results with Sepsityper were previously reported by Morgenthaler and Kostrzewa with 80% (n = 3320) species ID (17). Regarding the performance of the FAST System in terms of ID, Grinberg and colleagues reported an ID concordance of 94% (n = 201) using MALDI Biotyper as compared to colonies obtained by subculture (18). Similarly, Verroken and colleagues reported 89.5% (n = 266) concordant ID results using the FAST System LC and MALDI Biotyper including 80 blood cultures bottles spiked with multidrug-resistant bacteria (19). Likewise, Kuo and colleagues demonstrated an accurate ID of 94.1% (272/289) using LC generated by the FAST System and MALDI-TOF MS Microflex (20). Fifty-four of the 272 samples corresponded to initially negative blood culture bottles which were seeded with key organisms (e.g., *Streptococcus agalactiae*, *Streptococcus pyogenes*, *Klebsiella* spp., *Proteus mirabilis*, *Enterobacter* spp., *Escherichia coli.*, etc.) in order to have a more diverse and larger set of bacteria with highly resistant patterns. All 54 samples (100%) were correctly identified with the LC suspension. Hence, by using combined prospective and spiked data set, they demonstrated that a wide array of clinically relevant Gram-positive and Gram-negative bacteria were identifiable using the LC suspension produced by the FAST System (19, 20).

In our study, FAST-DD led to slightly better results (CA of 98.7% for Gram positive and 97.5% for Gram negative) when compared to directly inoculated DD from PBC, which are part of our routine workflow, than the automated method using FAST-MicroScan (CA of 96.1% for both Gram positive and Gram negative). Comparable CA values were previously reported for both Gram-positive [97.4% (18), 97.7%–100% (19), and 99.5% (20)] and Gram-negative bacteria [98.5% (18), 97.8%–99% (19), and 97.8% (20)] where automated AST systems other than MicroScan WalkAway (i.e., bioMérieux Vitek 2 or BD Phoenix) were utilized. Given these previous studies, our study though is the first to use MicroScan WalkAway demonstrating the compatibility of this automated AST technology with the FAST System. Nevertheless, a few VmajE and majE were reported, which could have a clinical impact on patient treatment, especially for VmajE, where a patient might be treated with antibiotics that are supposed to be sensitive when they are resistant to that treatment. Most VmajE and majE were detected for staphylococcal and Enterobacterales species, which are also the most frequently detected pathogen groups, without any noticeable error accumulation for specific substances tested among those

groups (for details refer to Results: AST comparison or Supplemental material S2-Tables S1 to S3).

An important limitation of the FAST System is the lacking ability to process polymicrobial PBC. This has to be taken into account, since polymicrobial BSIs are considerably detected with a range of 6%–32% of all diagnosed BSI (21, 22). Another limitation can be seen in the fact that a single instrument can only process two PBC cartridges simultaneously. Therefore, for potential implementation in a routine workflow, one would consider having multiple instruments or selecting urgent samples (e.g., an ICU patient) that should be prioritized for processing. Only one instrument was utilized during the period of this study.

In addition, several limitations of our study are offered for consideration. First, it was a monocentric analysis with a moderate sample size. Second, the number of some pathogens (e.g., yeast) obtained during the investigation was too low to draw meaningful conclusions. Hence, we can simply rely on this limited number of samples, which limits the impact of the described findings. It is important to also mention that the FAST Prep cartridges utilized in this study were designed, primarily, for bacteria only. However, the results obtained with yeasts indicate a potential application to yeast samples, as well. Therefore, it would be interesting to perform additional investigations on a larger number of yeast samples to accurately evaluate the performance of the FAST System LC for yeast ID and AFST.

In summary, the FAST System LC enables a turnaround time reduction of approximately 24 hours for conclusive ID results. In addition, MicroScan AST results could be obtained with a reduction time of 24 hours from PBCs with similar accuracy compared to subculture-based reference techniques. The system connects well with Bruker Biotyper MALDI-TOF MS and MicroScan WalkAway *plus,* as it provides enough biomass to perform ID and inoculate DD and MicroScan AST plates. Our findings and those made in previous studies indicate that the FAST System LC approach is capable of delivering more timely results that can improve the outcomes of patients suffering from BSI.

