In vivo and *in vitro* characterization of two camelpoxvirus isolates with decreased virulence

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Key words

Dromedary - *Camelus dromedarius* - *Camelus bactrianus* - Orthopoxvirus - *In vitro* experimentation - *In vivo* experimentation - Vaccine.

Summary

Two camelpoxvirus (CPV) strains isolated from camels with generalized skin disease were serially passaged on Vero cells. Various phenotypic properties were investigated *in vitro* and *in vivo* and compared with those of the corresponding wildtype strains. In many aspects no differences were observed. However, in a mouse model both passaged strains proved to be highly attenuated. In addition, both strains failed to replicate in a cell line derived from camel skin cells. Comparison of physical maps established for enzymes HindIII and XhoI revealed deletions accounting for a total of 22 kbp in one attenuated strain. In the second strain only minor alterations were noted.

■ INTRODUCTION

Today almost 20 million camels (*Camelus dromedarius* and *Camelus bactrianus*) are kept in Africa, Asia, India and the Middle East for milk, meat and wool production, transport or racing (18). As the interest in husbandry and diseases of camels has increased, camelpox in particular has received attention (20). It is caused by the so-called camelpoxvirus (CPV) which represents a separate species within the genus Orthopoxvirus (19). Mainly camel calves are affected and develop a proliferative skin disease often complicated by bacterial infections (12, 23). The mortality can be as high as 30 % and recovering animals may develop lifelong immunity (12). With no therapy at hand, prophylactic vaccination seems to be a promising approach to protect camels. Higgins (8) reported about early attempts of nomads to protect their animals by exposing them to scabs from recent outbreaks ground in milk. In 1992, camels were vaccinated with vacciniavirus (*Orthopoxvirus commune*) (8), and in the same year homologous vaccines - derived from CPV isolates serially passaged in cell culture - were described by two groups (7, 10). According to both studies homologous vaccination proved to be effective after subsequent challenge in a limited number of animals. However, no data are available that would allow discrimination of the vaccine strains from wildtype isolates.

In this study data on biological characteristics of two serially passaged CPV isolates and their progenies are presented. In a mouse model, passagation is shown to have led to a significant decrease in virulence.

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■ MATERIALS AND METHODS

Viruses and cells

CPV strains *variole de dromadaire* 49 (VD49) and *variole de dromadaire de Maurétanie* (VDM) have been isolated from camels with generalized skin lesions in Niger (VD49) and Mauretania (VDM) (kindly provided by Dr. N. Ba-Vy, CIRAD-EMVT). Both strains were subcultured five times on MA-104 cells (African green monkey kidney cell line). In the following, these 5th passages will be referred to as wildtype virus (VDM wt and VD49 wt, respectively). After 107 passages on Vero cells the strains were plaque purified three times. Subsequently, the 114th passages (referred to as VDM 114 and VD49 114, respectively) were investigated.

Replication was assessed in the following cell lines: BHK 21 (baby hamster kidney), Dubca (camel skin; kindly provided by Prof. Dr. O.-R. Kaaden, Munich), E.Derm (equine dermal fibroblasts), L929 (murine subcutis), MA-104 (African green monkey kidney), MDBK (Madin Darby bovine kidney), MDCK (Madin Darby canine kidney) and Vero (African green monkey kidney). For microscopical inspection, cell lines were subcultured on cover slips in Leighton tubes, and infected with freeze-thawed cell culture material of VDM and VD49 wt and 114, respectively. After 1 h at 37°C, cells were washed once and culture continued. Cells were fixed at 6, 12, 24, 30, 36, 42, 48, 72 and 120 h *post infectionem* (p.i.) with ethanol-glacial acetic acid (3:1), stained with hematoxylin-eosin and embedded (16). In order to compare

the efficiency of replication in natural host cells, Dubca cells were incubated with VDM and VD49 (wt and 114, respectively), for one hour, washed and culture continued. After defined times *post infectionem* (1, 12, 24, 48, 72 and 120 h) cells were freeze-thawed twice and titrated on MA-104 cells. Titers in plaque forming units (pfu) were determined according to the method of Kaerber (11).

