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**REVIEW ARTICLE**

# Cross-species transfer of viruses: implications for the use of viral vectors in biomedical research, gene therapy and as live-virus vaccines

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## Summary

**Summary** All living organisms are continuously exposed to a plethora of viruses. In general, viruses tend to be restricted to the natural host species which they infect. From time to time viruses cross the host-range barrier expanding their host range. However, in very rare cases cross-species transfer is followed by the establishment and persistence of a virus in the new host species, which may result in disease. Recent examples of viruses that have crossed the species barrier from animal reservoirs to humans are hantavirus, haemorrhagic fever viruses, arboviruses, Nipah and Hendra viruses, avian influenza virus (AI), monkeypox virus, and the SARS-associated coronavirus (SARS-CoV). The opportunities for cross-species transfer of mammalian viruses have increased in recent years due to increased contact between humans and animal reservoirs. However, it is difficult to predict when such events will take place since the viral adaptation that is needed to accomplish this is multifactorial and stochastic.

Against this background the intensified use of viruses and their genetically modified variants as viral gene transfer vectors for biomedical research, experimental gene therapy and for live-vector vaccines is a cause for concern. This review addresses a number of potential risk factors and their implications for activities with viral vectors from the perspective of cross-species transfer of viruses in nature, with emphasis on the occurrence of host-range mutants resulting from either cell culture or tropism engineering. The issues are raised with the intention to assist in risk assessments for activities with vector viruses. Copyright © 2005 John Wiley & Sons, Ltd.

**Keywords** gene transfer vector; virology; gene therapy; adaptation; cross-species transfer; zoonosis

## Introduction

The last two decades have shown an increased awareness of the fact that viruses occasionally cross the species barrier leading to the emergence of viral diseases in new host species [1,2]. In the past such events may have been overlooked as the underlying cause of the emergence of a new disease. However, advances in diagnostic technologies now facilitate detection and recognition of new emerging viruses even at an early stage. Prominent examples are hantavirus, haemorrhagic fever viruses, arboviruses, Nipah and Hendra viruses, avian influenza virus (AI), monkeypox virus, and the SARS-associated coronavirus (SARS-CoV) [2–5].

Crossing the species barrier from one animal species to another is most readily noticed when it is associated with overt pathology. The cross-transfer

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is known as a zoonosis in the case where the virus is transmitted from non-human hosts to humans and causes disease [6].

Crossing the species barrier is an unpredictable event that involves complex interactions between the virus and the newly adopted host [7]. The HIV virus and contemporary human influenza viruses are prominent examples of viruses that have crossed the species barrier and established themselves permanently in the human population without further dependence on the presence of the original animal host reservoir. Fortunately, natural adaptation of a new virus leading to permanent establishment and dissemination within the human population is a rare event [8]. Often, the virus is not readily adapted to infect and spread efficiently from human to human. The emergence of new viral infections often follows environmental, ecological and technological changes caused by human activities [9]. These activities may lead to an increased contact between humans and animal hosts acting as reservoirs of zoonotic viruses. Agricultural development, an increased exploitation of environmental resources, growth and increase in the mobility of the human population and trade and transportation of food and livestock, have been identified as important factors contributing to the introduction and spread of a number of new viruses in the human population [10–13].

The road map for cross-species transfer may differ for individual viruses. However, some common underlying factors that affect the probability of zoonotic events can be identified (Table 1). The potential of viruses to adapt to new or changing cellular environments or ecological niches via genetic variation appears to be a key feature [8,14].

The advent of novel technologies for genetic modification of viruses offers new opportunities for biomedical research. Often, these activities involve handling of viruses and their genetically modified variants in large quantities. The use of viral vectors in experimental gene therapy or as live vaccines may be also a cause for concern. Indeed, such activities meet the primary requirements for cross-species transfer, i.e. contact between infectious viruses and a potential new host species. Therefore, it is important to identify those activities that have a finite risk of leading to new viral infections and to practice appropriate biosafety measurements. In this review risk factors associated with activities with viral vectors will be addressed from the perspective of emerging viruses that have crossed the host barrier in nature.

**Table 1. Examples of factors contributing to the recruitment of new host species**

| Event | Virus | Reference |
|---|---|---|
| Mutations that facilitate the use of alternative receptors in the newly adopted host species | Feline parvovirus | [29] |
| Transmission to humans after occupational exposure | Hendravirus | [135] |
| Intensified contact between natural and new host species due to climatic changes | Hantavirus | [136] |
| Immune evasion by genetic variation (e.g. antigenic drift and shift) | Influenza virus | [55] |
| Introduction in new geographic areas by migrating birds | West Nile virus | [137] |
| Initial close contact between natural and newly adopted host due to changes in natural infrastructures | Nipah virus | [135] |

## Molecular mechanisms involved in adaptation: RNA and DNA viruses

The processes that underlie cross-species transfer through host-range expansion and establishment of viruses in new host species depend on the accumulation of genetic changes [7,15]. These are likely to differ for various viruses and may affect virtually every aspect of the viral life cycle. This process of adaptation can occur by a variety of mechanisms including mutation, recombination and reassortment.

