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Viral discovery as a tool for pandemic preparedness

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Summary
Emerging diseases are frequently caused by novel or previously unrecognised zoonotic viral pathogens, which tend to originate in and emerge from wildlife. When human or animal cases are first recognised, molecular or serological diagnostic assays specific to them do not yet exist, causing a delay in the identification of an outbreak's aetiologic agent as well as its source. Preparing for the next virus to emerge is a major public health challenge, impeded by a poor understanding of the diversity of potential candidates that exist in wildlife reservoirs. Characterising the diversity of viruses in key wildlife species will help to reduce the time between detection and response in an outbreak situation, and inform public health strategies that reduce the risk of spillover from animal reservoirs. Pathogen discovery techniques such as consensus polymerase chain reaction (cPCR) and next-generation sequencing (NGS) have been used to identify known and novel viruses in animals and humans, but have not been widely used in surveillance programmes. Metagenomic studies have identified novel viruses, new strains of known viruses, and have characterised host microbiomes. While NGS represents an unbiased approach to viral sequence detection, it is constrained by lower sensitivity than conventional PCR, requires substantial bioinformatics capabilities, and is cost prohibitive and therefore not widely available in the regions of the world that are most vulnerable to zoonotic disease emergence. In contrast, consensus PCR uses standard and widely available technologies, has greater sensitivity than NGS, and has also been used to identify novel viruses in wildlife, livestock and humans, though it is limited to detecting target genetic sequences conserved across known groups of viruses. The use of cPCR, in combination, if possible, with NGS and serology, can offer a powerful approach to rapidly identifying aetiologic agents in an outbreak and characterising the virome of key wildlife known to carry zoonotic viruses. Here, the authors review pathogen discovery techniques currently being used in human and animal surveillance programmes and the challenges of using viral discovery to identify novel zoonotic pathogens.

Keywords
One Health – Pandemic – Pathogen discovery – Polymerase chain reaction – PREDICT – Serology – Virus – Wildlife – Zoonotic virus.

Introduction

Zoonotic viruses have caused a growing number of global epidemics. Severe acute respiratory syndrome (SARS) Coronavirus (CoV), H1N1 pandemic influenza (2009), human immunodeficiency virus (HIV), Ebola virus, and Middle East respiratory syndrome (MERS) CoV are examples of emerging zoonotic viruses that have spread globally and caused significant morbidity and mortality (1, 2, 3, 4, 5, 6). Pandemic viruses tend to be RNA viruses that originate in wildlife and emerge in regions of the world where humans and animals live in close association and where significant anthropogenic triggers for spillover exist (7, 8). Human activities, such as land-use change, agricultural intensification, urbanisation, global travel, wildlife trade and deforestation, are important drivers of zoonotic disease emergence (9, 10, 11). Zoonotic viruses may be transmitted by a variety of mechanisms, including direct contact (e.g. hunting, butchering, animal bites), indirect contact (e.g. food contamination, aerosolisation/ inhalation) or by arthropod vector (9). These viruses may jump directly to humans from wildlife reservoirs (e.g. HIV) via a secondary, non-reservoir wildlife species

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(e.g. Ebola virus), or via domestic animals (e.g. Nipah virus [NiV], highly pathogenic avian influenza H5N1) (2, 12, 13, 14, 15). Although it is wildlife and livestock that are principally involved in the process of zoonotic disease emergence, resources for disease surveillance are predominantly applied to human populations, and zoonotic disease outbreaks in animal hosts are rarely identified before human cases occur. Understanding the diversity of viral agents in local wildlife and livestock communities in a particular environment, and understanding the behavioural interactions between people and animals in that environment, may inform risk assessments for human health. This could ultimately improve the response to a novel zoonotic disease outbreak by reducing the time between detection and intervention, thereby reducing morbidity and mortality (16).

