MALDI-TOF MS in clinical parasitology: applications, constraints and prospects

NEELJA SINGHAL[1], MANISH KUMAR[2] *and* JUGSHARAN SINGH VIRDI[1]*

[1] *Department of Microbiology, University of Delhi South Campus, Benito Juarez Road, New Delhi-110021, India*
[2] *Department of Biophysics, University of Delhi South Campus, Benito Juarez Road, New Delhi-110021, India*

(*Received 15 January 2016; revised 1 June 2016; accepted 10 June 2016; first published online 8 July 2016*)

SUMMARY

Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) is currently being used for rapid and reproducible identification of bacteria, viruses and fungi in clinical microbiological laboratories. However, some studies have also reported the use of MALDI-TOF MS for identification of parasites, like *Leishmania, Giardia, Cryptosporidium, Entamoeba*, ticks and fleas. The present review collates all the information available on the use of this technique for parasites, in an effort to assess its applicability and the constraints for identification/diagnosis of parasites and diseases caused by them. Though MALDI-TOF MS-based identification of parasites is currently done by reference laboratories only, in future, this promising technology might surely replace/augment molecular methods in clinical parasitology laboratories.

Key words: MALDI-TOF, parasites, diagnosis, protozoans, ticks.

INTRODUCTION

Mass spectrometry was developed as an analytical technique to determine mass to charge (m/z) ratio of chemical compounds (Aebersold and Mann, 2003). With time, numerous variants of this technique evolved, which were based on different methods of ionization and detection systems. Of these, matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) was found to be highly suitable for analysis of biological molecules (Momo *et al*. 2013). In MALDI-TOF MS process, the sample or the analyte is co-crystallized within a matrix solution and ionized by irradiating with a laser beam. The matrix solution absorbs the photonic energy of laser beam, effectively desorbing and ionizing the biological molecules. The ionized biomolecules are then accelerated at a fixed potential through the fixed length of a flight tube. A detector located at the end of the flight tube measures very precisely the time of flight (TOF) of the molecular ions. The TOF of a particular molecular ion is decided by the degree of ionization, as well as its molecular mass. Based on the TOF information, a characteristic spectrum, unique for a given biomolecule is generated, which constitutes its specific fingerprint.

MALDI-TOF MS can simultaneously detect various molecules of different masses. Various researchers have therefore reported the usefulness of MALDI-TOF mass spectra of protein fragments (called peptide mass fingerprints – PMF) derived from intact cells of bacteria, viruses and fungi for their rapid and reliable identification (Fenselau and Demirev, 2001; Croxatto *et al*. 2012; Nenoff *et al*. 2013). Accordingly, the use of intact cell mass spectrometry-based molecular-phenotypic identification has been successfully reported for diagnosis of various bacterial, viral and fungal diseases (Emonet *et al*. 2010; Downard, 2013; Singhal *et al*. 2015). Depending on the cellular composition and architecture of different groups of microorganisms, researchers have evaluated different methods of sample preparation ranging from intact-cell to protein extraction-based methodologies. Both, Gram positive and Gram negative bacteria have been identified using 'direct bacterial profiling', whereby a single bacterial colony spotted on the MALDI plate is over layered with matrix solution and/or a 'preparatory extraction' with formic acid (Ilina *et al*. 2009; Stephan *et al*. 2011). Specialized procedures for sample preparation have been reported for *Nocardia*, Actinomycetes and Mycobacteria (Clark *et al*. 2013). These involve boiling of *Nocardia* and Actinomycetes in water to promote cellular lysis, precipitation of proteins by ethanol, air drying and resuspension in a solution of formic acid and acetonitrile before spotting the sample on MALDI plate (Verroken *et al*. 2010). Since biosafety is a major concern while working with Mycobacteria, a procedure combining inactivation and lysis was reported to be appropriate for identification of Mycobacteria (El Khéchine *et al*. 2011). For this, bacterial colonies collected in screw-cap tubes containing water and Tween 20 were inactivated by heating and vortexed with glass beads to facilitate complete cellular

* Corresponding author: Department of Microbiology, University of Delhi South Campus, Benito Juarez Road, New Delhi-110021, India. E-mail: virdi_dusc@rediffmail.com

Parasitology (2016), **143**, 1491–1500.
doi:10.1017/S0031182016001189

disruption. These were finally suspended in a solution of formic acid-acetonitrile before spotting on the MALDI plate (El Khéchine *et al*. 2011). For identification of fungi, various methods of sample preparation have been used based on both chemical extraction and mechanical lysis of fungal hyphae and spores (Hettick *et al*. 2008; Lau *et al*. 2013).

After an exhaustive study of the published literature we realized that MALDI-TOF MS has also been used for identification of protozoan parasites like *Leishmania* spp., *Giardia* spp., *Cryptosporidium* spp., ticks and fleas. The major aim of this review is to collate the information, howsoever limited, for applications of MALDI-TOF MS for identification of disease-causing human parasites. The usefulness and the constraints of this new technology in expanding our knowledge about identification of parasites are also discussed.

APPLICATIONS IN PARASITOLOGY

The parasites, which are known to cause diseases in human beings belong to three main categories: protozoa, helminths and ectoparasites. Protozoans, which are unicellular eukaryotes, are known to cause several diseases in humans such as malaria, leishmaniasis, amoebiasis, giardiasis, sleeping sickness *etc*. Helminths commonly known as worms are multicellular organisms. Many, but not all, live in the digestive tract of man and animals from where they may invade to other organs of the body, as part of their life-cycle. Ectoparasites e.g. ticks, mites and fleas are multicellular organisms, which live on the body surface of the host. The diagnosis of parasitic diseases is conventionally done by microscopic examination of blood, tissue and excreta samples of the host. The sample preparation for microscopic examination is labor-intensive, time consuming procedure and requires highly trained personnel for correct diagnosis. When tissue/biological samples are not available, or when patients exhibit low parasitaemia and/or are asymptomatic, diagnosis might be carried out using immunological assays (Ndao, 2009). The polymerase chain reaction (PCR)-based diagnostic assays are more specific and sensitive than both microscopic and immunological assays. These can detect parasites even when patients exhibit low parasitaemia and/or are asymptomatic (Mens *et al*. 2007). The relative advantages and disadvantages of conventional and modern methods in diagnosis of various parasites have been shown in Table 1.

