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# The Challenges and Advances in Diagnosis of Vector-Borne Diseases: Where Do We Stand?

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## Abstract

Vector-borne diseases (VBD) are of major importance to human and animal health. In recent years, VBD have been emerging or re-emerging in many geographical areas, alarming new disease threats and economic losses. The precise diagnosis of many of these diseases still remains a major challenge because of the lack of comprehensive data available on accurate and reliable diagnostic methods. Here, we conducted a systematic and in-depth review of the former, current, and upcoming techniques employed for the diagnosis of VBD.

Keywords: chip-based technology, diagnostics, mass spectrometry, molecular biology, serology, vector-borne diseases

## Introduction

Vector-borne diseases (VBD) are of major importance to human and animal health. Hematophagous arthropod vectors such as mosquitoes, ticks, and sand flies are responsible for transmitting bacteria, viruses, and protozoa between vertebrate hosts, causing diseases such as malaria, dengue fever, and Crimean-Congo hemorrhagic fever. Historically, diseases such as leishmaniasis and malaria have had a great impact on health and they are still posing a huge burden on public health in many countries (Hotez et al. 2006). Until the early 20th century, VBD were responsible for more deaths in humans than all other causes combined (Kalluri et al. 2007).

In recent years, VBD have emerged or re-emerged in many geographical regions, causing global health issues for humans, livestock, companion animals, and wildlife (Harrus and Baneth 2005). The eco-epidemiology of VBD is affected by an interplay between major factors such as the pathogen, the host (human, animal), the vector, and the environment. Major contributors in the spreading of VBD include eco-climatic changes, development of insecticide and drug resistance, globalization, and the significant increase in international trade and travel (Harrus and Baneth 2005). Altogether, these factors govern disease epidemiology and infection patterns in many geographic regions.

For example, invasive mosquitoes have become widely established across Europe, resulting in the emergence of malaria in Greece and West Nile virus (WNV) throughout parts of south-eastern Europe (Medlock and Leach 2015). Similarly, the prevalence of Lyme disease continues to increase throughout Europe, and tick-borne encephalitis and Crimean-Congo hemorrhagic fever viruses have changed their geographical distribution. Recently, the World Health Organization has declared that the Zika virus constitutes a public health emergency of international concern, and its spreading can also be expected in Europe. From a veterinary perspective, the emergence of Bluetongue and Schmallenberg viruses has shown that northern Europe is equally susceptible to transmission of VBD, despite the longer winter period compared with southern Europe (Medlock and Vaux 2015).

Lack of comprehensive information on existing and upcoming diagnostic methods for VBD makes the diagnosis and disease control complicated. In general, laboratory diagnosis of VBD can be divided into two categories: direct methods (microscopy, culturing of the causative agent, nucleic acid detection, etc.) and indirect methods (e.g., detection of organism-specific immune responses). Acute infections are usually identified by direct tools, whereas indirect tools are useful for the characterization of secondary or convalescent phases.

The majority of VBD have common issues in rapid and sufficient diagnostics (Table 1). Many of the diagnostic tools based on molecular techniques are applied for the finest detection and characterization of vector-borne infections; nevertheless, VBD may still be misdiagnosed. On the other hand, the advances in molecular techniques have brought to the discovery of previously unknown pathogens, distinguished closely related species and revealed new possible transmission mechanisms of vector-borne infections (Harrus and Baneth 2005, Colwell et al. 2011). Continuous development of the diagnostic technologies and their deployment are crucial to identify, control, and treat the VBD. High throughput technologies such as whole-genome sequencing, omics approaches (metagenomics and metaproteomics) and high-resolution analytical methods such as mass spectrometry (MS), open new opportunities for diagnostics of VBD.

Here, we conducted a systematic review of the former, current, and upcoming techniques employed for the diagnosis of VBD.

**Routine and Advanced Platforms for the Diagnosis of Vector-Borne Pathogens**

*Conventional techniques*

Microscopy is still one of the well-established, low-cost direct methods for identification of vector-borne pathogens. It is still the method of choice for blood parasites such as Plasmodium or Babesia, although its sensitivity is lower than that of molecular diagnosis (Solano-Gallego et al. 2016). However, in some cases, sample preparation can be time consuming and labor intensive, and in many cases, diagnosis may be hampered by sparseness of organisms. The method is subjective, especially in cases when differentiation of similar-appearing organisms is required. Thus, morphologic interpretation needs significant expertise (Ndao 2009). Despite these limitations, the microscopy represents a useful initial test for the patient with general signs of infection without diagnostic hallmarks, especially in endemic areas (Dumler et al. 2007).

Electron microscopy (EM) is still on the forefront of clinical diagnosis of viral and rickettsial diseases, study of ultrastructure, and unfolding of the basic principles of pathogenesis. In the diagnostic setting, it is particularly valuable in the surveillance of emerging diseases and potential bioterrorism viruses (Goldsmith and Miller 2009). Traditionally, negative staining for transmission electron microscopy has been the ‘‘gold standard’’ for imaging of microbial samples, for example, in diagnostic virology; on the other hand, improvements in the specimen preparation have enabled scanning electron microscopy (SEM) as an efficient diagnostic tool (Golding et al. 2016). Diagnostic EM has two advantages over enzyme-linked immunosorbent assay (ELISA) and nucleic acid amplification tests: after a simple and fast negative staining, EM (mainly SEM) enables fast morphological identification and differential diagnosis of infectious agents contained in the specimen without the need for special considerations and/or reagents. Nevertheless, EM has the disadvantage of being unsuitable as a screening method (Schramlova et al. 2010).

