Poxviruses
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Genus *Avipoxvirus*

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Abstract

Poxviruses identified in skin lesions of domestic, pet or wild birds are assigned largely by default to the *Avipoxvirus* genus within the subfamily *Chordopoxvirinae* of the family *Poxviridae*. Avipoxviruses have been identified as the causative agent of disease in at least 232 species in 23 orders of birds. Vaccines based upon attenuated avipoxvirus strains provide good disease control in production poultry, although with the large and intensive production systems there are suggestions and real risks of emergence of strains against which current vaccines might be ineffective. Sequence analysis of the whole genome has revealed overall genome structure and function resemblance to the *Chordopoxvirinae*; however, avipoxvirus genomes exhibit large-scale genomic rearrangements with more extensive gene families and novel host range gene in comparison with the other *Chordopoxvirinae*. Phylogenetic analysis places the avipoxviruses externally to the Chorodopoxvirinae to such an extent that in the future it might be appropriate to consider the *Avipoxvirus*es as a separate subfamily within the *Poxviridae*. A unique relationship exists between Fowlpox virus (FWPV) and reticuloendothelosis viruses. All FWPV strains carry a remnant long terminal repeat, while field strains carry a near full-length provirus integrated at the same location in the FWPV genome. With the development of techniques to construct poxviruses expressing foreign vaccine antigens, the avipoxviruses have gone from neglected obscurity to important vaccine vectors in the past 20 years. The seminal observation of their utility for delivery of vaccine antigens to non-avian species has driven much of the interest in this group of viruses. In the veterinary area, several recombinant avipoxviruses are commercially licensed vaccines. The most successful have been those expressing glycoprotein antigens of enveloped viruses, e.g. avian influenza, Newcastle diseases and West Nile viruses. Several recombinants have undergone extensive human clinical trials as experimental vaccines against HIV/AIDS and malaria or as treatment regimens in cancer patients. The safety profile of avipoxvirus recombinants for use as veterinary and human vaccines or therapeutics is now well established.

Introduction

Poxviruses identified in skin lesions of domestic, pet or wild birds are assigned largely by default to the *Avipoxvirus* genus within the subfamily *Chordopoxvirinae* of the family *Poxviridae* [1]. Disease is characterised by proliferative lesions of the skin ranging from small nodules to tumour or

wart-like masses and rarely with proliferative or diphtheric membranes on mucous membranes of the respiratory tract, mouth and oesophagus. *Avipoxvirus*es have usually been assigned species names on the basis of the bird species from which the virus was isolated or at least described by light or electron microscopy of lesions. Our understanding of the relationships of these avipoxvirus species to each other and to the type species – Fowlpox virus (FWPV) – is rudimentary since detailed genomic information is currently available for two FWPV isolates [2, 3] and one Canarypox virus (CNPV) isolate [4]. The detailed study of FWPV and CNPV has been driven largely by their use as vaccine vectors for poultry and human vaccines [5].

Avipoxviruses have been identified as the causative agent of disease in a wide range of avian species – a review of available literature reveals natural infections described in at least 232 species in 23 orders of birds [6]. In some instances these infections have been of considerable concern as a threat to endangered species or species in captive breeding recovery programs. Disease caused by FWPV in domestic poultry, while not one of the major diseases of concern to commercial poultry production, can cause significant problems from time to time when conditions are favourable for transmission, predominantly mechanical transmission by mosquitoes. Attenuated strains of FWPV and other avipoxviruses are successfully and widely used to vaccinate susceptible species for disease control.

A detailed understanding of the molecular virology and relationships of avipoxviruses is largely restricted to FWPV and CNPV for which full-genome sequences are available [2–4]. These studies have revealed characteristic poxvirus morphology with a large double-stranded DNA genome (266–288 kbp for FWPV, 365 kbp for CNPV), cytoplasmic replication with gene expression regulatory elements in common with the *Chordopoxvirinae* and a genome that encodes in excess of 250 putative genes. The genomes of avipoxviruses exhibit large-scale genomic rearrangements, more extensive gene families and novel host range genes in comparison with other members of the *Chordopoxvirinae*. A unique relationship exists between FWPV and the avian retrovirus, reticuloendotheliosis virus (REV). All FWPV vaccine strains carry a remnant long terminal repeat (LTR), while field strains carry a near full-length provirus integrated at the same location [7]. The provirus gives rise to REV infection when the FWPV infects susceptible poultry. REV sequences have not been detected in other avipoxviruses.

Perhaps the greatest interest in avipoxviruses has been in their use as vaccine vectors, firstly to deliver vaccine antigens to poultry and secondly as vaccine vectors for non-avian species [5]. Many of the techniques developed for the construction of orthopoxvirus recombinants were readily adapted to the construction of avipoxvirus recombinants with appropriate changes to the cell substrate and selection protocols. Approaching a billion doses of recombinant FWPV (rFWPV)-avian influenza H5 vaccine have been used in the control of avian influenza in Mexico [8, 9]. The observation that FWPV and CNPV infect a wide range of mammalian cells without productive repli-

cation, while gene expression occurs at a level sufficient to induce antibody, cellular and protective immune responses to the recombinant antigen, is the driver for substantial interest in the use of avipoxviruses as vaccine vectors in non-avian species, including man [10]. The utility of the avipoxvirus vaccine vectors has been extended in prime-boost vaccination regimens [11] and for the delivery of cancer and immune-stimulatory/modulatory molecules for treatment regimens [12]. A number of avipoxvirus recombinants have undergone extensive preclinical and clinical trials, including vaccine candidates for the control of HIV/AIDS and malaria [13, 14]. The safety profile of such recombinants is now well established [15, 16].

Taxonomy and origins

The avipoxviruses are classified in the family *Poxviridae* subfamily *Chordopoxvirinae* genus *Avipoxvirus* [17]. The type species is the well known and characterised FWPV; many different isolates of FWPV have been described, including a wide range of commercial vaccines available globally. There are now ten official species accepted with several more tentative species in the genus (Tab. 1) [1]. Species demarcation is poorly defined but criteria include disease characteristics, origin host, growth characteristics in the chorioallantoic membrane of embryonated eggs or avian cell cultures, cross-protection in chickens against classical FWPV and restriction endonuclease analysis of genomic DNA and cross-hybridisation. Given the size and complexity of the avipoxviruses at the genome and virion level, our understanding of the extent and nature of relationships of the official and tentative species is at best rudimentary. Nucleotide sequence determination of selected conserved genes might provide a means of presumptive classification, and has been attempted using the 4b gene [18, 19]. However, relationships defined on this basis will fail to take into account variations in gene content arising from gene loss and gain during evolution [20, 21]. Avipoxviruses may well be the largest and most diverse genus in the *Chordopoxvirinae* [6]. Although the origin of birds is controversial they have probably been around in some form for at least 150 million years. The avipoxviruses have almost certainly co-evolved with their hosts and in the process acquired genes from the host that have assisted the virus to counter the host cellular and immune responses [20, 21]. Avipoxviruses that are accepted as species tend to be those which have been successfully cultivated in the laboratory.