Infection of embryonated eggs

The pock morphology of VDM and VD49 wt and 114, respectively, was examined after infection of the chorioallantoic membrane (CAM) of 11 day old chicken eggs according to the method of Mayr *et al.* (16). Briefly, 0.1 ml of diluted (10^0 to 10^{-4}) freeze-thawed infected cell culture material was applied to four eggs. The titer of the virus material used was determined on Vero cells (11). Eggs inoculated with 0.1 ml phosphate buffered saline served as a control. Six days post inoculation the CAMs were collected and examined.

Animal experiments

Suckling F81 POP mice (27) were infected with 10-fold dilutions (10^0 to 10^{-6}) of purified virions of VDM and VD49 wt and 114, respectively, 36 ± 8 h after parturition. Six animals received 0.1 ml intraperitoneally (i.p.) and 7 to 8 animals received 0.02 ml intracerebrally (i.cer.). The titer of each virus preparation was determined on Vero cells (11). Untreated animals or animals inoculated with dilution buffer (2.5 mM Tris - 1 mM EDTA, pH 8.0) served as controls. Infected animals were inspected twice a day. Animal experiments were approved by the government of Oberbayern (reference nbr. 211-2531-28/93).

Restriction enzyme analysis

Viral DNA of both CPV strains was isolated from purified virions from passages 0 (wildtype), 30, 80 or 82, 110 (not plaque purified) and 114, respectively. DNA was cleaved with restriction endonucleases HindIII and XhoI according to manufacturer's instructions (Boehringer, Mannheim, Germany). Resulting fragments were analyzed in 0.4 % and 1.2 % agarose gels and their molecular weights were estimated by comparison with molecular weight standards (1 KB ladder and HMW marker; Pharmacia, Freiburg, Germany). The total size of each genome was obtained by summation. Southern blots were prepared on Hybond N+ membranes (Amersham/Buchler, Braunschweig, Germany) using 0.4 N NaOH as transfer buffer. For hybridization restriction enzyme generated DNA-fragments of CPV, vacciniavirus strain Elstree and cowpoxvirus strain Brighton were gel-isolated and labeled according to manufacturer's instructions (DIG-DNA-Labeling and Detection Kit Nonradioactive; Boehringer, Mannheim, Germany).

■ RESULTS

Replication of VDM and VD49 in vitro

Replication of VDM and VD49 wt and 114, respectively, was observed in cell lines derived from various hosts. Generally, 12 to 24 h p.i. rounding of cells could be seen. The formation of visible plaques became evident after 24 h p.i. On the second day plaque diameters averaged from 0.2 mm to 0.5 mm on most cell lines and up to 1.5 mm on Dubca cells. A characteristic feature of CPV is the formation of multinucleated giant cells. This was most distinct in African green monkey kidney cell lines Vero and MA-104 with 20 to 200 nuclei involved. Some differences in virus yield and plaque size were observed between wt and passage 114. The formation of comets was only seen with the highly passaged strains. Most obvious was the failure of both virus strains from passage 114 to replicate in MDCK cells and in Dubca cells. Forty-eight hours after infection the titer of wildtype viruses had increased at least 400-fold (VD49 wt) and 6000-fold (VDM wt), respectively, whereas no increase was observed for VDM 114 and VD49 114 infected cells as demonstrated for Dubca cells in figure 1. Furthermore, agglomerations of rounded cells was observed in BHK, E.derm (except VDM) and L929 cells after infection with wildtype strains and only in E.derm cells after infection with passages 114. Table I summarizes the differences in cytopathic effects of both strains in different cell lines (rounding up of cells, plaque formation and plaque diameter up to 0.5 mm are not noted).

