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Host switching pathogens, infectious outbreaks and zoonosis: A Marie Skłodowska-Curie innovative training network (HONOURs)

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ABSTRACT

The increase of the human population is accompanied by growing numbers of livestock to feed this population, as well as by an increase of human invasion into natural habitats of wild animals. As a result, both animals and humans are becoming progressively vulnerable to infections with known (zoonotic) pathogens, but are also increasingly exposed to novel viruses. Global trade as well as climate changes can contribute to pathogen transmission, e.g. through import of infected vectors or expansion of habitats for arthropod vectors such as mosquitoes and midges. Infectious disease outbreaks, especially those by novel viruses, are generally unexpected, and therefore we should be prepared with tools and abilities for immediate action, including the identification of the causative agent, the evaluation of its pathogenic potential for animals and humans, and the fast development of diagnostic assays to allow contact tracing and quarantine measures.

HONOURs is a Marie Skłodowska-Curie Actions Innovative Training Network (MSCA-ITN), teaching 15 talented young researchers to become "preparedness-experts". HONOURs, initiated in April 2017, involves 11 laboratories from 6 different European countries, all at the forefront of novel virus investigations and characterizations. The network includes surveillance experts in both the veterinary and the human health sector, who have developed and utilize highly sensitive virus discovery techniques, e.g. next generation sequencing based genomics and universal primers based PCR, to allow identification and characterization of novel viruses. Production of pure viral proteins, providing high-resolution structures, aids in the design of novel, fast and easy-to-use diagnostics. Organotypic *in vitro* cell cultures systems (e.g. pseudostratified human airway epithelia) provide tools for virus replication, if needed via a reverse genetics platform, and the production of virus stocks permits inoculation in animal models to examine disease, evaluate candidate vaccines, and fulfilment of the Koch's postulates. Scientists of the various institutes will provide training in the HONOURs network through

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specialized courses and workshops, combined with challenging research projects. The final aim of the network is to deliver 15 expert scientists, ready to act in case of the emergence of an epidemic.

1. Goals of HONOURs

Infectious diseases have always been a massive burden on the wellbeing of humans and animals, far beyond the consequences of war and natural disasters. Infectious outbreaks in animals and humans emerge unexpectedly, caused by known pathogens or by hitherto unknown pathogens. History has shown that the spread of unknown animal viruses is the main source of novel viral infections in livestock and humans. With a novel virus introduction, either in the veterinary or in the human field, it is important to address several essential research questions, e.g. the geographic location of the introduction and the size of the epidemic, the disease association, the mortality and morbidity, the species involved in a transmission chain, and the replication characteristics such as cell- and organ-tropism. Currently there is no comprehensive teaching programme for researchers in this area, therefore the main objective of the HONOURs MSCA-ITN is to train young researchers (Early Stage Researchers: ESRs) on different aspects of emerging viral infections and the threat of zoonotic viruses (Fig. 1). The ESRs will follow specialized courses supplied by the senior scientists in HONOURs (Box 1). Each ESR will also have an individual research topic leading to a thesis defence and the awarding of a PhD degree (Box 2).

1.1. Recognizing an introduction

In case of emerging or re-emerging epidemics, the recognition of an introduction is of utmost importance. In general, this depends on syndrome surveillance and differential diagnostics, operational in most European countries, with local and national syndrome notification protocols. However, infections causing milder disease, or less recognizable disease symptoms, remain below the surface and are likely missed when only syndrome surveillance is operational. Recognition of these introductions requires not only the close observation of disease symptoms, but also careful evaluation of unexpected negative results in diagnostic tests for known pathogens. There are possibilities within HONOURs to recognize also these milder or hidden outbreaks, as most institutes in HONOURs perform infectious disease diagnostics, and have access to data or information on diagnostic samples from patients with clinical symptoms of unknown etiology. For exactly this reason an Outbreak Antenna team has been formed within HONOURs, as a hands-on platform for the ESRs to develop, explore, and test their ideas, using the diagnostic results and data available at their institute as their playing ground.