Disease in production poultry

FWP in production chickens and turkeys tends to be slow-spreading with characteristic skin lesions ranging from small nodules to tumour or wart-

Table 1. Family *Poxviridae*, subfamily *Chordopoxvirinae*, genus *Avipoxvirus*

Genus *Avipoxvirus*	Type species	Other species	Assigned abbreviations	Full genome sequence: strain and accession no.
	Fowlpox virus		FWPV	FP9 – AJ581527[a] FPV – AF198100[b]
		Canarypox virus	CNPV	CNPV – AY318871[c]
		Juncopox virus	JNPV	
		Mynahpox virus	MYPV	
		Pigeonpox virus	PGPV	
		Psittacinepox virus	PSPV	
		Quailpox virus	QUPV	
		Sparrowpox virus	SRPV	
		Starlingpox virus	SLPV	
		Turkey pox	TKPV	
Tentative species		Crowpox virus	CRPV	
		Peacockpox virus	PKPV	
		Penguinpox virus	PEPV	

[a]Fowlpox virus FP9: plaque-purified, tissue culture-adapted, attenuated European virus [3].
[b]Fowlpox challenge virus; Animal Health Inspection Service Centre for Veterinary Biologicals, Ames Iowa [2].
[c]CNPV strain Wheatley; American Type Culture Collection (ATCC VR-111) [4].

like masses, which occur predominantly on unfeathered skin areas, e.g. the head and legs [22]. Mortality is low; however, a substantial transient drop in egg production can occur in laying birds. In young birds, growth rates can be significantly affected [23, 24]. The role that simultaneous REV infection arising from the provirus integrated into the FWPV genome might play in the expression of FWP is unknown. However, the potential effects of REV infection cannot be discounted since REV infection is known to lead to immunosuppression [25]. A severe diphtheritic form of FWP with proliferative lesions in the nasal, laryngeal and tracheal regions results in respiratory distress and higher mortality [23].

Disease control – vaccination and transmission control

Control of diseases caused by the avipoxviruses is best achieved by the prevention of transmission and by vaccination [23, 24]. There are no suitable or specific treatments available once infection is established. Transmission by biting insects, particularly mosquitoes, can be linked to seasonal and

geographic incidence of disease, so appropriate screening and insect control programs for commercial poultry production can reduce the impacts of disease. Infection *via* cutaneous injuries or inhalation can be reduced by control of crowding and decontamination of premises following outbreaks. FWPV can survive in dried scabs for extended periods (weeks if not months), so attention to sanitation of housing, feed and water is essential for effective disease prevention. FWP is distributed worldwide in domestic poultry with the incidence variable in different geographic regions – related to management and hygiene practices, mosquito control and the use of prophylactic vaccination.

Prophylactic vaccination for the control of FWP in commercial poultry has been practiced for a considerable period of time [22]. The early history of vaccines for FWP, pigeonpox (PGP), turkeypox (TKP) and CNP was well documented by Beaudette in 1949 [22]; this reference provides fascinating insights into early attempts to vaccinate against avipox and to understand the relationships amongst the avipoxviruses. Since the late 1960s and early 1970s, modern commercial vaccines have been available in most regions of the world for the control of FWP. Other vaccines, e.g. against TKP, quailpox (QUP), CNP and PGP, are available in specific regions [23]. The virus strains used in the vaccines have been derived empirically by the passage of field isolates in embryonated eggs or chicken embryo-derived cell cultures. Selected on the basis of immunogenicity (protection against challenge) and attenuation (reduced pathogenicity) in comparison with the original field strain and on the basis of freedom from other avian pathogens, the vaccines have found widespread use for the control of avipox disease. The origins and history of many named vaccine strains are obscure because of commercial consideration or the loss of information with the passage of time [22].

Avipox vaccines are most effectively applied by wing web inoculation using single or two pronged needle inoculators. This makes the vaccines expensive to administer in commercial poultry as each bird needs to be handled. Vaccine take can be assessed by inspection of the inoculation site for the development of a characteristic pox lesion 5–10 days after vaccination. Administration by other routes, e.g. drinking water or spray is far less effective in the induction of protective immunity with much higher virus concentrations needed to achieve an acceptable level of protection [22, 26, 27]. *In ovo* vaccination at close to hatch date with highly attenuated or rFWPV strains may be a viable alternative to individual chick vaccination [28, 29]. Revaccination may be necessary to sustain protection in chickens used for egg production or subject to heavy challenge because of high mosquito populations. FWPV vaccine strains range from highly attenuated suitable for vaccination of 1-day-old chicks to others that have residual pathogenicity and are recommended for first use at 3–6 weeks of age or for revaccination immediately prior to commencement of lay.

FWPV vaccines provide little if any effective protection against TKPV, QUPV and CNPV, consequently it is essential to use the appropriate vac-

cine for disease control in these species [22, 23]. The poor cross-protection is probably related to the antigenic differences that exist between these viruses. Vaccination is sometimes practiced in the circumstance where a small proportion of birds are showing disease in an endeavour to limit further spread within a flock.

Humoral and cell-mediated immune responses play a role in immunity, although their relative contributions have not been thoroughly investigated [30, 31]. Humoral responses can be assessed by ELISA or virus neutralisation [32]. The utility of commercial poultry vaccines for the control of poxvirus diseases in other avian species is questionable, although on occasions their use has been attempted. Such uses are not without risks as the vaccines themselves should always be considered as having the potential to cause disease in the vaccinated species.

Many of the commercial vaccine strains of FWPV and CNPV have been used as the basis for the development of recombinant poxvirus vector-based vaccines for control of other avian diseases or for use in non-avian species [5]. FWPV vaccines were associated with the inadvertent spread of REV because of apparent vaccine contamination. Modern poultry vaccines rarely have the risks of spread of adventitious agents as the quality assurance processes for their production are well established [24]. Nonetheless, the association of FWPV with REV has turned out to be a unique relationship with the REV provirus integrated in the FWPV genome [7]. The availability of full-genome sequences for one FWPV strain and one CNPV opens the possibility of rational attenuation of viruses for the development of new avipox vaccines or to enhance specific immunogenicity characters where the avipoxviruses are used for the delivery of antigens and/or immunomodulators to avian and non-avian hosts [5]. We have recently removed the integrated REV provirus from FWPV vaccine and field strains to generate new vaccine strains (D. Boyle, unpublished).

