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Pathogen detection and disease diagnosis in wildlife: challenges and opportunities

A.L. Michel [(1)*], H. van Heerden [(1)], D. Prasse [(2)], V. Rutten [(1, 3)], S. Al Dahouk [(2)] & B.M. Crossley [(4)]

(1) Department of Veterinary Tropical Diseases, Bovine Tuberculosis and Brucellosis Research Programme, Faculty of Veterinary Science, University of Pretoria, Private Bag X04, Onderstepoort 0110, South Africa
(2) Department of Biological Safety, German Federal Institute for Risk Assessment, Postfach 12 69 42, D–10609, Berlin, Germany
(3) Department of Biomolecular Health Sciences, Division of Infectious Diseases and Immunology, Section Immunology, Faculty of Veterinary Medicine, Utrecht University, Postbus 80163, 3508 TD, Utrecht, the Netherlands
(4) California Animal Health and Food Safety Laboratory, University of California Davis, 620 West Health Science Drive, Davis, California, 95616, United States of America
*Corresponding author: anita.michel@up.ac.za

Summary

Recent decades have witnessed an increase in the demand for pathogen detection and other diagnostic approaches for wild animal populations as interest has grown in infectious diseases that occur in wildlife. This is partially as a result of human population encroachment into wildlife habitats, efforts to protect vulnerable wildlife populations and an increased commercial use of wildlife. As contact rates increase, so does the spillover risk of pathogens between wildlife, domestic animals and humans.

The challenges encountered when developing and validating diagnostic tests for use in wildlife are manifold and primarily centre on issues concerning diagnostic samples and suitable test approaches. Under these constraints, it is tempting to resolve the situation by adopting diagnostic tests validated for domestic animal species. Numerous examples using this approach have been published in the literature and some are presented in this paper. The authors present scenarios highlighting advantages and disadvantages of different types of tests in wildlife and current impediments to their validation.

Special attention is drawn to future perspectives with regard to the potential of novel and innovative technologies to improve detection of existing, and discovery of unknown, pathogens as well as to accelerate our understanding of infectious wildlife diseases and their diagnosis.

Keywords

Diagnostic test – Emerging disease – Infectious disease – Novel diagnostic approach – Pathogen detection – Test validation – Wildlife – Zoonosis.

Introduction

The changing status and use of wildlife in modern society necessitates an increasing repertoire of tools to detect pathogens that affect them (1, 2). Surveillance for wild animal pathogens holds the key to demonstrating freedom from diseases, managing persistent diseases, detecting disease spillover and ensuring the early detection of emerging and re-emerging pathogens.

Test validation for wildlife is more complex than that for domestic animals, as there are a number of issues to resolve. These issues include: the emergency use of assays rapidly developed in response to emerging disease threats; new agents emerging from wildlife; and the often-limited information available on the pathogens and commensal agents existing in wildlife populations (3). Although tests validated for use in domestic species are often employed in testing wildlife, these tests require further validation to ensure quality of the results for the new species and sample matrices.

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The concept of test validation, which is linked to the requirement for diagnostic tests to be 'fit for purpose', has been introduced and promoted by the World Organisation for Animal Health (OIE) through a standardised validation pathway which serves to document the integrity and quality of test results while also allowing for the harmonisation of test protocols between trading partners (3). The guidelines for the validation of diagnostic tests for infectious diseases were initially intended for universal use in domestic and wild animal species, but due to specific challenges associated with test validation in wild animals, a dedicated set of guidelines applicable to wildlife was adopted in 2014 (3).

While the principles of validation, i.e. development, optimisation and standardisation, remain unchanged, the introduction of 'provisional recognition of a test' based on preliminary estimates of its performance is a new addition to the validation pathway for wildlife tests. Two scenarios are considered: *1)* a wildlife species for which a specific validated test exists in a taxonomically closely related domestic species, and *2)* a wildlife species without a validated test in a related species. In both scenarios 1 and 2, provisional recognition of a test can be achieved by demonstrating its analytical and diagnostic performance in the respective required minimum number of samples. It is not, however, intended as the endpoint of validation. Full validation remains a long-term goal that is achievable through continuous systematic data collection. Provisional recognition offers a compromise when a fully validated test in wildlife is unavailable; a provisional test with science-based validation data in line with the 'fit-for-purpose' requirement is valuable, even if the data are limited (3).

The authors review a series of recently published case studies that outline the most important limitations in determining diagnostic performance for tests in wildlife and provide examples of step-wise approaches towards test validation in different settings and different geographical regions. They also showcase how novel technologies may in future help close gaps in our understanding of wildlife infections and improve disease detection.