With recent advances in proteomics, MALDI-TOF MS has been proposed as an alternative approach for identification of bacterial and fungal pathogens (Fenselau and Demirev, 2001; Emonet *et al*. 2010; Croxatto *et al*. 2012; Downard, 2013; Nenoff *et al*. 2013; Singhal *et al*. 2015). The potential of this technology for accurate identification of many parasites has also been reported. The parasites which have been identified using MALDI-TOF MS have been enlisted in Table 2.

MALDI-TOF MS IN IDENTIFICATION OF DIAGNOSIS OF PROTOZOAN PARASITES

Leishmania

Leishmaniasis is a vector-borne protozoan parasitic disease, the clinical spectrum of which might range from mild cutaneous leishmaniasis to life-threatening visceral leishmaniasis (Dedet and Pratlong, 2009). Clinical presentation of the disease is influenced by the species of the genus *Leishmania* infecting the patient (Herwaldt *et al*. 1992). Also, different species of *Leishmania* produce disease symptoms of varied severity and respond to therapy in their own unique way. Currently, no vaccine is available for leishmaniasis, hence early identification of the infecting *Leishmania* species, and initiation of appropriate chemotherapy is the only way to control this disease. The conventional methods for diagnosis of infection are based on direct examination of smear and culture, which requires expertise. Culturing is labor-intensive, and the results are available only after weeks. The gold standard method for identification of *Leishmania* species, which has been recommended by WHO is multi-locus enzyme electrophoresis (MLEE). MLEE uses the relative electrophoretic mobilities of intracellular enzymes for characterization and differentiation of different organisms by generating their specific electromorph types. But this method is very labor-intensive, expensive and requires culturing of sufficient quantity of parasites, which may take several weeks (Pratlong *et al*. 2009). Hence, this method is confined to reference laboratories only.

The molecular methods based on real-time PCR, PCR amplification followed by analysis using restriction fragment length polymorphism or multilocus sequence typing, or sequencing of multiple genes (Mary *et al*. 2004; Rotureau *et al*. 2006; Montalvo *et al*. 2010) permit investigation of clinical samples, small-sized cultures and yield results in one or 2 working days (Foulet *et al*. 2007).

Recently, a few studies have reported the use of MALDI-TOF MS for identification of various *Leishmania* species from *in vitro* cultures (Cassagne *et al*. 2014; Culha *et al*. 2014; Mouri *et al*. 2014). Mouri *et al*. (2014) described a sample preparation method for accurate and efficient identification of various *Leishmania* species by MALDI-TOF MS. Needle aspirates of skin lesions from infected patients were cultured in suitable medium for a few days and observed under microscope for motile promastigotes. Positive promastigote cultures were washed in pure water, re-suspended in water and ethanol and centrifuged. The residual pellet was

https://doi.org/10.1017/S0031182016001189 Published online by Cambridge University Press

Table 1. Advantages and disadvantages of conventional and modern methods in clinical parasitology

Organism	Detection method	Advantages	Disadvantages
Leishmania spp. Gold standard method for diagnosis–visualization of amastigotes in splenic or bone marrow aspirates of patient	Microscopy	High specificity but low sensitivity	Highly trained and experienced technical persons required
	Serology-based methods	Serology based diagnostic tests are easy to perform, even field workers with minimal training can perform these reliably	Antibody detection is useful in visceral leishmaniasis, but is of limited value in cutaneous disease, since most patients do not develop significant antibody response. Antibody persistence for long periods after cure makes distinction between past and present infections difficult
	Nucleic acid-based methods	Detection rate is high; can be applied directly to the biological samples without the need to culture the parasite	Lack inter-laboratory standardization; require use of expensive reagents
	Matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS)	Easy sample processing, faster results and less cost per test	Need for cultivated parasites; initial cost of the equipment is high
Cryptosporidium spp. Gold standard method for diagnosis–direct fluorescent antibody (DFA) assay of the stool samples	Microscopic examination of the stained oocysts fixed on slides	Inexpensive	Staining procedure is time consuming; skilled technicians are required to reliably detect oocysts; detects only oocysts
	Serology-based methods	Relatively high specificity and sensitivity in detection of *Cryptosporidium* oocysts in fecal smears and environmental samples	Does not assess viability of oocysts. Variations in incubation conditions of the labeled antibodies might provide inconsistent results
	Nucleic acid-based methods	Highly sensitive in detecting low numbers of oocysts in environmental samples. Polymerase chain reaction (PCR) and Real time-PCR products help to determine parasite genotype (s) and thus the source of outbreak	Presence of inhibitors and sample contaminants might detrimentally affect the PCR efficacy; PCR does not directly assess viability of oocysts
	MALDI-TOF MS	Relatively rapid and simple process for parasite identification	Initial cost of the equipment is high
Giardia spp. Gold standard method for diagnosis–DFA assay of the stool samples	Microscopic examination of cysts or trophozoites in tissue, fecal, and environmental samples	Inexpensive	Trained technicians are required; species-specific identification is not possible; does not determine cyst viability
	Serology-based methods (Immunoassays to detect coproantigens)	Coproantigen detection methods can detect infections prior to excretion of cysts in host feces; useful for cost-effective and rapid screening of large numbers of fecal samples	Sensitivity of coproantigen detection assays can be lower than microscopic approaches. Also, due to antigen cross-reactivity or the use of formalin as a fixative, there can be variations in the performance of immunoassays
	Nucleic acid-based methods	Highly sensitive and specific. Useful for identification of different species of *Giardia*	Prone to contamination and non-specific DNA amplification
	MALDI-TOF MS	Relatively rapid and simple process for parasite identification	Initial cost of the equipment is high

Table 1. (*Cont.*)