Replication of VDM and VD49 in vivo

The pock morphology on the CAM was determined after six days of incubation. By then, small white-opaque pocks had developed with 0.2 to 1.0 mm in diameter. Comparison of either wt or passage 114 infected CAMs displayed no differences in pock morphology, and only a slight shift in the ratio of plaque forming units to pockforming units was observed. By day 6, 25 % of VDM and VD49 wt infected embryos and 20 % of VD49 114 infected embryos had died, whereas no losses were seen after infection with VDM 114.

In order to determine the effect of serial *in vitro* passage on virulence a mouse model was developed. Intraperitoneal (i.p.) application of 10^5 pfu of VDM wt or VD49 wt in baby mice caused death within 2 to 3 days. After application of as little as 10 pfu the animals died within 5 to 6 days. The LD_{50} per mouse (p.m.) was calculated to be 3 and 5 pfu, respectively, after i.p. inoculation of VDM and VD49 wt (table II and figure 2 et 3). An identical value (LD_{50} p.m. = 3 pfu) was calculated for VDM wt using the intracerebrally (i.cer.) route, whereas the LD_{50} p.m. for VD49 wt was 225 pfu (figure 2, 3 and table II). Furthermore, there

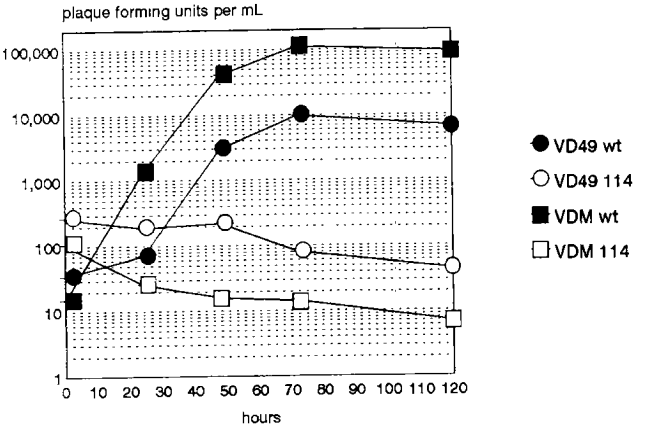

Figure 1: replication of camelpoxvirus (CPV) strains VDM and VD49 in Dubca cells. The values represent the titers (plaque forming units (pfu)) after 1, 24, 48, 72 and 120 h post infectionem *(determined on MA104 cells).*

TABLE I

Differences in cytopathic effects (cpe) observed in various cell lines after infection with camelpoxvirus (CPV) strains VDM and VD49 wt (wildtype) and 114 (passage 114), respectively

Cell line	VDM		VD49	
	wt	114	wt	114
BHK	AG(20-50)	-0-	AG(10-30)	-0-
Dubca	GC;Dia(1.3)	AG(20-50)	GC;Dia(1.0)	AG(5-10)
E.Derm	GC(5-10)	CF;GC(-10)	AG(5-10)	CF;GC(-10);Dia (0.7)
L929	AG(10-20)	-0-	AG(10-20)	-0-
MA-104	GC(50-200)	GC(5-15)	GC(50-100)	GC(5-20);Dia(0.7)
MDBK				
MDCK				
Vero	GC(50-200)		GC(20-50)	

cpe, like rounding up of cells, plaque formation and plaque diameters up to 0.5 mm are not particularly noted.
AG = agglomeration (in brackets: number of cells involved); CF = comet formation; GC = giant cell (in parentheses: number of nuclei involved);
Dia: = diameter (in parentheses: diameter in mm); -0- = no effect was seen, neither rounding up nor plaque formation

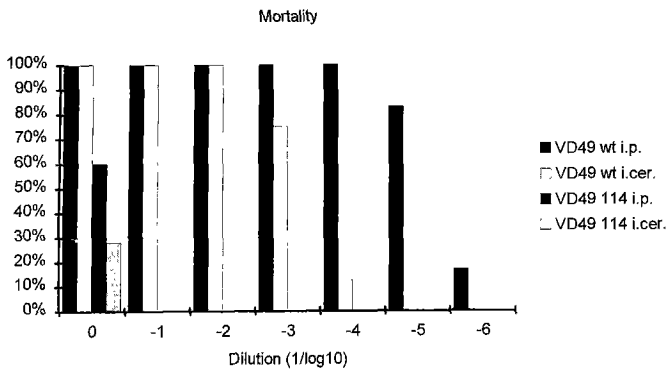

Figure 2: mortality of suckling mice after intraperitoneal (i.p.) or intracerebral (i.cer.) infection with camelpoxvirus (CPV) strains VD49 wt and VD49 114.