Challenges in validating diagnostic tests in wildlife

The ability to obtain sufficient and representative samples for estimating the test parameters, including diagnostic sensitivity and specificity, is a key challenge in test validation (4). Other common problems include poor sample quality and limited access to post-mortem samples and to samples that are representative of every stage of the disease, due to a lack of access to infected animals covering the full spectrum of disease stages (3). Experimental infection can be used in the test validation process, but although this meets the requirements for provisional validation, it rarely allows for more than an initial evaluation of the performance parameters of newly developed tests in the wildlife target species, mainly for ethical and financial reasons (5, 6). Physiological stress responses associated with captivity may significantly alter the course of the disease (7). Due to the biological stress response to captive conditions, a wild animal's immune response and the associated disease progression is likely to be measurably different from the natural situation. Experimental conditions may trigger the re-activation of a latent infection (8) or increase susceptibility to concurrent infections, which may potentially interfere with the test validation process.

Direct detection methods, including histopathology, culture, antigen capture enzyme-linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR), are all equally applicable to all animal species, but they are labour- and cost-intensive compared to serological tests measuring antibodies, which have the major advantages of being low cost and high throughput. Serological tests are more effective in supporting disease control strategies and certification of freedom from disease and are, therefore, likely to remain a cornerstone of disease surveillance in wildlife. Consequently, efforts to validate them are crucial. It is, however, a drawback that the most sensitive serological tests require species-specific reagents (9). At least for some pathogens, multi-species serological tests have been successfully used alongside standard assays in a range of wildlife species, e.g. the serum agglutination test (for brucellosis), competitive ELISA (for African horse sickness) (10) and lateral flow assay using protein G (an immune-globulin-binding protein) (tuberculosis, brucellosis) (11, 12, 13).

The detection of pathogen-specific antibodies by lateral-flow type tests can be a valuable low-cost tool in wildlife disease surveillance. In the case of tuberculosis diagnosis, preliminary validation has been reported for a lateral flow assay in wild boar (14) and for the Dual Path Platform (DPP) VetTB assay in badgers. The use of the latter assay could additionally facilitate the implementation of a greater variety of control strategies in European badgers (*Meles meles*) in a 'trap-side' setting (15, 16). Another example, the *Brucella* card test, is a traditional serological assay that has been shown by Schumaker *et al.* to provide 96.4% diagnostic sensitivity and 76.9% specificity for use in screening for brucellosis in wild elk and bison populations (17). In the Greater Yellowstone Area of the United States of America, a combined approach of screening by serology and confirmation by culturing of tissue collected at slaughter has been used for surveillance of brucellosis. A similar approach was followed during a devastating outbreak of peste des petits ruminants in the critically endangered Saiga antelope (*Saiga tatarica mongolica*) in Mongolia in 2017. In

that case, an immuno-chromatographic antigen detection assay, developed for small domestic ruminants, was used successfully in ocular and nasal secretions in parallel with other direct detection assays, including histopathology and reverse transcription polymerase chain reaction (RT–PCR) (18).

Where the introduction of a pathogen causes mortalities in wildlife, post-mortem examination is generally the most informative tool in providing the first disease diagnosis, but it is often compromised due to advanced decomposition of the index case. This was observed in an outbreak of canine distemper which reduced the lion (*Panthera leo*) population in a small wildlife reserve by 93% (19). The diagnostic approach depended on the euthanasia and pathological examination of other clinically affected animals. Antigen and antibody detection assays were unsuccessful due to the lack of conjugated anti-species antibodies. However, a real-time PCR test, which could be phased in, assisted in the further diagnosis of cases and management of the outbreak (20).

Tests based on PCR are confirmatory and can produce results quickly, making them valuable diagnostic tools in ruling out highly contagious diseases that have a huge economic impact and require immediate control measures. These include diseases such as foot and mouth disease (FMD), African swine fever (ASF) and important human zoonoses such as rabies and, most recently, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Recently, a portable, battery-operated quantitative RT–PCR platform has been introduced for the diagnosis of canine distemper (21). The on-site detection of ASF virus in suids has been demonstrated using a combination of a rapid PCR test and a lateral flow strip (PCR–LFS) (22) or a more cost-effective recombinase-based isothermal amplification assay (23). This transfer to a mobile technology platform has opened up opportunities to use PCR-based tests in point-of-care (POC) diagnostics in remote wildlife populations. Point-of-care applications have an important role to play in wildlife diagnostics because they eliminate disadvantages associated with sample handling, storage and long-distance transport.