Organism	Detection method	Advantages	Disadvantages
Blastocystis spp. Gold standard method for diagnosis–short-term xenic *in vitro* culture of the patient's stool samples	Microscopic examination of wet mounts and stained smears Short-term (24–72 hrs) xenic *in vitro* culture required	Inexpensive	Environmental conditions might deteriorate the samples, due to which *Blastocystis* spp. can be confused with other parasites. Culturing is time consuming and can bias subsequent genotyping due to the different ability of the isolates to grow in selective medium
	Serology-based immunoassays are currently not used for diagnosis of *Blastocystis* infections	–	–
	Nucleic acid-based methods-subtype-specific primers using feces directly or after culture of fecal specimens	Highly sensitive and specific. Also, useful for epidemiological studies.	Expensive, not available widely
	MALDI-TOF MS	Rapid and simple processing before analysis; gives reliable results in short time	Procedure optimization and validation are required before incorporating this technique in clinical laboratories for daily use
Entamoeba histolytica Gold standard method for diagnosis–classically it was analyses of isoenzymes; currently Real Time PCR using species-specific primers for amplification of DNA extracted from patient's stool samples is used	Microscopic examination of stools for detection of trophozoites or cysts	Inexpensive	Sensitivity is less even when viewed by expert technician; samples need to be examined within a short period of collection time; differentiation from protozoa with similar morphological features is not possible
	Serology-based assays	High sensitivity; time- and cost-effective	The antigens are denatured during fixation of the stool specimens, limiting testing to fresh samples. Serological tests fail to distinguish past from current infections in regions of high endemicity; hence are more useful for identification in regions where amoebiasis is less common
	Nucleic acid-based	High sensitivity and specificity than serological tests	Expensive, not easily affordable for developing countries where amoebiasis is endemic
	MALDI-TOF MS	Less labour intensive; cost-effective	Initial cost of the equipment is high
Tick spp. Gold standard method for diagnosis–morphological identification of specimens removed from patient/animal body	Morphological examination	Inexpensive, can be conducted in the field	Good expertise required for accurate identification; integrity of the specimens is necessary to avoid loss of species specific characteristics; immature, engorged ticks and lack of developed morphological characters makes identification difficult
	Serology-based assays are not used	–	–
	Nucleic acid based-methods	Highly sensitive and specific. It is compatible with several sample preservation modes and time	Involve DNA amplification and sequencing; expensive, typically limited to well-equipped laboratories. PCR assays for distinguishing ticks in different stages of development are not available
	MALDI-TOF MS	Simple and minimal sample processing. Mass spectra are generated rapidly, which can be immediately compared against reference spectra, allowing high-throughput	Identification is best for fresh specimens and gets compromised in long-term preserved specimens
Flea spp. Gold standard method for diagnosis–morphological identification of specimens removed from patient/animal body	Morphological examination using reference or identification keys	Inexpensive, can be conducted in the field	Good expertise and time required, hence not suitable for identification of large populations of fleas
	Serology-based assays are not used	–	–
	Nucleic acid-based methods	Highly sensitive and specific	PCR assays, which can distinguish flea species, or identify unique gene fragments have not been developed
	MALDI-TOF MS	Simple, rapid and cost-effective	Reliable identification was obtained for fresh samples only, samples stored in ethanol for long periods gave insignificant results

Table 2. Summary of the methods used for sample preparation of parasites for their identification by matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS)

Organisms	Sample preparation for MALDI-TOF MS	Matrix	References
Leishmania spp.	Promastigotes obtained from *in vitro* cultured needle aspirates of skin lesions were suspended in a solution of formic acid and acetonitrile	α-cyano-4 hydroxycinnamic acid	Mouri *et al.* (2014)
	Cryopreserved samples of cutaneous, mucocutaneous and visceral leishmaniasis cultured *in vitro* to obtain promatigotes were suspended in saline solution	α-cyano-4 hydroxycinnamic acid	Cassagne *et al.* (2014)
	Samples of cutaneous leishmaniasis cultured *in vitro* to obtain promastigotes were suspended in a solution of formic acid and acetonitrile	α-cyano-4 hydroxycinnamic acid	Culha *et al.* (2014)
Cryptosporidium spp.	Oocysts obtained from fecal samples of experimentally infected mice were extensively washed with de-ionized water, and subjected to freeze- thaw cycle for five times	3,5-dimethoxy-4-hydroxy-cinnamic acid	Magnuson *et al.* (2000)
	Oocysts from fecal samples of experimentally infected mice were extensively washed with de-ionized water, mixed with the matrix solution and allowed to stand for 45 min before spotting on the MALDI plate	3,5-dimethoxy-4-hydroxy-cinnamic acid	Glassmeyer *et al.* (2007)
Giardia spp.	Cysts obtained from fecal samples of experimentally infected mice were extensively washed and suspended in de-ionized water, mixed with matrix solution and incubated for 60 min before spotting on the MALDI plate	3,5-dimethoxy-4-hydroxy-cinnamic acid	Villegas *et al.* (2006)
Blastocystis spp.	Parasites were obtained after axenically culturing the fecal samples on solid media. Protein extract was prepared by treatment with ethanol/formic acid	α-cyano-4 hydroxycinnamic acid	Martiny *et al.* (2014)
E. histolytica	Trophozoites obtained from fecal samples were grown in xenic and axenic cultures. Protein extract was prepared by treatment with ethanol/formic acid	α-cyano-4 hydroxycinnamic acid	Calderaro *et al.* (2015)
Ticks spp.	Various body parts of laboratory-bred and field-collected ticks were treated with guanidinium chloride solution, homogenized and subjected to sonification. The resulting protein extract was acidified with trifluoroacetic acid.	α-cyano-4 hydroxycinnamic acid	Karger *et al.* (2012)
	Legs of ticks obtained from wild or removed from patients were used. The protein extract was prepared after homogenization of the specimens in formic acid and acetonitrile solution	α-cyano-4 hydroxycinnamic acid	Yssouf *et al.* (2013, 2015*a*)
	Protein mixture of hemolymph recovered from distal portion of amputated leg of ticks mixed in a solution of formic acid and acetonitrile was used.	α-cyano-4 hydroxycinnamic acid	Yssouf *et al.* (2015*b*)
	Legs of ticks were mixed with formic acid and homogenized. The homogenate was centrifuged and the supernatant was mixed with the matrix solution	α-cyano-4 hydroxycinnamic acid	Rothen *et al.* (2016)
Flea spp.	Various body parts of laboratory-reared adult fleas including both fresh and preserved specimens were used. The protein extracts were prepared by homogenizing the specimens in formic acid and acetonitrile solution	α-cyano-4 hydroxycinnamic acid	Yssouf *et al.* (2014)

suspended in a solution of formic acid and acetonitrile, vortexed and centrifuged. The supernatant was spotted on the MALDI plates and after the sample had air dried; it was over layered with the matrix solution (α-cyano-4- hydroxycinnamic acid, CHCA). Analysis of PMF of each sample revealed that isolates could be identified down to the subgenus level of *Viannia* or *Leishmania*, and also to the species level based on the presence/absence of mutually exclusive peaks in their PMFs. The researchers confirmed that identification of *Leishmania* spp. by MALDI-TOF MS was comparable with reference methods in specificity and sensitivity (Mouri *et al.* 2014).