Mutations occur in the genomes of DNA as well as RNA viruses. In general, mutations occur slower in DNA viruses than in RNA viruses because of the proofreading function of many DNA polymerases. This corrects mistakes made by the polymerase during replication. RNA genomes are replicated by RNA polymerases that lack proofreading. Therefore, mutations in RNA viruses can occur up to a million-fold more frequently than in DNA viruses [16,17]. As a consequence, RNA viruses generally evolve more rapidly, and lead to genetic heterogeneity and the presence of so-called viral quasispecies [18]. The concept of quasispecies states that in an infected host, the virus exists as a population of genetically related but divergent variants defined by a master sequence and a complex and dynamic series of mutant sequences. While the master sequence remains the predominant sequence present within the population, the spectrum of mutants may shift in response to selective pressures. From the quasispecies population a variant may be selectively expanded [19–21].

Although the major mechanism that drives adaptation is based on accumulation of point mutations, evolution of viruses also occurs through recombination. Recombination occurs in both DNA and RNA viruses leading to the exchange of parts of genomes. This may result in the emergence of new virus variants. For recombination to take place, at least co-infection of a cell by two different virus variants is required. Recombination plays an important role in evolutionary changes of DNA viruses. In some cases, recombination between viruses and cellular nucleic acid can lead to the capture of cellular coding sequences [22].

Reassortment is another important evolutionary mechanism in RNA viruses with a segmented genome, such as influenza viruses and reoviruses. After co-infection of a cell with different strains or subtypes, genomic segments may be shuffled and rearranged in progeny virus particles

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resulting in the generation of new viruses with different biological properties. This offers viruses a large adaptive potential by facilitating evolutionary leaps in response to changing cellular environments without the need for gradual accumulation of favourable mutations [23].

## Examples from nature: adaptation via genetic changes

In this section a number of examples of animal viruses that have crossed the species barrier are chosen to illustrate how they have evolved in nature through genetic changes. The examples illustrate that under the right environmental conditions host-range variants evolve that may establish themselves in the newly recruited host as new viruses causing disease. After the initial cross-transfer to the new host species, a period of further adaptation may be required. The examples also show how the evolutionary processes continue while an epidemic evolves.

### Cats to dogs

In the late 1970s, a new syndrome of viral enteritis and myocarditis emerged in dogs and subsequently swept rapidly across the world, killing thousands of dogs within a few years after its initial appearance. The virus was named canine parvovirus type 2 (CPV-2). Phylogenetic analysis revealed that this virus was remarkably similar to feline parvovirus-like viruses such as feline panleukemia virus (FPV) which infects cats, mink, and raccoons, but not dogs [24]. Therefore, CPV-2 presumably emerged as a natural host-range mutant of a feline parvovirus [25,26].

Host-range properties are determined by the capsid protein for both CPV-2 and FPV. Although CPV-2 is capable of infecting feline cells in culture, the virus does not replicate in cats. FPV, on the other hand, is able to replicate in dogs in a restricted fashion, i.e. in bone marrow and thymus, but not in cultured dog cells [27]. CPV-2 differs from FPV by only two nucleotide substitutions within the capsid gene resulting in two amino acid substitutions [28]. These changes are associated with the ability of CPV-2 to bind to the canine transferrin receptor with high affinity [29]. As a result, CPV-2 acquired the capacity to infect canine intestinal tissue. It is thought that subsequently this virus acquired transmissibility between dogs and further adapted to replicate more efficiently in dogs as it became pandemic.

Interestingly, within three years after its initial appearance in 1978, CPV-2 was replaced worldwide by an antigenically and genetically variant virus CPV-2a [25]. This indicates that CPV-2a had a strong selective advantage over CPV-2. Another antigenic variant derived from CPV-2a, CPV-2b which differs by only two amino acids, arose in 1984. This implies that variants of CPV gradually arose due to further adaptation and selection in dogs, in reaction to selective, most likely immunological, pressure. The two new variants (types 2a and 2b) differ at 5 or 6 amino acid from CPV-2 isolates [30]. At present, the two variants are still endemic in the canine population. These two variant viruses have an expanded host range compared to the original CPV-2 since they replicate in cats in both experimental settings and in the wild although with no or relatively low pathogenicity [31,32]. Remarkably, the prevalence of CPV-2a and CPV-2b and new antigenic variants (CPV-2c) has now been demonstrated in a wide range of feline populations worldwide (reviewed in [33]).