Small wildlife populations managed in fenced reserves are more vulnerable to disease introductions than cohorts in large ecosystems (24). To avert a negative impact from disease on conservation, pathogen screening of animals and early, accurate detection are imperative before stocking or exchange of breeding individuals. An outbreak of bovine tuberculosis in African buffalo (*Syncerus caffer*) in a newly founded conservation area in South Africa illustrated the spectrum of economic and conservation implications as veterinary quarantine measures put an indefinite halt on the movement of buffalo out of this reserve (25). Epidemiological investigations revealed that the outbreak did not originate from buffalo, for which provisionally validated immunoassays exist (21), but from introduced, untested game species (25). A similar situation is found in the United States of America, where both farmed elk (22, 23) and bighorn sheep (26, 27) contracted disease from other species of unknown disease status.

Such examples show that, in the absence of suitably validated tests, a combination of multiple, especially direct, detection methods should be the recommended diagnostic approach. Not only is such an approach the best option for diagnosis in the interim, but it also goes a long way towards test validation.

Spillover and spillback of diseases, including zoonoses

Human population growth, commercial wildlife farming, the encroachment of human settlements into previously uninhabited wildlife areas and the implementation of new agricultural activities in these areas have created an interface between domestic and wild animals on the one hand, and humans on the other. Increasing contact rates between these populations and their pathogens facilitate the transmission and exchange of infectious diseases previously thought to be host specific. In countries with a rich biodiversity, wildlife resources play an important role in the economy, contributing to employment, the tourism industry and food production. In South Africa, the resources are divided between state-owned wildlife conservation areas and the wildlife industry. The latter includes wildlife ranching, focused mainly on breeding and live animal sales; wildlife ecotourism and hunting; and wildlife products, e.g. venison, trophies and hides. Control programmes for diseases affecting both livestock and wildlife are mandatory. In the case of commercially used African buffalo, the diseases monitored by control programmes include FMD, theileriosis caused by *Theileria parva*, bovine brucellosis and bovine tuberculosis. As financially challenging as these control programmes are for the owners, they have provided unique opportunities for diagnostic data collection. If well coordinated and approached strategically, these management interventions can effectively drive the test validation process for trade-sensitive diseases in wildlife (11, 21, 28, 29).

Monitoring of the wildlife reservoir population for the purpose of managing the risk of pathogen spillover to cattle is more complex if it necessitates the development of diagnostic tests for a new target species (scenario 2). This is the case with Eurasian wild boar, red deer and white-tailed deer, which are all widely considered to be members of the *Mycobacterium bovis* maintenance host community.

Serological approaches using ELISA have been developed and shown to have diagnostic merit (12, 13, 30). A similar situation, requiring test development for a new species, exists for *Mycobacterium tuberculosis*. Tuberculosis can be spread as a reverse zoonosis from humans to elephants and can be transmitted amongst animals in zoos and in African and Asian elephant camps (both inter- and intra-species transmission). In spite of the fact that analytically sound tests have been established (31, 32, 33), a clear-cut validated protocol for diagnosis of tuberculosis in elephants is still not yet in place. This is mostly due to the complex progression of the disease, concomitant immune responsiveness and lack of samples representative of all disease stages. Clearly, a combination of the available assays seems by far the best option for coming to a trustworthy diagnosis, as reported for bovine tuberculosis in buffaloes (34) and African lions. In these cases, assays have been described for measuring *M. bovis*-specific antibody titres, T-cell reactivity *in vitro* (interferon gamma release assay) (35) and T-cell reactivity *in vivo* (tuberculin skin test) (36).

Anthrax, bovine brucellosis and rabies are classical examples of 'neglected' zoonoses which can be transmitted between wild animals and livestock or humans. These diseases challenge Veterinary Services to strike a balance between wildlife conservation, disease control in livestock and the protection of human health. In acute diseases, such as anthrax and rabies, indirect detection methods are of little use; direct antigen or nucleic acid assays form the gold standard for diagnosis and can be used effectively in wildlife species which succumb to clinical disease (37, 38). Serological analysis has only rarely been used in studies of anthrax, possibly due to the perception that most infected animals will not survive to produce an antibody response. However, serological tests for anthrax-specific antibodies in carnivores were employed to ascertain the disease pattern in anthrax-endemic regions (39, 40).

Surveillance of rabies infection in wild mammals requires the use of serological tests, which are extremely difficult to validate, as results from corresponding antigen detection tests may yield negative results due to clearance of the rabies virus following exposure (41, 42).