Another research group described a simple method of sample preparation for identification of

https://doi.org/10.1017/S0031182016001189 Published online by Cambridge University Press

human species of *Leishmania* by MALDI-TOF MS. The cryopreserved isolates procured from reference centers were thawed and washed with Rosewell Park Memorial Institute (RPMI) medium and incubated in the traditional Novy-MacNeal-Nicolle medium followed by incubation in RPMI medium for 1 week each. The motile promastigotes were harvested from RPMI medium, washed in saline solution and 1 μL of the saline promastigote solution was spotted on the MALDI plate and air dried. These were over layered with matrix solution (CHCA) and analysed. The researchers recorded PMFs of each isolate and constructed a reference database of main species of *Leishmania* which inflict humans. Applying the same procedure of washing a rich culture of promastigotes with saline, they successfully identified 66 of the 69 strains of *Leishmania* isolated from clinical samples (Cassagne *et al*. 2014).

In an another study, investigators (Culha *et al*. 2014) employed the same sample preparation method for MALDI-TOF MS, as used by Mouri *et al*. (2014) and accurately identified *Leishmania* spp. which were isolated from Turkish patients after *in vitro* culture.

Although, all the researchers reported that MALDI-TOF MS was highly successful in identification of *Leishmania* spp., most of them reported that the need for cultivation of parasites before identification was a major hinderance for integration of this technique in clinical diagonostics.

Enteric protozoans

Water-borne protozoan parasites are usually diagnosed by microscopic examination of stool or water samples, which can neither predict the species nor indicate the viability of the infecting protozoans (U.S. Environmental Protection Agency, 1999). In research laboratories, the application of MALDI-TOF MS to human intestinal parasites has been limited to obtaining general parasitic proteome data and discovery of novel biomarkers (Thézénas *et al*. 2013). The first study which reported the use of MALDI-TOF MS for identification of *Cryptosporidium parvum* – a chlorine resistant protozoan parasite was carried out in 2000 (Magnuson *et al*. 2000). *Cryptosporidium parvum* is found in surface waters and causes cryptosporidiosis, a parasitic infection known characterized by intractable or persistent diarrhoeal illness and potential mortality among immunocompromised individuals. Magnuson *et al*. (2000) investigated two types of *C. parvum* oocysts for analysis by MALDI-TOF MS – whole-oocysts and freeze-thawed oocysts. The oocysts were obtained from fecal samples of experimentally infected mice. For whole-oocyst analysis, the samples were extensively washed with de-ionized water and spotted on the MALDI plate. For samples subjected to MALDI analysis after freeze-thaw, washed oocyst samples from above were alternately frozen (in liquid nitrogen) and thawed (at 60 °C) for 1 min each for five times. The sample was centrifuged and the supernatant was spotted on the MALDI plate. Before the sample dried, it was over layered with matrix solution containing 3, 5-dimethoxy-4-hydroxy-cinnamic acid and left for air-drying. Another 0·25 ml of matrix solution was applied over it and mass spectra were acquired. The researchers observed that the PMFs of *Cryptosporidium* oocysts, which were both washed and freeze-thawed were highly reproducible and had increased sensitivity. They further compared the PMFs of *C. parvum* with that of *Cryptosporidium muris* – another member of the same genus and found that PMFs of *C. parvum* were quite distinct from those of *C. muris*. They further investigated oocysts from various lots and found that the same washing and freeze-thaw procedure gave highly reproducible mass spectra. They suggested that MALDI-TOF MS was a simple and rapid supplement method for quality controls for production of *C. parvum* oocysts (Magnuson *et al*. 2000). Later, Glassmeyer *et al*. (2007) described an improved method of sample preparation for acquiring mass spectra of *C. parvum* oocysts by MALDI-TOF MS. Intact oocysts obtained from fecal samples of experimentally infected mice washed thrice in high performance liquid chromatography (HPLC) grade water and finally suspended in it were used as the sample for analysis. They used 3, 5-dimethoxy-4-hydroxy-cinnamic acid as the matrix solution and suggested that if the oocyst sample was mixed with the matrix solution and allowed to stand for 45 min before spotting on the MALDI plate, it was easier to obtain spectra with more number of clear peaks.

Members of the genus *Giardia* are protozoan parasites, which cause giardiasis, characterized by asymptomatic illness to severe abdominal pain, acute diarrhea and sometimes death. Currently, the *Giardia* cysts are detected in water by microscopic examination, which can neither predict the species nor indicate the viability of the cysts (U.S. Environmental Protection Agency, 1999). An independent research group described a MALDI-TOF MS-based approach for identification of *Giardia lamblia* and *Giardia muris* from intact cysts (Villegas *et al*. 2006). The intact cysts were washed thrice in distilled water and suspended in distilled water. An equal volume of the matrix solution (3, 5-dimethoxy-4-hydroxy-cinnamic acid) was mixed with the sample and incubated for 60 min, spotted on the MALDI plate and air-dried. Mass spectra were acquired for each isolate and it was observed that the mass spectral fingerprints of *G. lamblia* and *G. muris* were sufficiently distinct to allow their identification by MALDI-TOF MS (Villegas *et al*. 2006).

https://doi.org/10.1017/S0031182016001189 Published online by Cambridge University Press

The potential of MALDI-TOF MS has also been reported for identification of intestinal protozoan parasites of the genus *Blastocystis* from clinical samples (Martiny *et al*. 2014). The pathogenicity of *Blastocystis* in humans has been related to different subtypes of this parasite (Souppart *et al*. 2010). Martiny *et al*. (2014) described the use of MALDI-TOF MS for identification and differentiation of various subtypes of *Blastocystis*, which are frequently present in clinical samples. Two methods of sample preparation were evaluated–ethanol/formic acid extraction and direct deposition. In ethanol/formic acid extraction procedure, parasite colonies were suspended in distilled water and absolute ethanol and centrifuged. The pellet was washed with distilled water and re-suspended in formic acid and pure acetonitrile, centrifuged and spotted onto the MALDI plate. In direct deposition method, parasite colonies were directly deposited on the MALDI plate without any pre-treatment. When air-dried, deposit in each method was covered with the matrix solution (CHCA). The researchers reported that the mass spectra of *Blastocystis* isolates, which were obtained after ethanol/formic acid extraction were better than direct deposition method. Their results indicated that MALDI-TOF MS might serve as a valuable tool for identification and determination of various subtypes of *Blastocystis* (Martiny *et al*. 2014).