ACKNOWLEDGMENTS

The FAST System and consumables (FAST PBC Prep cartridges) for this study were provided by Qvella (Richmond Hill, Canada). Qvella had no influence on evaluation and data interpretation.

The authors did not receive any honoraria.

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ADDITIONAL FILES

The following material is available online.

Supplemental Material

Supplemental file S1-Table S1 (JCM00569-23-s0001.pdf). AST results in the Area of technical uncertainty (ATU)
Supplemental file S2-Tables S1-S4 (JCM00569-23-s0002.pdf). Discrepant AST results and overview of global AST results

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VI
DISCUSSION

6. Discussion

An accurate diagnosis of infections such as those caused by helminthic parasites or severe BSI caused by bacteria and fungi is a major challenge for clinical microbiology laboratories (Elsheikha, 2014; Song, Neff and Gyarmati, 2022). Standard diagnostic methods, mainly based on microscopy and/or culture, often require time-consuming and laborious techniques such as e.g. blood cultures (1-7 days of incubation) for the detection of BSI, the Koga-agar plate method for detection of specific helminths (requiring at least 48 h of incubation), the Kato-Katz thick smear or stool concentration and/or filtration for the detection of STH eggs, (Glinz *et al.*, 2010; B. Speich *et al.*, 2014; Kristanti *et al.*, 2018; Lamy *et al.*, 2020). Other methods such as serology and, to a lesser extent, molecular methods (e.g., PCR) are available, but their utilization is still limited due to their low sensitivity and/or expensive prices (Feucherolles *et al.*, 2019a).

The introduction of MALDI-TOF MS in routine microbiology laboratories has revolutionized the field, particularly in HICs where it is implemented in many routine microbiology laboratories, enabling rapid, reliable, and cost-effective bacterial identification (Seng *et al.*, 2009b). Indeed, this technology, based on measured spectra of protein profiles in comparison to reference databases, enables microorganisms identification at the species level within seconds on a variety of clinical samples (Feucherolles *et al.*, 2019a). However, the availability of databases containing species-specific spectra is crucial to obtain reliable results.

6.1. Enhancing helminthic parasite detection by MALDI-TOF MS

As no helminth-specific commercial database has yet been released, it is currently necessary to implement "in-house" libraries for helminth identification by MALDI-TOF. With the commercial MALDI-TOF MS platforms already commercially available (e.g., Bruker MALDI Biotyper), it is possible to include further MSPs (reference spectra) to expand the database by adding newly created spectra and/or libraries generated from various organisms. Hence, once the protein spectra of a new helminth isolate of the same species are subjected to the newly expanded database, they can easily be examined for identification (Sy, Conrad and Becker, 2022).

In this thesis, we investigated and confirmed the applicability of MALDI-TOF MS in helminth species identification through three research articles presented in **section V** (**results: articles n°1, 2, and 3**). In fact, with various helminth species (*Fasciola* spp., *Schistosoma* spp., and *T. saginata*) belonging to different classes (trematodes, and cestodes), we demonstrated in different scenarios (**i**) the applicability of MALDI-TOF MS in identifying adult parasites (*Fasciola* spp., *Schistosoma* spp., and *Taenia* spp.); (**ii**) and we evaluated the effect of different conservation media (water, ethanol, formalin, and water) on *T. saginata* proglottids for MALDI-TOF identification.

Unlike bacteria and fungi, helminths are multicellular organisms having different developmental stages (eggs, larvae, and adults). The application of MALDI-TOF MS for helminth identification is a newly

developing field, and only a few studies have been published in this research context. Most of these pertain to the investigation of specific helminth biomolecules, adults and/or larval stages (Feucherolles *et al.*, 2019a; Sy, Conrad and Becker, 2022). In clinical settings, many helminthic parasites, especially those living in the intestines (e.g., STH) or mesenteric veins (e.g., schistosomes), are most easily accessible through detection of their eggs [i.e., they are expelled with feces (e.g., *Ascaris*, hookworms, *S. mansoni* etc.) or urine (e.g., *S. haematobium*)] (Gryseels *et al.*, 2006; Loukas *et al.*, 2016; Else *et al.*, 2020). However, we started to work on adult worms as a prerequisite for subsequent identification of eggs.