Emergence of new strains

The scale and intensity of global production of commercial poultry has led to the emergence of new diseases principally caused by viruses and the emergence of variants of existing viruses. With this have come the pressures to develop new vaccines or to select new strains for inclusion in vaccines. Most of the challenges have come from the RNA genome viruses [e.g. infectious bronchitis virus, infectious bursal disease virus (IBDV) and avian influenza virus]; however, Marek's disease virus (MDV) has undergone substantial variation with many older vaccines providing poor or limited protection against the emerging MDV strains [33]. There are some reports of emerging FWPV strains causing unusual disease patterns or disease against which current vaccine may be ineffective [34–36]. The potential for emergence of new FWPV strains or the spread of other avipoxviruses into commercial

poultry production systems is real; however, the timing and consequence of emergence of such viruses is not predictable.

Disease in wild birds and in threatened avian species

Avipoxviruses have been identified as the causative agent of disease in a wide range of avian species (for review see [6]). Mortalities approaching 80–100% on occasions have been reported in pigeons, quails and canaries with highly pathogenic strains [22–24]. Natural disease in wild and caged birds ranges from mild cutaneous lesion on feet and other unfeathered areas to severe disease and high mortality associated with cutaneous and diphtheritic disease. Poxvirus infections along with avian malaria are considered to be important factors in limiting and threatening endangered and unique populations of birds on the Hawaiian, Galapagos and Canary Islands [37, 38]. In other circumstances the introduction of poxvirus infections into captive breeding programs for endangered bird species has been of significant concern [39].

Poxvirus infection is generally diagnosed on clinical signs, histopathology, e.g. characteristic cytoplasmic inclusion bodies in infected cells, and electron microscopic detection of virus in lesions [24]. Where viruses have been isolated by inoculation of embryonated eggs or avian cell cultures, further studies have been possible [23, 24]. These studies usually involve cross-protection and pathogenesis studies in chickens in comparison with FWPV [37], restriction endonuclease analysis of genomic DNA [37, 39–42] or sequencing of PCR amplified genome regions [18, 19]. Complex patterns of relationships to FWPV are revealed with the avipoxviruses that are rarely pathogenic in chickens, providing poor cross protection against FWPV challenge and with significant differences in the restriction endonuclease profiles of the genomes [23]. The sources of poxvirus infections in such a wide range of avian species can only be speculated upon; virus infection could be enzootic and only manifests as disease under stress or other environmental factors; virus and disease might spill over from related species or from domestic poultry; and there is the possibility of extended persistence of virus in cutaneous warty lesions in some avian species [43]. Spread of poxvirus from one avian species to another because of habitat disruption or insect transmission has the potential to cause severe disease in the newly infected species. The full nature and complexity of the relationships of these avipoxviruses will only be revealed with detailed genome sequence analysis of a greater range of isolates.

Whole genome sequences

Genome sequences with analysis of putative gene functions and relationships are now available for a pathogenic FWPV US (FWP challenge virus;

Animal Health Inspection Centre for Veterinary Biologics, Ames, Iowa), a plaque-purified, tissue culture-adapted, attenuated European virus FWPV (FP9) and a CNPV virulent strain (Wheatley C93, American Type Culture Collection VR-111) [2–4]. Additionally, genome wide differences between the FWPV US and FP9 have been characterised in the HP1 strain, which is the progenitor virulent FWPV of FP9 [3]. FWPV genome sizes ranged from 266 kbp for FP9 to 288 kbp for the pathogenic FWP challenge strain. The CNPV genome (365 kbp) is 80–100 kbp larger than the FWPV genomes. Given the size and complexity of the genomes, it is not proposed to review individual genes and their relationships in detail. Readers are best referred to the original manuscripts for this level of analysis.

In the case of FWPV US, there is a resemblance to *Chordopoxvirinae* in overall genome structure and function with a centralised conserved core of genes whose functions are involved in the basic replicative mechanisms such as viral transcription and RNA modification, genome replication and the structure and assembly of mature virions; there are 65 conserved gene homologues involved in these functions [2]. The FWPV US genome contains inverted terminal repeats approaching 10 kbp in length. Gene expression regulatory elements, e.g. early, intermediate and late promoters, contain typical *Chordopoxvirinae* sequences. The early poxvirus transcription termination sequence (T5NT) is identifiable near the translational stop codon of many predicted early genes. However, there are marked differences in that genome co-linearity in comparison with the *Chordopoxvirinae* is disrupted in FWPV US by translocations and inversions, multiple and large gene families and novel cellular homologues. Much of the marked size difference between FWPV US and other *Chordopoxvirinae* is accounted for by the large numbers of cellular homologues and 10 multi-gene families. Most notably, in FWPV US, the ankyrin repeat family (31 genes), N1R/p28 family (10 genes) and the B22R family (6 genes) represent ~32% of the total genome [2]. There are a large number of putative cellular homologue genes involved in immune evasion, apoptosis, cell growth and tissue tropism. Other cellular homologues are involved in steroid biogenesis, antioxidant functions and vesicle trafficking. All of these suggest that there is a substantial modification of host cell function occurring upon virus infection. There is also a suggested photo-reactivation DNA repair pathway encoded by FWPV US. Gene acquisition by horizontal transfer appears to have played a significant role in the adaptation of FWPV US to its avian host [2].

A comparison of the attenuated, tissue culture-adapted European FP9 strain with FWPV US identified just 118 differences; 71 genes were affected by deletion (26 of 1–9334 bp), insertion (15 of 1–108 bp), substitution, termination or frame-shift [3]. FWPV FP9 is a derivative of a virulent European FWPV HP1 by passage in embryonated eggs and chicken embryo fibroblast cell culture (over 400 passages). Sequence determination of the HP1 at loci where differences exist between the FP9 and FWPV US showed that 68 of 118 loci differed from the FWPV US but were identical to FP9. More

than half of the differences between the two geographic FWPV lineages represented differences between the parent virulent viruses – HP1 and FWPV US. Thus, more than half of the differences between FWPV US and FP9 represent differences between different viruses of the two geographic origins. A comparison of the attenuated European FP9 with its virulent parent FWPV HP1 showed that 50 of 118 loci were different – representing changes/mutations accumulated during the egg and tissue culture passage for attenuation. Twelve of the 46 open reading frames affected by the apparent passage-specific mutations encoded members of the ankyrin repeat family. The mechanisms by which such mutations lead to attenuation are as yet unclear [3].

