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Virus discovery: one step beyond

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Recent advances in the metagenomics field have had huge effects on the identification and characterization of newly emerging viral pathogens. To allow timely and efficient responses to future viral threats, an integrated multidisciplinary approach utilizing expertises in several areas, including clinical assessment, virus surveillance, virus discovery, pathogenesis, and the molecular basis of the host response to infection, is required. It requires the scientific community involved in virus discovery to go one step beyond.

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Introduction

After an initial decrease in industrialized countries in the first half of the last century, the burden of infectious diseases on human health worldwide has markedly increased in the past decades [1]. A complex mix of predisposing factors in our modern world has created new opportunities for the emergence of infectious diseases in animals and humans alike. This is largely related to globalization, environmental and anthropogenic changes, and the changing nature of contacts between animals and humans [1,2•,3••,4]. Most of these emerging events in humans are caused by viruses that have their origin in the animal world [2•,5–8,9•,10,11]. Following initial zoonotic transmissions these viruses usually go through a period of adaptation to the new human host, after which they may spread to cause epidemics or eventually a true pandemic. The most devastating examples of pandemics caused by initially zoonotic viruses are the acquired immunodeficiency syndrome (AIDS) pandemic caused by human immunodeficiency virus (HIV) that spilled over from chimpanzee reservoirs and Spanish influenza that spilled over from bird reservoirs [12–14]. Other examples are severe acute respiratory syndrome (SARS) coronavirus from bats and civet cats, swine influenza A virus H1N1 2009, and Nipah virus from bats and pigs [15–17,18•]. The human mortality figures associated with these emerging viral diseases ranged from hundreds in limited epidemics to many millions in pandemics. For example, the ongoing AIDS pandemic caused the deaths of tens of millions of people, whereas SARS that had apparently spilled over from a bat reservoir, and caused a starting pandemic could for the first time in history be halted before more than one thousand people had died [19]. A major impact of newly emerging and re-emerging virus infections is also seen on animals. In wildlife it poses a direct threat to biodiversity, and in domestic animals it causes huge losses to livestock production. The latter may be due to direct mortality or to culling strategies for disease control [2•].

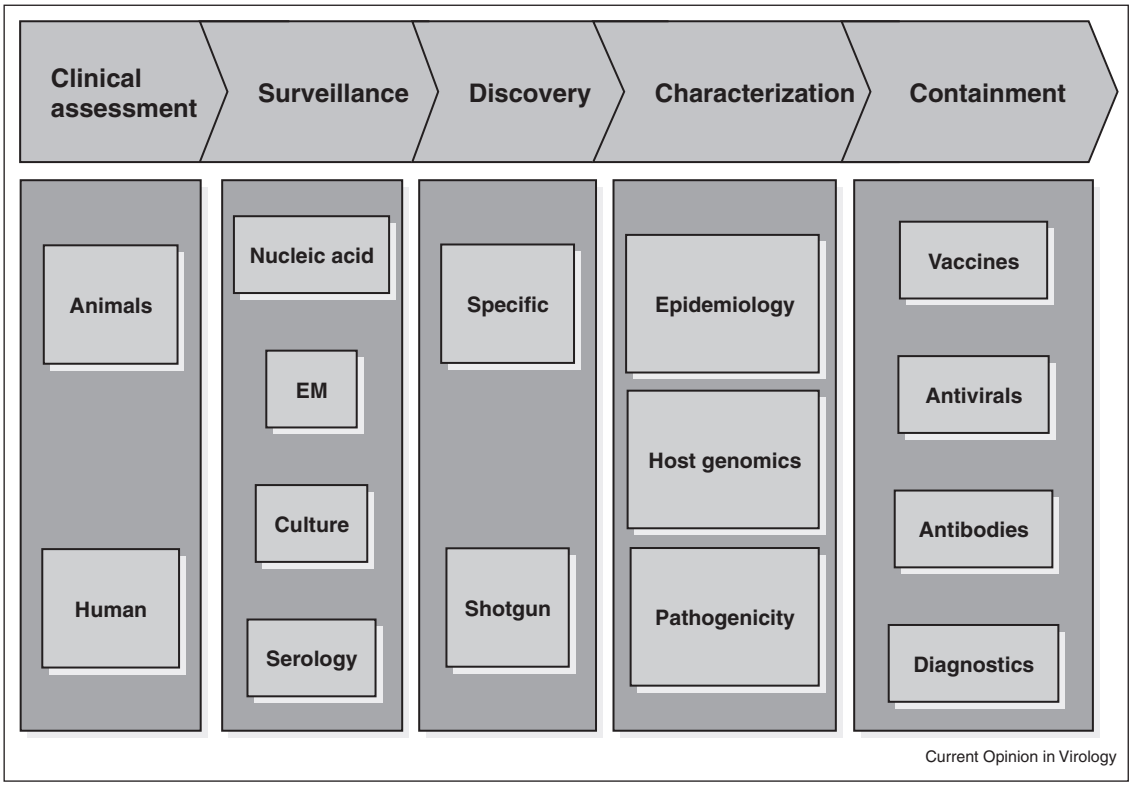
In this light it is important to create a well coordinated global effort to monitor viral pathogens to gain a thorough understanding of the diversity of viruses in animals and humans, virus transmission routes, and virus tropism providing information about potential pathogenic threats from animal reservoirs to human health [2•,18•,20]. Thus, an integrated multidisciplinary approach utilizing expertises in several areas, including clinical assessment, virus surveillance, virus discovery, virus diversity, evolutionary processes, epidemiology, pathogenesis, and the molecular basis of the host response to infection, will be required to understand the dynamics of infection and to mitigate potential effects of future infectious threats (Figure 1).