The scarcity of data on the detection of helminth parasite eggs investigation by MALDI-TOF MS can be explained by various limiting factors, such as the limited MALDI-TOF devices and specially trained staff in endemic areas of LMIC (Sy, Conrad and Becker, 2022); also, the complexity of biological samples that need specific protocols for accurate processing (e.g., stool) constitute additional challenges (Feucherolles *et al.*, 2019b). Indeed, for egg-positive stool samples, the creation of egg-specific spectra would require a preliminary egg purification step to isolate the eggs from the stool debris (Sy, Conrad and Becker, 2022). Additionally, the lack of commercial platforms including helminth databases, and the absence of standardized protocol for sample preparation play a major role in slowing down the wider use or application in routine microbiology laboratories (Feucherolles *et al.*, 2019a; Sy, Conrad and Becker, 2022).

To overcome these limitations, the following aspects should receive particular attention and be further investigated:

- Development, optimization, and standardization of simple, and reproducible protocols for sample preparation from various matrices (stool samples, isolated parasites, etc.), as well as critical program parameters associated with instrument optimization (i.e., frequent maintenance) to improve spectral quality, as demonstrated for other organisms (Lau *et al.*, 2019; Nellessen and Nehl, 2023).
- Thorough assessments of the impact of prolonged sample storage on the quality of MALDI-TOF spectra with various storage solutions (ethanol, water, saline, formalin, etc.), and storage conditions (ambient temperature, fridge, freezer, etc.).
- Implementation of helminth-specific databases with many possible spectra generated from various samples at different developmental stages and from different geographical locations in order to cover possible intra-species variations that could be reflected in protein spectra profiles (Sy, Conrad and Becker, 2022).
- Strengthen collaboration between endemic regions in LMIC and non-endemic areas in HICs (where MALDI-TOF MS devices are routinely available in microbiology laboratories and staff is well-trained to work with these machines) for MALDI-TOF MS training, capacity building

and elaboration of research studies aimed at investigating the possible utility of MALDI-TOF MS in-depth directly on site with a large number of samples.

- Intensified research activities on this topic in order to generate funding opportunities and, subsequently, encourage the inclusion of helminth-specific spectra into commercial databases. This would expedite the diagnostic process by avoiding the time-consuming step of implementing an in-house library. As MALDI-TOF MS devices are increasingly being introduced in laboratories of LMIC, while the experience regarding the microscopic recognition of parasite eggs in HIC is waning, this could become a major advantage for improved diagnostics across these heterogenous groups of settings.

Overall, despite the promising results pertaining to the applicability of MALDI-TOF MS for helminths identification using protein profiling approaches (Schubert and Kostrzewa, 2015; Yssouf *et al.*, 2016; Solntceva, Kostrzewa and Larrouy-Maumus, 2020; Li *et al.*, 2022), a series of limitations associated with the herein presented MALDI-TOF studies are offered for consideration:

(i) The limited number of samples and the lack of diversity of sample origin (i.e., samples tested for database validation were isolated at the same location as the samples used to create the database, also only adult worms were tested), which could explain the sometimes lower LSVs, especially regarding the species level. Indeed, pathogens belonging to the same species from different geographical areas might differ in specific characteristics. Hence, a more diverse, complete, and representative spectral database could enable better ID results (Yssouf *et al.*, 2016).

(ii) Thus far, not all types of clinically relevant biological samples have been tested. Indeed, applying MALDI-TOF MS to e.g. urine or serum from infected individuals in the case of schistosomiasis would be the next important step on the way to clinical application and validation. (Feucherolles *et al.*, 2019b). With such samples (i.e., non-purified and containing proteins and other molecules from host body fluids), the approach would certainly be different than the one used in the proof-of-concept study (i.e., implementing an in-house database including MALDI-spectra generated from isolated worms) as protein spectra generated by MALDI-TOF MS will also display proteins patterns stemming of the host. To overcome these limitations and to test the performance of MALDI-TOF MS on clinical samples, it would require more advanced investigations aiming to detect, e.g. specific biomarkers (discriminant peaks) representative of parasite-secreted antigens or other patterns characterizing the infectious status, which enable discriminating infected from non-infected groups (Robijn *et al.*, 2008). However, challenges related to the variability of antigenic infectious markers, of which the most studied are circulating cathodic antigens (CCA) or circulating anodic antigens (CAA) (Van 't Wout *et al.*, 1995) in the case of schistosomiasis, should be considered. Nevertheless, special pattern characteristics of the infectious status (e.g., over or under-expression due to up

or down-regulation of the secretion of certain proteins like collagen isoforms, cytokeratin 18, hydroxyproline) could still be observed in the serum (Manivannan *et al.*, 2011). Hence, once these variations are reflected on the spectra profiles, comparison analysis with spectra profiles from sera of healthy patient could allow the discrimination between infected and non-infected groups as previously reported in animal experiments (Huang *et al.*, 2016, 2020).