Entamoeba histolytica causes amebiasis, which ranks second after malaria as a common protozoan parasitic disease. Conventionally, the parasite is detected by microscopy and culture, which detect pathogenic *Entamoeba histolytica* and nonpathogenic *Entamoeba dispar*. *Entamoeba dispar* is a non-pathogenic species which is morphologically identical to *E. histolytica* (Tanyuksel and Petri, 2003). The PCR and real-time PCR-based molecular methods can differentiate between the two *Entamoeba* species (Calderaro *et al*. 2006). Recently Calderaro *et al*. (2015) described the applicability of MALDI-TOF MS for identification and differentiation of *E. histolytica* and *E. dispar* grown both axenically and in xenic cultures from clinical samples. Proteins were extracted from cultures containing 10^6 trophozoites mL^{-1} using ethanol/formic acid extraction procedure and spotted on the MALDI plate. Mass spectral analysis of *E. histolytica* and *E. dispar* isolates showed presence of at least five peaks capable of clearly discriminating *E. histolytica* and *E. dispar*. The researchers reported that the main advantage of MALDI-TOF MS (detection limit 10^6 trophozoites g^{-1} of feces) over molecular methods such as real-time PCR (detection limit 10 trophozoites g^{-1} of feces) is that it is less laborious and inexpensive (excluding the instrument cost) for diagnosis of *E. histolytica* in using *in vitro* xenic cultures (Calderaro *et al*. 2015).

MALDI-TOF MS IN IDENTIFICATION OF ECTOPARASITES

Ticks

Ticks are obligate parasites, which serve as vectors for many bacterial (Parola and Raoult, 2001), viral (Hubálek and Rudolf, 2012) and protozoan pathogens (Gray *et al*. 2010). Since, certain tick species are specific vectors for certain microbial pathogens, an early identification of the tick species, which might have bitten an individual may give an early clue to the disease transmitted by it and initiation of post exposure therapy. Only, expert and experienced entomologists do morphological identification of the ticks' species, and that too not in damaged specimens or specimens in immature stage of life-cycle (Parola and Raoult, 2001). Currently, PCR assays for distinguishing tick species in damaged specimens or in different stages of the development are not available (Yssouf *et al*. 2013). An elaborate review on advantages and disadvantages of morphological, molecular and MALDI-TOF MS-based techiques in identification of ticks has been recently published (Yssouf *et al*. 2016).

Several research studies have shown the potential of MALDI-TOF MS for rapid and reliable identification of the tick species (Karger *et al*. 2012; Yssouf *et al*. 2013, 2015*a*, *b*). Karger *et al*. (2012) described the use of whole-animal mass spectrometry for reliable determination of various species of ticks. Adult ticks and nymphs homogenized separately in a plastic pestle were treated with guanidinium chloride solution and subject to sonification in a water bath. The sample was centrifuged and the extract was acidified with trifluoroacidic acid (TFA). The acidified extracts were concentrated and spotted directly on the MALDI plate and allowed to dry. Dried spots were overlaid with the matrix solution (CHCA) and again allowed to dry. Mass spectra were acquired for each sample and a reference database of spectra was constructed. Comparison and analysis of mass spectra of unknown samples with the reference database allowed determination of the species of the ticks. The method was reproducible and capable of identifying the correct species of the ticks when adults or nymphs or even larvae were used as the starting material. Cluster analysis indicated that the primary determinant of the MALDI mass spectra was the species. The developmental stages formed distinct clusters within the given species. It was observed that species identification of ticks was also possible using body parts and engorged ticks. Only a small, but substantial part of the tick's body (except leg specimens) was required to yield spectra sufficient for species determination.

Yssouf *et al*. (2013) further described the use of MALDI-TOF MS for rapid identification of tick species using only leg specimens. The legs of the

https://doi.org/10.1017/S0031182016001189 Published online by Cambridge University Press

ticks were homogenized in formic acid and acetonitrile solution, centrifuged and the supernatant over layered with the matrix solution (CHCA), was deposited on the MALDI plate. Mass spectra were acquired and a reference database of spectra was constructed. Comparison of test samples against the spectral database revealed that all organisms obtained from wild or removed from patients were accurately identified using MALDI-TOF MS. The researchers observed that with the help of MALDI-TOF MS, identity of tick species could be established in less than an hour. They proposed a procedure by which MALDI-TOF MS could be used to identify ticks species and determine the presence of *Rickettsia* in them. Interestingly, the PMF profiles of protein extracts prepared from tick legs were successful in discriminating the infected and uninfected ticks (Yssouf *et al*. 2015*a*). In another study the same research group reported that protein mixture of hemolymph recovered from distal portion of amputated leg of ticks mixed in a solution of formic acid and acetonitrile, spotted on the MALDI plate and overlaid with the matrix solution (CHCA) was mixed with the samples by pipetting was also useful for identification of tick species and the associated pathogens (Yssouf *et al*. 2015*b*).

Recently Rothen *et al*. (2016) reported the usefulness of MALDI-TOF MS for reliable identification of species of closely related afrotropical ticks. Two to eight legs of a tick were detached, placed in a microcentrifuge tube containing formic acid, homogenized and centrifuged. The supernatant was mixed with the matrix solution and spotted on the MALDI plate. The mass spectra profiles were highly successful in identifying the various tick species. The researchers reported that MALDI-TOF MS proved to be a reliable tool in identifying morphologically and genetically, highly similar tick species.