Restriction endonuclease enzyme profiles of avipoxvirus genomes show significant variation in genome arrangements, suggesting the potential for marked differences in genome content [37, 40, 41]. Limited gene sequence data from CNPV Tokyo CG-2 strain indicated that, while CNPV and FWPV appeared to share regions of similar gene order, there are marked differences at the deduced amino acid level. Gene homologies between CNPV and FWPV ranged from 55% to 74%, a divergence that is comparable to that seen between the different *Chordopoxvirinae* genera [44]. The substantial differences between CNPV and FWPV were confirmed by full sequencing of the CNPV genome [4]. The CNPV is markedly larger than FWPV, with 365 kbp *versus* 266–288 kbp. Central regions of the CNPV genome contain homologues of the *Chordopoxvirinae* genes involved in the basic replicative mechanisms such as viral transcription, RNA modification, viral DNA replication, structure and assembly of virions. It has been shown that there are 106 conserved *Chordopoxvirinae* genes shared between CNPV and FWPV with an average of 70% amino acid identity. CNPV genome encodes 39 genes, the homologues for which are absent from the FWPV genome or fragmented, while CNPV lacks 15 genes present in the terminal genome regions of FWPV. Internal genome regions exhibit considerable variability between CNPV and FWPV in contrast with the relative overall conservation of central regions of genomes in other *Chordopoxvirinae*. Major genome variability is located near the junctions of genome rearrangements relative to the other *Chordopoxvirinae*. These regions contain genes that appear to be involved in virus-host interactions. The CNPV Wheatley C93 strain has a close relationship at the nucleotide (98%) and amino acid (91% to 100%) level to the CNPV Tokyo CG-2 strain in those regions for which there is comparable sequence available [4, 44]. This provides a level of confirmation of genome conservation for different isolates causing CNP and supports the designation of CNPV at the species level in the *Avipoxvirus* genus. Tulman et al. [4] concluded that "the genomic data and phylogenic analysis of individual open reading frames support a monophyletic origin of the two avipoxviruses relative to the other *Chordopoxvirinae*." The divergence between FWPV and CNPV appears to be as great as that between other *Chordopoxviridae* genera. The apparent divergence established by

restriction endonuclease analysis of avipoxvirus genomes [37, 39–42] and nucleotide sequence of the 4b gene [18, 19] supports this conclusion, and suggests that there is potentially substantial and far ranging genomic diversity amongst the viruses that have been reported as causing disease in at least 232 species in 23 orders of birds. With substantial sequence data from a range of avipoxviruses, our understanding of the *Avipoxvirus* genus may be such that they constitute a separate subfamily within the *Poxviridae*.

Relationships to poxviruses in general

Complete genome sequences for 20 poxviruses have allowed genome wide analysis of phylogeny, genome structure and evolutionary pathways [20, 21]. Gene order and gene spacing are highly conserved within the *Chordopoxvirinae* with the exception of FWPV and CNPV [2, 4]. Phylogenetic analyses placed FWPV externally to the *Chordopoxvirinae* (and presumably CNPV, although a similar analysis has not yet been reported). Notwithstanding the conservation of overall genome composition and structure, including a central core of genes, inverted terminal repeats and a large numbers of functionally important orthologs, the FWPV and CNPV genomes exhibit large-scale genomic rearrangements with more extensive gene families and novel host range genes in comparison with the other *Chordopoxvirinae* [2, 3]. Gene loss and gain appears to be the dominant mechanism in the evolution of *Chordopoxvirinae* genomes. Many genes have been acquired by horizontal gene transfer from the host. FWPV has gained 94 genes since divergence from a common ancestor of the *Chordopoxvirinae* [20]. Of 34 gene families shared by *Chordopoxvirinae* with animal genomes, 8 are found only in FWPV [21]. Acquisition by horizontal gene transfer has been an important and perhaps dominant source of new genes for avipoxviruses in their evolution. Many of the acquired genes enable the virus to escape host cellular and immunological defences. It is perhaps an important challenge in poxvirus biology to understand the rate and mechanisms of gene transfer.

FWPV and REV

A unique relationship exists between the avian retrovirus, REV and FWPV [7]. A near full-length, infectious provirus of REV has been found in the genome of most if not all pathogenic isolates of FWPV. At least one vaccine strain, FPV-S, whose use was discontinued because it was the source of REV in poultry in Australia also carries the infectious provirus. Other FWPV vaccine strains known not to cause REV infection carry either a complete or partial LTR [7, 45–49]. The presence of full or partial LTRs in FWPV vaccine strains can be explained by the presence of tandem

repeated LTRs of the provirus. Such structures are inherently unstable in poxvirus genomes with the intervening sequences rapidly lost by inter- or intramolecular recombination [50, 51]. Instability of tandem repeated sequences in poxviruses is exploited to construct recombinants using transient dominant selection methods [52, 53]. Since the 5' and 3' LTRs of the integrated provirus are different, the presence or absence of a full or partial LTR upon loss of the REV provirus would be dependent upon the cross-over site during the recombination event in the LTR leading to the provirus loss [45, 48].

A number of features of the REV integration into the FWPV genome suggest an ancient and unique event [46]. Provirus and LTR sequences have only been found at a single location in the FWPV genomes examined (between FPV201 and FPV203, FPV202 being mainly encompassed by the LTR sequences) [3]. Integration site sequence duplications that normally occur during provirus integration are absent. The 5´ LTR is complete, while the 3' LTR has deletions and rearrangements when the near full-length provirus is present. Provirus or LTRs have been identified in FWPV strains isolated well before the widespread use of FWPV vaccines in commercial poultry [46]. In Australia, FWPV field isolates made in late 1940s to early 1950s all carry the REV provirus (unpublished observations). FWPV strains carrying the REV sequences appear to be globally distributed.

Re-integration or recombination of REV into vaccine strains carrying LTR remnants appears an unlikely scenario. It has been speculated that this might lead to pathogenic FWPV arising from vaccine strains in the field and perhaps explain apparent vaccine failures. Given the complexity of the FWPV genome and the undoubted role of multiple gene products in determination of virulence and pathogenicity, the re-integration of REV alone is unlikely to be sufficient to restore full virulence to FWPV vaccine strains – many of which have undergone multiple passages in culture to generate attenuation with consequent gene loss, rearrangement and disruption [3]. There is no evidence for this occurring and it is important to remember the biology of FWPV and REV. Retrovirus integration takes place in the nucleus of infected cells by a well-defined pathway. FWPV DNA replication takes place in the cytoplasm of the infected cell and is unlikely to be a readily accessible target for REV integration. Given the physical and functional separation of REV and FWPV infection cycles in cells, integration is an unlikely rare event following co-infection of cells with FWPV and REV. Attempts to generate re-integration or recombination between the vaccine strain FPV-M3 and REV by co-infection and passage in CEF cells have been unsuccessful (Boyle, unpublished). It is difficult, however, to construct selection regimens that might allow rare events to be detected. In contrast, co-infection in cell culture with MDV and REV or avian leukosis virus (ALV) leads to rapid and numerous integration events in the MDV genome – a reflection that MDV replicates in the nucleus of the infected cell where it can be a ready target for REV or ALV integration [54].