The emergence of CPV-2 serves as an example of rapid global distribution and establishment of host-range mutants in an immunologically naïve new host. Whereas CPV-2 most likely arose as a natural host-range mutant derived from cats, some other scenarios on its emergence have been suggested [34]. It was suggested that CPV-2 may have emerged after cross-transfer from a yet unidentified animal host to dogs, or CPV-2 may have arisen under selective growth conditions during FPV live-virus vaccine production in canine cells and subsequently spread via vaccination. The high titers of the virus shed in faeces and its resistance to inactivation may explain its initial rapid dissemination, also into countries with strict quarantine regulations for dogs. Human activity may have stimulated the spread through mechanical transport, presumably aided by long-distance air travel [25,35].

### The HIV pandemic

The AIDS pandemic is now generally accepted to stem from a viral zoonosis. Human immunodeficiency viruses 1 and 2 (HIV-1 and HIV-2) emerged separately around the same time in distinct geographically areas as the result of multiple zoonotic transmissions from simian immunodeficiency virus (SIV)-infected non-human primates to humans [36]. Based on their genomic organization and phylogenetic analyses it is clear that HIV-1 and HIV-2 fall into two different SIV lineages [37]. This implicates that both viruses must have had distinct origins. Both phylogenetic and epidemiologic evidence indicate that HIV-1 evolved as a consequence of SIVcpz transmission from chimpanzees to humans in central Africa [38,39]. However, to date, no serological or genetic evidence of widespread prevalence of HIV-1-related strains exists in chimpanzees in the wild in Africa. Transmission of SIV from sooty mangabeys in West Africa most probably caused the emergence of HIV-2 since SIV strains derived from sooty mangabeys are phylogenetically closely related to HIV-2. HIV-2 is found at a high prevalence in sooty mangabeys [40,41].

Both HIV-1 and HIV-2 show enormous genetic diversity. HIV-1 comprises three genetically distinct virus groups (M, N, and O) of which the predominant group M consists of 11 subtypes or clades of which all but two have spread throughout the world. In contrast, HIV-2 is mainly confined to the African continent and comprises seven distinct phylogenetic lineages, subtype groups A through

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G, which can be categorized in epidemic subtypes A and B and non-epidemic subtypes C through G [37,42]. It has been estimated from phylogenetic and epidemiological data that initial cross-species transfers of both the M group of strains of HIV-1, and the progenitor group of subtypes of HIV-2, may have taken place around 1930 in West Africa [43,44]. HIV-1 may have initially started to spread in Africa at the beginning of the 1960s [45,46]. Therefore, more than two decades of 'silent' human-to-human transmission may have occurred in Africa before AIDS became apparent and HIV was identified as its causative agent in the early 1980s.

In the advent of the HIV epidemic early SIV strains initially may have crossed the species barrier as a result of an increase of contact between humans and SIV-infected simian species. Apparently, activities such as hunting, handling and consumption of contaminated uncooked simian meat led to direct exposure to animal blood and body fluids [47]. The following years passaging between infected humans of partially adapted SIV strains may have resulted in series of cumulative mutations and genetic changes. The large genetic differences that exist between SIV and HIV indicate that the initial SIV strains that crossed the species barrier must have undergone adaptation in humans in a relatively short period of time. This suggests the involvement of some modern iatrogenic event. Massive vaccination programs carried out at that time using non-sterile injection needles may have provided opportunities for transmission and further adaptation of the virus to humans in Africa [48,49].

The genetic and phenotypic evolution of the HIV virus still proceeds at a high pace not only between individuals worldwide, but also within infected individuals. During the time-course of infection the extensive genetic diversity originates from the rapid viral turnover and replication errors caused by reverse transcriptase [50,51]. In addition, among the globally pandemic HIV-1 M group, several circulating recombinant forms (CRFs) exist. These CRFs result from recombination events between two different strains within the same individual and now constitute 10–20% of newly characterized circulating strains [52]. This diversity allows the HIV virus to rapidly adapt under selective pressure generated by antiretroviral drugs and host immune responses. Selection of HIV variants has been implicated in the use of different co-receptor molecules and selection for different cell types and tissues and body compartments such as lymph nodes and the brain during late stages of infection and manifestation of different disease patterns [53,54].