A literature review on brucellosis caused by *Brucella melitensis* in wildlife up to 2018 (B. Glover and H. van Heerden, unpublished data) highlights the fact that most of the diagnostic methods used (serology and/or culture) are validated for use in livestock (scenario 1). This raises concerns due to possible differences in the suitability of test reagents (9), in the serological responses of wild animals, and in *B. melitensis* transmission in wildlife, which may require adapted approaches to diagnosis. Figure 1 illustrates the culture-confirmed occurrence of brucellosis in wildlife per country as identified by the literature review (the results indicate some level of under-reporting, when compared to the official OIE records for brucellosis caused by *B. melitensis*). One of the examples of testing wild animals with tests validated in livestock came from a report of *B. melitensis* infection in sable antelope (*Hippotragus niger*) on two wildlife ranches in South Africa that kept both livestock and wildlife (43). On both ranches, brucellosis was diagnosed using serological tests validated for cattle, and *B. melitensis* biovars 1 and 3 were subsequently isolated from culled reactor animals. The persistent infection is consistent with the disease circulating within small, ranched populations and being spread through the keeping and trading of high-value animals (43). *Brucella melitensis* was isolated from ibex (*Capra ibex*) in the French Alps using a test validated for use in livestock species (44). France was bovine brucellosis-free from 2003 until 2012, when the disease re-emerged in bovines and humans through wildlife (Alpine ibex) (45). Genetic analysis indicated spread from wildlife to livestock and humans (46).

Systematic surveillance and epidemiological investigation of wildlife infections using validated tests are crucial, particularly for those wildlife species that are reservoir hosts and disease vectors, as demonstrated by the numerous reports concerning, amongst others, swine (47), sheep (48) and elk and deer (5, 49, 50). In the Greater Yellowstone region in the United States of America, factors such as increased population and animal density, changes in land management, and the reintroduction of wolves are believed to have contributed to the increase of brucellosis due to *Brucella abortus* among wild elk and, thus, to the related elevated risk of transmission to domestic livestock (5).

Pathogen exchange between wild and domestic animals is also facilitated through migration of wildlife hosts or vector expansion into livestock farming areas as a consequence of climate change. As a result of the expansion of *Culicoides* midges, the vector of bluetongue virus (BTV), some wild ruminant species in Europe can maintain BTV, with the host and virus living in symbiosis. In the Mediterranean Basin, cattle and deer both drive a cycle of bluetongue virus, with both cycles linked through *Culicoides* midge species (51). Bluetongue disease causes great economic losses due to trade restrictions associated with outbreaks. Bluetongue virus cross-reacts with many antigenically related viruses, including some that are economically important, so reliable tests to detect BTV are used (and others are being developed), with RT–PCR being the most widely employed method (52). Diagnostic approaches used in domestic and wild ruminants are the virus neutralisation test, other serological assays, isolation of virus from blood and semen, and identification with PCR. Moreover, there is increasing interest in next-generation sequencing, as detecting the presence of diagnostically relevant DNA fragments is a fast and relatively cheap alternative to other tests.