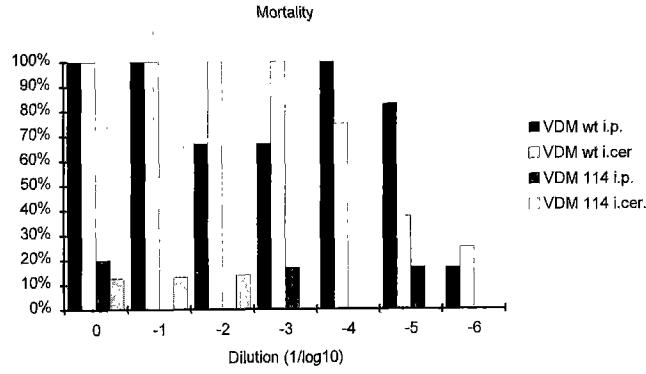

Figure 3: mortality of suckling mice after intraperitoneal (i.p.) or intracerebral (i.cer.) infection with camelpoxvirus (CPV) strains VDM wt and VDM 114.

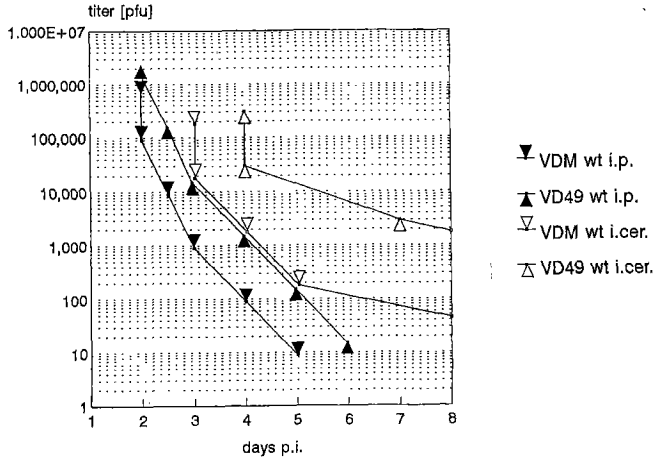

Figure 4: correlation of applicated virus titer and time of death of camelpoxvirus (CPV) infected mice (abscissae, time in days, and ordinates, pfu per dose). Criterion for time is the day, when more than 50 % of animals were dead.

was a difference when comparing mode of application and mean time interval until the animals died. Using an identical amount of pfu, i.p. infected mice died earlier than i.cer. infected animals (figure 4).

A marked attenuation was seen after application of the highly passaged viruses VDM 114 and VD49 114. The LD_{50} p.m. was calculated to be in the range of 1 to 7 x 10^5 pfu for both strains and both modes of application (figure 2, 3 and table II).

Genome analysis

The restriction fragments obtained after digestion of different passages of VDM and VD49 with either XhoI or HindIII are shown in figure 5. The majority of fragments were comigrating. However, some fragments present in the wt strain (i.e. 11, 14.5 and 18 kilobasepair (kbp) HindIII-fragment of VDM) were absent in subsequent passages. In contrast, some new fragments could be

TABLE II

Comparison of LD_{50} values for one mouse after application of camelpoxvirus strains VDM and VD49 wt (wildtype) and 114 (passage 114), respectively