The emergence of HIV exemplifies how multiple independent cross-species transmissions of simian viruses that are not associated with disease in their natural hosts eventually resulted in the establishment of two types of HIV in the human population. While adapting to its new host the virus underwent a myriad of molecular changes. Changes in social behaviour of humans may well have offered opportunities for newly evolved HIV strains to become pandemic.

## Influenza A

Pandemic influenza A is a zoonotic disease caused by cross-species transfer of influenza A viruses from animal reservoirs. The twentieth century has witnessed three influenza pandemics, Spanish influenza (1918), Asian influenza (1957), and Hong Kong influenza (1968), that killed millions of people worldwide. Although influenza A viruses have been isolated from a variety of vertebrates, including pigs, horses, seals, and whales, birds serve as the main reservoir and are a potential source for new pandemic strains [55].

Influenza A viruses contain eight negative-sense RNA segments that code for at least ten polypeptides of which eight are structural viral proteins and two non-structural proteins. Influenza A viruses are divided into subtypes based on both serological and genetic differences between the surface proteins and their encoding genes, respectively. To date 15 hemagglutinin (HA) subtypes (H1–H15) and nine subtypes (N1–N9) of the neuraminidase (NA) proteins have been identified. Influenza A viruses containing all different combinations of the HA and NA subtypes have been identified in aquatic birds. In humans only influenza A viruses of hemagglutinin subtypes H1 through 3 and neuramidase subtypes N1 and N2 have established permanent lineages. These viruses are considered human influenza A viruses [56].

In nature new influenza A viruses emerge via two mechanisms of antigenic variation. The first, antigenic drift, is caused by accumulation of point mutations in both the NA and HA surface proteins enabling new antigenic variant viruses to evade the human immune system and emerge via selection. Influenza A viruses that emerge via antigenic drift are responsible for the yearly epidemics in the human population. Antigenic shift occurs when a variant influenza A virus arises that is antigenitically completely distinct from former circulating influenza A viruses. The new virus is a reassortant that is characterized by the presence of a novel hemagglutinin gene segment alone or in combination with a complete novel neuraminidase gene segment. Influenza A variants that emerge through antigenic shift are potentially capable of causing novel pandemics in an immunologically naïve human population [55].

The avirulent nature of avian influenza virus infections in ducks and waterfowl results from adequate adaptation to their hosts [55]. Avian influenza viruses do not replicate efficiently in humans. Similarly, human influenza A viruses do not replicate efficiently in birds [57,58]. The trachea tissue of the pig contains receptors for both avian and human influenza A viruses. Therefore, they are permissive to both human and avian viruses and thought to function as a 'mixing vessel' for reassortment of not only human and avian, but also swine influenza A viruses [59]. Pigs are therefore also considered to be ecological niches important for the emergence of new influenza A viruses in humans. In pigs, newly reassorted viruses may further evolve by accumulation of additional mutations,

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further adapt to a mammalian host and eventually be transmitted to humans. Ample data indicate that further adaptation to the human cellular environment is necessary for replication and efficient transmission in humans. Adaptation has a polygenic basis and may involve multiple viral gene segments. It should be noted, however, that only in rare cases will cross-species transmission lead to permanent establishment of new lineages of influenza A viruses in humans [56,60].

Analyses of the viruses that caused the Asian and Hong Kong pandemics revealed that these were caused by reassortants that contained a mixture of avian and human genome segments [61,62]. Genetic analysis of the 1918 Spanish influenza virus initially suggested that the epidemic originated from a whole avian influenza virus that had been transmitted from infected pigs to humans [63]. However, the origin of the 1918 pandemic strain still remains an enigma since the presence of its HA molecule did not originate from any known avian strain. In addition, there was no evidence of adaptation to a mammalian host [64]. It was not until the 1997 Hong Kong epidemic that direct transmission of whole avian viruses to humans was observed. Analysis of the virus that caused this epidemic revealed that a reassorted influenza A virus (H5N1) of entirely avian origin had crossed the species barrier, apparently without adaptation to a mammalian host. Interestingly, the virus was able to replicate in humans but had not acquired human-to-human transmissibility, preventing efficient spread and, potentially, a global epidemic (reviewed in [65]).

Receptor specificity is considered to be a major determinant of the host range of influenza A viruses. The HA protein plays a pivotal role in host-cell receptor recognition and attachment. It binds sialic acid (SA) on the host cells. Avian influenza A viruses preferentially bind to terminal SA which is joined by an alpha2,3-linkage to the sugar chain of the glycoprotein or glycolipid in the gut. However, human influenza A strains bind to terminal SA through an alpha2,6-bond to cells in the respiratory tract as a result of acquired mutations [66–68]. The 1997 Hong Kong avian H5N1 strain, however, possessed avian binding properties [69]. This indicates that receptor specificity alone is not an absolute requirement for bird-to-human transmission. The host range of influenza A viruses is determined by a complex interplay of multiple factors [70].