Clinical assessment and surveillance

One of the most overlooked but crucial aspects in identifying new infectious disease threats is the role that clinicians, veterinarians and epidemiologists play in the recognition of idiopathic cases of newly emerging virus infections. These professionals are the first to recognize relatively uncommon or completely new infectious diseases, on the basis of changing clinical and epidemiological trends. They should participate in well-coordinated collaborative networks that may function as our first line of defence against newly emerging viruses. These networks should be involved in syndrome surveillance in combination with routine surveillance activities for known pathogens in both animals and humans to identify at a very early stage, emerging viral infections that would otherwise fly under the radar [16] This is essential for a timely response that ideally could even abrogate the viral threat altogether.

Key human target populations for these syndrome and virus surveillance activities are those with either a high exposure to wild or domestic animals, such as hunters,

Figure 1



Viral genomics aims at identification and characterization of emerging and re-emerging viruses. It requires different disciplines in an integrated response to allow viruses to be discovered and their potential pathogenic threat to be effectively countered.

butchers, farmers, veterinarians, and zoo workers, or populations with increased susceptibility, such as injecting drug users and immunocompromised individuals. In addition, syndrome and virus surveillance should focus on domestic animals as well as wildlife with key reservoir species that have previously shown to represent an imminent health threat to humans like domestic poultry, pigs and ruminants, as well as wild birds rodents and bats, respectively. Such efforts will lead to the identification of future viral threats and allow early detection of emergence in humans and control of their eventual spread [2•,3••,18•,20]. In combination with knowledge about the molecular basis of pathogenicity and transmissibility in the respective species, this information may even allow prediction of the viruses that are most likely to cross the species barrier and what is required for the evolutionary transition of an animal virus into a human pathogen.

Virus discovery — state of the art techniques

Despite the use of a wide range of sensitive diagnostic assays, in a relatively large proportion of patients and animals suffering from apparently infectious disease, no pathogens can be detected, suggesting the presence of unidentified viruses in human and animal populations. In the pre-genomics era, new viruses were first identified by animal experiments, virus isolation in tissue culture or standard molecular detection methods. Nowadays, in order to discover and isolate new or (re-)emerging viruses, it is crucial to develop and implement a set of novel molecular techniques, which, when applied as a technology platform, increase the success rate of finding new viruses in humans and animals that may or may not be associated with new or already identified disease entities.

Besides classical continuous cell cultures, in which many of the recently discovered viruses fail to replicate, several new ex vivo culture systems are being developed. Natural viral replication circumstances may be best mimicked by infection of organ cultures of surgery-derived by-product tissues. An example of the success of this approach is human rhinovirus C (HRVC), recently implicated in upper and lower respiratory tract infections of children and individuals with chronic respiratory diseases. Although HRVC was discovered in 2006 [21], efforts to propagate the virus culture failed until Bochkov and coworkers [22] employed human organ culture of sinus mucosa to serially propagate human rhinovirus C, which ultimately allowed complete full-length genome sequencing and cloning [22]. Likewise, ex vivo swine respiratory

and enteric tract cultures have been used to study influenza virus infections in their natural target cells [23–25].

With the development of PCR and novel sequencing techniques, we have seen an enormous increase in the identification and characterization of viral genomes. This has lead to development of new species-specific assays for routine diagnostic surveillance in animals and humans. In addition, generic PCR assays, that is PCR assays specific for a broader taxonomic range than one virus species (e.g. a whole virus genus or family) were developed which allow the identification of new virus species within already known virus families [26–29]. A method for comprehensive and unbiased analysis of viral prevalence in biological specimens was devised using long oligonucleotide (70-mer) DNA microarrays with the potential to simultaneously detect hundreds of viruses [30]. The 'pan-viral microarray' not only allows detection of known viruses, but also discovery of new viruses if they are sufficiently related to those already known to permit specific hybridization [31–34].