Cell culture is another gold-standard diagnostic method (Portillo 2015). These procedures are time consuming, and the isolation of pathogens is not always successful. Cultivation of slow-growing and fastidious bacteria, or even noncultivable bacteria, has always been a limitation. Bacteria transmitted by ticks are fastidious and difficult to grow in axenic media; many of them are obligate intracellular pathogens, as well as viruses, rickettsiae, and some protozoal pathogens. However, cultivation methods have improved considerably over the past several decades, with advances in the scope and diversity of media components,

control of environmental conditions, use of heterologous host cells, and use of growth-promoting factors (Mukamolova et al., 1998).

Xenodiagnosis, the feeding of a natural arthropod vector on a patient to detect evidence of infection, has provided definitive evidence for the etiology of diverse vector-borne infections, including malaria, yellow fever, epidemic typhus, and Lyme disease (Telford et al. 2014). The obvious infections for which xenodiagnosis may be useful would be those that are known to be vector borne, maintain a chronic state, and are characterized by sparse organisms that are very difficult to detect.

As molecular diagnostic techniques progress in scope and magnitude, it is critical to retain and use classical techniques, including cell culture and EM, that complement the advances in molecular methods. There is a continuing need to train young scientists in these traditional methods to maintain an underlying expertise.

*Serology-based assays*

Serological tests are widely employed to diagnose human and animal VBD, at the screening level, for both surveillance and research. They have a wide diagnostic time window, as antibodies for a pathogen may persist for months or even years. That makes these assays valuable to investigate past exposure to vector-borne pathogens. Advantages of serology include the ease of sample collection, mainly blood, and an ability to detect current infections by sero-conversion. They are often used as confirmatory tests in conjunction with molecular methods or cytology (Otranto et al. 2010).

A poor sensitivity of serological tests is observed in the early stage of infection in the majority of vector-borne infections. The demonstration of sero-conversion is also possible by retrospective confirmation of recent infection between antibody titers of acute and convalescent samples (Nilsson et al. 2005). Another limitation of serologic assays is the potential cross-reactivity within or between genera of viral or bacterial pathogens (Costa et al. 2005, Rawlins et al. 2005, Vermeulen et al. 2010). Thus, there is a need of testing with other related microorganisms to ensure no cross reactions and to avoid false positives. To improve the diagnostic specificity, various recombinant or purified antigens are being widely used (Maggi et al. 2014). Fast development of serological assays often relies on culture for antigen production. The use of purified antigens is limited by the inability of producing proteins in a sufficient amount. Therefore, to overcome this limitation in the cases where cultures are not available, the production of recombinant proteins or synthetic peptides offers an alternative if genetic information of potentially antigenic proteins is known.

The choice whether to use monoclonal or polyclonal antibodies depends on the context in which the application is being used. The principal advantages of monoclonal antibodies are their homogeneity and consistency, whereas polyclonal antibodies can be generated much more rapidly, at less expense, and with less technical skill than is required to produce monoclonal antibodies. Monoclonal antibodies are not generally useful for assays that depend on antigen cross-linking (e.g., hemagglutination) unless dimeric or multimeric antigens or antigens bound to a solid phase are used. In addition, they may not activate complements readily, because activation requires the close proximity of Fc receptors (Lipman et al. 2005). Modification of antibodies by covalently linking a fluorochrome or radionuclide may also alter antibody binding. This potential is of less concern when using polyclonal antibodies, which recognize a host of epitopes.

ELISA, immunofluorescence assays (IFA), and immunoblotting are the most widely used serological tests. Results of such assays must be interpreted with caution since a positive serological test could be associated with a past infection, and unrelated with current clinical signs. Moreover, clinicians should consider limitations of each commercially available test due to different specificity and sensitivity that may be affected by various factors, including antigen use. Besides conventional single-pathogen assays, some serological tests detect multipleVBD (Volgina et al. 2013). Multiplexed Luminex-based immunoassay demonstrates a prospective detection of viral hemorrhagic fever-associated immunoglobulin G antibodies

by using a panel of recombinant antigens of pathogens; for example, Crimean-Congo hemorrhagic fever virus, dengue virus (DENV), Rift Valley fever virus, and Hantaan virus (Wu et al. 2014). Luminex xMAP technology seems to be more sensitive and time saving than widely employed ELISA or IFA. A short overview of the recommended serology-based methods for VBD is given in Table 2.

*Nucleic acid-based assays*

With the upcoming of molecular diagnostic techniques in the 1990s, a new era of diagnostics was initiated in the field of VBD. By now, molecular diagnostic tools are used in almost all facilities dealing with VBD. Molecular diagnostic devices allow a fast detection of DNA or RNA of pathogens —even of small amounts and submicroscopic infections.