Virus passage	Virus titer [pfu][1]	Application[2]	LD_{50} p.m. [pfu][3]	Increase in LD_{50}[4]
VDM wt	9.0×10^5	i.p.	3	
VDM 114	8.4×10^5	i.p.	7.8×10^5	260,00
VDM wt	1.8×10^5	i.cer.	2.5	
VDM114	1.7×10^5	i.cer.	2.1×10^5	90,000
VD49 wt	15.0×10^5	i.p.	5	
VD49 114	4.1×10^5	i.p.	3.3×10^5	65,000
VD49 wt	3.0×10^5	i.cer.	225	
VD49 114	0.8×10^5	i.cer.	1.3×10^5	550

[1] amount of plaqueforming units (pfu) in the applicated volume (0.1 ml i.p.; 0.02 ml i.cer.) of stock virus (10^0) as determined on MA-104 cells

[2] kind of application: i.p. (intraperitoneal) or i.cer. (intracerebral)

[3] LD_{50} per mouse (p.m.) in pfu (based on virus titer and mortality rate, figures 2 and 3)

[4] increase of the LD_{50} in the ratio of passage 114 to wt

detected (i.e. 7.9 kbp fragment of VDM and 10.1 kbp fragment of VD49). The total size of each genome was obtained by summing up. One hundred fourteen passages did not result in any changes of the length of VD49 as compared to the wt strain (about 166.5 kbp). However, the size of the genome of VDM 114 had decreased by about 22 kbp (172 kbp *versus* 194 kbp) compared to the wt virus. Physical maps were established for wildtype and passage 114 of both strains (figure 6) and the difference could be attributed to deletions in the right and left termini (reducing their size 17.5 and 4.5 kbp, respectively).

■ DISCUSSION

The pheno- and genotype of two CPV isolates (VDM and VD49) were examined before and after serial *in vitro* passages. Both wildtype viruses displayed similar characteristics as compared to data previously described (1, 2, 4, 13, 14, 23, 24, 26). However, after passagation differences were noted *in vivo* and *in vitro*. It seems remarkable that both strains have lost the ability to replicate in Dubca cells that originally were derived from camel skin cells. The second difference noted is the formation of comets that could be seen with the passaged viruses in MA-104 cells. Formation of comets is caused by a unidirectionally spread of extracellular enveloped virions (EEV). It is well accepted that EEV plays an important role in the pathogenesis *in vivo* and might contribute to a protective immune response (25). This is of course essential for an effective vaccine.

However, the essential part of this study was the comparison of virulence of VDM and VD49 before and after cell culture passagation in a mouse model. According to data described in references 2, 4, 13, 14, 26, the susceptibility of mice seems to be age dependent. In this study, 36 ± 8 h old mice proved to be highly susceptible for both VDM wt and VD49 wt. After intracerebral application, the pathogenicity of VD49 wt was slightly lower than the pathogenicity of VDM wt. Comparing the modes of application, intraperitoneal inoculation leads to a higher mortality and results can be obtained within six days, which should be of help in future experiments.

However, a striking low pathogenicity of cell culture passaged virus could be demonstrated. Comparing the LD_{50} p.m.-values, the amount of pfu increased up to 260,000-fold. Only a 10,000-fold increase was described for recombinant vacciniaviruses with a thymidin kinase negative phenotype (3). It can only be assumed that during passagation mutations and/or deletions have occurred. It is tempting to speculate that failure to replicate in Dubca cells (the reasons are unknown) point to a highly attenuated phenotype in camels too.

Of interest are two animals which received a dilution (10^{-3} and 10^{-5} fold) of VDM 114 i.p. and were eaten by their dams. Therefore, an examination was not possible. However, in both cases only one animal from a group of seven was missing and because no animal of the other groups died (10^{-1}, 10^{-2} and 10^{-4} fold virus dilutions) these two animals were not included in the survey on the LD_{50} value.

Investigation of the viral genome of VDM and VD49 indicates two different ways of genomic evolution during cell culture passages: VDM wt displays migration patterns and an overall genomic size similar to other CPV strains (5, 17, 24). Continuous passagation leads to a loss of 22 kbp in the terminal regions and thereby probably to a loss of genes which are not essential for replication *in vitro*. Many authors (6, 9, 15, 22) assume that in the variable-sized termini proteins are encoded which interfere with the host's immune response.