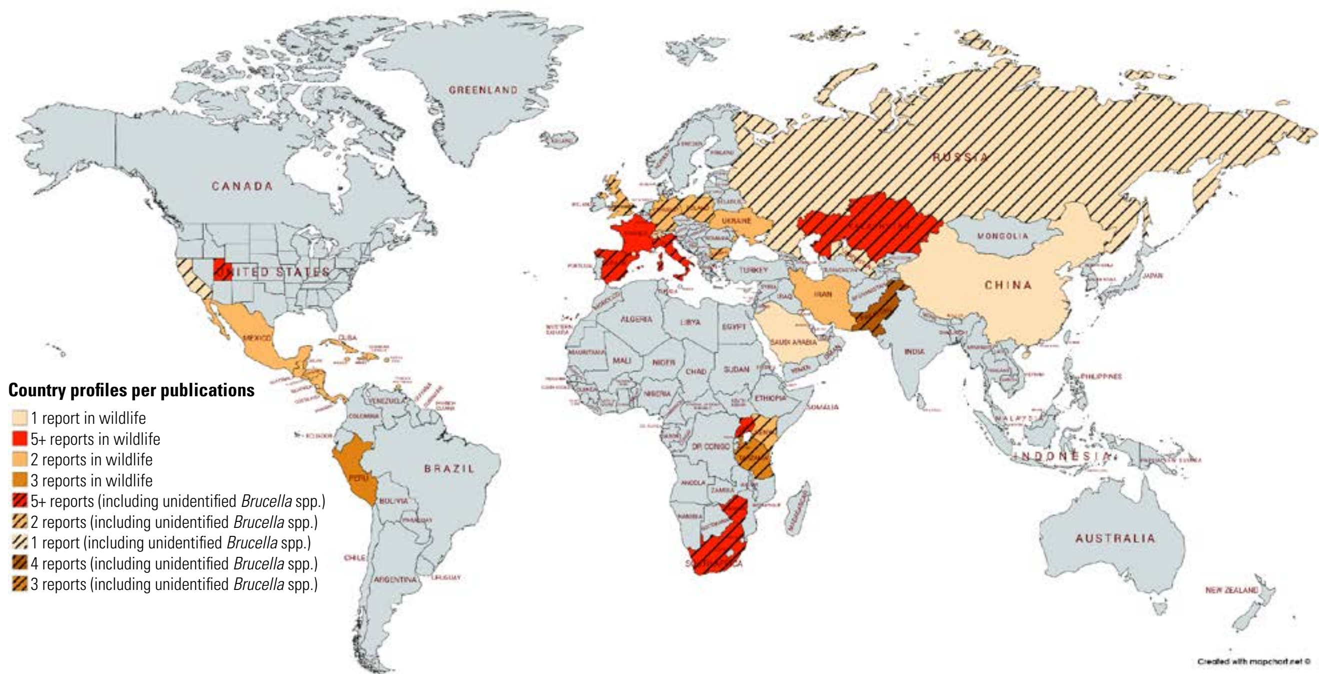

Fig. 1

A geographical representation of countries where *Brucella melitensis* has been isolated from wildlife, based on literature from 1962 to 2018

The map includes areas where the *Brucella* species was unidentified but was suspected to be *B. melitensis*. The literature search was carried out using the keywords '*Brucella melitensis*' and 'wildlife' on Pubmed from 1962 to 2018

Wildlife and emerging diseases

Due to minimal surveillance of endemic agents and pathogens in wild animal populations, the early detection of emerging diseases is limited. *Batrachochytrium dendrobatidis*, which causes a fungal disease that has decimated amphibian populations around the world (53), is believed by some to be the first emerging wildlife pathogen to have caused widespread species extinctions. Other emerging diseases that have been associated with dramatic population declines in wild animals include white-nose syndrome, a fungal disease that has caused a sharp decrease in bat populations in North America since 2006 (54), and snake fungal disease (caused by *Ophidiomyces ophiodiicola*), which leads to high mortality in multiple species of snake (55). In some situations, the source of an emergent disease is unknown, while in other situations the pathogen can be traced to spillover from one species to another, as occurred with Hendra virus, which spilled over from a fruit bat reservoir to horses and humans in 1994 (56, 57). Similarly, Menangle virus spilled over from fruit bats to pigs and humans in 1997 (58), and Nipah virus crossed from fruit bats to pigs, dogs, and humans in 1999 (56). In addition to causing mortality in the host species, emerging diseases have been shown to also threaten associated predator populations. For example, a new variant of rabbit haemorrhagic disease virus affecting wild rabbits was reported in the Iberian Peninsula in 2011, and long-term monitoring programmes in northern Spain subsequently documented a decline not only in the wild rabbit population, but also in the highly endangered Iberian Lynx (*Lynx pardinus*), which feeds on wild rabbits (59).

Once a new disease emerges, post-mortem examination (diagnostic pathology) combined with epidemiological and ecological evidence is used to verify the source and impacts of the disease. These studies are supported by laboratory testing, using techniques that may range from classic tissue examination (60) and agent isolation in *in vitro* cultures with subsequent identification (61), to molecular evaluation of the pathogen's genome (62, 63). Genome and transcriptome analysis of agents circulating in wildlife can play an important role in understanding and predicting public health risks. For example, analysing influenza viruses circulating in avian species (64) can alert us to possible outbreaks in human populations, and analysing SARS-CoV viruses circulating in bats, civet cats and pangolins can help us understand more about COVID-19 in humans (65, 66).

The process of test development through validation ideally involves a diverse team of scientists knowledgeable of the species affected, microbiologists with an understanding of the agent, and diagnosticians with applied expertise in the

laboratory and test approaches being used (67). Whoever is involved, a key concern is the time delay between disease recognition and the development of new tests (or modification of existing tests) for effective diagnosis and surveillance. The process may be quick or disjointed and prolonged for years due to perceived infrastructure or skills needs, and cost considerations. The pace at which tests are developed, validated, and implemented largely depends on who is affected by the pathogen. The tests that are developed most rapidly are for diseases that pose a risk to human populations, e.g. Hendra and Nipah viruses (68), pandemic influenza (69) and SARS-CoV (65). Tests for agents posing risks to commercial food animals (70), such as Schmallenberg virus and emerging food-borne parasites, or those that pose a risk to wild animals, including both endangered and non-endangered species (as mentioned above), usually take longer to develop. In the case of Hendra virus, Nipah virus and SARS-CoV, prescribed laboratory testing began within months of the first human outbreaks (68). In contrast, following the detection of the first cases of the aquatic animal disease caused by *B. dendrobatidis*, it took years for diagnostic tests to be developed, documented and validated (71).