Breakthroughs in the field of metagenomics have had far-reaching effects on the identification and characterization of newly emerging viral pathogens and on the recognition that a growing number of diseases that were once attributed to unknown causes are actually caused or triggered by infectious agents [1,35]. Viral metagenomics assays rely on sequence-independent amplification of nucleic acids from clinical samples, in combination with next-generation sequencing platforms and bioinformatics tools for sequence analysis [3••,10,18•,35–37]. They are relatively simple and fast, and allow detection of new viruses even if they are highly divergent from those that are already known [3••]. Since, the first application of metagenomics to the field of virology [38], it has been increasingly applied for virus discovery purposes resulting in an >100-fold increase in related publications in this field in 2010 and a substantial increase in the identification of new viruses [3••,39,40••]. With the development of these new techniques, the identification of a new virus and its complete genomic characterization can be done in a matter of days and at a fraction of the costs compared to only a few years ago [41,42•].

It is unlikely, however, that genomics-based tools will be used in a clinical diagnostic setting within the next decade. Methods need to be cost–effective and in a high-throughput format, which requires investment in bioinformatics tools, databases, and data management. Molecular diagnosticians need to gain experience in, and have the ability to interpret the data generated by these radically different genomics-based techniques and hurdles regarding patient privacy issues may need to be resolved before transition of genomics-based tools from a research setting to the clinic.

One step beyond …

After virus discovery and initial genomic characterization of the viral genome, an integrative approach to determine epidemiology and pathogenicity of the virus and the disease it causes is required in order to develop and tailor effective intervention and containment strategies. Many factors, among which immune status, age, and nutritional status, play an important role in the clinical outcome of infection. This underlines the importance of studying both host and pathogen parameters in an integrated way in the combat against virus infections. Comparisons between closely related viruses in different but related host species provides crucial information into various host pathways involved in different outcomes of disease. This is perhaps best exemplified by simian immunodeficiency virus (SIV) infections in different primate host species. Generally, non-human primate species that are naturally infected by SIV do not develop AIDS whereas non-natural hosts, like, for example humans when infected with HIV, do develop immunodeficiency and AIDS. Genomics-based approaches to delineate host responses to infection in both natural and non-natural hosts revealed that non-natural hosts have a higher viral load, immune activation, loss of certain T-cell subsets, and higher production of interferon-α in response to SIV infection than natural hosts [43–45]. Thus, the study of key interactions between host and pathogen may unravel possibilities for development of (multi-targeted) therapies designed to limit virus replication and to mitigate (immune)pathology and transmissibility. A few examples of recent important zoonotic events and the role of an integrative approach to elucidate their pathogenesis in order to combat such viral infections will be discussed below.

Highly pathogenic H5N1 avian influenza virus may cause infection of the lower respiratory tract and severe pneumonia in humans [46]. In contrast, human influenza A H1N1 and H3N2 viruses are important causes of upper respiratory disease, but rarely cause pneumonia [47]. Viral attachment to the host cell is a critical determinant for infection and tropism of the virus and studies into receptor attachment of human influenza A viruses and avian H5N1 influenza A virus revealed that the pattern of attachment in the respiratory tract coincides well with the difference in disease outcome. H5N1 influenza A virus primarily attaches to type II pneumocytes, alveolar macrophages, and nonciliated cuboidal epithelial cells in terminal bronchioles [48], whereas seasonal human influenza A viruses attaches primarily to tracheal and bronchial epithelium and type I pneumocytes in the alveoli [49]. Essentially, human seasonal influenza viruses mainly bind to the upper respiratory tract whereas H5N1 avian influenza viruses have a predilection for the lower respiratory tract [50].

The pattern of receptor attachment also correlates with marked differences in the efficiency of human-to-human

transmission of influenza A viruses, with H5N1 influenza A viruses displaying a notoriously low level of human-to-human transmission. There are concerns that the H5N1 virus could acquire molecular characteristics that would allow it to become more readily transmissible between humans and initiate a pandemic. The binding preference of the HA surface glycoprotein of H5N1 influenza A virus can be changed from avian α-2,3-linked sialic acid receptors to the human α-2,6-linked sialic acid receptors resulting in the associated change in tropism for the upper respiratory tract [51]. To study the conditions that would indeed allow H5N1 influenza A virus to acquire the ability of aerosol transmission *in vivo*, H5N1 influenza A virus was genetically modified to study whether the virus could be transmitted through air. Five amino acid substitutions proved to be consistently present in airborne-transmitted H5N1 influenza A viruses in ferrets [52–54]. The collective data suggest that highly pathogenic avian influenza H5N1 viruses have the potential to evolve directly in ways that allow airborne transmission between mammals, without reassortment in any intermediate host, illustrating the risk that these viruses may eventually become pandemic in humans. The next step is to perform a risk assessment of the likelihood of such a virus to arise and spread using computer simulations and associated laboratory experiments. This will ultimately allow informed decision making to justify efforts for pandemic preparedness for highly pathogenic H5N1 avian influenza A virus.