In contrast, the mouse pathogenic VD49 wt displays a significantly shorter genome than VDM wt and other CPV strains

(165 *versus* 190 kbp) (5, 17, 24) due to a truncated right terminus, thus resembling the VDM 114 strain with a highly attenuated phenotype. Passagation of VD49 wt leads only to a loss of approximately 1 kbp but is associated with a marked attenuation.

The mechanisms leading to attenuation by cell culture passagation are not yet understood. Maybe minute deletions or even point mutations in genes interfering with the host's immune response are responsible for the loss of pathogenicity (15, 22). Thus, further detailed investigations like marker rescue experiments would be helpful to identify the gene(s) involved in virulence. However, at present molecular investigations cannot replace animal experiments for the proof of apathogenicity for animals or man.

The results show that VDM 114 and VD49 114 might be candidates for live vaccines against CPV infection. However, this can only be evaluated in the natural host and must include subsequent challenge experiments. Thereby, two characteristics (failure to replicate in Dubca cells and formation of comets) could serve as important *in vitro* markers to distinguish the attenuated strains from field strains.

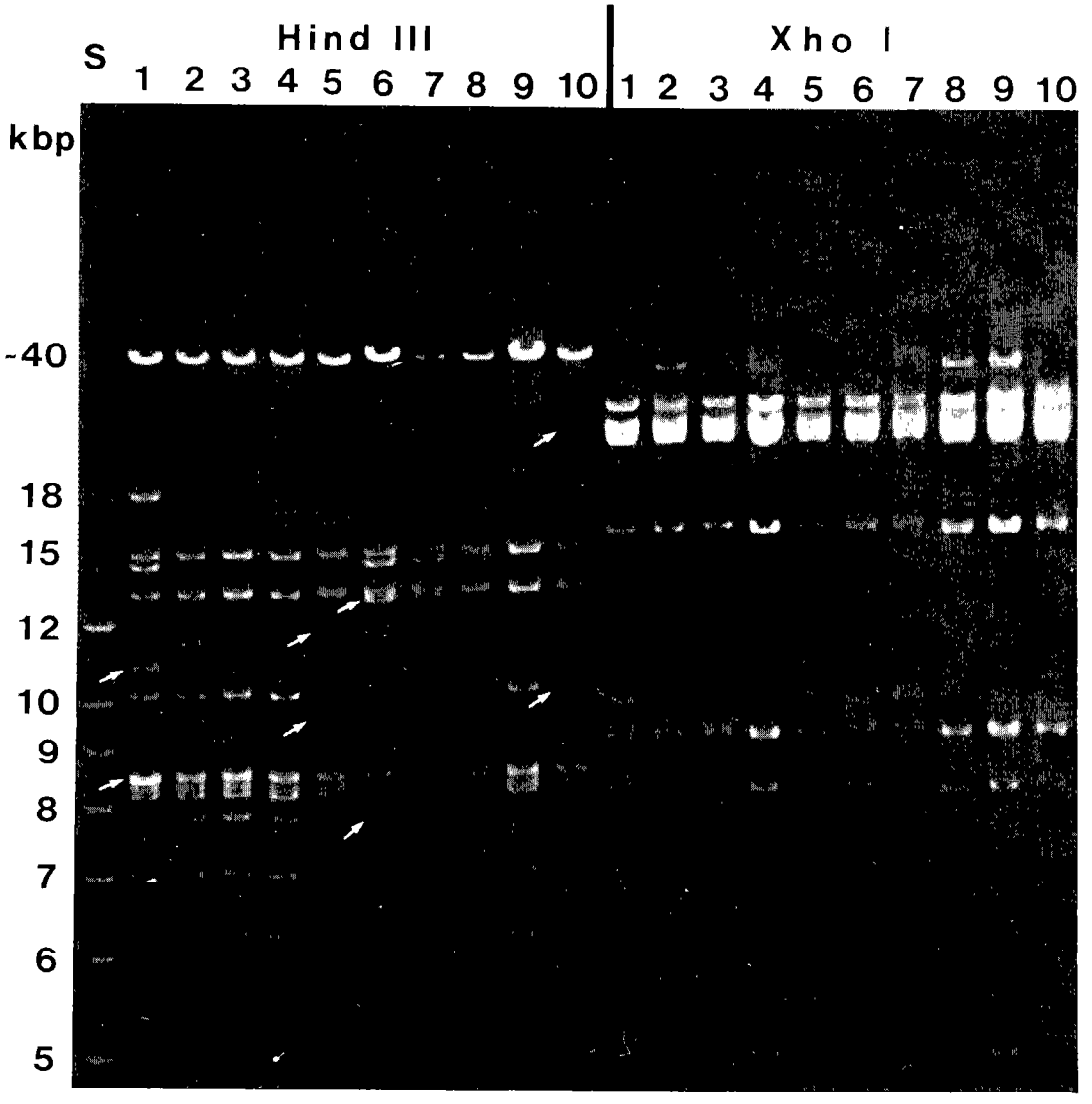

Figure 5: electropherogram of HindIII or XhoI digested DNA of camelpoxvirus (CPV) strains VDM and VD49 obtained from different passages. Arrows mark the terminal fragments.

S = molecular weight standard

1 = VDM wt
2 = VDM passage 30
3 = VDM passage 80
4 = VDM passage 110
5 = VDM passage 114
6 = VD49 wt
7 = VD49 passage 30
8 = VD49 passage 82
9 = VD49 passage 110
10 = VD49 passage 114