Novel technology platforms

Classical microbiological culture and PCR approaches are currently the gold (reference) standard for the direct detection of pathogens in domestic and wild animals (4, 72). However, these methods are often time-consuming and laborious and may lack sensitivity in the case of fastidious or as yet understudied microorganisms.

In this section, the authors present novel and innovative targeted and untargeted detection methods, which have great potential to overcome current diagnostic limitations. Some of these techniques are still under study and have only been tested *in vitro*, others have already found their way into field applications in both livestock and wild animals.

Significant improvements have been made to enhance and refine targeted molecular methods for direct and rapid detection of a pathogen from clinical samples (i.e. POC). As an emerging technology, digital droplet (dd) PCR enables quantification without the need for internal references or calibration curves. It also facilitates high-throughput screening and has a higher sensitivity and specificity than real time PCR (PCR). It is robust due to the fact that it is not affected by inhibitors and DNA contamination (73). A recently published *in vitro* study demonstrated the usefulness of ddPCR for direct detection of bacteria and antibiotic resistance genes (engineered *Escherichia coli* with low copy number plasmid containing an antibiotic resistance marker) from whole blood samples without prior DNA isolation, on a microfluidic device called IC3D (74). Spike-in experiments showed a limit of detection of the IC3D assay of 1–10 colony-forming units (CFUs) per millilitre (ml). In comparison, the real-time PCR showed a limit of 1,000 CFU/ml and a commercial platform had a limit of 50–100 CFU/ml. The IC3D method is still in its infancy and improvements are needed to reduce costs and hands-on time for field applications.

A second novel POC platform, based on the CRISPR-Cas system, has already been used for direct detection of human pathogenic viruses such as papilloma, Zika and dengue (75, 76, 77). CRISPR–Cas, originally described as a prokaryotic defence system against foreign molecules, is nowadays a powerful tool for genome editing and bioengineering (78, 79, 80). By making use of new Cas enzymes (77, 81, 82, 83), paper-based lateral flow assays were developed combining isothermal recombinase polymerase amplification and cleavage activity of the endonuclease (DETECTR and SHERLOCK approach) (75, 76, 77). Direct heating of diagnostic samples to destroy nucleases before processing (HUDSON) (84) rendered nucleic acid extraction unnecessary. Hence, this test system is fast and inexpensive and allows for highly sensitive detection of pathogenic nucleic acids at molar levels directly from clinical samples (76).

However, methods based on ddPCR and CRISPR-–Cas are targeted approaches, and pathogen detection depends on known primer combinations and species-specific CRISPR RNA guides, respectively. Simultaneous identification of various pathogens in a clinical sample remains difficult, and novel or uncommon pathogens go undetected.

A change in strategic thinking and the use of technological advances for *de novo* identification of hitherto unknown pathogens will expand our knowledge about unexpected disease transmission and (re-)emerging infectious diseases in wildlife (85). Although still in the very early phase of clinical implementation, metagenomics (DNA) and metatranscriptomics (RNA sequencing [RNA-seq]) are attractive tools to screen broadly, and without bias, for clinically relevant microbes in complex sample matrices and for host reactivity. In this way, biomarkers can be discovered, which may promote the development of early-stage diagnostic tests and alternative vaccines (based on messenger RNA [mRNA], DNA or immunogenic antigens) (86, 87). Transcriptome profiling is a highly advantageous method for direct detection of live pathogens and RNA viruses and for the identification of host biomarkers of active infection (86). RNA sequencing of animal samples could thus unravel unique RNA biosignatures specific for the host response to a certain infection (88). Circulating secreted RNAs (seRNAs) in the blood, but also differentially expressed mRNAs, microRNA (miRNA) and long non-coding RNA will be attractive diagnostic targets, as they

can be detected using microfluidic chips. This technology is already applied in human diagnostics (89).

To expand our knowledge about pathogens emerging in wildlife, Wu *et al.* (90) monitored and compared the viral population landscape of different rodent and other small mammal species that are widely distributed within habitats in close proximity to humans and livestock throughout China. Metagenomic virome analysis revealed a high prevalence and great diversity of viruses in rodents and shrews, including viruses that are known to cause severe animal and human diseases, e.g. haemorrhagic fever (*Arenaviridae*), indicating the importance of rodents as potential zoonotic reservoirs (90).