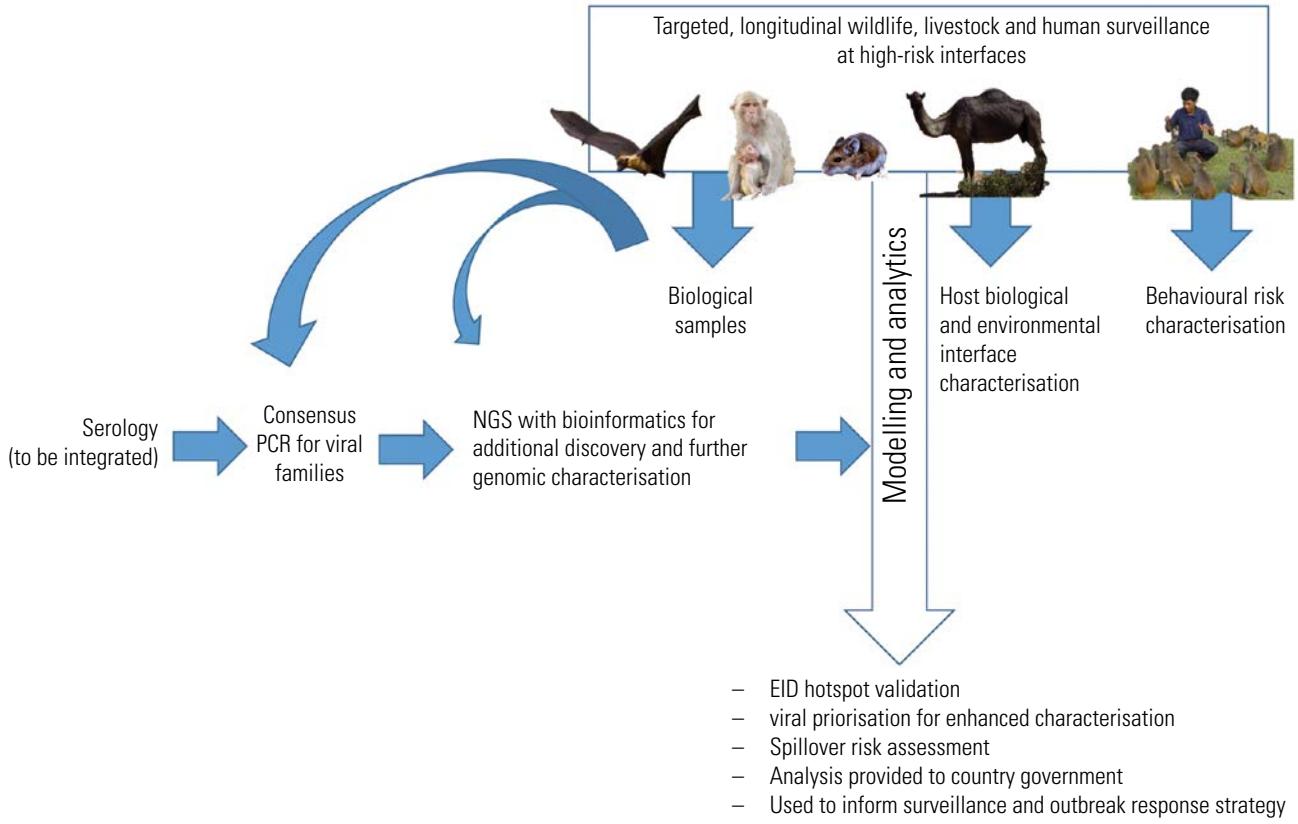
Little is known about viral dynamics in wildlife reservoirs as they relate to risk of spillover. Disease outbreaks in wild animal populations are rarely observed due to lack of surveillance (17). However, when observed, wildlife die-offs can indicate the circulation of zoonotic pathogens. West Nile virus caused crow die-offs as it spread across the United States in 1999–2000 (18). Significant gorilla and chimpanzee mortality was observed ahead of the movement of human Ebola virus (Zaire) outbreaks in Gabon and the Republic of Congo in 2003–2004 (19). Although Member Countries of the World Organisation for Animal Health (OIE) must report cases of OIE-listed diseases occurring in wildlife (they also submit information on non-listed wildlife diseases on a voluntary basis), there is currently no intergovernmental organisation, akin to the OIE, the World Health Organization or the Food and Agriculture Organization of the United Nations, that is dedicated to active wildlife disease surveillance. Also, most countries do not have a federal agency with that mandate. This gap limits the global health community's ability to detect and identify potential zoonotic threats prior to human or domestic animal outbreaks.

Disease outbreaks in livestock may be more readily observed than those in wildlife, but the aetiologic agent responsible may initially be misidentified, particularly when the pathogen is novel. This can lead to ineffective interventions and delayed outbreak control, as occurred when NiV was initially misidentified as Japanese encephalitis virus in Malaysia (20). Outbreak interventions that focus on defining a case, identifying the source(s) of infection, and interrupting further transmission to prevent new cases necessarily rely on an initial cluster of human (or livestock) cases before a response can be initiated. A significant impediment to preventing outbreaks of novel zoonotic viruses is a poor understanding of the diversity and quantity of zoonotic viruses in animal reservoirs. Less than 1% of the vertebrate virome has been described, and it has been estimated that a minimum of between 300,000 and 1.3 million viruses exist in mammalian and migratory waterfowl hosts which have not yet been discovered (21; Carroll *et al.,* in review). Identifying unknown viruses in wildlife reservoirs, by obtaining either the whole viral genomic sequence or a partial sequence, is an important step in assessing their potential to infect humans. Having an *a priori* understanding of the molecular characteristics of the virus and the biological and ecological context in which the virus was identified (host species abundance and distribution, viral prevalence, sample type in which the virus was detected, etc.) may help to reduce the time it takes to identify its wildlife reservoir and mechanism of spillover in the event that it or a related virus emerges in people or livestock.