1.2. Identification of an introduction

With standard diagnostics, like cell culture, serology, or PCR, it is difficult to identify unexpected or novel viruses. Therefore, generic detection and identification assays building on the latest developments in sequencing and sequence analysis are needed to identify all potentially viral sequences. HONOURs has multiple beneficiaries on-board that are at the forefront of novel pathogen detection and characterization. Unbiased sequencing techniques have been developed by three members in HONOURs: 1. Direct next-generation sequencing (Direct-NGS) (Hoffmann et al., 2012), 2. VIDISCA-NGS (de Vries et al., 2011), and 3. The NetoVIR method (Conceição-Neto et al., 2015). These three techniques complement each other. The VIDISCA and NetoVIR techniques include an amplification step during library preparation and are thus more suitable for virus detection in samples containing low concentrations of nucleic acids, like serum, plasma, cerebrospinal fluid, respiratory and anal swabs. Direct-NGS is a shotgun sequencing approach lacking an amplification step, thereby reducing the bias of the sample library preparation step. Moreover, the direct-NGS is capable of virus detection in complex materials like solid tissues. Another difference among the techniques is how DNA fragmentation is performed. VIDISCA is based on restriction enzyme digestion at fixed positions, having the advantage that repeated detection of fragments provides the certainty of presence in a

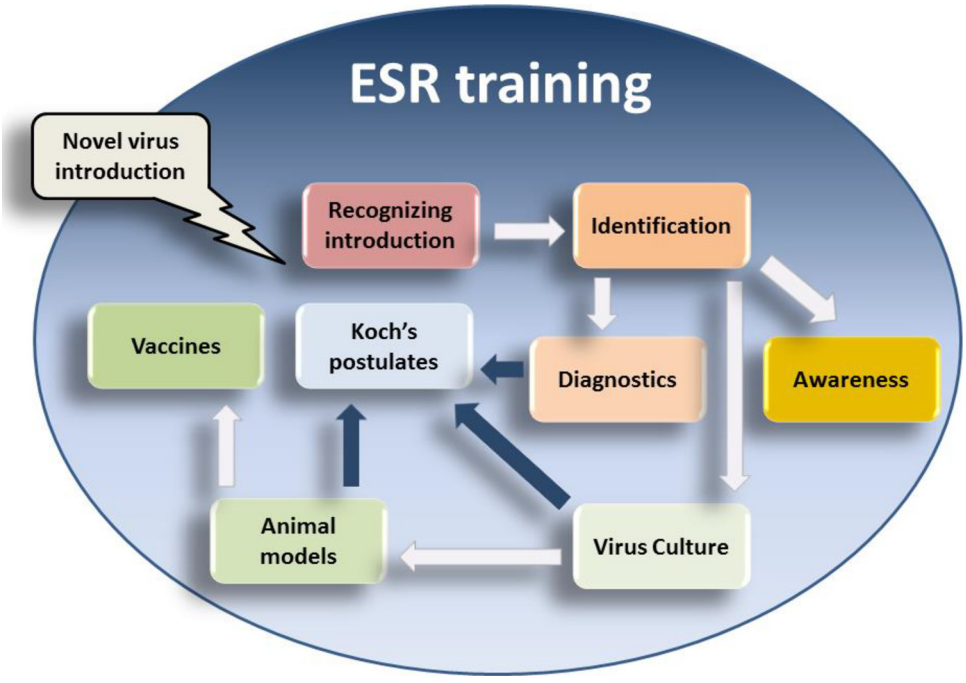

Fig. 1. Scheme of the research and training topics in HONOURs. In case of a novel introduction of a virus into the human or animal field, the first step in HONOURs is recognition of the introduction followed by identification of the virus, communication to the relevant stakeholders (awareness), generating fast and easy to use diagnostics, culturing of the virus, setting up of an animal model system and vaccine development.

Box 1
Research Work Packages and Network-Wide Training Events in HONOURs.