The influenza A virus illustrates the unpredictability of virus variation as well as the virus's great potential for adaptation. Regular close contact between birds, pigs and humans offers opportunities for reassortment and cross-species transfer. Hence, the live-bird markets in south-east Asia are considered a risk [71].

## Adaptation in the laboratory: selective pressures and changing environments

A variety of experimental conditions are applied in the laboratory for propagation and isolation of viruses and their genetically modified derivatives. As a result, these viruses are subject to selective forces that are likely to differ from those experienced in nature. Although conditions may be well defined and controlled, various selective pressures are generated in culture due to, e.g., changes of concentrations of nucleotide substrates, the addition of mutagenic substances, the use of different incubation temperatures, incubation with antibodies, or a change of host cells. These different selective forces have unpredictable influences on the virus.

Since cell culture conditions can have profound effects on the composition of viral populations, viral stocks consist of genetically heterogeneous populations (reviewed in [20,72]). Therefore adaptation of a virus to cell culture inevitably results in selection of new virus variants. Thus, under experimental conditions, cell cultures represent ecological mini-environments in which viruses are subjected to selective forces and can readily evolve, yielding new variants with an altered cell tropism or host range.

## Host-range mutants in cell culture: RNA and DNA viruses

The following examples demonstrate that upon persistent infection and passage in cell culture, cross-species transmissibility may be promoted by selection of virus variants with an altered host range. Adaptation in cell culture may result in changes in receptor specificity and tropism, and leads to the emergence of host-range mutant viruses.

### RNA viruses

The mouse hepatitis virus (MHV) is characterized by a narrow host-range and tissue specificity, both *in vivo* and *in vitro*. This specificity is primarily determined by the virus's surface Spike (S) glycoprotein, which is responsible for attachment to specific host-cell receptors [73]. MHV virus variants with both an altered receptor specificity and a broadened host range were selected during continued passaging in murine or mixed cultures consisting of murine and non-permissive hamster cells. Here MHV acquired the ability to infect human, hamster and monkey cells. MHV host-range expansion has been attributed to the presence of virus variants recognizing homologues of the normal receptor. Adaptation required mutations in the surface S protein and selection of host-range mutants for the use of the alternative cellular receptor [74–77].

Selection of variants with a changed receptor specificity resulting from passage in cell culture has also been demonstrated for foot-and-mouth disease virus (FMDV). Host-cell specificity of the parental virus is based on an RGD motif-dependent integrin-mediated entry pathway [78]. The RGD motif is an arginine-glycine-aspartic protein sequence within the virus capsid that recognizes

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and binds to some integrins on the cell surface of the host cell [79]. Yet, upon multiple passages in BHK-21 cells, FMDV variants emerged that acquired the ability to infect several initially non-permissive human and animal cell lines via an alternative entry pathway. Analysis of these variants revealed that adaptation of FMDV in cell culture led to an enhanced affinity for heparan sulfate as a receptor, independent of the RGD motif. Interestingly, these host-range variants were able to maintain infectivity in cell culture not only independent of an RGD motif, but also without the requirement to bind to heparin sulfate [80,81]. This implies the use of alternative receptors. Selection of the FMDV host-range mutants was associated with amino acid substitutions in or near the capsid RGD motif [82–84]. Recently it was demonstrated that passaging of FMDV in BHK-21 cells led to an expansion of the host-cell tropism to non-human primate and human cell lines. Selection of these host-range variants was also associated with amino acid substitutions in the viral capsid proteins [85].

## DNA viruses

There is less data on mutation frequencies and adaptation of DNA viruses in cell cultures compared to RNA viruses. However, there are a number of illustrative examples. Adaptation of SV40 and polyomavirus to different cellular environments resulted in the emergence of host-range mutants in cell cultures (reviewed in [86]). Random mutagenesis of human adenovirus followed by repeated passaging in certain cell lines allowed isolation of adenovirus host-range mutants [87,88]. Human adenovirus 2 (HAdV2) mutants with altered specificity resulting from reduced binding affinity of the adenovirus penton-base protein for the integrins on the cells were selected in persistently infected cell lines [89].