By 1997, the PCR became one of the most commonly used techniques in the field of VBD diagnostics. Nowadays, many standardized protocols are available with high specificity and sensitivity (higher compared with serology and/or microscopy). Various optimized techniques of PCR were established, for example, nested PCR, touchdown PCR, multiplex PCR, and RT-PCR. Quantitative PCR (qPCR) and its variants are also exploited successfully to measure the copy numbers of nucleic acid of the given pathogen in real time.

Loop-mediated isothermal amplification (LAMP), an isothermal DNA amplification (DNA amplification at a constant temperature without the need of a thermal cycler), is tuned for several infectious diseases mainly in poor and underdeveloped countries, thanks to its low cost and relatively easy-to handle procedure. The diagnosis of VBD such as malaria (Vallejo et al. 2015), leishmaniasis (Gao et al. 2015), and sleeping sickness (Njiru et al. 2008) was successfully performed by LAMP.

Similar to all diagnostic tools in the field of VBD, PCR and associated techniques also have their limitations. It is essential that the material to be analyzed must be stored and handled correctly (e.g., elimination of PCR inhibitors in blood, formalin). Care must be taken to use adequate nucleic acid extraction techniques depending on the sample material. False-positive PCR results constitute a major problem (e.g., because primers may nonspecifically bind to DNA of vertebrate hosts and/or other pathogens/microbiota). Verification by sequencing of amplicons can correct false-positive PCR results, but it is costly and needs additional working steps. Positive results in molecular diagnostics, although they indicate the presence of a nucleic acid of pathogen, do not necessarily mean that an acute infection is present at the time of sampling. On the one hand, asymptomatic cases might be diagnosed; on the other hand, post-treated samples can give positive results even days and months after treatment because of the circulation of nucleic acids of pathogens (Moody 2002).

Fluorescence in situ hybridization. Fluorescence in situ hybridization (FISH) is based on specific nucleic acid hybridization, which is revealed by the fluorescence of a targeted DNA sequence in situ, without an amplification or a cultivation step (Moter and Gobel 2000). A DNA probe of 15–30 nucleotides covalently linked with a fluorescent molecule, is hybridized to its complementary target DNA sequence in situ. The detected fluorescence indicates the presence of the targeted nucleic acid of the pathogen in the sample. FISH can provide spatial information and distribution of organisms, contrary to PCR-based reactions, notably by targeting ribosomal RNA (rRNA) genes, and it can be multiplexed by different fluorescent molecules. FISH avoids technical limitations of both immunohistochemistry (antibodies access to complex structures) and PCR-based reactions (lack of spatial information), whereas it exploits the most interesting properties of both assays; that is, specificity of PCR and fluorescent visualization of immunohistochemistry FISH offer a more accurate view of the microbial community and can be applied in a lot of different environments (Moter and Gobel 2000). In immunohistochemistry, antibodies used to detect microorganisms can be blocked by bigger structures such as biofilms or tissues, which is not the case with DNA probes. One of the limitations of FISH is the auto-fluorescence of some proteins, which can introduce false positivity. Another limitation is the ability of probes to penetrate into the cells.

The tridimensional structure of the cells, conserved by the technique, could block the access to the DNA target. Photobleaching could be also considered a problem and could decrease the sample fluorescence.

In case of VBD, *Plasmodium falciparum* and *Babesia* were identified by FISH (Shah et al. 2015). For the direct detection of Babesia in a thin blood smear fluorescent labeled oligomer probe targeted to *B. microti*, 18S rRNA was used. FISH can also be applied to detect viruses, as demonstrated by the study of Raquin et al. (2012), who successfully detected DENVs in mosquitoes. Their technique is also applicable to detect both dengue and chikungunya viruses. In situ nucleic acid amplification is being developed to increase FISH sensitivity, notably to make FISH able to address the physiological state of the cell, by targeting all the RNAs (Porter and Pickup 2000).

Wire-guided droplet manipulation. The wire-guided droplet manipulation is a new technique in which a wire manipulates a microliter-sized droplet in a hydrophobic milieu (Harshman et al. 2014). It allows a molecular partitioning, that is, the separation of complex sample components. This technique can be useful for blood screening, because the different cells form no aggregate and can be targeted by a PCR reaction for the identification of microorganisms in situ, such as *Klebsiella pneumoniae*. Although this technique is not used extensively for VBD, evolution of the technique and materials could enhance the diagnostic possibilities of this tool in the coming years.

Next-generation sequencing. Next-generation sequencing (NGS) provides fast and reliable DNA sequencing, without a priori knowledge of the targeted genome (Van Borm et al. 2015). Alignment tools are a crucial component of NGS. Using NGS, it is possible to rapidly sequence DNA directly from the vectors to generate a broad overview of the microbial community inside it, without the bias introduced by standard identification methods based on cultivable pathogens. By targeting different regions of the parasite genomes (from less to more conserved), NGS can perform identifications at different levels, from strains to families of parasites. NGS can also detect unknown parasites. Moreover, by sequencing some mRNA, NGS can provide a quantitative and qualitative evaluation of the microbial community. The analytical sensitivity of NGS approaches that of standard qPCR assays, enabling the detection of pathogen genomes at concentrations as low as 1 · 104 genome copies/mL (Frey et al. 2014).