The current COVID-19 pandemic is a reminder of the devastating impact that an emerging zoonotic disease can have. The disease-causing agent is SARS-CoV-2, a coronavirus showing high similarity to the RaTG13 virus from bats, which are assumed to be the reservoir hosts (91). Many efforts have been made to identify potential intermediate animal hosts that could be involved in the spillover from wildlife to humans. Various studies, including high-throughput metagenomic sequencing of wildlife samples in combination with comparative phylogenomic analysis, confirmed the close relationship between SARS-CoV-2, the pangolin-CoV and RaTG13 (sequence identity between 80% and 98%). This led to the hypothesis that SARS-CoV-2 originated from a recombination event between a virus similar to pangolin-CoV and a virus similar to RaTG13. All these studies emphasise the importance of fundamental research for a better understanding of zoonotic pathogens in wildlife.

Alonso-Hearn *et al*. (92) provided an example of the indirect diagnosis of paratuberculosis (Johne's disease) based on the host's response to *Mycobacterium avium* ssp. *paratuberculosis* (MAP). Reliable detection of the causative agent in silently shedding, asymptomatic carrier animals is only possible at later stages of infection, impeding surveillance efforts (92). Therefore, to be able to contain outbreaks in livestock populations, it is vital to find novel biomarkers to detect MAP before it spreads within and between herds. Current studies focus on the miRNA landscape of the host by analysing its blood transcriptome. In a differential RNA-seq study on MAP-infected Holstein cattle, transcriptome profiles of cows were compared and it was found that some of the identified differentially expressed genes seem to play a specific role in immune response during MAP infections, and may serve as novel biomarkers for targeted detection, which is also useful for surveillance in wildlife herds (92).

In the development of POC diagnostics for human tuberculosis, transcriptomics and proteomics analyses have identified biomarkers suitable for laminar diffusion assays that are used to assess cytokine profiles in sera and supernatants of white blood cells stimulated *in vitro*, thus enabling the differentiation between the various stages of disease progression (91, 93, 94). Because of the huge degree of homology between *M. tuberculosis* and *M. bovis,* and similarity in disease development, diagnosis of bovine tuberculosis may benefit from a similar approach. Moreover, as in the case of paratuberculosis, transcriptomic analyses of circulating small RNA molecules will support the diagnosis of bovine tuberculosis.

Proteomic approaches are also suitable for direct pathogen detection in complex clinical samples and to confirm the presence of the live agent. Proteotyping, for instance, uses high-resolution liquid chromatography tandem mass spectrometry (LC–MS/MS) for peptide profiling (95). Major advantages over whole-cell matrix-assisted laser desorption/ionisation time-of-flight (MALDI–TOF) mass spectrometry, which requires bacterial culture and isolation, are that LC–MS/MS enables in-depth analysis of microbial metabolic patterns and identification of virulence and antibiotic resistance signatures (95).

A study on the detection of *Francisella tularensis* from infected hare carcasses has already demonstrated the applicability of LC–MS/MS in wildlife without prior cultivation (96). High-resolution electrospray ionisation (ESI) LC-MS/MS analysis on liver and spleen tissue samples of infected and uninfected animals defined 4,223 species-specific marker peptides, which correctly identified *F. tularensis* without cultivation. Spike-in experiments showed that the limit of detection for pathogen-specific peptides correlated with the number of spiked-in genome equivalents.

Conclusions

Efforts to establish diagnostic tools and pathways for infectious diseases in wildlife are more complex than those for diseases in domestic animals, and they are invariably met with many more challenges. Some of these challenges cannot be overcome or require immense inputs in terms of expertise, finances and infrastructure. Several examples mentioned in this paper emphasise the advantages of using a test which exists for a related domestic animal species. While it is important to alert users to the potential pitfalls associated with this approach, it is no longer a 'doomed' approach, as the OIE's provisional validation pathway for diagnostic tests provides guidance on how test validation for wildlife diseases can be achieved in a phased approach, recognising challenges which are beyond the laboratory's control.

For many wildlife species and pathogens, adopting existing tests is not an option, but novel and high-throughput methods, although currently on a small scale, have

successfully entered the spectrum of veterinary diagnostic applications and their use in strategic approaches is likely to be of great benefit in future. Metagenomics and metatranscriptomics have found their first applications in human and livestock diagnostics, but are still not applicable in routine microbiology laboratories. However, with regard to spillover events or conservation efforts for endangered wildlife species, these techniques can help to improve herd management through the early detection of zoonotic pathogens and diseases and consequent early intervention, which helps to avoid species decline, unnecessary culling or economic losses. For the future, comprehensive knowledge about the background microbiome of livestock and wild animals will be crucial to better understand the difference between normal diversity in a healthy herd and disease in individual animals (97). The bottleneck for the routine application of comprehensive high-throughput methods in the diagnosis of infectious diseases in wildlife will be the shortage of well-equipped laboratories, trained staff (both for wet and dry laboratories), and high-quality reference databases. Recent experiences have shown that the technology response to new disease events is strongly biased towards emerging zoonoses with a high perceived threat to human health. Therefore, it is speculated that rapid progress will be made in the refinement and validation of novel technologies in the field of emerging wildlife diseases with a suspected or confirmed zoonotic nature.