Fleas

Fleas are small insects, which serve as vectors for several pathogens in animals and humans like, *Yersinia pestis* (causes plague), *Rickettsia typhi* (causes murine typhus), *Rickettsia prowazekii* (causes rural epidemic typhus), *Rickettsia felis* (causes flea-borne spotted fever) and many species of human-disease causing *Bartonella* (Bitam *et al*. 2010). Fleas are identified at the species level by morphological examination with the help of reference (identification) keys. This requires great expertise and is a time consuming process, hence not suitable for identification of large population of fleas. Molecular methods like sequencing of the 18S rRNA gene are available (Michael, 2002), but a PCR assay, which can distinguish flea species, or ideal PCR primers, which can identify the relevant gene fragments are still unavailable (Yssouf *et al*. 2013).

Yssouf *et al*. (2014) described the use of MALDI-TOF MS for rapid identification of various species of fleas using specimens from legs, heads only or body without abdomen. The specimens were homogenized in a solution of formic acid and acetonitrile, and centrifuged. Equal volumes of supernatant and matrix were spotted on the MALDI plate and mass spectra of each sample were acquired. Since the spectral profiles obtained from flea body without abdomen provided consistent and reproducible data, these were used to create the database of PMFs. Comparison of the test samples against the library of spectral database confirmed a rapid and reliable discrimination of flea species (Yssouf *et al*. 2014).

Concluding remarks

Until sometimes back, for most microbiologists it was difficult to comprehend that MALDI-TOF MS might be suitable for identification of whole microorganisms, and replace the conventional methods used in routine clinical microbiological laboratories. But, the development of microorganism-databases, paved the way for progression of MALDI-TOF MS from a protein identification tool to a diagnostic technique. The regulatory approval of MALDI-TOF MS by government agencies like US Food and Drug Administration and China Food and Drug Administration for *in vitro* diagnosis has provided the required impetus for development and commercialization of this exciting new technology (Luo *et al*. 2015; Patel, 2015).

It is quite obvious that research on application of MALDI-TOF MS in identification of various parasites has not advanced as it has for identification of bacteria, viruses and fungi. It would be worthwhile to recount some of the reasons underlying it. One of the primary reasons for this might be the complex biological nature of the parasite life-forms (oocyst/promastigote/trophozoites etc.) from which the protein sample may be extracted for MALDI-TOF MS analysis. Another reason might be the complex biology of the life cycles of the parasites as well as the diseases caused by them. For identification of parasites, the need to culture parasites *in vitro* is a major bottleneck for rapid MALDI-TOF-based identification of parasites (Mouri *et al*. 2014). The MALDI-TOF MS method is currently not available for field diagnosis and is carried out only in laboratories due to the high costs involved in initial procurement of the instrument.

Despite such constraints, MALDI-TOF MS has nevertheless significant advantages over the morphological, immunological and molecular detection methods. The main advantages of MALDI-TOF MS are the rapidity and accuracy associated with this technology. Also, it does not require highly trained laboratory personnel (Graça *et al*. 2012).

https://doi.org/10.1017/S0031182016001189 Published online by Cambridge University Press

From the literature it is also clear that the technology is not constrained by sample size and contamination by the host proteins. Since, MALDI-TOF MS has a wide spectrum of applications, cheaper versions of this instrument would definitely increase its affordability and application in field diagnosis. The efforts are already underway to develop simplified versions of the instrument using microfluidics technology (Yang *et al*. 2012). There is no denying the fact that the proof of the principle for application of MALDI-TOF MS for identification of parasites has already been demonstrated.

ACKNOWLEDGEMENTS

The authors acknowledge the financial support received from Science & Engineering research Board of Department of Science & Technology, DU-DST-PURSE II grant and University R & D research grant to University of Delhi South Campus.

FINANCIAL SUPPORT

Financial assistance was received as Start up Research Grant for Young Scientists (SB/YS/LS-156/2014) from Science & Engineering Research Board of Department of Science & Technology (NS), DU-DST-PURSE II grant and University R & D research grant for doctoral and post-doctoral studies to University of Delhi South Campus (JSV).

REFERENCES

Aebersold, R. and Mann, M. (2003). Mass spectrometry-based proteomics. *Nature* **422**, 198–207.

Bitam, I., Dittmar, K., Parola, P., Whiting, M. F. and Raoult, D. (2010). Fleas and flea-borne diseases. *International Journal of Infectious Diseases* **14**, e667–e676.

Calderaro, A., Gorrini, C., Bommezzadri, S., Piccolo, G., Dettori, G. and Chezzi, C. (2006). *Entamoeba histolytica* and *Entamoeba dispar*: comparison of two PCR assays for diagnosis in a non-endemic setting. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **100**, 450–457.

Calderaro, A., Piergianni, M., Buttrini, M., Montecchini, S., Piccolo, G., Gorrini, C., Rossi, S., Chezzi, C., Arcangeletti, M. C., Medici, M. C. and De Conto, F. (2015). MALDI-TOF mass spectrometry for the detection and differentiation of *Entamoeba histolytica* and *Entamoeba dispar*. *PLoS ONE* **10**, e0122448.

Cassagne, C., Pratlong, F., Jeddi, F., Benikhlef, R., Aoun, K., Normand, A. C., Faraut, F., Bastien, P. and Piarroux, R. (2014). Identification of *Leishmania* at the species level with matrix-assisted laser desorption ionization time-of-flight mass spectrometry. *Clinical Microbiology and Infection* **20**, 551–557.

Clark, A. E., Kaleta, E. J., Arora, A. and Wolk, D. M. (2013). Matrix-assisted laser desorption ionization-time of flight mass spectrometry: a fundamental shift in the routine practice of clinical microbiology. *Clinical Microbiology Reviews* **26S**, 547–603.

Croxatto, A., Prod'hom, G. and Greub, G. (2012). Applications of MALDI-TOF mass spectrometry in clinical diagnostic microbiology. *FEMS Microbiology Reviews* **36**, 380–387.

Culha, G., Akyar, I., Yildiz Zeyrek, F., Kurt, Ö., Gündüz, C., Özensoy Töz, S., Östan, I., Cavus, I., Gülkan, B., Kocagöz, T., Özbel, Y. and Özbilgin, A. (2014). Leishmaniasis in Turkey: determination of *Leishmania* species by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS). *Iranian Journal of Parasitology* **9**, 239–248.

Dedet, J. P. and Pratlong, F. (2009). Leishmaniasis. In *Manson's Tropical Diseases* (eds Cook, G. C & Zumla, A.), pp. 1341–1365. Elseiver Science Limited, Saunders, London.