Some studies have used NGS to identify microbial composition of the tick *Ixodes ricinus*, by sequencing the 16S rRNA gene (Vayssier-Taussat et al. 2013), and for identification of tick *Nuttalliella namaqua* (Mans et al. 2015). NGS can provide a global survey of the microbial composition of vectors, and it is, therefore, useful in epidemiology (Carpi et al. 2011). Tissue samples of animals can also be investigated directly, to detect their pathogens and survey herds or wildlife (Wittekindt et al. 2010). Detection of a pathogen that is present in low copy numbers can be hampered by the background of the host's genomic material. To circumvent this issue, a recent study (Carpi et al. 2015) used an enrichment system to enhance the efficiency of NGS with *Borrelia burgdorferi*, using ticks directly harvested on the field, and provided a cheap and fast technique for pathogen identification. Because of its advantages (fast, reliable, and circumvent culture bias), NGS are considered as a valuable tool to monitor pathogens in ticks and animals, both quantitatively and qualitatively. As the required equipment could still be too expensive for many laboratories, biotechnology companies are continuously improving their technologies to offer cheaper, faster, and easier NGS techniques.

*Multiplex assays*

Multiplex assays have been developed based on nucleic acid or protein detection technologies to enable the simultaneous detection in a high-throughput manner. Several new strategies for multiplex PCR including incorporating tags to amplicons, suspension microsphere arrays, and pyrosequencing have increased both multiplexing and high-throughput capabilities of detection techniques. On the other hand, multiplexing for protein detection has evolved from traditional ELISA assays, with the purpose of measuring multiple analytes in the same sample at the same time. Protein multiplex assays are available in several

different formats based on the utilization of flow cytometry, chemiluminescence, and array-based technology. Compared with traditional ELISA and PCR, multiplex arrays have a number of advantages, including (1) high-throughput multiplex analysis, (2) less sample volume requirements, (3) efficiency in terms of time and cost, (4) ability to evaluate the levels of the given analyte in the context of multiple others, and (5) ability to perform repeated measures of the multiplex panels in the same experimental assay conditions (Leng et al. 2008).

Multiple pathogens or species can be detected by using multiplex PCR assays, but coinfecting pathogens may cause competition in the PCR reaction. Significantly higher concentrations of one pathogen compared with the others can result in the detection of only one organism. However, a recent study reported a real-time multiplex PCR assay to detect the presence of *B. burgdorferi, B. microti*, and *Anaplasma phagocytophilum* simultaneously even when they are present in very low copy numbers (Chan et al. 2013). Elsewhere, for the detection of *B. burgdorferi, A. phagocytophilum*, and a protozoan pathogen *B. microti*, two real-time multiplex PCR assays were reported as a fast and costeffective method for pathogen detection (Hojgaard et al. 2014). Another study used multiplexing for the detection of *Anaplasma spp., B. burgdorferi* sensu lato, and *Bartonella* spp. (Hegarty et al. 2014). The detection of *Ehrlichia canis* and *A. platys* based on 16S rDNA by nested PCR was successfully achieved by using generic primers for *Anaplasmataceae* in the first round of PCR, followed by a second round of PCR using species-specific primers (Rufino et al. 2013).

For the detection of arthropod-borne viruses, a RT-PCR was designed to target S genomic segments of 47 viruses, including 29 arthropod-borne human pathogens, of the family RNA viruses Bunyaviridae (Lambert and Lanciotti 2009). After amplification, DNAs were subjected to a novel multiplex nucleotide sequencing for further species identification within the Bunyaviridae. Similarly, a multiplex qRT-PCR has been developed for the rapid detection and identification of eight medically important Flaviviruses (yellow fever virus, Japanese encephalitis virus, WNV, St. Louis encephalitis virus, and DENV serotypes 1–4 [DENV-1 to DENV-4], respectively) from mosquitoes, by using consensus amplimers located at the RNA-dependent RNA polymerase domain of nonstructural protein 5 (Chao et al. 2007).

Recently, new platforms for the rapid diagnosis of pathogens from direct clinical specimens have been introduced, such as RT-PCR-electrospray ionization-MS (ESI-MS). Here, ESI-MS is used to measure mass of the amplicons amplified by PCR to determine the base composition (Jordana-Lluch et al. 2014). This approach has been successfully used for detecting flaviviruses in biological samples (Grant-Klein et al. 2010, De Filette et al. 2012), *Rickettsia rickettsii, Babesi*a spp., and *Borrelia* spp. (Eshoo et al. 2012, Jordana-Lluch et al. 2014).

Mass spectrometry based assays MS has recently emerged as a diagnostic tool in clinical laboratories (Grebe and Singh 2011). As a diagnostic tool, MS is used for pathogen protein profiling (Sauer and Kliem 2010), protein identification (Kuleš et al. 2014), characterization of post-translational modifications (Bag et al. 2014), and relative/absolute protein quantification (Angel et al. 2012). There are two most important MS platforms used in pathogen diagnostics based on ionization type: matrix-assisted laser desorption/ionization (MALDI) and ESI. Although MALDI is used predominantly in gel-based approaches, ESI can be used in both gel-based and liquid chromatography (LC)-based proteomic approaches.

