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PORCINE ADENOVIRUSES: AN UPDATE ON GENOME ANALYSIS AND VECTOR DEVELOPMENT

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Although porcine adenoviruses (PAdV) are present in the swine populations worldwide, they usually do not cause any disease, or the infection is only manifested in a mild diarrhoea or respiratory signs. The importance of adenoviruses, however, is constantly growing as there is a possibility of developing them into viral vector vaccines against more significant swine pathogens. A short summary of the well-established facts of porcine adenoviruses is given and recent developments of the genetic analysis of these viruses are discussed in detail. The possibilities of vector development and examples of vector vaccines already reported in the literature are mentioned.

Key words: Porcine adenovirus, sequence analysis, vectors

Viruses of the family *Adenoviridae* are present all over the world. They have been isolated from humans, several other mammalian species, birds, amphibians, and they have also been described in fish. Porcine adenoviruses (PAdV) belong to the genus *Mastadenovirus* of the family.

The virion is non-enveloped, icosahedral, with an outer protein capsid embedding the compact DNA-protein complex of the viral core. The most abundant capsid proteins of the virion are also the best characterized ones, such as the hexon, the penton and the fibre proteins. The typical surface projections of the fibre, attached to the penton base, are characteristic electron microscopic features of the virion. The lack of a lipid membrane envelope means that these viruses are resistant to physical changes in the environment, and the infectivity of the virus can be preserved for extended periods even under harsh conditions.

The genetic material of the virus is a single, linear copy of non-segmented, double-stranded DNA. The size of the DNA molecule, depending on the PAdV serotype, varies between 32 and 35 kilobase pairs (kb). The genome organization of mastadenoviruses is well established and the PAdV genomes fall perfectly into this group (see later). The genome is usually divided into early (E) and late (L) regions (Fig. 1), depending on the onset of the activity of genes during the viral replication cycle. In a simplified version, the early regions generally function

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as parts of the replication machinery and the late regions code mostly for structural proteins. The early regions as the main targets of vector vaccine development will be discussed later, in more detail.

The PAdVs are usually present in pig populations without causing any disease. There are only few occasions reported when they were involved in some form of illness without any secondary infection, but in most cases the infection is usually only manifested in a mild diarrhoea or mild respiratory signs (Derbyshire et al., 1975; Hirahara et al., 1990).

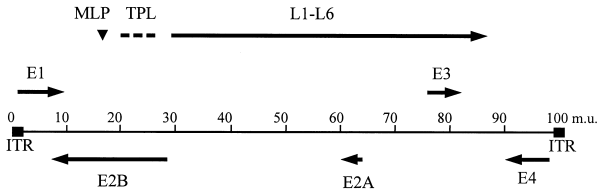

Fig. 1. Genome organization of porcine adenoviruses. The genome is indicated by the line divided into 100 map units (m.u.). The approximate size and location of early (E1-E4) and late (L1-L6) regions are shown by the arrows. MLP: major late promoter. TPL: tripartite leader. ITR: inverted terminal repetition

At least five, perhaps seven, PAdV serotypes are known to exist (Table 1). According to the latest report of the International Committee on Taxonomy of Viruses (Regenmortel et al., 2000), there are only 5 PAdV serotypes but 2 additional serotypes had been proposed by Kadoi et al. (1994 *a,b*). The serotypes are defined by traditional virus neutralization (VN) assays using serotype-specific, standardized polyclonal antibodies. Antigenic homology among the different serotypes can be detected by classic serological methods like complement fixation, immunodiffusion, or ELISA.

Table 1

The reference strains of recognized and putative PAdV serotypes

Serotype	Reference strain	Origin	Clinical signs
1	25R	England, 1964 (a)*	diarrhoea
2	A47	England, 1966 (b)	–
3	6618	England, 1966 (b)	–
4	F618	USA, 1965 (c)	encephalitis
	Munich	Germany, 1969 (d)	–
5	HNF61, HNF70	Japan, 1990 (e)	respiratory
6	TG/K79	Japan, 1994*a* (f)	enteritis
7	GK/K92	Japan, 1994*b* (f, g)	enteritis

*Letters indicate the references. a: Haig et al., 1964; b: Derbyshire et al., 1975; c: Kasza, 1966; d: Bibrack, 1969; e: Hirahara et al., 1990; f: Kadoi et al., 1994*a* (reported as serotype 5), 1994*b* (reported as serotype 6); g: Kadoi et al., 1995