6.2. Clinical application of new tools for detection of BSI-causing pathogens

Blood cultures are normally incubated for up to 7 days, even though most blood cultures in septic patients turn positive within 24 hours (Quirós *et al.*, 1996; Ransom *et al.*, 2021). It would be a major step forward to have a technique that is capable of avoiding this pre-incubation step. While promising techniques (e.g. direct detection of cell-free DNA in peripheral blood samples) are on the horizon, these are not yet available for daily practice in routine diagnostics (Dubourg, Raoult and Fenollar, 2019). In the meantime, it is important to shorten the subsequent steps once the blood culture has turned positive. While in the past, species identification and susceptibility testing took at least some additional 24 hours, this could now be substantially reduced by e.g. the Bruker SepsiTyper for direct identification from positive blood culture broths (Lagacé-Wiens *et al.*, 2012; Chen *et al.*, 2013). The herein evaluated FAST™ system is an alternative, which might be applicable in laboratories that are e.g. not using the SepsiTyper technique (Lamy *et al.*, 2020).

Recently, an innovative automated system (FAST™ System) for purification and isolation of microbial suspension from PBC has been released (Grinberg *et al.*, 2022; Kuo *et al.*, 2022). In this work, we evaluated the performance of MALDI-TOF MS as well as automated (MicroScan WalkAway) and manual (disk diffusion) AST for species identification and AST using the generated FAST™ System-Liquid colony. Compared with standard methods, ID and AST results obtained with FAST™ System-LC were concordant, proving the usefulness of this alternative method. In addition, this method reduces the time needed to obtain ID and AST results (see **section V**, **published articles: article n°5**). Thus, added to other automated systems already commercialized (e.g., Biofire, FilmArray, etc.), the FAST™ System represents an additional method offering an alternative option for improving and speeding up the diagnosis of BSI. Nevertheless, reported ID and AST results obtain with the FAST™ system have showed a few discrepancies when compared to standard method. Also, it does not allow the analysis of polymicrobial blood cultures (Sy *et al.*, 2023).

When compared to other direct methods such as Biofire (Altun *et al.*, 2013; Payne *et al.*, 2018), which target preselected molecular markers, the FAST™ System offers greater flexibility in that it can rapidly generate enough microbial suspension enabling ID and resistance detection in combination with automated and/ or manual systems (e.g., MALDI-TOF MS, MicroScan WalkAway, disk diffusion, BD Phoenix, etc.) (Grinberg *et al.*, 2022; Kuo *et al.*, 2022; Verroken *et al.*, 2022; Bonaiuto *et al.*, 2023; Maddalena *et al.*, 2023). Thus, with the microbial suspension obtained within few minutes, it is possible

to test any antibiotic using viable microorganisms. In fact, despite the tremendous benefits brought by these innovative automated systems such as Biofire (i.e., less hands-on-time, more rapid ID and resistance detection, enabling more timely and effective treatment, which could impact positively the outcome of the patient by reducing the time of hospital stay, and/or the mortality rates) (Devrim *et al.*, 2024), it is known that automated systems used for BSI identification resistance detection can lead to false-positive results (bioMérieux, 2024). For example, the detection of non-viable microorganisms and/or nucleic acids of *Enterococcus*, *P. aeruginosa*, or *Proteus* were reported when using the BioFire® FilmArray® BCID panel. Moreover, Gram staining results may differ from the BioFire® FilmArray® BCID panel results. Hence, the standard grown colonies-based methods are still necessary in such cases whenever discrepancies occur (bioMérieux, 2024).