Using a pathogen discovery approach to surveillance that combines advanced and established molecular and serological assays to detect and characterise viral diversity in wildlife can be an effective mechanism for reducing the impact of emerging zoonotic diseases. The United States Agency for International Development is using just such an approach in the 'PREDICT' project, which is part of its Emerging Pandemic Threats programme. Here, the authors use the PREDICT project to illustrate how pathogen discovery techniques are being used in multiple countries as part of a coordinated, targeted surveillance strategy, to try to identify and characterise novel zoonotic threats to humans and livestock before they emerge.

Surveillance for novel zoonotic viruses in wildlife, livestock and humans: the PREDICT project

The PREDICT project is a large-scale global surveillance initiative which is led by a consortium of institutions (www.predict.org). The project is active in more than 25 countries, and one of its major aims is to develop an early warning system for emerging viral zoonoses by building laboratory and field capacity in countries that are most vulnerable to spillover and emergence. PREDICT viral discovery activities centre around screening biological samples from animals and people at high-risk interfaces where people, livestock and wildlife have a high degree of contact (e.g. via hunting, farming, live animal markets, etc.) (Fig. 1). Biological sampling targets key wildlife taxa: bats, rodents and non-human primates – hosts which have a disproportionately high likelihood of carrying viruses that can be shared with people (22, 23). Additionally, livestock and human populations are sampled at these interfaces to identify instances where wildlife viruses may have already jumped into these populations. Molecular techniques such as consensus polymerase chain reaction (cPCR) and



EID: emerging infectious disease
NGS: next-generation sequencing
PCR: polymerase chain reaction

Fig. 1

Flow chart illustrating the use of pathogen discovery tools in PREDICT surveillance protocols

Biological specimens are collected from wildlife, livestock and human populations at high-risk interfaces, such as at live animal markets or in communities where people and animals have high degrees of contact. Additional syndromic surveillance occurs in select hospitals where patients may have a history of frequent or close animal contact. Samples are screened at partner laboratories using viral family-level consensus PCR protocols (PREDICT protocols are available at www.predict.org) and next-generation sequencing, where available. Information is analysed alongside host and environmental data and assessments are made as to which novel viral sequences should be prioritised for further characterisation. This process may include additional sequencing, functional sequence analysis, and *in vitro* or *in vivo* experiments to determine host breadth, cell entry, and pathogenesis. Results from surveillance and laboratory activities are regularly provided to points of contact in partner-country government wildlife, livestock and human health agencies to inform surveillance and outbreak response strategies. With consent from partner-country government agencies, PREDICT publicly disseminates results from surveillance via the Internet (Healthmap, Genbank) and via peer-reviewed scientific publications and public fora

next-generation sequencing (NGS) are used to detect viral sequences, which are then analysed using phylogenetic and ecological approaches to estimate the likelihood that they have zoonotic potential. The cPCR techniques use degenerate primers designed to detect conserved regions of viral genomes common to all members of a particular viral genus or family. NGS is used as an auxiliary tool to either probe for additional viral sequences in biological samples, or extend the length of the short sequences (~200 nucleotides) obtained by cPCR. Viral sequence data, spatial and epidemiological data, and ecological and environmental data are analysed and used to assess the potential risk of spillover and emergence for the novel viruses identified. Viral sequences detected by PREDICT are used to develop a baseline library of viruses and their associated hosts, and are linked to information related to the environment and human–animal interface at the site of sampling. PREDICT data are analysed both at country-specific and regional scales, and results are provided to each host country's human and animal health and wildlife agencies. This information may be used to inform surveillance and outbreak response strategy and help to improve local capacity to rapidly identify emerging pathogens and recognise their natural reservoir (24). This approach to surveillance may ultimately decrease the time between the diagnosis of an emerging zoonotic virus and intervention, reducing the magnitude of the outbreak (16).

Consensus polymerase chain reaction

Consensus PCR is the primary pathogen discovery tool used by PREDICT. It uses degenerate primers that bind to short

conserved domains flanking stretches of variable sequence. This allows for the 'universal' amplification of viruses within a given viral family, and for strains (both known and novel) to be discerned from the variable region within. This method is inexpensive, highly implementable, and has been used successfully to identify hundreds of novel viruses (or rather, fragments of sequences representing viruses) in wildlife in resource-poor settings (25). It is, however, limited by the ability of the degenerate primers to bind successfully to the target (conserved) domains and therefore has the potential to miss viruses that show divergence in this region. This technique allows for biological samples to be screened for broad groups of viruses by genus (e.g. *Henipavirus*) or viral family (e.g. *Paramyxoviridae*) using a single assay. Consensus PCR can be highly sensitive (26); however, the degenerate primers more often result in reduced sensitivity compared with pathogen-specific assays, and for that reason it is important to view cPCR as a strict discovery tool, and not as a diagnostic tool. The strength of this approach is its broad applicability, generating initial sequence data that can be further characterised where possible.