The prototype strains of the first four PAdV serotypes were isolated in the 1960s (Haig et al., 1964; Kasza, 1966; Clarke et al., 1967). A new serotype was identified in Japan in 1990 and designated PAdV-5 (Hirahara et al., 1990). The two reference strains, HNF-61 and HNF-70, of PAdV-5 were isolated from the same swine herd. In 1994, again in Japan, a different research group reported the isolation of two more serologically (VN) distinct adenoviruses linked to haemorrhagic enteritis cases (Kadoi et al., 1994*a*; Kadoi et al., 1994*b*). Based on serological comparisons with PAdV-1 to -4 but not with PAdV-5, one of these isolates (TG/K79) was also designated PAdV-5 and the other one (GK/K92) described as PAdV-6 (Kadoi et al., 1994*b*). Shortly after the first report of the new isolates a limited restriction endonuclease (RE) characterization of the genome of TG/K79 (Kadoi et al., 1995) showed that this strain had a different RE pattern compared to the first four serotypes. Based on the published data it is likely that the TG/K79 virus is not identical with the PAdV-5 isolated in 1990. The reference strains of the different PAdV serotypes are listed in Table 1.

As indicated earlier, the first four PAdV serotypes were isolated approximately at the same time, and the identification of new serotypes started only almost 25 years later. In the meantime, besides the serological studies, researchers dealt mostly with the characterization of the virulence and replication of the viruses *in vitro* and also *in vivo*. With the development of molecular virological methods the possibility of drawing new and more profound relationships of these viruses was born. RE analysis itself is a perfect tool for the purpose, and it was this method that helped to prove that the PAdV-1–3 serotypes described in England, despite their different VN reactions, were genetically very similar to each other (Garwes and Xuan, 1989).

The proposed use of the PAdVs as viral vector vaccines (Tuboly et al., 1993), especially where mucosal immune response is required, led to the extensive study of the PAdV genomes. The first limited RE studies were extended to detailed physical mapping of the PAdV-3 (Reddy et al., 1993) and PAdV-4 (Kleiboeker et al., 1993) genomes. Shortly after these analyses the PAdV-1–2 (Reddy et al., 1995*c*) and PAdV-5 (Tuboly et al., 1995) genomes were also mapped. The comparison of the RE physical maps confirmed the close genetic relationship of PAdV-1–3, and indicated that PAdV-4 and PAdV-5 represented distinct lineages of PAdVs. The marked difference of serotype 4 and 5 is not surprising, considering the geographical and temporal distances of the isolates.

The establishment of RE physical maps created the basis for further, more detailed studies on the genetic material of the virus. The sequencing of the genome started with the early regions. These are the regions that, based on human adenovirus experiences, might be able to support foreign genes in vector vaccines. The E3 region has been sequenced in all serotypes (PAdV-4, Kleiboeker, 1994; PAdV-3, Reddy et al., 1995*a*; PAdV-1–2, Reddy et al., 1996; PAdV-5, Tuboly and Nagy, 2000*a*). E3 is supposed to be the only part of the genome that

is non-essential in virus replication *in vitro*, although it may play an important role *in vivo* by helping to hide the virus from immune surveillance. The size of this region is an important feature of the virus from the perspective of vector vaccine development. There is a general rule established for helper independent human adenovirus (HAdV) vectors, i.e. that the size of foreign DNA inserted into the genome can not exceed the original genome size by more than 5% (Bett et al., 1993). This is thought to be the approximate upper limit of the virion's packaging capability. It is possible to delete the non-essential E3 region, or at least part of it, from the genome and to replace it with foreign DNA. The size of this deletion determines how much more than the original extra 5% can be inserted into the genome without losing stability. The E3 sizes for each serotype are: PAdV-1: 1162 base pairs (bp), PAdV-2: 1222 bp, PAdV-3: 1179 bp, PAdV-4: 1879 bp, PAdV-5: 2020 bp. Based on the length of the E3 region of these viruses serotypes 4 and 5 have the greatest potential of packaging foreign DNA, but the use of PAdV-4 as a vector vaccine is questionable due to its potential to cause severe neurological disorders. Experimental deletions of the E3 region have been done in PAdV-3 (Reddy et al., 1999*a*) and PAdV-5 (Tuboly and Nagy, 2000*b*). The maximum length of the E3 DNA removed from PAdV-3 was 595 bp and it was 1237 bp in PAdV-5. The large deletion of the PAdV-5 brings the theoretical vector capacity up to 2.9 kb. Foreign DNA was inserted into both viruses and expressed (Reddy et al., 1999*b*, Tuboly and Nagy, 2000*a*), surprisingly, the size of the largest foreign gene (4.4 kb) carried by PAdV-5 well exceeded the calculated maximum capacity.