The presence of REV provirus or LTR in other avipoxviruses has been explored to some extent. LTR sequences are absent from the completed genome sequence of CNPV in which the orthologous genes flanking REV sequences in FWPV are separated by 64 bp [4]. USA FWPV commercial vaccines (12 vaccines plus a parent strain and a recombinant FWPV) and 3 PGPV vaccines contain complete or incomplete REV LTRs [47], while 1 QUPV and 2 CNPV vaccines did not contain integrated REV sequences [47]. None of the FWPV vaccine strains contained a REV provirus. In contrast, 6 of 7 field isolates of FWPV made between 1949 and 1978 appeared to contain an integrated REV provirus – the remaining isolate appeared to have only a LTR remnant [46]. In this study, a CNPV and PGPV isolated in 1968 lacked integrated REV sequences. Our observations (unpublished) on 25 avipoxviruses isolated from native avifauna of Australia and New Zealand have shown that REV is absent from these isolates. The isolates were tested by both PCR and hybridisation for LTR and gag region sequences, which showed that REV sequences were absent throughout the genome, not just the homologous site identified in FWPV. Australian avipoxvirus isolates from poultry (chickens, turkeys, geese and pigeons) carried the provirus or a LTR.

Rapid loss of the REV provirus upon passage of field isolates in embryo cell culture might be expected due to the inherent instability of the provirus structure as discussed above. This has probably occurred in existing commercial vaccine strains during passage and selection. For the maintenance of the REV provirus in field strains of FWPV, a selective advantage must be conferred on the FWPV strains carrying the REV provirus. Equally intriguing is the mechanism of recovery of REV infection in chickens infected with FWPV strains carrying the REV provirus. We were unable to detect free REV in the vaccine strain FPV-S, yet this virus when inoculated into chickens gave rise to REV infection in all chickens [7]. Other FWPV isolates are undoubtedly contaminated with free infectious REV – perhaps a reflection of the co-isolation of FWPV and REV in the chicken embryo or cell culture used for isolation [55]. The integrated near full-length REV provirus is infectious since transfection of EcoRI-digested FWPV DNA into CEF cells results in the recovery of infectious REV [7]. Expression of the REV genome from the provirus 5' LTR promoter is unlikely since the promoter would not be recognised by the poxvirus transcription machinery. Expression of REV protein has been reported; however, the co-isolation of REV and FWPV in the culture systems could be an explanation for the apparent expression of REV genes from the FWPV carrying integrated REV provirus [45, 49].

The selective advantage conferred on FWPV strains carrying the REV provirus is probably related to the immunosuppression caused by the concurrent REV infection [25], leading to a longer and more severe FWP infection, thus extending the duration of possible transmission by contact or mosquitoes. Although it has been suggested that widespread FWPV vac-

cination might provide selection pressure for the retention of REV provirus in FWPV [48], this seems unlikely since REV provirus has been detected in isolates made before the widespread use of FWVP vaccines in poultry. Upon removal of the REV provirus from two field strains and FPV-S vaccine strain of FWPV, we have not been able to identify marked differences in disease produced in chickens infected at 3–4 week of age (Boyle, unpublished).

Removal of the REV LTR has been considered desirable by some for the use of FWPV strains for poultry vaccines or as vaccine vectors [5]. In the process of constructing complex FPV-M3/HIV vaccine vectors, we have removed the remnant LTR from FPV-M3 by using this locus for the insertion of HIV vaccine antigens. We did not observe any apparent impacts upon virus replication *in vitro* [53].

The relationship between FWPV and REV leads to a mechanism by which a retrovirus is transmitted through the infection cycle of a poxvirus, including mechanical transmission by biting insects. Earlier observations of REV transmission by biting insects might better be explained by transmission *via* FWPV. With FWPV, we are perhaps observing one example of a poxvirus gaining genetic information to its advantage from another virus. Equally, REV has gained significant advantage by being transmitted by mosquitoes.

Avipoxvirus vaccine vector technology

Upon the development of techniques for the construction of recombinant vaccinia viruses [56, 57] to deliver heterologous antigens as vaccines, a great deal of interest was generated in the potential to use species-specific poxviruses, e.g. FWPV and CNPV, in a similar manner. At the time there was a paucity of knowledge regarding the molecular biology of the avipoxviruses, consequently it was not obvious that the techniques developed for vaccinia virus recombinants would be directly applicable to the construction of avipox recombinants. The first attempts to construct rFWPV and rCNPV were directed to their use as vectors for the delivery of poultry vaccines [58, 59]. The novel finding that rFWPV and rCNPV could enter non-avian cell types, undergo an abortive (non-productive) replication cycle, express the foreign encoded vaccine antigen and thus induce immune responses in mammals led to an expanded interest in the avipoxviruses as vaccine vectors [10, 60–62]. It is generally accepted that avipoxviruses can cause productive infection and thus disease only in avian species. Early studies indicated that upon intranasal inoculation of mice there was an absence of replication and limited pathology [63] and that in cell cultures cytopathology occurs without replication [64]. For those avipoxviruses examined, there appears to be a general ability to enter most non-avian cells; however, the stage at which replication is blocked appears different depending upon cell type [65, 66].

Table 2. rFWPV and rCNPV delivered vaccines and therapeutics

Poultry vaccines	Veterinary vaccines (not poultry)	Human vaccines (non-cancer)	Cancer antigens and immuno-stimulatory/ modulatory molecules
Avian influenza virus H5, H7, H9, N1, NP	Bovine respiratory syncytial virus	Cytomegalovirus glycoprotein B	Bladder cancer
Avian leukosis virus	Bovine viral diahorrea virus	Hepatitis B virus	B7-1
Coccidiosis	Canine distemper virus	Hepatitis C virus	Melanoma
Duck hepatitis B virus	Equine herpes virus 1	HIV-1, HIV-2, SIV, SHIV	
Haemorrhagic enteritis of turkeys	Feline coronavirus	Japanese encephalitis virus	P53
Infectious bronchitis virus (avian coronavirus)	Feline leukaemia virus	Malaria *Plasmodium falciparum* *Plasmodium berghei*	Prostate antigen
Infectious bursal disease virus	Rabbit haemorrhagic disease virus	Measles irus	
Marek's disease virus	Rabies virus	*Mycobacterium* BCG	
Mycoplasma gallisepticum	West Nile virus	Rabies virus	
Newcastle disease virus			
Reticuloendotheliosis virus			
Turkey rhinotracheitis virus			

Detailed reference relating to studies using avipoxviruses expressing these antigens can be obtained by suitably structured search: http://www.ncbi.nlm.nih.gov/entrez

There has been a recent unconfirmed report of FWPV productive infection of baby hamster kidney (BHK-21) cell line [67]. A consequence of the abortive replication cycle is that avipoxvirus recombinants as vaccine vectors offer significant safety advantages when used to deliver vaccine antigens to mammalian species, in comparison with replication competent poxvirus vectors, e.g. vaccinia virus. A large number of rFWPV and rCNPV have now been described designed to express vaccine antigens for delivery to mammalian hosts. Many have progressed through veterinary and human clinical trials, including vaccine candidates against HIV/AIDS and malaria [13, 68] (Tab. 2). The safety profile of such recombinants is now well established, as a significant number of such recombinants have been subjected to regulatory

required toxicology and safety trials in animals and man without reports of significant adverse events [69–71].