The use of methods allowing for direct identification from PBCs, followed by rapid AST and appropriate treatment, offers many advantages, such as improved 30-day mortality, reduced hospital stay in adults, etc. (Bhavsar, Dingle and Hamula, 2018). However, these clinical impacts are only enhanced when antibiotic stewardship policies are associated (Beganovic, Costello and Wieczorkiewicz, 2017; Niwa *et al.*, 2019). Indeed, it has been shown during studies pertaining to Gram-positive cocci studies using a FISH assay, that the absence of an antibiotic stewardship program does not reduce the time to appropriate treatment (Cosgrove *et al.*, 2016). In contrast, the combination of the FilmArray BCID Panel and real-time antimicrobial stewardship recommendations significantly reduced the median time to optimal antibiotic therapy and time of exposure to broad-spectrum antimicrobials with a reduced prescription rate of unnecessary antibiotics (Messacar *et al.*, 2017) as well as the length of the hospital stay (Ray *et al.*, 2016). However, a more recent study reported by Agnetti and colleagues (Agnetti *et al.*, 2023) showed that only the time of optimal antimicrobial therapy (OAT) (i.e., the time to determine the most narrow-spectrum antimicrobial for the identified microorganism) was reduced by 20 h; but no impact on the time to effective treatment, length of hospital stay, admission to ICU, or on mortality was observed. Hence, further studies are warranted, and these must not be limited to diagnostic facilities, but should include and specifically address the communication with the treating clinical departments.

The major limitation of the FAST™ System is the lack of ability to process polymicrobial PBC. Indeed, the number of polymicrobial BSI may range between 6 and 32% of all diagnosed BSI, depending on the setting (Lin *et al.*, 2010; Bouza *et al.*, 2013). Another shortcoming is the limited throughput of samples processed in daily routine. A single instrument can only process two samples per run. Hence, the use of a single instrument could slow down the laboratory's routine workflow if other instruments or methods are not utilized in parallel. Furthermore, all studies reporting the use of MALDI-TOF MS combined with the FAST™ System were monocentric studies (Grinberg *et al.*, 2022; Kuo *et al.*, 2022; Verroken *et al.*, 2022; Bonaiuto *et al.*, 2023; Maddalena *et al.*, 2023; Sy *et al.*, 2023). A multicentric study with a larger dataset including data from various locations would certainly be more interesting to

investigate enabling a precise assessment of the real impact and performance of this MALDI and FAST™ System approach, particularly for samples like yeasts (Sy *et al.*, 2023).

6.3. Future perspectives

In a broader perspective to combat infectious diseases and in alignment with the United Nations Sustainable Development Goals (SDGs), improving the accurate diagnosis of helminth infections is an important aspect in the global strategy to control and, subsequently, eliminate NTDs. Indeed, WHO guidelines and global strategies such as the NTD roadmap 2021-2030 recommend diagnostic tools with improved sensitivity, because much progress has been made through the use of PC and improved hygiene (WHO, 2021b). Hence, many infections are now of only low intensity and the currently available diagnostic tools thus significantly underestimate the 'true' prevalences (WHO, 2021a; Lo *et al.*, 2022). Besides MALDI TOF-based diagnostic tools, also field-deployable techniques should be further investigated for their potential applicability to helminth diagnostics.

In addition to detection methods, control and elimination programs should more comprehensively consider integrated approaches combining improvement of diagnostic tools, especially their sensitivity and specificity; and also, their multiplex capacity (i.e., ability to detect many species simultaneously), drugs and vaccines development, as well as better hygiene and sanitation conditions, and reduction of poverty to prevent infection and/or re-infection (WHO, 2021a).

Digitalization, artificial intelligence (AI) or machine learning (ML) have great potential for future applications in resource-constrained settings of the Global South (Wang *et al.*, 2019; Burns, Rhoads and Misra, 2023). Ample evidence has shown that AI or ML associated with MALDI spectral data analysis can further enhance the accuracy, rapidity, reproducibility, and effectiveness of microbiological diagnostics (Goodswen *et al.*, 2021). Hence, AI or ML can reinforce the MALDI capacity in the analysis of blood culture and helminth samples, especially for complex samples like stool, or blood (Goodswen *et al.*, 2021; Komorowski *et al.*, 2022; Yu *et al.*, 2023). Indeed, by training AI or ML algorithms on MALDI spectra data generated from infected and non-infected samples, subsequent comparison analysis may enable distinguishing between different classes (infected vs non-infected). Further, such results can be saved, and the trained data utilized as a model to be validated for future tests. This has been reported for different types of samples (e.g., saliva, serum, etc.) and for various diseases (e.g., COVID-19, cancer, etc.) (Park *et al.*, 2019; Yan *et al.*, 2021; Costa *et al.*, 2022; Zambonin and Aresta, 2022).