Recently, a previously unknown coronavirus HCoV-EMC was isolated from a patient presenting with acute pneumonia and renal failure with fatal outcome in Saudi Arabia [55]. The clinical presentation of the patient was remarkably similar to that caused by the SARS coronavirus in SARS patients during the outbreak in 2002/2003 [55,56], whereas most infections caused by other human coronaviruses are relatively mild. The functional receptor for SARS coronavirus in humans is angiotensin-converting enzyme 2 (ACE2) [57], but it was soon established that HCoV-EMC uses a different receptor [58]. Fourteen human HCoV-EMC cases have been identified to date, resulting in eight fatalities from respiratory disease. The transmission chain of HCoV-EMC remains unclear and could be explained by human-to-human transmission as well as repeated introductions from a reservoir animal host. The complete genome of the virus was characterized soon after the discovery of the virus [41]. On the basis of the genetic relatedness between HCoV-EMC and bat coronaviruses, it is most likely that this virus emerged from bats. Such host species switching is plausible in light of the recent identification of dipeptidyl peptidase 4 (DPP4), an evolutionary well-conserved protein, as the functional receptor of HCoV-EMC and the ability of HCoV-EMC to use bat DPP4 as a functional receptor [59]. The identification of the receptor will contribute to the understanding of the pathogenesis and epidemiology of HCoV-EMC and may facilitate development of antiviral strategies.

Interestingly, SARS coronavirus is also thought to originate from bats, by recombination between two bat viruses. The resulting bat virus supposedly was transmitted first to palm civets (*Paguma larvata*) or other carnivores, and subsequently to humans at live animal markets in southern China [17]. Studies into the pathogenesis of the SARS coronavirus revealed that cynomolgus macaques (*Macaca fascicularis*) show lung pathology similar in nature to that observed in human adults with SARS upon infection [60,61]. Using a combined approach of animal experiments with immunohistochemistry and functional genomics, it was shown that the induction of early interferon signaling may be critical in conferring protection against SARS coronavirus [61]. This notion was enforced by showing that aged SARS coronavirus-infected macaques show more severe pathology under similar viral replication levels, which is associated with an increase in differential gene expression, inflammation, and reduced type I interferon expression compared to young adult macaques [62]. Interestingly also in humans a clear age-related susceptibility to developing clinical symptoms and severe disease has been noted. Subsequent therapeutic treatment of aged macaques with type I interferon reduces pathology in aged SARS coronavirus infected macaques without affecting virus replication levels [62], suggesting that not only prophylactic, but also therapeutic treatment with pegylated interferon-α should be considered as antiviral treatment [63]. Of note, however, is that subsequent studies showed that different non-human primate species develop pathology upon SARS coronavirus infection through distinct acute lung injury pathways [64]. This should be taken into account when analyzing outcomes of intervention strategies.

Conclusions

The increased threat of emerging and re-emerging virus infections to human and animal health asks for a timely and effective response to counteract these viral infections. This has been recognized and has resulted in the formation of quite a number of initiatives focusing on improved surveillance, virus discovery, and effective response management. For example, the European funded Seventh Framework Project (FP7) entitled European Management Platform for Emerging and Re-emerging Infectious disease Entities (EMPERIE) contributes to effectively countering the potential public health threat caused by new and emerging infectious diseases in Europe by establishing a powerful network capable of structural and systematic prediction, identification, modeling and surveillance of infectious diseases, health threats and pathogens. A nationally funded initiative in The Netherlands — the VIRGO consortium — focuses on providing the infrastructure for virus discovery and for

progressing our knowledge of viruses and the mechanisms of mutual virus-host interactions which will feed into the subsequent development of novel interventions, diagnostics and prognostics at the levels of both individual patients and populations. The FP7 European funded PATHSEEK consortium will set up a disruptive diagnostic technological pathogen sequencing platform that will deliver in 24–48 hours, all possible drug resistance mutations as well as data on nosocomial infection, from one patient sample in one single assay. Another FP7 European funded consortium called ANTIGONE (ANTIcipating the Global Onset of Novel Epidemics). These and comparable or related consortia which become increasingly interlinked, provide international collaborations with different expertise areas to allow broad range responses to mitigate potential effects of future infectious threats (Figure 1) with quite some success stories already.

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