Construction technologies

A substantial understanding of the molecular biology of the orthopoxviruses was essential to the construction of the first vaccinia virus recombinants. It was subsequently shown that the avipoxviruses share many basic features with the orthopoxviruses, particularly in the control of gene expression, e.g. promoters and transcription termination sequences. The essential features required for the construction of avipoxvirus recombinants can be summarised as: (1) a poxvirus promoter for gene expression, (2) sites for insertion of foreign genes either within a non-essential gene or between genes, and (3) a suitable method for identification and selection of recombinants. Additionally, it is prudent to consider the removal of early poxvirus transcription terminator (T5NT) sequences from the gene(s) to be expressed, as their presence may abort or significantly attenuate early gene expression in cells. Impacts upon the induction of cell-mediated responses may also occur as it has been demonstrated in vaccinia virus that late gene expression may not induce cell-mediated immune responses [72]; this impact has not formally been shown for avipoxvirus recombinants to my knowledge. It might be argued that late gene expression in FWPV is less likely to affect cell-mediated immune responses since FWPV does not shut down host cell protein synthesis to the extent that vaccinia virus does [73]. Antigen processing into MHC class I antigen-presenting pathways has been shown to be the mechanism by which vaccinia virus inhibits cell-mediated immune response induction from late expressed gene products [74].

If avipoxvirus recombinants are intended for animal or human clinical trials, then it is essential to use a cell substrate acceptable for this purpose. Consequently, growth and plaquing must be undertaken in chicken embryo cell cultures derived from certified sources of specific pathogen-free embryonated eggs. Additionally, full documentation and traceability of all biological materials used during the construction and growth of the recombinants will be required for regulatory approval to test the recombinants in humans – the work essentially needs to be conducted under GLP (Good Laboratory Practice) protocols. Other cell substrates, e.g. transformed quail cell lines, are not acceptable for vaccines contemplated for clinical use, although they are suitable for the construction of recombinants for research purposes [75, 76]. The construction of recombinant avipoxviruses should not be embarked upon lightly since the time required to construct recombinants is many months in contrast with vaccinia virus recombinants which can be constructed in a few weeks. The time difference is a reflection of the much longer replication cycle of avipoxviruses in comparison with vaccinia virus [77].

Promoters

It has been generally demonstrated that promoter sequences from one poxvirus will operate across the genera in the *Chordopoxvirinae* retaining temporal regulation [78]. Promoters such as the vaccinia virus P 7.5 early/late promoter have been widely used for the construction of recombinant poxviruses including rFWPV and rCNPV. The choice of promoter appears to have been largely driven by convenience and access with endogenous FWPV and CNPV promoters frequently used along with vaccinia virus optimised synthetic early or early/late promoters. A rational choice of promoter for optimal gene expression in recombinant avipoxviruses is not entirely clear as only a few studies have attempted to compare promoters for levels of expression [78–82] and promoter optimisation by sequence modification has been undertaken only in vaccinia virus. There is a paucity of evidence to support the belief that higher gene expression levels necessarily lead to better immune responses. With certain antigens the nature of the antigen rather than the expression level achieved from recombinant avipoxviruses has a greater impact on the immunogenicity [83].

Insertion sites

The key features of insertion sites are that they do not disrupt gene functions that might affect *in vivo* or *in vitro* replication or gene expression, and that stable recombinants can be plaque purified. The large genome size [2–4, 84] of the avipoxviruses suggests that there are many potential insertion sites (far more than have been described to date [44, 85–87]) and that there is a large capacity to carry multiple gene insertions either at individual or multiple sites [53]. The thymidine kinase gene of FWPV has been used as a locus for the construction of recombinants; however, in some circumstance stable recombinants have proven difficult to obtain [88–90]. We have been able to obtain stable recombinants within the thymidine kinase gene; however, this may be a reflection of the FWPV strain (FPV M3) and cell type used (chicken embryo skin cells) [58]. Others have shown that inactivation of the thymidine kinase gene can affect efficient replication of rFWPV [88]. Use of the thymidine kinase site for insertions is perhaps best avoided as there are many other potential sites including immediately downstream of the thymidine kinase gene [53]. We have encountered difficulties in generating stable recombinants on very few occasions for the approximately 150 recombinants constructed. Instability appears related to the gene being inserted, although with so few unstable recombinants a common factor is difficult to identify.

Recombinant selection

Following the recombination event generated by infection of cell cultures with parent virus and transfection with suitably constructed plasmid, recombinants can be identified by gene hybridisation or expression; however, the proportion of recombinants is low (less 1/1000 of the virus yield), making plaque purification of recombinants challenging. Co-expression of the *Escherichia coli* xanthine guanine ribosyl transferase gene conferring resistance to mycophenolic acid is a convenient selection marker for amplification of recombinants [58]. Additionally, co-expression of the Lac Z gene allows convenient identification and plaque purification of recombinants on the basis of blue staining of plaques with suitable β-galactosidase enzyme substrate [53, 58]. With dominant selection, the selection and marker genes are retained in the final recombinant [53]. Their presence in recombinants intended for human clinical trials may be problematic at the stage of regulatory approval, although rFWPVs carrying such genes have been approved in some jurisdictions for human clinical trials. The use of transient dominant selection for insertion of vaccine or therapeutic genes into avipoxvirus recombinants should be considered, as the selection and marker genes are not retained in the final recombinant and the selection and marker genes can be reused to make additional insertions at different loci [53]. This allows the construction of complex recombinants carrying multiple antigen genes and immune modulators. Multiple rounds (at least three or four) of plaque purification are generally required to generate homogenous stable recombinants. Thereafter, in our hands, recombinants have been stable through multiple generations required for master and working seeds lots, and final trial vaccine batches in preparation for human clinical trials [91]. The availability of plasmid vectors and general selection and amplification techniques, for both dominant and transient dominant selection, facilitates the construction of complex rFWPVs for use in vaccine trials [53, 91].

Avipoxviruses for the recovery of other poxviruses from naked DNA

The description of bacterial artificial chromosome vectors for the construction of vaccinia virus recombinants is dependent upon the use of FWPV to recover infectious vaccinia virus from poxvirus DNA [92, 93]. Poxvirus DNA is non-infectious; however, non-genetic reactivation, whereby an infectious virus can be recovered from an inactivated poxvirus by co-infection with an unrelated poxvirus (infectious or inactivated in a different manner) provides the mechanism for recovery of infectious virus from naked poxvirus DNA [94, 95]. Since productive avipoxvirus infections are restricted to avian cells, FWPV non-genetic reactivation of poxviruses in avian or non-avian cells provides a facile mechanism for recovery of infectious poxvirus from naked DNA – the contaminating FWPV is simply removed by passage on non-

avian cells [96]. Recovery of FWPV from DNA has not been demonstrated to date; however, it should be possible by the use of a poxvirus whose replication is non-permissive in avian cells or whose infectivity has been suitably inactivated. Conservation of poxvirus promoter and transcription elements across the poxvirus genera suggests a ready explanation for this mechanism. On occasions FWPV has been incorrectly described as providing packaging or helper virus function [97].