More recently, upon passaging in cell culture, adaptation led to the emergence of herpesvirus host-range mutants. These viruses use alternative receptors and replicate in different cell types in the natural host, and in cells from different species that were previously non-permissive. Several glycoproteins are essential for the entry of alphaherpesviruses such as pseudorabies virus (PrV), herpes simplex virus (HSV) and bovine herpesvirus 1 (BHV-1) (reviewed in [90]). Interaction between, e.g., the viral glycoprotein C (gC) and heparan sulfate mediates primary attachment. For infection, however, a secondary interaction between glycoprotein D (gD) and one of several entry receptors is required. Single amino acid substitutions in the gD glycoprotein of herpes simplex 1 (HSV-1) as well as complete ablation of this glycoprotein in the swine pseudorabies virus (PrV) led to a gD glycoprotein-independent entry mode using alternate receptors [91–93]: at least three classes of cell-surface proteins are now thought to be involved in alphaherpesvirus entry [94]. Also, cell culture adaptation of human cytomegalovirus (HCMV), a betaherpesvirus, resulted in the selection of phenotypic variants that had lost their endothelial tropism [95].

# Identification of risk factors in laboratory practice

## Adaptation through variability

In cell culture viruses may readily adapt by mutation, selection and competition. These processes are stochastic in nature. Adaptation is therefore an unpredictable process, strongly influenced by the experimental setting, e.g. the multiplicity of infection used, the number of passages employed, and the type of selection employed. One should be aware of the potential adaptation when working with virus-infected cell cultures.

## The advent and contribution of recombinant DNA technology and genetic modification

Recombinant DNA technology, including 'reverse genetics' and the availability of complete (infectious) clones for a large number of RNA and DNA viruses, allows genetic modification of viral genomes and generation of recombinant [90,90] viruses *in vitro*. These technologies offer the possibility to deliberately change the tropism or host range of the viruses. Some of the latest technologies are discussed in the context of two important virus groups, i.e. influenza A viruses and coronaviruses.

### Influenza A viruses

The advent of reverse genetics systems now allows the generation of recombinant infectious influenza A viruses entirely from cloned cDNAs in cell culture. These systems are based on transfection of at least eight plasmids, each containing a copy of one of the eight influenza A virus genomic segments [96,97]. The technology permits the generation of custom-made recombinant influenza A viruses (reassortants) containing specific (heterologous) gene segments of interest and offers the possibility to study their biological properties in cellular and animal model systems. In addition, reverse genetics can be used in the development of vaccine strategies.

The use of reverse genetics allows the deliberate introduction of specific mutations in viral genes allowing selective evaluation of the contribution of individual genes or segments to, e.g., the virus's virulence/pathogenicity, transmissibility and host range. This approach has already been shown to be pivotal for the generation and characterization of reassortants containing heterologous influenza A segments from, e.g., highly pathogenic avian influenza (HPAI) H5N1 strains [98] or the 1918 pandemic strain [99,100]. In addition, this technology allowed the generation from cloned segments of the HPAI H5N1 strain that caused the deadly 1997 Hong Kong outbreak [101].

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Reverse genetics systems can also be used as an alternative for the production of both live attenuated and inactivated vaccines in preparing for pandemic influenza A virus threats. Conventional annual (inactivated) vaccine production is based on simultaneous infection of chicken eggs with two different influenza A strains followed by selection of the desired vaccine virus. This reassorted virus then contains the NA and HA segments of the relevant circulating influenza A virus against the background of six complementary segments derived from an attenuated reference strain (e.g. A/Puerto Rico/8/34 H1N1), which is safe for humans [102]. To overcome the difficulties of selecting such reassortants and subsequent laborious time-consuming passaging of these viruses, plasmid-based reverse genetics can be used for fast and directed generation of vaccine strains [103]. For vaccines based on HPAI viruses the use of reverse genetics has another important advantage. Such highly pathogenic viruses are lethal to chicken embryos and cannot be grown in large quantities in this way. The virus's high pathogenicity is associated with the presence of basic amino acids adjacent to the cleavage site within the HA molecule [104]. By using recombinant DNA technology this sequence can be eliminated. Plasmid-based reverse genetics can then be used to generate the desired vaccine strain containing the attenuated HA molecule [105,106].

In conclusion, plasmid-based reverse genetics enables the generation of defined reassorted influenza A viruses consisting of, e.g., human and avian viral gene segments of interest. However, it is usually not possible to predict the biological properties from the gene constellation of such variant viruses. Such activities therefore pose potential risks, in particular when the gene constellation is not based on characterized isolates.