- **WP2-Outbreak Antenna**: Infectious diseases; Outbreak Antenna Meeting. Sites involved: FLI, EDI-IVI, MSD-AH, AMC, KU Leuven, UBern, Spiez, Charité (see legend of Fig. 2 for institute abbreviations).
- **WP3-Pathogen Discovery:** VIDISCA-NGS; Random priming-NGS (NetoVIR); Direct-NGS; Metagenomics and microarrays for virus detection; High throughput nested RT-PCR and degenerative oligonucleotide design; High throughput analysis of ticks and mosquitoes by pan-virus family/ real-time RT-PCR. Sites involved: FLI, AMC, KU Leuven, Spiez, Charité, UBern, BPRC.
- **WP4-Virus Culture and Animal Models:** Nonhuman primates in biomedical research; Reverse genetics; Virus-host interactions on airway epithelia; BSL3/3+ trans-boundary animal course; Commercial vaccine development. Sites involved: AMC, FLI, BPRC, MSD-AH, EDI-IVI.
- **WP5-Advanced Diagnostics:** Novel diagnostics; Protein production and serology; Flavivirus serology; High throughput nested RT-PCR and degenerative oligonucleotide design. Sites involved: Charité, ULeeds, INGENASA, UBern.
- **WP6-Awareness**: Infectious diseases; Media training. Sites involved: AMC, KU Leuven, FLI, Charité.

Box 2
ESR Individual Research Projects.

(1)

(1) **Unknown viruses in animal/human diseases (AMC).** Diseases like pathogen negative-diarrhoea, -respiratory tract infections, and AIDS-related chronic immune activation, may be influenced by unexpected or unknown virus infections. Investigation by VIDISCA-NGS and antibody capture-VIDISCA, will reveal the influence of viruses and vectors in disease.
(2) **Human gut viral metagenome in the first years of life (KU Leuven).** Reveal whether the gut virome dynamics of infants is influenced by factors, such as hygienic and socio-economic environments, antibiotics, disease, diet, and vaccination.
(3) **Optimized cascaded next-generation virus detection and molecular characterization (FLI).** Analyse and characterize all novel pathogens via a one-fits-all platform. Direct metagenomics platforms and assays will be further optimized through best selection of samples, sample preparation, and data analysis.
(4) **Prevalence and disease association of newly detected microorganisms (Charité)**. Genomic characterization and evolutionary reconstructions of novel viruses can elucidate potential viral reservoirs. Zoonotic origins of new viruses will be investigated by phylogenetic inference, ancestral state reconstructions and hypothesis testing.
(5) **Isolation and molecular analysis of zoonotic emerging respiratory viruses (EDI-IVI).** Genetically modified primary airway epithelial cell cultures have been established that are transduced with lentivirus constructs to express specific genes of interest. The virus-induced host responses on the (transduced) pseudo-stratified primary airway epithelia cell cultures will be investigated.
(6) **Replication of novel viruses in epithelial cells (AMC).** Virus discovery using organotypic *in vitro* cultures. Replication will be investigated via single cell transcript sequencing and aspecific infection-staining techniques.
(7) **Establishment of a reverse genetics platform for emerging viruses (EDI-IVI).** Emerging viruses are often difficult to isolate and sometimes only the genomic sequence is available. Cloning of full-length cDNA from sequencing information combined with the expertise to rescue recombinant viruses will bypass the virus isolation step.
(8) **Pathogenic potential of novel flaviviruses (BPRC).** The early pathological events following Usutu and Zika virus infection will be investigated by infection of cell lines, and primary cells from a natural host: nonhuman primates.
(9) **New approaches to confirm involvement in disease (MSD-AH).** Identify novel viral pathogens, design 2D and 3D culture platforms, confirm involvement in disease and work towards a vaccine solution.
(10) **Development of diagnostic methods for newly discovered viruses (INGENASA).** Detection of zoonotic viruses of the Bunyvirales, such as Crimean-Congo hemorrhagic fever virus and Rift Valley fever virus. Multiplex serology tests will be developed for the differential diagnosis between these two and other related viruses.
(11) **Viral nucleocapsid proteins, their structure and novel diagnostic assays (ULeeds)**. Elucidate molecular structures of viral nucleoproteins and nucleocapsid complexes of bunyaviruses and paramyxoviruses.
(12) **In vitro and in vivo characterization of viral pathogens (FLI).** Novel viruses will be cultured on suitable cell cultures from the collection at the FLI (> 1250 different cell lines). An animal model will serve as an alternative approach to promote virus replication, collect polyclonal antisera, and achieve a first challenge model.
(13) **Detection and sequencing methods for the identification of new and emerging viruses (UBern).** Evaluate sample preparation and sequencing protocols for adenovirus-containing clinical samples (model system) supplemented with virus family wide PCR assays to identify novel virus family members in ticks, mosquitos, and rodents.
(14) **Development of impedimetric sensors for emerging arena- and bunyaviruses (ULeeds).** Generate impedance-based detection assays by protein expression/purification, antibody and Affimer production, biosensor fabrication and testing. The structure of the viral antigen used in the biosensor device will be determined by electron microscopy.
(15) **Full genomic sequencing of viral pathogens with large genomes (KU Leuven).** Optimising ultra-long read sequencing protocols (e.g. MinION™) to investigate intra-host and inter-host genetic diversity of human cytomegalovirus and relate to the prognosis and treatment of a patient.