Reverse genetics of RNA viruses – T7 system

The use of T7 RNA polymerase for transient gene expression and for negative-strand RNA virus rescue was pioneered using vaccinia virus expressing T7 polymerase [98]. Replacement of vaccinia virus (wild type or modified vaccinia Ankara) with FWPV expressing T7 has the advantages of reduced cytopathic effects in non-avian cells, comparable levels of expression, handling safety and lack of productive infection. Recovery of the rescued virus is simplified since removal of the FWPV-T7 is achieved by passage on non-avian cell cultures [99–103].

Poultry vaccines

Avian influenza

Vaccination of poultry with rFWPV expressing H5 or H7 avian influenza haemagglutinin (HA) induces protection against experimental or natural infection with highly pathogenic avian influenza (HPAI) [59, 104–106]. Clinical disease and mortality are reduced or prevented even though haemagglutination inhibiting (HI) antibody titres following vaccination are low or undetectable [59, 104, 105]. rFWPV-expressed influenza nucleoprotein (NP) fails to provide protection [105]. Protection is antibody mediated [105] and HA-type specific [104, 105]. Interestingly, a recent report showed that rFWPV-H5-N1 provided protection against H5N1 and H7N1 HPAI challenge. Presumably the cross-protection was mediated *via* immunity to the common neuraminidase (N1) [107]. Shedding of avian influenza virus *via* respiratory and enteric routes is significantly reduced in vaccinated birds [9], thus reducing the potential for spread. Antibody responses to HA and NP rise rapidly following challenge, suggesting a substantial level of replication of challenge virus even though disease does not occur [104]. The restriction of antibody responses to the HA following vaccination and the induction of high titres of antibodies to both HA and NP following infection can be used to discriminate vaccinated birds or flocks from those in which avian influenza (HPAI or LPAI) may have circulated since the latter flocks will have antibodies to both HA and NP – so-called DIVA tests (dif-

ferentiation of infected from vaccinated animals). Optimisation of the HA insert may not be necessary to provide effective field protection against H5 avian influenza, as a single rFWPV-H5 recombinant provided adequate protection against H5 influenza virus isolates from four continents over a 38-year period [108]. Prior vaccination or field exposure to FWPV may limit the usefulness of rFWPV-influenza vaccines as the protection afforded against avian influenza challenge is inconsistent in such circumstances [109, 110]. rFWPV-H5 alone or in combination with other avian influenza vaccines has been widely used in Mexico – approaching a billion doses of vaccine have been used [109]. HPAI H5N1 in Asia has had profound impacts on poultry production and is currently considered the greatest threat of emergence as pandemic human influenza [111]. rFWPV-influenza vaccines have the potential to find widespread application for poultry vaccination in Asia. Their use to date has only been documented in chickens and turkeys. Vaccine efficacy in ducks and water birds needs to be demonstrated.

Newcastle disease

rFWPV and rPGPV expressing haemagglutinin-neuraminidase (HN) and/or fusion (F) proteins from Newcastle disease virus (NDV) provide protection against challenge with virulent NDV [86, 112–118]. Efficacy may be enhanced by the expression of both HN and F and by the use of rFWPV in conjunction with conventional NDV vaccines in a prime-boost vaccination regimen [113]. NDV HI antibody responses were markedly elevated in chickens vaccinated with live or inactivated NDV vaccine prior to vaccination with rFWPV-HN (geometric mean NDV HI titres were 10–100-fold higher). In contrast, chickens previously vaccinated with non-recombinant FWPV vaccine failed to develop NDV (HI) antibodies following vaccination with rFWPV-HN and were not protected against challenge with virulent NDV.

Other poultry vaccine candidates

Candidate rFWPV vaccines against a number of poultry pathogens have been evaluated (Tab. 2). Vaccine successes have been achieved predominantly with glycoproteins from enveloped viruses, e.g. avian leukosis, avian influenza, MDV, NDV, REV, and turkey rhinotracheitis virus [119–126]. Exceptions have been infectious bursal disease virus (IBDV) and haemorrhagic enteritis of turkeys where rFWPVs expressing the VP2 protein or hexon, respectively, have been shown to induce protective immunity [127–131]. rFWPV vaccine candidates against coccidiosis [132] and infectious bronchitis virus ([133] and Boyle, unpublished observations) have had variable or limited success. Host genetics have been shown to play a role in the

efficacy of rFWPV candidate vaccines in chickens against IBDV [128] and MDV [121]. Since these studies have been conducted using inbred chickens, it is not clear if genetic effects play a role in limiting efficacy in commercial production breeds. It is also not clear if the observed differences are related to an inherent feature of the antigens or to their delivery by rFWPV.

Enhancing poultry vaccines based on rFWPV

Heterologous prime-boost vaccination regimens and co-expression of immune-stimulators/modulators have found favour as mechanisms to improve immunogenicity of rFWPV-based vaccines [12, 134]. NDV HI antibody responses were markedly elevated in chickens vaccinated with live or inactivated NDV vaccine prior to vaccination with rFWPV-HN (geometric mean NDV HI titres were 10–100-fold higher) [113]. Sequential vaccination with recombinant MDV and rFWPV expressing the VP2 gene of IBDV markedly improved protection from gross lesions upon challenge with very virulent IBDV [135]. Chicken IL-18 co-expressed with VP2 gene of IBDV has been reported to significantly improve protection afforded against IBDV [5]. Co-expression in rFWPV of chicken type I interferon and NDV HN and F genes reduced post-vaccination body weight loss when the vaccine was used *in ovo* or shortly after hatching; however, the antibody responses to NDV were reduced by the co-expressed interferon [136]. *In ovo* vaccination of turkeys with rFWPV expressing HN and F of NDV and chicken type I or II interferons demonstrated earlier induction of antibodies to NDV without any adverse effects on hatchability. Treatment of chickens with rFWPV expressing chicken myelomonocytic growth factor (cMGF) prolonged survival times and reduced viraemia and tumour incidence when highly susceptible chickens were challenged with virulent MDV. In addition, rFWPV cMGF treatment improved vaccination protection provided by herpes virus of turkey vaccine. Both innate and acquired immune responses appeared enhanced following rFWPV cMGF treatment [137, 138].