### Coronaviruses

Recombinant DNA technology has allowed the construction of infectious cDNA clones of large RNA viruses such as coronaviruses, including the SARS-associated coronavirus (SARS-CoV) [107–109]. These reverse genetics systems can now be used as tools for the production of defined genetically modified coronaviruses [110]. This allows the introduction of specific mutations into the genome of coronaviruses, and, e.g., the exchange of specific viral genes between different coronaviruses to study their pathogenesis, replication strategy, and cross-species transmissibility. The possibility to engineer tissue and host tropism using these technologies makes coronaviruses potential vectors for vaccine development and possibly for gene therapy [111–113].

Host-range specificity of coronaviruses is primarily determined at the virus entry level. Several studies have demonstrated that sequence changes in the gene encoding the coronavirus surface Spike (S) glycoprotein can lead to a change of tropism and host-range specificity [73]. This is illustrated by the generation of a chimeric coronavirus by targeted recombination, in which the ectodomain of the S glycoprotein of mouse hepatitis virus (MHV) was replaced by the ectodomain of the S glycoprotein of feline infectious peritonitis virus (FIPV). This substitution conferred specific tropism for feline cells, while the ability to infect murine cells was lost [114]. Vice versa, a reverse genetics strategy for FIPV was developed conferring the ability to infect murine cells [115]. Similar techniques may aid the studies on the pathogenicity of the SARS-CoV.

The examples mentioned above indicate that the use of recombinant DNA technology now provides for powerful systems to generate and modify, e.g., highly pathogenic viruses such as pandemic influenza A viruses and SARS-CoV.

## Identification of risk factors for therapeutic and experimental settings

### Vaccine production

Live attenuated virus vaccines are among the most successful viral vaccines known to date. Traditionally, attenuation is achieved by the 'Jennerian approach', i.e. serial passaging in cell culture [116]. By this method a number of useful vaccines currently in use have been generated. Still, the mechanism by which the attenuated phenotype evolves is largely unknown. For instance, the nature and degree of genetic variation present at different stages of the attenuation process is usually not known. The presumed mechanism of attenuation is based on host-range restriction due to accumulation of changes in surface (glyco)proteins [117]. Thus selection of variants seems inherent to the process of generating the desired level of attenuation and genetic stability to prevent reversion to the wild-type virus Therefore, dependent on the passage history, diversity within the virus population is likely to represent adaptations to growth in cell culture. As a result, genetic variants with different host-range phenotypes may be present in the vaccine strain of the virus. Examination of substrains of live attenuated vaccine lots based on the yellow virus 17D strain and measles virus Edmonston strain demonstrate that these virus stocks indeed consist of a heterogeneous population of variants [118–121]. A number of adverse consequences of the use of such virus stocks have been reported and associated with possible selective growth advantage of host-range variants in the recipient [122,123]. Therefore, caution should be taken before releasing live attenuated viral vaccines based on non-human animal viruses.

### Development of vector viruses for gene therapy

Significant progress has been made in approaches to genetically modify the tropism of vector viruses. Such strategies have been used in the development of cancer gene therapy. Initially, replication-deficient vectors were used for this purpose. However, to improve efficacy, tumor-targeted replication-competent viruses have been

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developed for the use of viral therapy of cancer (virotherapy) [124,125]. Here we discuss some of the developments with human adenovirus type 5 (HAdV5).

HAdV5 has been widely used for a number of vector applications [126]. However, the use of genetically modified adenoviral vectors has some limitations [127]. Their efficacy relies on the presence of its receptor, CAR (coxackievirus and adenovirus receptor for HAdV5), on target cells. Primary binding of the virus to CAR is mediated by the knob domain of the adenoviral fiber protein. Subsequent internalization is mediated by the interaction between the RGD motif in the penton base of the virus and secondary host-cell integrin receptor molecules [128,129]. To achieve cell-type specificity and a high efficacy in the absence of the CAR receptor different strategies have been developed. These include redirecting adenoviral binding to alternative cellular receptors by genetic modification of genes coding for the capsid proteins fiber, hexon and penton base [130]. This may result in either an expanded tropism or in abolishment of the adenoviral native tropism. Table 2 summarizes a number of properties contributing to the relative risk for the use of vector viruses. For this purpose a numerical hazard score was assigned to each property. Table 3 summarizes a number of adenoviral vectors with altered properties and their relative risks. Such modified viruses are now being evaluated in a clinical setting for experimental gene therapy. Table 3 illustrates that a change of cell tropism, tissue tropism, or host range of a viral vector should be considered as factors in risk assessment for activities with genetically altered vector viruses. In general, the use of replication-competent viral vectors poses special concerns with regard to unintended spread to new and undesired cell types, as well as horizontal transmission of the vector [131,132]. A replication-competent vector virus with an altered tropism or host range virtually constitutes a new viral pathogen with the potential of a new disease manifestation.