Downard, K. M. (2013). Proteotyping for the rapid identification of influenza virus and other biopathogens. *Chemical Society Reviews* **42**, 8584–8595.

El Khéchine, A., Couderc, C., Flaudrops, C., Raoult, D. and Drancourt, M. (2011). Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry identification of mycobacteria in routine clinical practice. *PLoS ONE* **6**, e24720.

Emonet, S., Shah, H. N., Cherkaoui, A. and Schrenzel, J. (2010). Application and use of various mass spectrometry methods in clinical microbiology. *Clinical Microbiology and Infection* **16**, 1604–1613.

Fenselau, C. and Demirev, P. A. (2001). Characterization of intact microorganisms by MALDI mass spectrometry. *Mass Spectrometry Reviews* **20**, 157–171.

Foulet, F., Botterel, F., Buffet, P., Morizot, G., Rivollet, D., Deniau, M., Pratlong, F., Costa, J. M. and Bretagne, S. (2007). Detection and identification of *Leishmania* species from clinical specimens by using a real-time PCR assay and sequencing of the cytochrome B gene. *Journal of Clinical Microbiology* **45**, 2110–2115.

Glassmeyer, S. T., Ware, M. W., Schaefer, F. W., III, Shoemaker, J. A. and Kryak, D. D. (2007). An improved method for the analysis of *Cryptosporidium parvum* oocysts by matrix-assisted laser desorption/ionization time of flight mass spectrometry. *Journal of Eukaryotic Microbiology* **54**, 479–481.

Graça, G. C., Volpini, A. C., Romero, G. A., Oliveira Neto, M. P., Hueb, M., Porrozzi, R., Boité, M. C. and Cupolillo, E. (2012). Development and validation of PCR-based assays for diagnosis of American cutaneous leishmaniasis and identification of the parasite species. *Memórias do Instituto Oswaldo Cruz* **107**, 664–674.

Gray, J., Zintl, A., Hildebrandt, A., Hunfeld, K. P. and Weiss, L. (2010). Zoonotic babesiosis: overview of the disease and novel aspects of pathogen identity. *Ticks and Tick Borne Diseases* **1**, 3–10.

Herwaldt, B. L., Arana, B. A. and Navin, T. R. (1992). The natural history of cutaneous leishmaniasis in Gautemala. *Journal of Infectious Diseases* **165**, 518–527.

Hettick, J. M., Green, B. J., Buskirk, A. D., Kashon, M. L., Slaven, J. E., Janotka, E., Blachere, F. M., Schmechel, D. and Beezhold, D. H. (2008). Discrimination of *Aspergillus* isolates at the species and strain level by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry fingerprinting. *Analytical Biochemistry* **380**, 276–281.

Hubálek, Z. and Rudolf, I. (2012). Tick-borne viruses in Europe. *Parasitology Research* **111**, 9–36.

Ilina, E. N., Borovskaya, A. D., Malakhova, M. M., Vereshchagin, V. A., Kubanova, A. A., Kruglov, A. N., Svistunova, T. S., Gazarian, A. O., Maier, T., Kostrzewa, M. and Govorun, V. M. (2009). Direct bacterial profiling by matrix-assisted laser desorption-ionization time-of-flight mass spectrometry for identification of pathogenic *Neisseria*. *Journal of Molecular Diagnostics* **11**, 75–86.

Karger, A., Kampen, H., Bettin, B., Dautel, H., Ziller, M., Hoffmann, B., Süss, J. and Klaus, C. (2012). Species determination and characterization of developmental stages of ticks by whole-animal matrix-assisted laser desorption/ionization mass spectrometry. *Ticks and Tick Borne Diseases* **3**, 78–89.

Lau, A. F., Drake, S. K., Calhoun, L. B., Henderson, C. M. and Zelazny, A. M. (2013). Development of a clinically comprehensive database and a simple procedure for identification of molds from solid media by matrix-assisted laser desorption ionization–time of flight mass spectrometry. *Journal of Clinical Microbiology* **51**, 828–834.

Luo, Y., Siu, G. K., Yeung, A. S., Chen, J. H., Ho, P. L., Leung, K. W., Tsang, J. L., Cheng, V. C., Guo, L., Yang, J., Ye, L. and Yam, W. C. (2015). Performance of the VITEK MS matrix-assisted laser desorption ionization-time of flight mass spectrometry system for rapid bacterial identification in two diagnostic centers in China. *Journal of Medical Microbiology* **64**, 18–24.

Magnuson, M. L., Owens, J. H. and Kelty, C. A. (2000). Characterization of *Cryptosporidium parvum* by matrix-assisted laser desorption ionization-time of flight mass spectrometry. *Applied and Environmental Microbiology* **66**, 4720–4724.

Martiny, D., Bart, A., Vandenberg, O., Verhaar, N., Wentink-Bonnema, E., Moens, C. and van Gool, T. (2014). Subtype determination of *Blastocystis* isolates by matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS). *European Journal of Clinical Microbiology and Infectious Diseases* **33**, 529–536.

Mary, C., Faraut, F., Lascombe, L. and Dumon, H. (2004). Quantification of *Leishmania infantum* DNA by a real-time PCR assay with high sensitivity. *Journal of Clinical Microbiology* **42**, 5249–5255.

Mens, P., Spieker, N., Omar, S., Heijnen, M., Schallig, H. and Kager, P. A. (2007). Is molecular biology the best alternative for diagnosis of malaria to microscopy? A comparison between microscopy, antigen detection and molecular tests in rural Kenya and urban Tanzania. *Tropical Medicine and International Health* **12**, 238–244.

Michael, F. W. (2002). Phylogeny of the holometabolous insect orders basedon 18S ribosomal DNA: when bad things happen to good data. *Experimentia Supplementum* **92**, 69–83.

Momo, R. A., Povey, J. F., Smales, C. M., O'Malley, C. J., Montague, G. A. and Martin, E. B. (2013). MALDI-TOF mass spectrometry coupled with multivariate pattern recognition analysis for the rapid biomarker profiling of *Escherichia coli* in different growth phases. *Analytical and Bioanalytical Chemistry* **405**, 8251–8265.

Montalvo, A. M., Fraga, J., Monzote, L., Montano, I., De Doncker, S., Dujardin, J. C. and Van der Auwera, G. (2010). Heat-shock protein 70 PCR-RFLP: a universal simple tool for *Leishmania* species discrimination in the New and Old World. *Parasitology* **137**, 1159–1168.