Next-generation sequencing

Next-generation sequencing is more expensive than traditional cPCR, but sequencing costs have decreased dramatically over the past decade, making the technology more accessible (27). Next-generation sequencing technologies offer advantages over PCR in terms of pathogen discovery, as they are able to capture a very broad diversity of viral sequences because they do not rely on specific primers and target sequences, making them unbiased assays. Rather than relying on primers, NGS techniques use adaptors that are ligated onto double-stranded DNA for the indiscriminate amplification of all nucleic acids present in the sample. Following unbiased amplification, viral sequences are parsed out *in silico* and identified based on genetic homology to known viruses (see [28, 29] for a detailed review). This approach has been used widely as a discovery tool on samples from humans (30), domestic animals (31), wildlife (32, 33), and even insects (34). However, NGS is not routinely used in resource-poor settings because of the size and complexity of the data generated (often thousands or millions of sequences that must be run through algorithms that compare each one to known sequences and provide matches with associated probabilities), which require bioinformatics expertise to be interpreted appropriately. PREDICT uses NGS to augment cPCR screening of high-interest samples or to obtain additional or full-genome sequences from samples shown by cPCR to contain novel viruses. NGS constitutes a relatively small proportion of testing done under PREDICT, and is limited to a few laboratories that already have the appropriate technologies in place. Despite its potential breadth of discovery, NGS lacks the sensitivity of PCR, leading many to combine both approaches when hunting for unknown viruses, which may be present in trace amounts in a clinical sample (35). More recently, capture-based methods have been developed that promise to revolutionise NGS as a discovery tool by increasing sensitivity levels so that they match those of PCR (36). These methods use a set of probes to 'capture' viral sequences from cDNA libraries with complex backgrounds (i.e. host material, bacteria, etc.), and enrich the sample specifically for viral products. This selection process reduces the sequencing depth required to find viruses in clinical samples, improves genome coverage, and allows more samples to be run in a single lane (reducing the cost) (36). A large proportion of the PCR testing for PREDICT has been conducted in in-country partner laboratories. Through targeted training and technology transfer, the PREDICT project has enhanced both field and laboratory surveillance capacity in wildlife, livestock and human health government agencies (7). Engagement with a range of professionals (e.g. government officials, field officers and laboratory technicians) and technology transfer to government institutions have been essential in promoting the sustainable adoption of a 'One Health' approach for surveillance and pathogen discovery techniques and to encourage the use of these techniques for both routine surveillance and as part of outbreak response. The capacity-building approach used will allow governments to continue to employ these surveillance strategies beyond the lifetime of the PREDICT project.

Understanding risk from viral discovery data

Describing novel viral sequences obtained from wildlife can be useful for characterising overall viral diversity, particularly in taxonomic groups associated with emerging zoonoses. Bats (order Chiroptera) and rodents (order Rodentia) comprise 60% of mammal species and have been identified as key wildlife reservoirs for zoonotic viruses (23). The viruses recently identified from bats and rodents using cPCR and NGS techniques include a novel bat influenza virus, poxvirus, and a large diversity of paramyxoviruses. This has prompted Drexler *et al*. to hypothesise that all paramyxoviruses originated in bats (32, 37, 38, 39, 40, 41). A large number of studies have identified various novel coronaviruses in bats, some of which are very closely related to SARS CoV, MERS CoV, or human CoV 229, which has led Hu *et al*. to hypothesise that all human coronaviruses come from bats (42, 43, 44, 45, 46, 47, 48, 49). Using the combined cPCR/NGS approach to screen bats, rodents and non-human primates in more than 25 countries, the PREDICT project has, to date, identified more than 800 novel viral sequences (25). Using PREDICT cPCR protocols, Anthony *et al*. detected a set of 55 novel viruses, representing nine viral families, in a single bat species, namely *Pteropus medius*, a common frugivorous bat (and a

reservoir for NiV) found throughout the Indian subcontinent (21, 50). The authors also estimated the sampling effort and resources that would be required to discover the majority of viruses that would be present in any mammalian species.

These studies have significantly advanced our understanding of viral diversity in important wildlife taxa. However, RNA sequences do not, by themselves, provide information about a virus's pathogenicity or any specific zoonotic threat it may pose. Closely related viruses such as NiV and Hendra virus (HeV) may elicit similar disease (humans infected with NiV experience the same symptoms as those infected with HeV, and animals infected with one of these viruses display the same clinical signs as those infected with the other) (51, 52), but relatedness alone is not a reliable predictor of zoonotic or pathogenic potential. For example, Cedar virus, which is genetically and structurally similar to HeV and NiV, does not cause pathology in animal models (53). Similarly, although Ebola virus and Marburg virus are each highly pathogenic viruses causing severe disease in humans (and laboratory-animal models), the related Ebolavirus Reston does not appear to cause disease in humans (or pigs), although it does cause disease in macaques (54). Natural reservoirs for zoonotic viruses that cause significant disease in other animal or human hosts tend not to experience appreciable disease when infected (55). For this reason, targeting apparently healthy wild animals, rather than sick ones, is critical to an effective surveillance strategy for zoonotic viruses (55). What makes the risk assessment of novel viruses particularly challenging is that, until a newly discovered virus from wildlife is found in a sick animal or person and is shown to be associated with the observed disease, it is very difficult to determine its risk to human health.