clinical sample. The random shearing-based methods (NetoVIR and Direct-NGS) have the benefit of producing sequence reads that can form contigs via overlapping reads. Contigs are generally larger than single sequence reads and thus more suitable for phylogeny and identity searches. Not only metagenomics techniques, but also sensitive generic PCRs (Drexler et al., 2015) and microarrays (Abendroth et al., 2017) provide powerful tools to identify new members within known virus families, and both techniques are part of HONOURs.

1.3. Fast detection and association with disease

Once a novel or newly emerging virus is identified and involvement in a disease is suspected, establishment of the association with disease is vital. To study this association, sensitive and specific detection assays are needed, not only for the pathogen, but also for the antibody response elicited by the viral infection. Within HONOURs several institutes are involved that develop sensitive and specific detection assays for the virus and/or antibodies to the virus. The laboratories provide sensitive, high throughput, real time PCR assays and serological tests (Oechslin et al., 2017; Corman et al., 2016; Sastre et al., 2016). Furthermore, assay development within HONOURs also includes impedimetric sensoring, which offers rapid and highly sensitive detection of virus particles, viral proteins and also anti-viral antibodies, independently of PCR or immunostaining techniques (Ahmed et al., 2013). The purified viral proteins needed for the diagnostic assays will also be used for both Titan Krios cryo-EM imaging and crystallization trials with high-resolution structures as a result. These structures provide information regarding the function of the proteins in the viral replication cycle, the evolutionary relatedness when compared to other known structures, and a rationale of exposed antigenic sites, important for monoclonal antibody production (Carter et al., 2012; Surtees et al., 2015).

1.4. Fulfilling Koch's postulates

Apart from the first detection of a novel viral introduction and establishing the association with disease, it is important to fulfil the Koch's postulates as part of the virus characterization steps (Loeffler, 1884):

- The microorganism must be found in abundance in all organisms suffering from the disease, but should not be found in healthy organisms.
- The microorganism must be isolated from a diseased organism and grown in pure culture.
- The cultured microorganism should cause disease when introduced into a healthy organism.
- The microorganism must be re-isolated from the inoculated, diseased experimental host and identified as being identical to the original specific causative agent.

A necessity for the fulfilment of the postulates is a virus culture stage to acquire pure virus stocks for inoculation in an animal model system. HONOURs will focus on novel strategies for virus culture using primary cells. Airway epithelia is the main portal of respiratory virus entry, thus primary epithelial cells will be the first choice to be used in virus culture. The epithelial cells will be stimulated to differentiate, providing an elegant system that mimics the site of virus entry (e.g. the differentiated epithelia of the respiratory tract) (Jónsdóttir and Dijkman, 2016; Kindler et al., 2013).

The target cells will not only be cultured from humans but also from various animals e.g. pig, monkey, birds, bats. These advanced virus culture systems may allow propagation of viruses that cannot be cultured in conventional cell line-based culture systems (Pyrc et al., 2010). In case of virus replication, the virus harvests are to be used in animal models with the aim to prove involvement in disease by fulfilling the Koch's postulates.