The gel-based proteomic approach is mostly employed for proteome mapping of circulating serum proteins that are indicative for a specific pathogen infection and/or identification of altered expression of proteins with a potential role as diagnostic tools (biomarkers) (Kuleš et al. 2014), development of therapeutics (vaccines) (Renesto et al. 2005, Kuleš et al. 2016), and antigen-based detection tests (Ndao 2009). Despite some limitations (time consuming, protein properties considerations), the gel-based approach gives the insight of differentially expressed proteins. ESI, for example, LC-ESIMS/ MS is mostly used for large-scale pathogen profiling by using the bottom-up shotgun proteomic approach (Paape et al. 2010) or peptide

mass fingerprinting (Brinkworth et al. 2015), and for quantification purposes rather than for direct microbial identification (Ho and Reddy 2010).

Since the introduction of ‘‘biotyping’’ pathogen identification based on protein profiling directly from clinical samples or colonies made MALDI-time-of-flight (TOF) MS a powerful high-throughput diagnostic method (Seng et al. 2013). MALDI-TOF MS combined with a reference database search (Table 3) has also been used for the rapid identification of vectors and vector-borne pathogens from ticks (Fotso Fotso et al. 2014). However, in case of viral and parasitic diseases, a limitation of the method remains the cultivation of pathogens that are necessary to reduce the confounding background by increasing specific protein concentration in the sample (Mouri et al. 2014).

Using MALDI-TOF MS, different pathogens were identified directly from the ticks, such as *Borrelia* spp. and *Rickettsia* spp. (Calderaro et al. 2014, Yssouf et al. 2015). MALDI-TOF MS has also been employed for the identification of *Leishmania* promastigotes at the species level, consistent with the existing reference molecular method (PCR), but there are still some limitations of the MS method due to principal component analysis (PCA) cluster analysis, which is unable to properly identify subgenera species (Mouri et al. 2014).

For the detection and diagnosis of the malaria Plasmodium parasite both in vitro and in vivo (directly from the blood), a routine, rapid, and high-throughput MS method has been developed. The method is based on the detection of intact ferriprotoporphirin IX (heme) sequestered by parasites in molecular crystal hemozoin by direct ultraviolet laser desorption MS. The intensity of MS signal of the pigment hemozoin is correlated with the number of parasites per unit volume of blood (limit of detection is 10 parasites/lL of blood), making the hemozoin a qualitative and quantitative biomarker for malaria (Demirev et al. 2002, Demirev 2004). For pathogen diagnostics, chip-based MS platforms, such as surface-enhanced laser desorption/ionization time-offlight (SELDI-TOF) MS, can be applied to detect biomarker patterns (Ndao et al. 2010). An advantage of the SELDI is that target proteins can be retained, purified, and characterized on an affinity chip surface depending on protein properties (Protein Chip Array) (Ndao 2009). The limitation of SELDI MS compared with MALDI MS is that SELDI has lower resolution and accuracy, and it is unsuitable for highmolecular- weight proteins (above 100 kDa). The SELDI technique has been used in the diagnostics of African trypanosomiasis (Papadopoulos et al. 2004) and DENV (Poole- Smith et al. 2014).

Apart from proteomic methods, diagnostic MS platforms for volatile organic compounds have emerged, based on odor modification in hosts and vectors due to parasite infection. Head-space solid-phase microextraction/gas chromatographymass spectrometry (GC-MS) combined with multivariate PCA has been used for leishmaniasis diagnosis from canine hair as a noninvasive and painless method that is acceptable for dog owners (de Oliveira et al. 2008). Volatile biomarkers discriminated by this method could be classified as aldehydes, ketones, and hydrocarbons (benzaldehyde, 2-hexanone, and 2,4-nonadienal).

*Chip-based technologies and point-of-care diagnostics*

The desired characteristics of chip technology-based Point-of-Care (POC) diagnostic technologies include (1) disposability, (2) cost-effectiveness, (3) ease of use, and (4) portability (Huckle 2008). POC diagnostics is able to analyse small volumes of body fluids. The cost of diagnostics is also one of the important parameters for global health applications. Systems that can be automated and miniaturized offer enormous advantage over others, as they may be used in field situations requiring less complicated protocols.

For example, disposable dipstick tests seem to indicate the most promising advancement for POC, rapid, sensitive, and cost-effective microbial detection (Doria et al. 2012). A disposable plastic chip and a low-cost portable device have been reported for Plasmodium-specific PCR (Taylor et al. 2014). These chips are run on a custom-built instrument containing a Peltier element for thermal cycling and a laser/ camera setup for amplicon detection of SYBR Green fluorescence, representing an important step toward POC development for malaria control.

For many VBD, the chip-based techniques based on nucleic acid biosensors (NABs) and antibody-/aptamer-based sensing designs are developed (Table 4) (Foudeh et al. 2012). Nucleic acid-based biosensors primarily use DNA, RNA, peptide nucleic acid, and aptamers (both DNA and RNA) as oligonucleotide probes (Bora and Sett 2013). Depending on the transduction platform used, NABs can be optical, electrochemical (either label-based indirect or label-free direct), or piezoelectric. The major advantage of DNA-based probes is their ability to amplify specifically a targeted DNA of pathogen from the host genomic DNA.