Traditionally, the most robust evidence of a causal relationship between microbe and disease has been the fulfilment of Koch's postulates: demonstrating the isolation of a pathogen from one or more sick individuals and showing its ability to infect human cells *in vitro* and cause the originally observed disease in a naïve animal model. However, viral isolates are often difficult or impossible to obtain from clinical samples, even with significantly ill patients, which would make Koch's postulates impossible to fulfil (56). Molecular detection of viral RNA is often faster and more successful than attempts at isolation. The increasing use of molecular technologies as research and diagnostic tools, particularly with viral pathogens, has required a rethinking of what constitutes acceptable evidence that a microbe identified in one or more sick patients (human or animal) is the cause of the observed disease. Lipkin proposed three stages of certainty with respect to confirming causation of disease by a suspected microbe: possible causation; probable causation; and confirmed causation (27). Detection of nucleic acid from tissue samples from a sick patient signifies possible causation. Detection of nucleic acid from tissues exhibiting pathology and a known association between the observed disease process and related pathogens create more certainty of plausible causation – which is enhanced by *i)* detection of specific antibodies (e.g. IgM) against the identified microbe, *ii)* a cluster of patients with the same disease and microbe, and *iii)* a high concentration of microbial nucleic acid in clinical samples. Finally, fulfilment of Koch's postulates or clinical improvement following treatment with specific antimicrobial therapeutics confers the highest level of certainty of a causal relationship between the detected microbe and the observed disease (27). While these new criteria allow for molecular detection to provide suitable evidence for disease causation, they do not apply when a novel microbe is detected in the absence of disease. In this instance, *in vitro* and *in silico* approaches may be helpful to better understand the zoonotic potential of a newly discovered virus.

PREDICT uses both modelling and experimental approaches to estimate spillover potential and therefore prioritise novel viral sequences for further characterisation. This ecological analysis makes use of surveillance data collected on a range of factors, such as host breadth (e.g. in how many different species was a given sequence identified); geographical range; type of human–animal interface at which the virus was detected; and host abundance (as a measure of likelihood of contact with other animals and people). Phylogenetic analyses are used to assess sequence relatedness to other pathogenic and zoonotic viruses. Viral sequences deemed to be a high priority will be further characterised, which may involve obtaining additional genomic sequence, viral isolation, and experimental methods that use viral sequence to make recombinant viruses and that test their ability to enter other animal and human cells (57). The PREDICT global team is in the process of prioritising newly discovered viruses for additional characterisation based on the aforementioned properties.

Challenges to using molecular techniques for surveillance

While using PCR has been effective in discovering novel viruses in wildlife, using a primarily molecular approach has limitations in terms of fully capturing viral diversity in taxonomically important host species. PCR protocols used to create pan-viral family or viral group assays are designed to amplify regions of the viral genome which are highly conserved, and tend to generate very short sequences (~200 nucleotides). Short sequences can be used for phylogenetic analysis; however, they limit one's ability to make accurate inferences about evolutionary relationships among viral strains or functional differences between a novel virus and other closely related viruses, as compared

to whole-genome sequences. For example, when estimating viral diversity based on the number of nucleotide changes, an analysis of a 357-nucleotide sequence within the NiV N gene was found to overestimate viral diversity compared to an analysis of the entire N gene open reading frame (ORF) (8% versus 6%), whereas a 729-nucleotide sequence in the N gene region more accurately reflected whole N gene mutation rates (5%) (58). Understanding genetic diversity within a viral group is important, as genotypic variation may result in differences in pathogenicity. Related strains of NiV have shown differences in clinical outcomes in both people and animal models. Compared to the Malaysia strain, the Bangladesh strain has shown a more substantial respiratory component to human disease and more oral shedding of virus in ferrets, which may relate to transmissibility; whereas the Malaysia strain has shown greater pathogenicity than the Bangladesh strain in hamsters (59, 60, 61). As more and more sequences are obtained within a viral group, especially those derived from people or animals that are experiencing disease, the relationship between genotype and phenotype will become clearer. However, at present, determining the pathogenicity of a newly recognised virus obtained from its natural wildlife host (which typically does not show signs of disease), based on its sequence alone, is a major challenge. While there is no agreed standard for the minimum sequence length needed for identifying evolutionary relationships among pathogens, whole-gene ORF sequences or whole-genome sequences allow for a more robust analysis of nucleotide and amino-acid differences among sequences. NGS has made whole-genome sequencing more efficient and faster than PCR; however, sample quality and low viral quantity can affect the amount of sequence obtained by high-throughput methods (62).