Other veterinary vaccines delivered by recombinant avipoxviruses

The safety profile offered by rFWPV and rCNPV for delivering vaccines to non-avian species make them attractive vaccine vectors for a wide range of animal species (Tab. 2). In experimental studies, successful induction of protective immune responses has predominantly occurred with glycoprotein antigens of enveloped viruses. rFWPV and/or rCNPV expressing antigens from rabies, canine distemper, feline leukaemia and West Nile viruses provide effective protection against disease [139–147]. Expression of the rabies glycoprotein by rFWPV provided protection against disease in mice, cats and dogs [10, 139]. In a comparative study of vaccinia virus, rFWPV

and rCNPV expressing the rabies glycoprotein, the rCNPV elicited better neutralising antibodies and was approximately 100 times more effective in inducing a protective immune response than rFWPV. Protection provided by immunisation with rCNPV was not significantly different from that induced by the replication competent vaccinia virus rabies glycoprotein recombinant [139]. Although the level of rabies glycoprotein expression was slightly higher from the rCNPV than rFWPV, the difference was not sufficient to account for the marked difference in protection induced. The greater efficacy of rCNPV was probably the motivation for the extensive development of CNPV (ALVAC) as a vaccine vector in preference to FWPV [148, 149]. It is not clear if these differences in immunogenicity would necessarily hold for other antigens expressed by rCNPV and rFWPV.

Canine distemper is an important disease and it provides a useful model for vaccine studies for the other morbilliviruses, e.g. rinderpest and measles. rCNPV (ALVAC) expressing HA and F of canine distemper virus provides high levels of protection against symptomatic disease in a ferret challenge model and in dogs [142, 143]. The rCNPV vaccine was safe and could be used in combination with other canine vaccines without detrimental effects on the performance of any of the vaccines [143]. Oral vaccine delivery was found to be an effective vaccination route inducing protective immunity in highly susceptible Siberian pole cats as a model for potential vaccine use in the endangered black-footed ferret [150]. Intranasal vaccination with rCNPV (ALVAC) and recombinant vaccinia virus expressing HA and F in young ferrets induced lower levels of neutralising antibodies and provided poorer protection than animals vaccinated parenterally [151]. rFWPV and vaccinia virus vaccines expressing rinderpest HN and F genes provided a modest level of protection against canine distemper in ferrets, demonstrating the ability to generate cross-reacting immunity to morbilliviruses [152].

The spread of West Nile virus into North America in 1999–2000 has led to substantial veterinary and public health issues. An effective vaccine for the control of West Nile disease in horses based upon rCNPV expressing prM/E proteins has been licensed for use in horses [145–147]. A single intramuscular dose of vaccine provided protection against the development of viraemia (eight out of nine horses) following challenge (day 26 post vaccination) using West Nile virus-infected mosquitoes even in the absence of measurable antibody responses in some of the horses [146]. Two doses of vaccine provided effective protection against the development of mosquito-transmitted viraemia for at least 1 year post vaccination [145]. A marked amnestic antibody response was observed in horses previously vaccinated with an inactivate West Nile virus vaccine and subsequently boosted with the rCNPV vaccine – a prime-boost vaccination regimen [147].

rCNPV expressing *env* and *gag* genes of feline leukaemia virus provides high level protection against oro-nasal challenge with feline leukaemia virus [144, 153]. Protection lasted for at least 1 year, was effective against severe contact challenge and was obtained in the absence of detectable antibodies

to the env antigen. A high proportion of the cats failed to develop latent infections following challenge. When used in combination with other feline vaccines there were no impacts upon the performance of the rCNPV vaccine or the other vaccines.

Preclinical and clinical human vaccine trials

The search for an effective HIV/AIDS vaccine is perhaps the greatest biomedical research challenge existing today. It is in this area that rFWPV and rCNPV have been explored in great detail. It is not proposed to review this area extensively as it is well covered in specialised reviews [154]. Underlying this interest is the safety profile of avipoxviruses in non-avian hosts [70, 155, 156], the observations that heterologous prime-boost vaccination regimens can both enhance and direct the immune response to DNA vaccines and other poorly immunogenic vaccines [11, 13, 157, 158], and that co-expression of immunostimulatory/modulatory molecules can enhance or modify the nature of responses [159–161]. Much of the emphasis on rFWPV and rCNPV has been in their use in prime-boost vaccination regimens to generate enhanced cell-mediated immune responses [13, 157]. Studies in non-human primates have shown that this approach induces elevated levels of cellular immunity and provides effective levels of immunity against HIV/SHIV that can reduce peak and set viral loads, albeit without preventing infection. Regrettably, to date early phase human clinical trials with DNA/rFWPV prime-boost vaccination regimens have provided disappointing results [162, 163]. Co-expression of immunostimulatory/modulatory molecules in conjunction with HIV antigens, while attractive scientifically has, in our hands, faced substantial regulatory hurdles when proposed for use in non-HIV infected individuals. rFWPV expressing HIV antigens and human interferon-γ has been tested in Phase I/IIa therapeutic vaccination trials in HIV-positive individuals; however, the results have once again been disappointing [164]. In contrast to HIV/AIDS, the prime-boost vaccination regimen involving DNA vaccine and rFWPV has provided promising levels of T cell-mediated immunity to malaria including *Plasmodium falciparum* in pre-clinical and human clinical trials [14, 71, 165, 166]. rFWPV and rCNPV have been explored for the delivery of vaccines against cytomegalovirus, hepatitis B and C viruses, Japanese encephalitis virus, measles virus, rabies virus and mycobacterium (Tab. 2) [5].

In the cancer therapy area, rFWPV and rCNPV are being explored to see whether they can express cancer antigens and immunostimulatory/modulatory molecules to develop novel treatment regimens [167–171]. This is a large and growing area of research and is well covered in specialised literature. It is an area in which immunostimulatory/modulatory molecules can be explored since the safety concerns are overridden to the extent that, in the absence of any other treatment regimen, a higher level of risk can be

accepted. This can include the direct intra-tumour injection of rFWPV or rCNPV expressing tumour antigens and immunostimulators in attempts to break self tolerance.

General conclusions

The avipoxviruses have gone from neglected obscurity to important vaccine vectors in the past 20 years. The seminal observation of their utility for delivery of vaccine antigens to non-avian species has driven much of the interest in this group of viruses to the extent that rFWPV and rCNPV have undergone extensive clinical trials in humans for vaccines against HIV/AIDS and in treatment regimens for cancer patients. Their application as vaccine vectors in avian and non-avian species has been most successful where glycoprotein antigens of enveloped viruses have been expressed. Interest in the human area has been driven by their safety profile, generation of enhanced and directed responses in prime-boost vaccination regimens and the ability to co-express immunostimulatory/modulatory molecules. Exploration of the basic molecular virology of the avipoxviruses has thrown light on the evolutionary pathways of the *Poxviridae* and in the future it may be necessary to consider the *Avipoxvirus* genus as a separate subfamily within the *Poxviridae* but outside the *Chordopoxvirinae*. The intriguing and unique relationship that exists between FWPV and REV is one of those fascinating stories that have emerged from our studies of this group of viruses.

To provide consistency and accuracy this manuscript has adopted the nomenclature and abbreviations used by the ICTV: 7th Report [1]. However, where referring to specific isolates or strains of virus, the nomenclature or abbreviation adopted by the publication where the isolate or strain was described have been used, e.g. FPV-M3, FP1.

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