Apart from the MALDI-TOF MS analysis based on intracellular biomolecules, mainly ribosomal proteins (Solntceva, Kostrzewa and Larrouy-Maumus, 2020), recent evidence has shown that MALDI-TOF MS analyses are not restricted to proteins. Other substances such as lipids can also be utilized for MALDI-TOF MS analysis (Solntceva, Kostrzewa and Larrouy-Maumus, 2020). Reported studies using

lipid-specific biomarkers have demonstrated their practical use in the diagnosis of certain bacterial species (Leung *et al.*, 2017), and further research is warranted on other pathogen classes.

In summary, this thesis investigated and proved the capacity of MALDI-TOF MS as an innovative technology to improve the diagnosis of helminthic parasites and BSI in different scenarios. These findings reveal that MALDI-TOF MS is a rapid and powerful microbiological diagnostic tool with multiple applications (e.g., in bacteriology, parasitology, etc.) and various functionalities enabling the identification of different types of samples such as helminth parasites or PBCs, and can also be combined with other tools (ML algorithms, FAST™ System, MicroScan WalkAway, etc.) for better performances or more advanced applications.

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Publications and conferences

- **Publications (research and review articles published in peer-reviewed journals)**

1) **Sy I**, Margardt L, Ngbede EO, Adah MI, Yusuf ST, Keiser J, Rehner J, Utzinger J, Poppert S, Becker SL. Identification of Adult *Fasciola* spp. Using Matrix-Assisted/Laser Desorption Ionization Time-of-flight (MALDI-TOF) Mass Spectrometry. *Microorganisms*. **2021**; 9(1):82. https://doi.org/10.3390/microorganisms9010082 (**Research article**)

2) Ebersbach JC, Sato MO, de Araújo MP, Sato M, Becker SL, **Sy I**. Matrix-Assisted laser desorption/ionization time-of-flight mass spectrometry for differential identification of adult *Schistosoma* worms. *Parasit Vectors.* **2023;** 16(1):20. https://doi.org/10.1186/s13071-022-05604-0 (**Research article**)

3) Wendel TP, Feucherolles M, Rehner J, Poppert S, Utzinger J, Becker SL, **Sy I**. Evaluating Different Storage Media for Identification of *Taenia saginata* Proglottids Using MALDI-TOF Mass Spectrometry. *Microorganisms*. **2021**; 9(10):2006. https://doi.org/10.3390/microorganisms9102006 (**Research article**)

4) **Sy I**, Conrad L, Becker SL. Recent Advances and Potential Future Applications of MALDI-TOF Mass Spectrometry for Identification of Helminths. *Diagnostics*. **2022**; 12(12):3035. https://doi.org/10.3390/diagnostics12123035 (**Review article**)

5) **Sy I,** Bühler N, Becker SL, Jung P. Evaluation of the Qvella FAST™ System and the FAST-PBC cartridge for rapid species identification and antimicrobial resistance testing directly from positive blood cultures. *J Clin Microbiol*. **2023**; 61(10):e00569-23. https://doi.org/10.1128/jcm.00569-23 (**Research article**)

6) Abdrabou AMM, **Sy I**, Bischoff M, Arroyo MJ, Becker SL, Mellmann A, von Müller L, Gärtner B, Berger FK. Discrimination between hypervirulent and non-hypervirulent ribotypes of *Cloistridioides difficile* by MALDI-TOF mass spectrometry and machine learning. *Eur J Clin Microbiol Infect Dis*. 2023; 42(11):1373-1381. https://doi.org/10.1007/s10096-023-04665-y (**Research article**)

7) Ngbede EO, **Sy I**, Akwuobu CA, Nanven MA, Adikwu AA, Abba PO, Adah MI, Becker SL. Carriage of linezolid-resistant enterococci (LRE) among humans and animals in Nigeria: coexistence of the *cfr*, *optrA*, and *postA* genes in *Enterococcus faecium* of animal origin. *J Glob Antimicrob Resist*. **2023**; 34:234-239. https://doi.org/10.1016/j.jgar.2023.07.016 (**Research article**)