Another challenge with applying pathogen discovery techniques to surveillance – particularly in healthy wildlife species – is that different biological samples may yield different results, which can influence sampling strategy. In wild-caught bats, for instance, some viruses have been reliably detected in organ tissue but not in excreta; for example, in free-ranging Egyptian fruit bats (*Rousettus aegyptiacus*) Marburg virus, a filovirus related to Ebola, was detected by PCR and isolated from liver and spleen, but not from saliva or faeces (63). Marburg was, however, subsequently detected in oral secretions from experimentally infected Egyptian fruit bats (64). Henipaviruses have been detected in urine and saliva from wild-caught pteropid bats and also from experimentally infected bats (65, 66). The determination as to whether or not to conduct destructive sampling should include consideration of the conservation status of the species being sampled. Non-destructive sampling may be more acceptable to wildlife authorities and the public, particularly if working on species which may be protected by local or international laws. Non-destructive clinical sampling tends to be limited to blood samples or samples taken from possible routes of viral excretion (swabs of the oropharynx, urogenital tract, rectum, and of urine, faeces); however, these samples may contain very low amounts of viral RNA compared to organ tissue, which can limit the ability of cPCR and NGS assays to detect a virus or resolve extended lengths of sequence.

Using molecular tools for surveillance can be particularly challenging, because they rely on there being detectable levels of viral nucleic acid in the clinical samples obtained at the time of sampling. Many zoonotic viruses have acute viraemic periods in animals and people, which limit opportunities for detecting viral nucleic acid. In cases where clinical disease is apparent, and surveillance is adequate, it may be possible to collect samples early enough in the course of disease to detect a viral genome. However, if a patient is sampled late in the course of illness or during a convalescent phase, viral loads may be too low to detect. Ebola virus, which has an incubation period in humans of 2–21 days, can be readily detected in bodily fluids from symptomatic patients, but only for a limited time. Viral particles were detectable in blood by PCR from day three of infection up to day 7–16 after the onset of symptoms (67).

Surveillance for zoonotic viruses in natural wildlife reservoirs can present a different challenge, as hosts are frequently asymptomatic when infected (55). For example, in experimental infections, Marburg virus can be detected in the natural reservoir species, the Egyptian fruit bat, up to nine days post-infection, but does not cause appreciable disease (63, 64, 68). Similarly, henipaviruses (NiV and HeV) do not cause pathology in the reservoir host, but HeV RNA has been detected up to 19 days post-inoculation in experimental infections (65). The prevalence of henipaviruses in wild bat populations tends to be low (~1%–3%) (69, 70, 71), and the combination of low prevalence and a short viraemic period makes it particularly difficult to detect virus in wild bats. A cross-sectional sample must be large enough to provide statistical (>95%) confidence of detecting virus in at least one infected individual, assuming that bats are equally likely to be infected at any time of the year. Obtaining large sample sizes can be particularly challenging with free-ranging wildlife, further limiting the likelihood of viral detection (9). If there are seasonal patterns associated with infection, then longitudinal studies with repeated sampling in a given population may be necessary to maximise the opportunity for viral detection. Once a virus of interest is identified and prevalence is estimated based on empirical data, more targeted epidemiological studies can be implemented.

Serological approaches to pathogen discovery and surveillance

Standard serological assays which are commonly used and widely available for the detection of antibodies against known viruses include enzyme-linked immunosorbent assays (ELISAs), Western blots, and serum neutralisation assays (reviewed in [72]). While these are important tools for detecting antibodies against specific agents and, in the case of serum neutralisation assays, determining neutralising ability and providing a quantitative output, they are limited in their use for unknown agents. ELISAs and Western blots require validation for each host species whose serum is screened, and positive controls, which means having serum known to contain target antibodies – another challenge when the pathogen of interest is yet unknown.

The development of a serological assay for a novel virus, once adequately characterised, can substantially augment surveillance efforts and help to confirm whether or not a host species is a natural reservoir. Specific antibodies can persist in the serum for years, and assuming that mortality from an outbreak does not reach 100%, one may detect serological evidence of infection more easily than the virus due to a longer window of opportunity for detection. For example, the prevalence of NiV in *Pteropus* species has been estimated at less than 1% (50, 71), while the prevalence of anti-NiV antibodies has been found to be 20%–56% in the same species (50, 71, 73). Given the challenges associated with the capture of bats and other wild animals, and low detection rates of viral pathogens, serological studies have been extremely useful in epidemiologic and ecological studies of viral dynamics in reservoir hosts.