An optical sensing-based microfluidic technology was combined with RT, PCR amplification, and microarray hybridization to develop a silicon-based micro-electromechanical system integrated lab-on-chip that can simultaneously detect and differentiate between 26 pathogen species (including bacteria, parasites, and viruses, most of which are vector borne) that cause 14 tropical diseases (Tan et al. 2014). Elsewhere, an oligonucleotide DNA microarray design for multi-gene detection and identification of mosquito-borne RNA viruses was recently developed based on an amplification of three genes from different viral genera for electrochemical detection on a portable, field-tested microarray platform (Grubaugh et al. 2013).

*Metagenomics and metabolomics*

Metagenomics applied to VBD research aimed at characterizing the microorganisms present in the environment (e.g., vectors, media) and at understanding the association of the microbiota with pathogens. Metagenomic analyses are most often undertaken by sequencing the bacterial 16S rRNA subunit or by whole-metagenome shotgun sequencing, typically in a massively parallel pyrosequencing platform (Preidis and Hotez 2015). High-throughput pyrosequencing has been suggested as an improved means of detecting arthropod borne viruses among entire populations of vectors, such as DENV detection in mosquitoes. Sequencing the metagenome of multiple tick vectors revealed known tick-borne pathogens, including *Anaplasma, Bartonella, Borrelia, Ehrlichia, Francisella,* and *Rickettsia* (Nakao et al. 2013).

Large-scale analyses of metabolites produced during the course of infection, by both the parasite and the vertebrate host, may represent a gold mine for the identification of novel diagnostic biomarkers. Metabolites produced by microbial and host cells contain an extraordinary array of physicochemical properties, may be present in virtually any tissue or body fluid, and are found in concentrations differing by multiple orders of magnitude (Preidis and Hotez 2015). MS coupled to GC (GC-MS) can easily detect volatile, thermally stable metabolites with less than micromolar sensitivity, whereas LC (LC-MS) is used to detect nonvolatile polar and nonpolar compounds with nanomolar resolution.

The major drawback of all metabolomics approaches is cost, in terms of both data acquisition and labor intensity of data analysis. Moreover, a fully annotated, comprehensive metabolite library, especially for microbial-derived compounds, is still not complete. Metabolomics has recently resulted in the discovery of biosignatures for several VBD, including diagnostic approaches for malaria and Lyme disease (Tritten et al. 2013, Molins et al. 2015).

Multiple steps along the ‘‘omics’’ approaches (metagenomics, transcriptomics, proteomics, and metabolomics) had to be employed simultaneously to increase the yield of exploratory studies. Just as an example, the initiative launched to compile *L. major* genes evolved enormously and now provides a freely accessible LeishCyc database that houses a comprehensive bank of gene products, metabolites, and biochemical pathways from transcript, protein, and metabolome profiling studies in an integrated format (Doyle et al. 2009).

**Gold Standards in the Diagnostics—Time for Revising?**

Identification of pathogens in biological samples has been dominated by the use of culture-dependent methods, conventional molecular approaches, and serological tests. However, these methodologies suffer from major limitations. Microscopy remains the important part of laboratory testing for the diagnosis of

most VBD, especially in resource-limited settings, but it is highly subjective and dependent on experience and training. Cell culture procedures are time consuming, and isolation of pathogens is not always successful. The specificity and the sensitivity of serological tests are not always optimal, and cross-reactions are a common problem.

The upcoming of molecular diagnostic techniques resulted in several paradigm shifts: viability of the organism is no longer necessary, assays can take hours instead of days, and the prevalence of some diseases is shown to be much higher than previously believed based on culture results alone (Baron 2011).

Molecular assays have become widely available for most VBD, including infections with significant worldwide morbidity and mortality, such as malaria and leishmaniasis. It is noteworthy, however, that most of the molecular tests are based on non standardized, laboratory-developed methods, requiring significant maintenance demands and quality control measures to ensure optimal assay performance. Testing systems are often complex and expensive, requiring sophisticated instrumentation, molecular-grade reagents, highly skilled operators, consistent electricity sources, temperature and humidity controls, and highly regulated transportation and storage capabilities for patient specimens and reagents (Vasoo and Pritt 2013). As a result, the use of laboratory developed tests is generally limited to centralized reference laboratories and specialized research facilities.

With new applications for molecular assays, new questions arise. Can these tests have enough sensitivity and reproducibility to replace traditional methods? What kind of changes in sample collection, transport, and storage are necessary to conduct? Can laboratory personnel, physicians, and veterinarians perform some of these highly technically complex assays, interpret results correctly, and apply them in clinical practice? Are the decreased turnaround time and improved sensitivity and specificity worth the additional cost? Is it the time to replace old-fashioned techniques with modern and high-throughput ones?

New molecular test applications such as LAMP and nucleic acid amplification show promise for future widespread implementation in resource-poor settings, because the need for a thermocycler is obviated, although additional challenges remain, such as specificity of LAMP. Chip technology-based diagnostic technologies also deliver major breakthrough, with the main advantages of disposability, cost-effectiveness, ease of use, portability, and possibility of multiplexing. Hitherto, much progress has been made with molecular multiplexing enabling simultaneous identification and discrimination of a large number of pathogens. It is, undoubtedly, a potential turning point in the molecular diagnostics of VBD.