- **Conferences (Posters and Oral presentations)**

1) Conrad L, Ngbede EO, Adah MI, Yusuf ST, Becker SL, and **Sy I.** Application of Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry for identification of adult tapeworms: Moniezia spp. and Thysaniezia spp. The 30th meeting of the German Society for Parasitology (DGP). **15-17 March 2023**. Gießen, Germany. (**Poster presentation**)

2) **Sy I,** Bühler N, Becker SL, and Jung P. Prospective evaluation of the Qvella FAST™ System and the FATS PBC Prep cartridge for species identification and antimicrobial resistance detection in positive blood cultures. The 74th annual meeting of the German Society for Hygiene and Microbiology e.V (DGHM). **05-07 September 2022**, Berlin, Germany. (**Oral presentation**)

3) Ebersbach JC, Sato MO, de Araújo MP, Kirinoki M, Sato M, Becker SL, and **Sy I.** MALDI-TOF MS for differential identification of adult *Schistosoma* worms: diagnostic accuracy and effects of storage media on the spectra profiles. The 74th annual meeting of the German Society for Hygiene and Microbiology e.V (DGHM). **05-07 September 2022**, Berlin, Germany. (**Poster presentation**)

4) **Sy I.** Online symposium entitled "**What's up MALDI-TOF Mass Spectrometry in Microbiology**" by the Luxembourg Institute of Science and Technology (LIST). **15-16 April 2021**→ published in [REPLAY] Day 2 |"What's up with MALDI-TOF Mass Spectrometry in Microbiology?" | 2021 (youtube.com) (from 1:16:08). (**Oral presentation**)

5) **Sy I.,** Wendel TP, Feucherolles M, Nimmesgern A, Stuermann A, Endriss Y, Utzinger J, Poppert S, and Becker SL. Application of MALDI-TOF MS for identification of helminths in clinical samples. The 30th European Congress of Clinical Microbiology and Infectious Diseases (ECCMID). **17-21 April 2020**, Paris, France. (**abstract accepted for Poster presentation → published in ECCMID 2020 abstract Book**)

Curriculum Vitae

For data protection reasons, the curriculum vitae is not included in the electronic version of the dissertation.

Acknowledgments

First and always, I must thank الله (**God**) S.W.T, the Greatest, Who has graced me with His blessings. For sustaining me throughout this project, and for putting in my way the people who have lifted me up and contributed in one way or another to the completion of this work.

I extend my heartfelt thanks to my supervisor, and head of the Institute of Medical Microbiology and Hygiene (IMMH), **Prof. Dr. Dr. Sören L. Becker,** who gave me the opportunity to work in his laboratory and for closely supervising this present work. Thank you for your wise counsel, advice and steady guidance.

I would like to thank all my colleagues from the research team of IMMH: **Prof. Markus Bischoff, Dr. Philipp Jung, Dr. Gubesh Gunaratnam, Dr. Mohamed Elhawy, Dr. Ahmed Mostafa Abdrabou, Ben Wieland, Jacqueline Rehner, Judith Fürstenberg, Tabea Wendel, Jurena Ebersbach, and Lucie Conrad.**

I also thank all the laboratory members from the diagnostic team of IMMH, especially **Dr. Sophie Schneitler, Dr. Alexander Halfmann, Dr. Franziska Roth, Sophie Müller, Anna Nimmesgern, Nina Bühler, Diana Velten, Lena Margardt, Anika Berndt, Julian Haupenthal, Martina Birke, Monika Schmitt, Sandra Rauch.**

I thank all the collaboration partners: **Prof. Jürg Utzinger, Prof. Brama Koné, Dr. Maureen Feucherolles, Dr. Kibafori D. Silué, Dr. Jean Tenena Coulibaly, Dr. Marcello Otake Sato, Dr. Emmanuel Ngbede, Fateneba Koné.**

I would like to extend the thanks to all my friends, especially **Badara Sène.**

Last but not least, I thank my family for everything they have done and continuing to do for me. My parents **[Diaguily Sy** (rest in peace, Dad), and **Salamata Samba Kébé],** my brothers and sisters **(Ibrahima Sy, Aboubacar Sy, Aminata Sy, Aly Sy, Aissatou Sy,** and **Ousmane Sy),** my lovely wife **(Oumou Hawa Diallo),** and my daughter **(Amina Sy).** Thank you for being around during this journey.