Mouri, O., Morizot, G., Van der Auwera, G., Ravel, C., Passet, M., Chartrel, N., Joly, I., Thellier, M., Jauréguiberry, S., Caumes, E., Mazier, D., Marinach-Patrice, C. and Buffet, P. (2014). Easy identification of *Leishmania* species by mass spectrometry. *PLoS Neglected Tropical Diseases* **8**, e2841.

Ndao, M. (2009). Diagnosis of parasitic diseases: old and new approaches. *Interdisciplinary Perspectives on Infectious Diseases* 278246. http://dx.doi.org/10.1155/2009/278246

Nenoff, P., Erhard, M., Simon, J. C., Muylowa, G. K., Herrmann, J., Rataj, W. and Gräser, Y. (2013). MALDI-TOF mass spectrometry - a rapid method for the identification of dermatophyte species. *Medical Mycology* **51**, 17–24.

Parola, P. and Raoult, D. (2001). Tick-borne bacterial diseases emerging in Europe. *Clinical Microbiology and Infection* **7**, 80–83.

Patel, R. (2015). MALDI-TOF MS for the diagnosis of infectious diseases. *Clinical Chemistry* **61**, 100–111.

Pratlong, F., Dereure, J., Ravel, C., Lami, P., Balard, Y., Serres, G., Lanotte, G., Rioux, J. A. and Dedet, J. P. (2009). Geographical distribution and epidemiological features of Old World cutaneous leishmaniasis foci, based on the isoenzyme analysis of 1048 strains. *Tropical Medicine and International Health* **14**, 1071–1085.

Rothen, J., Githaka, N., Kanduma, E. G., Olds, C., Pflüger, V., Mwaura, S., Bishop, R. P. and Daubenberger, C. (2016). Matrix assisted laser desorption/ionization time of flight mass spectrometry for comprehensive indexing of East African ixodid tick species. *Parasites and Vectors* **9**, 151.

Rotureau, B., Ravel, C., Couppié, P., Pratlong, F., Nacher, M., Dedet, J. P. and Carme, B. (2006). Use of PCR-restriction fragment length polymorphism analysis to identify the main new world *Leishmania* species and analyze their taxonomic properties and polymorphism by application of the assay to clinical samples. *Journal of Clinical Microbiology* **44**, 459–467.

Singhal, N., Kumar, M., Kanaujia, P. K. and Virdi, J. S. (2015). MALDI-TOF mass spectrometry: an emerging technology for microbial identification and diagnosis. *Frontiers in Microbiology* **6**, 791.

Souppart, L., Moussa, H., Cian, A., Sanciu, G., Poirier, P. E., Alaoui, H., Delbac, F., Boorom, K., Delhaes, L., Dei-Cas, E. and Viscogliosi, E. (2010). Subtype analysis of *Blastocystis* isolates from symptomatic patients in Egypt. *Parasitology Research* **106**, 505–511.

Stephan, R., Cernela, N., Ziegler, D., Pflüger, V., Tonolla, M., Ravasi, D., Fredriksson-Ahomaa, M. and Hächler, H. (2011). Rapid species specific identification and subtyping of *Yersinia enterocolitica* by MALDI-TOF mass spectrometry. *Journal of Microbiological Methods* **87**, 150–153.

Tanyuksel, M. and Petri, W. A., Jr. (2003). Laboratory diagnosis of amoebiasis. *Clinical Microbiology Reviews* **16**, 713–729.

Thézénas, M. L., Huang, H., Njie, M., Ramaprasad, A., Nwakanma, D. C., Fischer, R., *et al*. (2013). PfHPRT: a new biomarker candidate of acute *Plasmodium falciparum* infection. *Journal of Proteome Research* **12**, 1211–1222.

U.S. Environmental Protection Agency. (1999). Method 1623: *Cryptosporidium* and *Giardia* in water by filtration. /IMS/FA. EPA/821-R-99-006. Office of Water, U.S. Environmental Protection Agency, Washington, D.C.

Verroken, A., Janssens, M., Berhin, C., Bogaerts, P., Huang, T. D., Wauters, G., and Glupczynski, Y. (2010). Evaluation of matrix-assisted laser desorption ionization–time of flight mass spectrometry for identification of *Nocardia* species. *Journal of Clinical Microbiology* **48**, 4015–4021.

Villegas, E. N., Glassmeyer, S. T., Ware, M. W., Hayes, S. L. and Schaefer, F. W., III (2006). Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry-based analysis of *Giardia lamblia* and *Giardia muris*. *Journal of Eukaryotic Microbiology* **53**, S179–S181.

Yang, M., Chao, T. C., Nelson, R. and Ros, A. (2012). Direct detection of peptides and proteins on a microfluidic platform with MALDI mass-spectrometry. *Analytical and Bioanalytical Chemistry* **404**, 1681–1689.

Yssouf, A., Flaudrops, C., Drali, R., Kernif, T., Socolovschi, C., Berenger, J. M., Raoult, D. and Parola, P. (2013). Matrix-assisted laser desorption ionization-time of flight mass spectrometry for rapid identification of tick vectors. *Journal of Clinical Microbiology* **51**, 522–528.

Yssouf, A., Socolovschi, C., Leulmi, H., Kernif, T., Bitam, I., Audoly, G., Almeras, L., Raoult, D. and Parola, P. (2014). Identification of flea species using MALDI-TOF/MS. *Comparative Immunology, Microbiology and Infectious Diseases* **37**, 153–157.

Yssouf, A., Almeras, L., Terras, J., Socolovschi, C., Raoult, D. and Parola, P. (2015*a*). Detection of *Rickettsia* spp. in ticks by MALDI-TOF MS. *PLoS Negleted Tropical Diseases* **9**, e0003473.

Yssouf, A., Almeras, L., Berenger, J. M., Laroche, M., Raoult, D. and Parola, P. (2015*b*). Identification of tick species and disseminate pathogen using hemolymph by MALDI-TOF MS. *Ticks and Tick Borne Diseases* **6**, 579–586.

Yssouf, A., Almeras, L., Raoult, D. and Parola, P. (2016). Emerging tools for identification of arthropod vectors. *Future Microbiology* **11**, 549–566.