Over the past decade, advances in genomics and transcriptomics have contributed toward considerably enhancing our knowledge of the host–parasite–vector triangle. As the first vector genome to be sequenced, *Anopheles gambiae* genome heralded the ‘‘genomics era’’ for VBD research. Developments of omics methods, including genomics, transcriptomics, proteomics, metabolomics, and metagenomics, have shown promise for the in-depth research in VBD. The huge amount of omics data needs to be digested by the use of powerful bioinformatics tools. As innovative technologies are being more incorporated into the workflow of the VBD laboratory diagnosis, traditional diagnostics will slowly step aside and serve as a supplemental or confirmatory methodology.

**Authors’ Contributions**

Performed literature searches, wrote and revised the article: J.K., L.P., K.B., L.T., H.-P.F., A.H., A.G., N.G., P.N., V.M., and M.B. All authors read and approved the final version of the article.

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Table 1. Overview of General Advantages and Disadvantages of Diagnostic Methods for Vector-Borne Diseases.

| | *Methods*[a] | *Advantages* | *Disadvantages* |
|---|---|---|---|
| Conventional (classical) methods | Microscopy—blood/tissue smear examination<br>Pathogen isolation<br>Cell culture | Simple equipment and reagents<br>Suitable for resource-limited settings | Labor and time intensive<br>Lower sensitivity<br>Expertise required |
| Serology-based methods | IFA<br>ELISA<br>Immunoblotting<br>PRNT<br>SN<br>HI<br>CFT | Easy sample collection<br>Low cost and easy application | Potential cross-reactivity<br>Lower sensitivity<br>History of exposure (lag time) |
| Nucleic acid-based methods | PCR<br>RFLP-PCR<br>RT-PCR<br>FISH<br>LAMP | Increased sensitivity, specificity, and speed of diagnosis<br>Differentiation of morphologically similar parasites | Equipment and reagent cost<br>Lack of standardization and variability in performance<br>False-positive and -negative results |
| Omics-based methods | NGS<br>MS-based assays (LC-MS/MS, MALDI-TOF, iTRAQ, ICAT, SILAC);<br>Metagenomics (NGS)<br>Metabolomics (GC-MS, LC-MS, NMR) | High-throughput analysis<br>Increased sensitivity and specificity | Equipment and reagent cost<br>Labor intensity of data analysis<br>Lack of sequenced genomes |
| Multiplex methods | Multiplex PCR<br>Microarrays<br>PCR-ESI/MS<br>iTRAQ<br>MRM | Detection of coinfection<br>High-throughput analysis<br>Less sample volume requirements<br>Efficiency in terms of time and costs | Competition between coinfecting pathogens |

Diagnostic tools are divided as a part of conventional (classical) methods, serology-based methods, nucleic acid-based methods, omics-based methods, and multiplex-based methods. Note that performance and requirements of individual assays may vary.

[a]Not an exhaustive list.

IFA, immunofluorescence assay; ELISA, enzyme-linked immunosorbent assay; PRNT, plaque reduction neutralization test; SN, serum neutralization test; HI, hemagglutination inhibition test; CFT, complement fixation test; RFLP-PCR, restriction fragment length polymorphism PCR; FISH, fluorescence in situ hybridization; LAMP, loop-mediated isothermal amplification; PCR-ESI/MS, PCR electrospray mass spectrometry; iTRAQ, isobaric tags for relative and absolute quantitation; MRM, multiple-reaction monitoring; NGS, next-generation sequencing; MS, mass spectrometry; LC-MS/MS, liquid chromatography-tandem mass spectrometry; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; ICAT, isotope coded affinity tag; SILAC, stable isotope labeling by amino acids in cell culture; GC-MS, gas chromatography–mass spectrometry; NMR, nuclear magnetic resonance.

Table 2. Pathogen Identification by Recommended Serology-Based Methods

| *Pathogen* | *Disease* | *Serology-based methods* | *References* |
|---|---|---|---|
| *Borrelia burgdorferi* | Human borreliosis | Two-tiered tests | Branda et al. (2011) |
| | Canine/feline borreliosis | Two-tiered tests | Mead et al. (2011) |
| CCHFV | Human CCHF | ELISA | Mertens et al. (2013) |
| | Cattle-CCHF | ELISA | Mertens et al. (2015) |
| DENV | Human dengue fever | IgM capture ELISA | Hunsperger et al. (2009) |
| WNV | Human West Nile virus infection | IgM immunoassays | Shi and Wong (2003) |
| TBEV | Human TBE | IgM/IgG capture ELISA<br>Plaque reduction neutralization test (PRNT) | Lindquist and Vapalahti (2008), Levanov et al. (2014) |
| *Leishmania* spp. | Visceral leishmaniasis | Direct agglutination test (DAT) | Lockwood and Sundar (2006) |
| | Visceral leishmaniasis | rK39 dipstick test | Lockwood and Sundar (2006) |
| | Cutaneous leishmaniasis | Leishmanin skin test (Montenegro skin test) | Weigle et al. (1991) |
| *Babesia microti* | Human babesiosis | Indirect IFA | Krause et al. (1994) |
| *Babesia divergens* | Bovine babesiosis | IFA, ELISA | Zintl et al. (2003) |

CCHF, Crimean-Congo hemorrhagic fever; CCHFV, Crimean-Congo hemorrhagic fever virus; DENV, dengue virus; TBE, tick-borne encephalitis; TBEV, tick-borne encephalitis virus; WNV, West Nile virus.