The next most popular target area of the genome for foreign gene insertion is the E1 region. The E1 region of adenoviruses is essential for virus replication, therefore, the E1 deleted virus can only replicate under special conditions. The E1 functions have to be added to the environment somehow. The solution is the use of a helper virus, or as a more practical approach, the use of a special cell line, such as the 293 cells for human adenoviruses, carrying the E1 genes. The deletion of this region can be a useful tool in the construction of safe recombinant vaccines. The E1 deleted virus propagated in the special cell line to a certain titre, when injected into the animal, has the ability to get inside the cells and starts to express the required genes, but the genome can not replicate, so no newly generated virus leaves the infected cell. The abortive infection of such viruses guarantees the safety of the vaccine. The E1 region of PAdV-3 (Reddy et al., 1998*b*), PAdV-4 (Kleiboeker, 1995*a*) and PAdV-5 (Nagy et al., 2000) has been analyzed, but no E1 carrying cell line has been constructed so far for PAdVs.

Most recently, the E2 gene of classical swine fever virus was inserted into PAdV-3 and expressed (Hammond et al., 2000). The site for insertion was yet another region of the genome, between the E4 and the right inverted terminal repeat (ITR) sequence. The E4 region of the virus is also essential for the replica-

tion, but inserting a gene after the E4 is possible as it does not disrupt any viral gene function. The E4 region of PAdV-3 (Reddy et al., 1997) and PAdV-5 (Tuboly et al., 2000*c*) has been sequenced. The PAdV-3 E4 region is similar to that of other typical mastadenoviruses but the sequence and transcriptional analysis of PAdV-5 suggested that although PAdV-5 had an E4 region similar to other mammalian adenoviruses, it also had some unique characteristics. The most important differences were: (1) the size of the E4 region in PAdV-5 was about 50% larger than in most human adenoviruses and in PAdV-3; (2) the number of sequence elements involved in mRNA transcription and regulation was higher than in PAdV-3; (3) the deduced amino acid sequences of two open reading frames (ORF5 and ORF6) were similar to each other (both homologous to the 34 kDa E4 protein of HAdV-2; Herisse et al., 1981), indicating that the same function may be present in a duplicate form. The HAdV 34 kDa protein plays an important role in viral DNA replication, but the presence of the two copies of a similar gene in PAdV-5, raises the possibility of deleting part of the E4 region in order to further shorten the original size of the genome and increase the vector capacity of the virus.

The ITR sequences of PAdV-1 to -5 have been analyzed (Reddy et al., 1995*b*) and compared. Interestingly the first 17 nucleotides of PAdV-1, PAdV-2, PAdV-3 and PAdV-5 were identical but the PAdV-4 strain shared only the first 10 bases. The close relationship among the first 3 serotypes was confirmed and revised, PAdV-1 and PAdV-3 ITRs were similar in length and 90% homologous whereas the PAdV-2 ITR was approximately 50 nucleotides longer and only 67% similar to PAdV-1.

Analyses of some of the late gene sequences of PAdV strains have also been published. The fibre of the virion is involved in the attachment to cellular receptors and consequently the entry into the cells. The sequence of the fibre gene is decisive not only for the serotype but also for the cell- and tissue-specificity of the virus (Chroboczek et al., 1995). The analysis of the fibre gene and certain elements of the fibre protein is crucial for influencing the tropism of the virus. It is possible to target selected receptors by replacing parts of this gene, generating vector vaccines and gene therapy tools specific for certain organs or cell types. The fibre protein is usually divided into three distinct structural domains: the tail, the shaft and the head. The fibre gene of the PADV-4 NADC-1 isolate (Kleiboeker, 1995*b*) was analysed and reported to be different from known adenovirus fibre genes. Despite sequence homologies with other fibre genes, unique characteristics were also detected. The shaft region was shorter and the head was more than twice as long as that of known adenovirus fibre sequences. An unusual RGD motif was recognized in the head region and sequence similarities to S-lectin proteins were detected. The PAdV-3 and PAdV-5 fibre genes have also been sequenced (Reddy et al., 1995*a*; Nagy et al., 2000). The fibre gene of PAdV-3 had an unusually long (742 bp) tandem repeat sequence

starting within the shaft region, and the TLWT motif, usually starting the head region, was missing from the fibre of both PAdV-5 reference strains.

The pVIII genes of PAdV-1 to -5 have been sequenced (Kleiboeker, 1994; Reddy et al., 1996; Tuboly and Nagy, 2000*b*) and compared to each other and to known avian and mammalian adenovirus pVIII genes on the protein level. The results confirmed again that PAdV-1, -2 and -3 were close relatives and PAdV-4 and -5 were different on the genetic level as well. The phylogenetic analysis of the established pVIII protein sequences showed that PAdV-5 was more closely related to bovine adenoviruses (BAdV), namely to BAdV-1, than to any of the known PAdVs. The sequence similarity to other bovine and canine adenoviruses also suggested that PAdV-5 was not a descendant of other PAdVs. The sequence analysis of other early and late genes confirmed this suggestion.