**Table 2. Assignment of arbitrary hazard scores to properties contributing to the relative risk of vector viruses**

| Property | Hazard score |
|---|---|
| Replication-competent | +2 |
| Presence of transgene | +1 |
| Presence of host-range expanding modification | +1 |
| Replication defective | −1 |
| Presence of host-range restricting modification | −1 |

# Discussion

In nature many factors may contribute to the emergence of a new zoonotic viral disease. These factors consist of viral evolutionary processes such as mutation, natural selection and competition, host determinants, e.g., immune status and physiological factors, and environmental determinants such as ecological and climatological circumstances. As highlighted by the emergence of new viral diseases in the last two decades, the process of adaptation often involves the acquisition of an altered cell tropism or host range. Against this background the intensified use of viruses and their genetically modified variants as viral gene transfer vectors for biomedical research, experimental gene therapy and for live-vector vaccines is a cause for concern.

This review highlights the importance of identifying and evaluating the risks and consequences of activities that may generate host-range mutants with the capacity of cross-species transmission. The use of such viruses may lead to inadvertent introduction of vector viruses with a changed cell or tissue tropism and/or host range through an immunologically naïve and non-adapted hosts. Interactions between the virus and the cellular receptor often determine the host range of the virus and therefore constitute a species barrier [133]. Minor mutations in the viral capsid or surface glycoproteins may already result in profound changes in cell tropism or host range of a virus. In this review we have therefore focused on the level of virus entry to address some implications for activities with viral vectors, and in particular with host-range mutants. This could contribute to a rational and reasoned inventory of factors that should be considered in risk assessments of activities with viral vectors.

In considering possible risks involved in handling replication-competent vector viruses in the laboratory,

**Table 3. Properties contributing to the relative risk of adenoviral vectors compared to wild-type human adenovirus type 5 (HAdV5)**

| Properties | Example | Reference | Relative risk |
|---|---|---|---|
| Rep. comp. wt* | HAdV-5 | [138] | ++ |
| Rep. def. | HAdV-5 *dl*312 | [139] | + |
| Rep. def. + transgene | HAdV-5 HSVtk | [140] | ++ |
| Rep. def. + host-range restriction + transgene | HAdV-5-HSVtk-CAR ablated | [141] | + |
| Rep. def. + host-range expansion + transgene | HAdV-5-HSVtk-RGD | [142] | +++ |
| Rep. comp. + host-range restriction | ONYX-015 | [143] | + |
| Rep. comp. + host-range restriction + transgene | ONYX-tk | [144] | ++ |
| Rep. comp. + host-range expansion | CRAd-RGD | [145] | +++ |
| Rep. comp. + host-range expansion + transgene | CRAd-HSVtk-RGD | [145] | ++++ |

*point of reference.
Abbreviations: rep. comp., replication-competent; rep. def., replication-deficient; wt, wild-type; HAdV, human adenovirus; *dl*312, HAdV-E1A-deleted; HSVtk, thymidine kinase herpes simplex virus; CAR, coxsackievirus-adenovirus receptor; RGD, arg-gly-asp tripeptide motif; CRAd, conditionally replicating adenovirus; ONYX-015, HAdV-E1B-55kD-deleted CRAd.

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awareness for phenotypic selection of viruses in cell culture should be raised. Possible risks involved must be mitigated by adequate biosafety measures. Many viruses have the capability to use various receptors [134]. This implies that concepts such as host-range barrier and host-cell specificity may be rather flexible than rigid. If the host range and completion of the viral life cycle is exclusively restricted to the level of entry, forced entry may bypass important discriminatory host-cell restriction steps. This may result in distinct pathological phenotypes and new disease manifestations. Therefore, precaution should be taken to avoid inadvertent release and spread of such potential harmful vector viruses.

It goes without saying that live-virus vaccines are among the most effective modalities to control viral pathogens. It is evident that for such vaccines the benefit is higher than the possible adverse effects. Nevertheless, the risks associated with handling vaccine viruses, viral vectors, and exotic viruses are small but finite. We are just beginning to understand the mechanisms that drive the emergence of new viruses and viral diseases in nature. There are parallels between the patterns that are seen in emerging viral diseases and certain virus modifications that are generated, either deliberately or inadvertently, when handling viruses in the laboratory. Such patterns and the associated risks should be noted. The capacity of a virus to replicate and spread implies that in the risk evaluation, not only the risks to the laboratory personnel or the patient, but also the effects on the environment (including the human population) should be weighed against the evident benefits. Only then can viruses maintain and extend their prominent role as widely applicable entities for clinical and veterinarian use.

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