Isolation of emerging viruses in cell culture can be difficult due to limited quality, quantity or availability of sample material. However, often there is a genomic sequence available. In such cases it is possible to generate (c)DNA clones of a novel virus that can be introduced in a host cell via transfection. In combination with a rescue system – e.g. the differentiated epithelial system described above - the infectious virus might be obtained and subsequently used for inoculation in an animal model. If neither conventional cell lines nor any primary cell culture

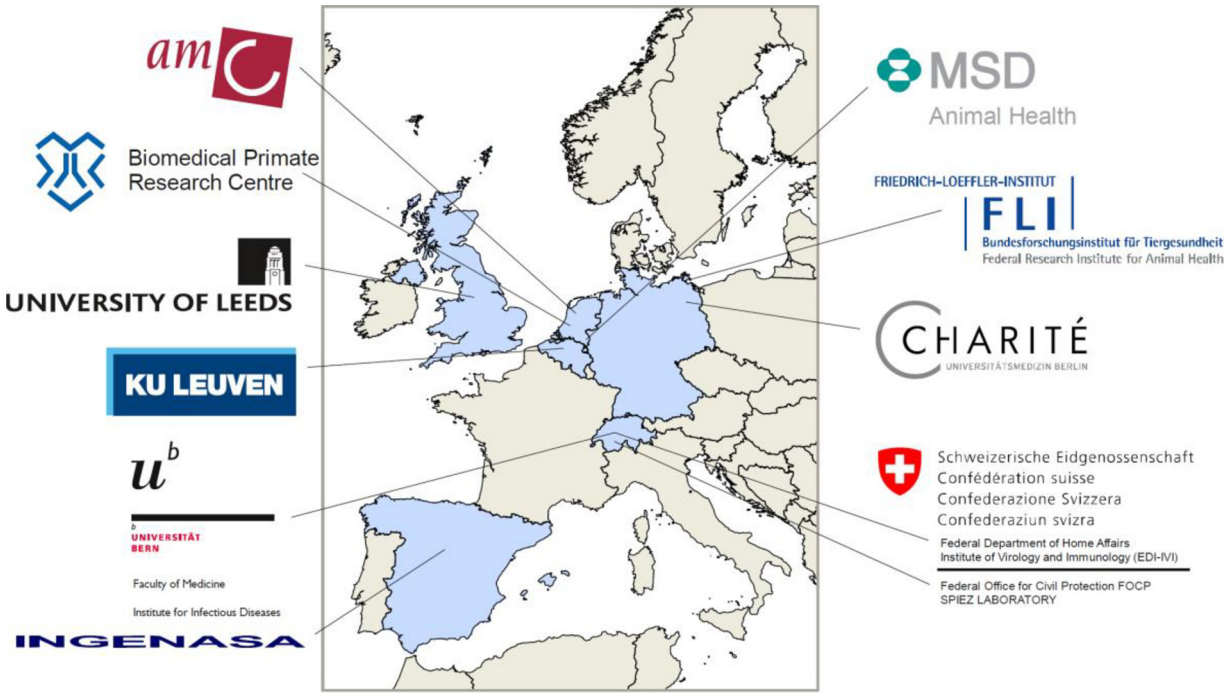

Fig. 2. Countries (blue) and institutes participating in HONOURs. The HONOURs consortium consists of 11 laboratories, from the following universities/companies/institutes: Academic Medical Center, University of Amsterdam (AMC); Rega Institute for Medical Research of the Katholieke Universiteit Leuven (KU Leuven); Institute of Virology at the Charité - Universitätsmedizin Berlin (Charité); Institute for Infectious Diseases, University of Bern (UBern); School of Molecular and Cellular Biology at the University of Leeds (ULeeds); MSD Animal Health (MSD-AH); Inmunología y Genética Aplicada SA (INGENASA); Friedrich-Loeffler-Institut (FLI); Eidgenössisches Departement des Inneren, Institute of Virology and Immunology (EDI-IVI); Biomedical Primate Research Centre (BPRC); and Labor Spiez (Spiez). The map was generated using QGIS (www.qgis.org) and open source data from Natural Earth (http://www.naturalearthdata.com) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

system is available for virus propagation, infectious virus can even directly be obtained following transfection of the genetic material. Once an animal model system is established, the disease and the viral pathogenicity can be studied, followed by studies on vaccine candidates.

1.5. Fifteen experts on host switching pathogens, infectious outbreaks and zoonosis

Apart from receiving education on all abovementioned subjects, each ESR will have an individual research project (see Box 2). The research topics addressed are based on the expertise of the ESR-employing institute or company (see Fig. 2). The wealth of the specialized trainings and the challenging research projects resulting in a PhD degree is the strength of HONOURs, the royal jelly preparing 15 promising scientists for the future.

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