The penton base gene (McCoy et al., 1996*b*), the 23K protein (McCoy et al., 1996*a*), the 100K protein (McCoy et al., 1997) and the hexon gene (McCoy et al., 1999) of PAdV-3 were published as separate papers, but most recently the sequence of the entire genome of PAdV-3 has been published (Reddy et al., 1998*a*), and the sequencing of the genome of the PAdV-5 HNF-70 strain has also been completed (Nagy et al., 2000). The results indicated that the late genes of both strains were similar to other mammalian adenovirus late genes and no unusual divergence could be observed. The phylogenetic comparison of hexon genes of several adenoviruses confirmed the findings based on such analysis of the pVIII sequences, namely that PAdV-5 was more closely related to bovine adenoviruses (BAdV-1 and -2) than to porcine adenoviruses.

Recombinant human adenoviruses have been shown to be efficient vector systems for the delivery of porcine coronavirus antigens, like the spike protein of the transmissible gastroenteritis virus (TGEV) or porcine respiratory coronavirus (Torres et al., 1996; Callebaut et al., 1996). Their widespread use in domestic animals, however, may be limited, mainly because of human safety concerns. Animal adenoviruses, on the other hand, are mostly species specific, presenting no risk for humans or other animal species, and they replicate more effectively in their host, providing a safer and more efficient gene delivery system.

The possibility of the development of PAdV vector vaccines has already been mentioned. The most likely candidates based on safety reasons and on the level of characterization of their genomes are PAdV-3 and PAdV-5. The potential sites for gene insertion on the genome are the E1 and E3 regions or between the E4 region and the right terminal ITR. The field of preventive veterinary medicine and the need for efficient vaccines against several viral diseases of pigs provide several possibilities for the development of recombinant porcine adenovirus vectors. This is especially true in the case of those infectious diseases where the local immune response in the gastrointestinal or in the respiratory tract is crucial in the protection. PAdV-3 and PAdV-5 have been demonstrated to in-

duce both systemic and local mucosal immune response after a single dose of orally administered live virus (Tuboly et al., 1993; Tuboly and Nagy, 2000*a*).

Recently PAdV-3 has been developed into helper-dependent (Reddy et al., 1999*a*) and helper-independent expression vectors (Reddy et al., 1999*b*, Hammond et al., 2000). So far two viral genes have been expressed by helper independent PAdV-3 vectors. Namely the gD gene of the Aujeszky's disease virus has been inserted into the E3 region (Reddy et al., 1999*b*) and the E2 gene of the classical swine fever virus inserted near the right hand terminus of the adenovirus genome (Hammond et al., 2000). Both recombinant viruses were able to express the foreign gene to some extent, proving that PAdVs could be used as vaccines or expression vectors.

The development of PAdV-5 into a recombinant TGE vaccine has been described (Tuboly and Nagy, 2000*a*). Helper-independent recombinant PAdVs have been constructed and tested for their ability to express the spike gene of TGEV. The viruses expressing the gene *in vitro* induced humoral immune response in pigs, not only of systemic but also of local nature in the intestine and in the lungs as well.

Both PAdV-3 and PAdV-5 are in the stage of vector development when the expression of practically any foreign gene is possible. The choice of the serotype to be used though is not so simple. The widespread occurrence of serotype 3 in the swine populations and the pre-existing PAdV-3 specific virus neutralizing antibodies may limit the use of this serotype as a vector vaccine. PAdV-5, however, has only been isolated in Japan (in 1990) and there are no reports on the occurrence of this serotype elsewhere in the world, so the presence of PAdV-5 neutralizing antibodies would not restrict the use of the vector vaccine. Another advantage of PAdV-5 over PAdV-3 is that the size of possible deletions in the E3 or E4 regions (see earlier) are much larger than in serotype 3.

The use of porcine adenoviruses as vector vaccines is only one of the areas, although the best developed, where they could be utilized. Besides using them as live vaccines they can also be *in vitro* expression systems for antigens of vaccines, diagnostics or for any other purpose where correct, mammalian type protein processing is required, like in the production of hormones, cytokines, etc. Adenoviruses are being investigated as tools for gene therapy and cancer therapy as well. PAdVs may also be developed into such tools and by directed mutagenesis of the fibre gene sequences involved in cell receptor binding, specific targeting of certain cell groups may become possible in he future.

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