Non-specific reactions (or 'cross-reactions') in serological assays, while typically confounding when looking for antibodies against a specific antigen, may in fact be useful indicators that one or more antigenically related viruses are circulating within a population. Combining specific and non-specific probes in a multiplex assay may help to identify novel but related strains of known viruses. The Luminex bead-based assay uses a fluorescent phycoerythrin reporter, microfluidics and lasers. It also uses multiple beads of different colours, each emitting a distinct wavelength of light when agitated by a laser, depending upon which bead binds antibodies in the serum sample. Luminex can screen for multiple targets in a single well using a relatively small volume of sera (5 µl), making it particularly advantageous when studying small (<20 g) mammals such as bats and rodents, from which a small volume of blood can be safely taken. A Luminex-based multiplex serological assay for the detection of IgG antibodies specific for NiV and HeV was first developed at the Commonwealth Scientific and Industrial Research Organisation's Australian Animal Health Laboratory (74) and has since been expanded to include novel nucleoprotein (N) antigens (75). It is currently used in Australia as a surveillance tool for HeV and other related bat-borne viruses. The panel now uses viral envelope glycoproteins from all known henipaviruses and has been used to screen human, livestock, and bat sera in a number of international HeV research projects (74, 75, 76, 77, 78, 79). Probes that use HeV nucleocapsid proteins are less antigenically specific than those using soluble surface glycoproteins, and therefore allow for the detection of novel yet related henipaviruses (74, 80). When an unknown virus is suspected in a population, a longitudinal study (repeated sampling over time) using cPCR approaches may ultimately identify a novel pathogen that would have otherwise been missed. Once a novel virus has been identified from a domestic or wild animal species, specific serological assays may be designed and used for screening 'high-risk' human populations to determine whether spillover has occurred.

An important advance in serological surveillance has been the use of molecular techniques to characterise the broad array of antibodies present in an individual, as a means to describe the sum of historical infections. High-throughput sequencing has been used to characterise antibody repertoires in response to specific vaccines, as well as natural repertoires elicited by exposure to pathogens (81, 82, 83, 84). To date, these techniques have mainly been applied to humans; however, their use in domestic animal or wildlife species could potentially help to identify host species for novel zoonotic viruses.

Discussion and future directions

The global community remains vulnerable to the emergence of zoonotic pathogens due to an increase in drivers responsible for zoonotic disease emergence and inadequate surveillance of human and animal populations for viral pathogens (16). The lack of understanding of the diversity of zoonotic viruses in natural reservoirs or source populations, coupled with an inability to rapidly identify novel aetiologic agents in an outbreak, will likely continue to contribute to local and regional vulnerability to epidemics unless current public health and surveillance systems are improved (3). The time it takes for public health systems to recognise and respond to the emergence of novel zoonotic pathogens is too long to sufficiently minimise the risk of widespread epidemics. Applying pathogen discovery techniques to surveillance systems that include wildlife, livestock, and human populations, along with the use of agent-specific PCR assays, can help to rapidly diagnose and respond to novel viruses causing disease in people or animals. It can also help to control an

outbreak of an emerging virus before it progresses from a localised outbreak to a broader epidemic via travel to urban centres or other countries (17, 85). Comprehensive laboratory-based molecular platforms have been developed with nucleic acid probes in a single test designed to detect every known vertebrate virus, and serological analogues are also in development (36). Portable field-based diagnostic platforms can minimise the time between collecting clinical samples and diagnosing the cause of a disease outbreak. Field-based diagnostics also address the difficulty of maintaining the cold chain when samples are transported from field to laboratory, particularly in tropical climates and remote locations. Pathogen discovery tools can also help in the development of specific molecular assays which can then be used in point-of-care or field situations, as was the case with a mobile laboratory unit used for Ebola virus diagnostics in the West Africa Ebola outbreak (86).

The PREDICT project has provided a proof-of-concept approach to identifying novel viruses and estimating viral diversity in key mammalian species where zoonoses are most likely to emerge. PREDICT has led to the advancement of the Global Virome Project, which is a new large-scale initiative that seeks to characterise the total diversity of viruses in all mammals and migratory waterfowl across emerging infectious disease (EID) hotspots (www.globalviromeproject.org). Engaging governments and scientific organisations in countries which are most vulnerable to EIDs and integrating pathogen discovery into a coordinated, comprehensive surveillance system can help the global health community to be better prepared to respond to emerging zoonotic viruses.

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La découverte des virus, un outil en appui de la préparation aux pandémies

J.H. Epstein & S.J. Anthony

Résumé

Les maladies émergentes sont souvent causées par des virus nouveaux ou précédemment inconnus, de portée zoonotique, qui ont généralement leur source dans la faune sauvage, à partir de laquelle s'effectue leur émergence. Lorsque le premier cas d'infection par un virus de ce type est détecté chez l'homme ou chez les animaux, il n'existe encore aucune épreuve moléculaire ou sérologique de détection de l'agent étiologique, ce qui retarde son identification ainsi que l'élucidation de la source du foyer. La préparation aux futures émergences virales est un véritable défi de santé publique et se voit entravée par les lacunes des connaissances sur la diversité des virus potentiellement candidats. La caractérisation des différents virus qui affectent les principales espèces sauvages permettra de réduire le délai entre le moment où un nouveau foyer est détecté et celui où une réponse lui est apportée, et d'élaborer en connaissance de cause des stratégies de santé publique visant à limiter le risque d'un franchissement d'espèce à partir des réservoirs animaux. Les techniques de découverte d'agents pathogènes, par exemple l'amplification en chaîne par polymérase (PCR) pan-générique ou consensus et le séquençage de nouvelle génération (SNG) sont utilisées pour identifier des virus connus ou nouveaux chez l'homme et l'animal, mais ne sont pas d'une utilisation courante dans les programmes de surveillance. Les études métagénomiques permettent d'identifier des virus nouveaux ainsi que les souches nouvelles de virus connus et servent également à caractériser le microbiome de l'hôte. Le SNG constitue une méthode de détection des séquences virales exempte de biais mais sa sensibilité moindre que celle des PCR classiques, les capacités bio-informatiques considérables qu'il requiert et son coût prohibitif sont des contraintes importantes qui en limitent l'utilisation dans les régions du monde les plus vulnérables à l'émergence des maladies