Table 3. Pathogen Identification by Mass Spectrometry-Based Diagnostic Methods

| *Pathogen* | *Species* | *Diagnostic MS-based method* | *Sample type* | *References* |
|---|---|---|---|---|
| *Flavivirus* | Mosquito-borne viruses: Dengue virus 1, Dengue virus 2, Dengue virus 3, Dengue virus 4, Japanese encephalitis virus, St. Louis encephalitis virus, Tembusu virus, West Nile virus, Yellow fever virus<br>Tick-borne viruses: Central European subtype TBEV, Far Eastern subtype TBEV Karshi virus, Kyasanur Forest disease virus, Langat, Omsk hemorrhagic fever virus, Powassan | RT PCR-ESI-MS | Infected vectors (Mosquito/Tick), blood, brain tissue, spiked samples | Grant-Klein et al. (2010), De Filette et al. (2012) |
| *Rickettsia* | *Rickettsia c. conorii*<br>*Rickettsia ricketsii* | Protein profiling by MALDI TOF MS<br>RT PCR-ESI-MS | Tick hemolymph<br>Culture, plasma | Yssouf et al. (2015)<br>Wolk et al. (2009) |
| *Borrelia* | *B. afzelii*,<br>*B. crocidurae*,<br>*B. burgdorferi*,<br>*B. recurrentis*,<br>*B. crocidurae*,<br>*B. duttonii*,<br>*B. lusitaniae*,<br>*B. japonica*,<br>*Borrelia* sp.,<br>*B. andersonii*,<br>*B. garinii*,<br>*B. californiensis*,<br>*B. valaisiana*,<br>*B. hermsii*,<br>*B. turcica*<br>*Borrelia* | Protein profiling by MALDI TOF MS<br>Protein profiling by MALDI TOF MS<br>RT PCR-ESI-MS | Plasma<br>Tick legs<br>Culture<br>Whole blood | Fotso Fotso et al. (2014)<br>Eshoo et al. (2012), Jordana-Lluch et al. (2014) |
| *Leishmania* | *L. major*<br>*L. tropica*<br>*L. killicki*<br>*L. guyanensis*/ *L. Panamensis*[a]<br>*L. braziliensis*/ *L. Peruviana*[a] | Protein profiling by MALDI TOF MS | Promastigotes cultured from skin lesions | Mouri et al. (2014)<br>Fotso Fotso et al. (2014) |
| *Plasmodium* species | *Plasmodium falciparum*<br>*Plasmodium yoelii* | Hemozonin assay by LDMS | Blood | Demirev et al. (2002) |
| *Babesia* | *Babesia canis* | MALDI TOF, RT PCR-ESI-MS | Plasma | Adaszek et al. (2014) |

[a]Not differentiated by dendrogram built from principal component analysis.

LDMS, laser desorption MS; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; RT PCRESI/MS, reverse transcription PCR-electrospray mass spectrometry.

Table 4. Examples of Chip-Based Methods in Vector-Borne Disease Diagnostics

| *Technique* | *Species* | *Chip type* | *Sample type* | *References* |
|---|---|---|---|---|
| Nucleic acid-based biosensors (NABs) | *Trypanosoma brucei gambiense*, *T. brucei rhodesiense*<br>*Trypanosoma cruzi*<br>*Plasmodium falciparum* (*P. falciparum*), *P. knowlesi*, *P. malariae*, *P. ovale*, and *P. vivax*<br>Chikungunya virus (CHIKV)<br>Dengue virus (DENV) (serotype 1 to 4)<br>Japanese Encephalitis virus (JEV)<br>West Nile virus (WNV)<br>Rift Valley virus (RVV) | Integrated lab-on-chip microelectromechanical systems (MEMS) | Plasma | Tan et al. (2014) |
| | *Plasmodium falciparum* | Microfluidic (nucleic acid based) | Blood | Warkiani et al. (2015) |
| | *Flavivirus* (*Flaviviridae*), *Alphavirus* (*Togaviridae*), *Orthobunyavirus* (*Bunyaviridae*), and *Phlebovirus* (*Bunyaviridae*) | ArboChip5.1 DNA microarray | Mosquitoes | Grubaugh et al. (2013) |
| | *Leishmania donovani* | ITO-coated glass plate DNA biosensor (18S rRNA) | Blood | Mohan et al. (2011) |
| Antibody/aptamer based biosensors | *Leishmania* spp. | rK39 dipstick test, ELISA-based dipstick | Blood | Bern et al. (2000) |
| | Dengue virus | Paper-based stacking flow to detect dengue-specific immunoglobulins | Saliva | Zhang et al. (2015) |
| | Dengue virus | Magnetic bead-based assay to detect Dengue virion and antibodies | Serum | Lee et al. (2009) |
| | *P. falciparum*, *P. vivax* (variants 210 and 247) | VecTest™ Malaria ELISA-based dipstick | Ground mosquitoes | Ryan et al. (2002) |
| | *Anaplasma phagocytophilum*, *A. platys*, *Borrelia burgdorferi*, *Ehrlichia canis*, *Ehrlichia chaffeensis*, and *Ehrlichia ewingii* | SNAP® Multi-Analyte assay | Serum | Hegarty et al. (2015) |