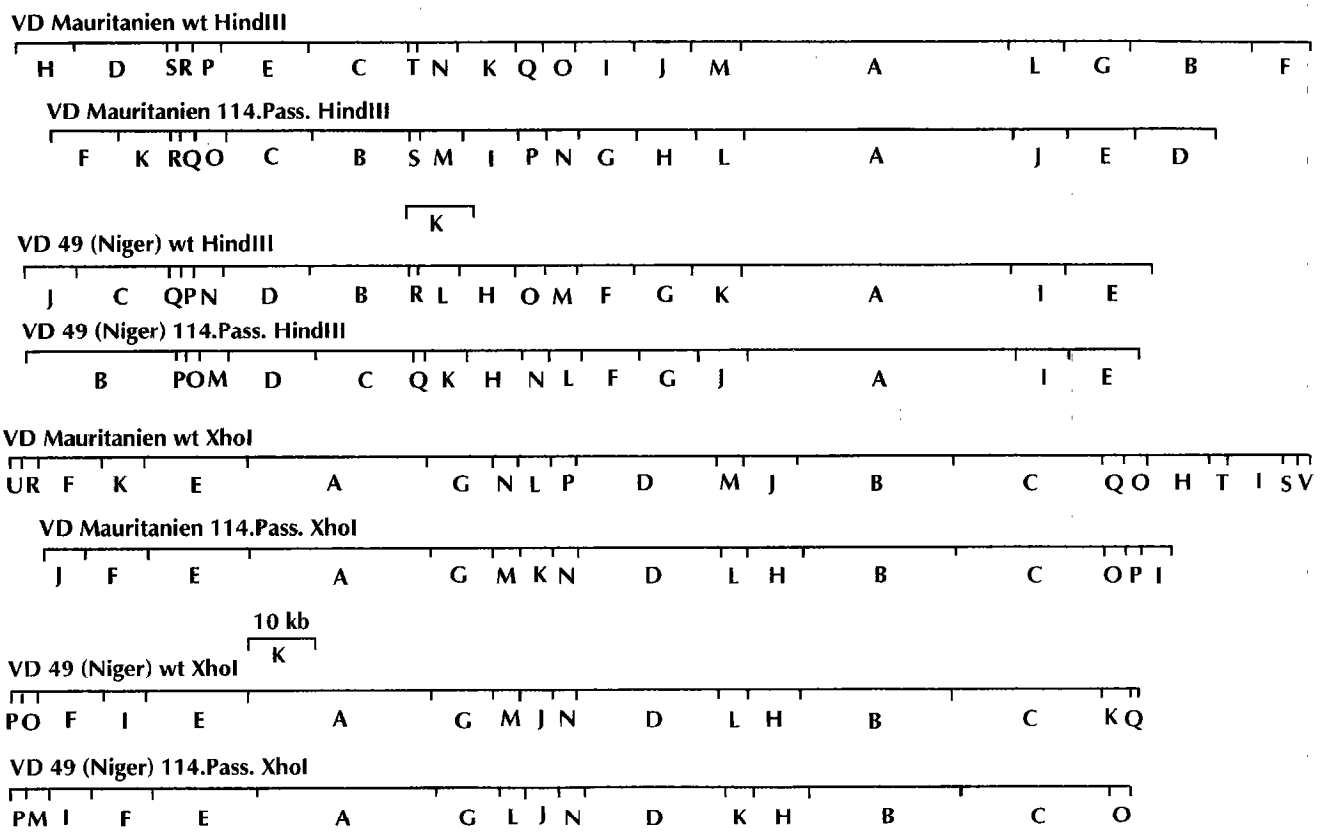

Figure 6: physical maps of HindIII or XhoI digested DNA of camelpoxvirus strains VDM and VD49 wt and 114, respectively.

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Résumé

Otterbein C.K., Meyer H., Renner-Müller I.C.E., Munz E.
Caractérisation *in vitro* et *in vivo* de deux souches de virus de la variole des dromadaires à virulence atténuée

Deux souches de virus de la variole des dromadaires (CPV) isolées de dromadaires affectés d'altérations cutanées généralisées ont été soumises à des passages en série de cellules Vero. Différentes caractéristiques phénotypiques furent étudiées *in vivo* comme *in vitro* et comparées à celle des souches sauvages correspondantes. A beaucoup d'égards, aucune différence ne put être constatée. Toutefois, dans un modèle de souris, les deux souches soumises au passage s'avèrent sensiblement atténuées. De plus, ces souches ne sont pas en mesure de se reproduire dans une culture dérivée de cellules cutanées de dromadaire. Par comparaison des cartes génomiques physiques établies pour les enzymes HindIII et XhoI, des délétions totalisant 22 kilobasepaires furent constatées pour l'une des souches atténuées. Le génome de la deuxième souche ne comportait que des altérations mineures.