zoonotiques. En revanche, la PCR consensus fait appel à des technologies normalisées et largement disponibles, tout en présentant une meilleure sensibilité que le SNG ; elle permet également d'identifier des virus nouveaux présents dans la faune sauvage, chez les animaux domestiques ou chez l'homme, bien qu'elle ne détecte que des séquences génétiques cibles conservées d'un groupe connu de virus à l'autre. Le recours à la PCR consensus, si possible associé aux techniques de SNG et à la sérologie se révèle une stratégie puissante qui permet d'identifier rapidement les agents responsables d'un foyer et de caractériser le virome d'espèces sauvages jouant un rôle majeur en tant que réservoirs de virus zoonotiques. Après avoir passé en revue les techniques de découverte d'agents pathogènes actuellement utilisées dans les programmes de surveillance des maladies animales et humaines, les auteurs font le point sur les enjeux de ces techniques pour l'identification de nouveaux agents pathogènes zoonotiques.

Mots-clés
Amplification en chaîne par polymérase – Découverte d'agents pathogènes – Faune sauvage – Pandémie – PREDICT – Sérologie – Une seule santé – Virus – Virus zoonotique.

El descubrimiento de virus como herramienta de preparación para pandemias

J.H. Epstein & S.J. Anthony

Resumen
La causa de las enfermedades emergentes reside muchas veces en virus zoonóticos de aparición reciente o hasta entonces no descritos, que en general se originan y surgen en animales salvajes. Cuando se detectan los primeros casos en personas o animales aún no existen pruebas específicas de diagnóstico, ya sea molecular o serológico, y ello retrasa la identificación del agente etiológico del brote y la determinación de su origen. Prepararse para el próximo virus que vaya a aparecer es un gran objetivo de salud pública, lastrado en la práctica por el escaso conocimiento de la gran diversidad de posibles candidatos que moran en los reservorios de la fauna salvaje. La caracterización de los diversos virus que existen en las principales especies de animales salvajes ayudará a reducir el lapso que media entre la detección y la respuesta en caso de brote y a fundamentar a partir de ahí estrategias de salud pública que reduzcan el riesgo de diseminación desde los reservorios animales. Hasta ahora las técnicas de descubrimiento de patógenos, como la reacción en cadena de la polimerasa (PCR) de consenso (PCRc) o la secuenciación de próxima generación, han servido para identificar virus nuevos o ya conocidos en personas y animales, pero no se han aplicado de forma generalizada a los programas de vigilancia. Gracias a estudios de metagenómica se han podido detectar virus recién aparecidos o nuevas cepas de virus ya conocidos y caracterizar los microbiomas de los organismos anfitriones. Aunque la secuenciación de próxima generación constituye un método exento de sesgos para detectar secuencias víricas, adolece de una menor sensibilidad que la PCR convencional, exige una considerable capacidad de gestión informática de datos biológicos y tiene un costo prohibitivo, por lo que no suele aplicarse en las regiones del mundo que están más expuestas a la aparición de enfermedades zoonóticas. La PCR de consenso, en cambio, reposa en técnicas habituales y muy extendidas, ofrece mayor sensibilidad que la secuenciación de próxima generación y también ha sido utilizada para identificar virus nuevos en personas o animales salvajes y domésticos, si bien solo permite detectar las secuencias

genéticas «diana» conservadas de entre todos los grupos conocidos de virus. El uso de la PCRc, combinado con la secuenciación de próxima generación y técnicas serológicas cuando sea posible, puede ofrecer un potente método para identificar con rapidez los agentes etiológicos de un brote y caracterizar el viroma de los principales animales salvajes de los que se sabe que son portadores de virus zoonóticos. Los autores pasan revista a las técnicas de descubrimiento de patógenos que se utilizan actualmente en los programas de vigilancia sanitaria y zoosanitaria y exponen las dificultades que presenta el uso del descubrimiento de virus para identificar nuevos patógenos zoonóticos.

Palabras clave
Descubrimiento de patógenos – Fauna salvaje – Pandemia – PREDICT – Reacción en cadena de la polimerasa – Serología – Una sola salud – Virus – Virus zoonótico.

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