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Détection des agents pathogènes et diagnostic des maladies dans la faune sauvage : difficultés et perspectives

A.L. Michel, H. van Heerden, D. Prasse, V. Rutten, S. Al Dahouk & B.M. Crossley

Résumé

Nous assistons depuis quelques décennies à une augmentation de la demande en matière de détection des agents pathogènes et d'approches diagnostiques applicables aux populations de la faune sauvage, parallèlement à un intérêt accru pour les processus infectieux chez les animaux sauvages. Cette évolution résulte en partie de l'empiètement des populations humaines sur les habitats de la faune sauvage, des efforts déployés pour protéger les populations d'animaux sauvages vulnérables et d'une intensification du commerce axé sur la faune sauvage. À mesure que les contacts se multiplient, le risque d'un franchissement de la barrière d'espèces et d'une transmission des agents pathogènes des animaux sauvages aux animaux domestiques et à l'homme s'accroît également.

La mise au point et la validation d'épreuves diagnostiques destinées à la faune sauvage se heurtent à de multiples difficultés dont en premier lieu la problématique des échantillons à analyser et des méthodes d'essai appropriées. Compte tenu de ces contraintes, la tentation est grande de résoudre le problème en faisant appel à des épreuves diagnostiques validées pour les espèces animales domestiques. Le recours à cette solution est illustré par d'abondants exemples dans la littérature, dont certains sont rappelés ici. Les scénarios présentés par les auteurs mettent en avant les avantages et les inconvénients d'un certain nombre de tests de différents types utilisés chez les animaux sauvages, ainsi que les obstacles qui empêchent de les valider.

Une attention particulière est accordée aux perspectives ouvertes par le potentiel d'innovation des nouvelles technologies, qui annoncent une meilleure détection des agents pathogènes existants ainsi que la possibilité d'en découvrir de nouveaux, d'accroître notre connaissance sur les maladies infectieuses de la faune sauvage et d'accélérer leur diagnostic.

Mots-clés

Détection des agents pathogènes – Faune sauvage – Maladie émergente – Maladie infectieuse – Nouvelle méthode diagnostique – Test de diagnostic – Validation d'une épreuve – Zoonose.

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Detección de patógenos y diagnóstico de enfermedades en la fauna silvestre: dificultades y oportunidades

A.L. Michel, H. van Heerden, D. Prasse, V. Rutten, S. Al Dahouk & B.M. Crossley

Resumen
En los últimos decenios, a medida que crecía el interés por las enfermedades infecciosas que se dan en la fauna silvestre, también iba aumentando la demanda de soluciones para poder detectar patógenos o aplicar otros métodos de diagnóstico en las poblaciones de animales silvestres. Este interés se explica, en parte, por la intrusión humana en los hábitats de la fauna silvestre, los esfuerzos por proteger a las poblaciones silvestres vulnerables y la creciente utilización comercial de la fauna silvestre. A medida que el frote se intensifica, también aumenta el riesgo de transmisión de patógenos entre animales silvestres, animales domésticos y personas.

Las diversas y numerosas dificultades que han surgido a la hora de concebir y validar pruebas de diagnóstico para animales silvestres tienen que ver primeramente con las cuestiones de las muestras de diagnóstico y los métodos analíticos adecuados. Ante semejantes limitaciones, es tentador salvar el obstáculo recurriendo a pruebas de diagnóstico validadas para especies de animales domésticos. La bibliografía abunda en ejemplos de este tipo de soluciones, algunos de ellos descritos aquí por los autores, que también presentan situaciones hipotéticas para exponer las ventajas y desventajas de distintos tipos de prueba en la fauna silvestre y los impedimentos que hoy frenan su validación. Los autores destacan las perspectivas futuras que se abren con nuevas y novedosas tecnologías que traen consigo la posibilidad de mejorar la detección de los patógenos existentes y el descubrimiento de otros por ahora desconocidos y de ahondar rápidamente en nuestro conocimiento de las enfermedades infecciosas de la fauna silvestre y su diagnóstico.

Palabras clave
Detección de patógenos – Enfermedad emergente – Enfermedad infecciosa – Fauna silvestre – Nuevo método de diagnóstico – Prueba de diagnóstico – Validación de pruebas – Zoonosis.

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