Mots-clés : Dromadaire - *Camelus dromedarius* - *Camelus bactrianus* - Orthopoxvirus - Expérimentation *in vitro* - Expérimentation *in vivo* - Vaccin.

Resumen

Otterbein C.K., Meyer H., Renner-Müller I.C.E., Munz E.
Caracterización *in vivo* e *in vitro* de dos aislamientos de Camelpoxvirus con virulencia disminuida

Se aislaron dos cepas de Camelpoxvirus (CPV) en camellos con enfermedad cutánea generalizada, las cuales fueron luego sometidas a pasages en serie en células Vero. Se investigaron varias propiedades fenotípicas *in vitro* e *in vivo*, en comparación con aquellas correspondientes a las cepas de tipo salvage. En muchos aspectos no se observaron diferencias. Sin embargo, en un modelo en ratones, ambos pasages de las cepas demostraron ser altamente atenuantes. Además de esto, ninguna de las cepas logró replicarse en una línea celular derivada de células de piel de camello. La comparación de los mapas físicos establecidos para las enzimas HindIII y XhoI revelaron errores de un total de 22 kbp en una cepa atenuada. En la segunda cepa se notaron solamente alteraciones menores.

Palabras clave : Dromedario - *Camelus dromedarius* - *Camelus bactrianus* - Orthopoxvirus - Experimentación *in vitro* - Experimentación *in vivo* - Vacuna.