Genetische Variabilität, Wirt-Assoziation und Pathogenität von Kuhpockenviren (CPXV)

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INHALTSVERZEICHNIS

1 EINLEITUNG

1.1 HISTORISCHE EINFÜHRUNG

Der bedeutendste Vertreter der Familie der Pockenviren (*Poxviridae*) ist das *Variola Virus* (VARV), das eine der ältesten und bekanntesten Infektionskrankheiten auslöst: die Menschenpocken (*Smallpox*). Das VARV ist ausschließlich humanpathogen, hoch ansteckend und verursachte schwere bis tödliche Krankheitsverläufe [1, 2].

Es wird angenommen, dass bereits im alten Ägypten der damalige Pharao Ramses V an Menschenpocken erkrankte und verstarb (1157 vor Christus). Seine Mumie weist etliche pockenähnliche Läsionen auf [3, 4]. Erste zuverlässige Berichte von Erkrankungen an Menschenpocken sind aus China überliefert und bis in das 4. Jahrhundert nach Christus zurückzuverfolgen. Es gibt jedoch zahlreiche Hinweise darauf, dass die Menschenpocken bereits seit dem 3. Jahrhundert vor Christus in China zirkulierten [1]. Überlieferungen aus Indien reichen bis ins 7. Jahrhundert nach Christus zurück. Anschließend, im 10. Jahrhundert nach Christus, breitete sich die Erkrankung nach Südasien bis zum Mittelmeer aus. Eine Verschleppung nach Europa erfolgte wahrscheinlich im 13. Jahrhundert nach Christus [4-6]. Im 16. Jahrhundert nach Christus traten die Menschenpocken dann bereits endemisch auf dem europäischen Kontinent auf; pro Jahr verstarben nach groben Schätzungen ca. 400.000 Menschen [5, 7]. Zeitgleich breitete sich das VARV durch die europäischen Siedler auch in Nordamerika aus [4-6].

Erste Impfungen gegen Menschenpocken wurden bereits um 1000 vor Christus in Asien durchgeführt. Diese als Variolation bezeichnete Impftechnik wurde aber erst durch Lady Mary Wortley Montegue im 18. Jahrhundert bekannt. Sie erlernte die Impftechnik auf einer Reise nach Konstantinopel im Jahr 1720 und brachte ihr Wissen mit in ihre Heimat, dem heutigen Großbritannien. Nachdem Lady Mary Wortley Montegue ihrer 4-jährigen Tochter Krustenmaterial von Menschenpocken inokulierte, breitete sich die Technik in London und im gesamten Königreich aus [7, 8]. Zu dieser Zeit wurde die Technik der Variolation im westlichen Europa stetig weiterentwickelt. Bekannt wurde zum Beispiel Daniel Sutton, der nur noch eine oberflächliche Schnittführung durchführte, um so starke Blutungen und Schmerzen zu vermeiden [2]. Auch Edward Jenner, dessen Eltern an den Menschenpocken verstarben, wandte die Technik der Variolation an und entwickelte sie weiter. Jenner beobachtete, dass Melkerinnen, die Kuhpocken induzierte Läsionen an ihren Händen besaßen, immun gegen Menschenpocken waren. Mit diesem Wissen entnahm er im Jahr 1796 Material aus einer Pockenläsion der Melkerin Sarah Nelms und

gab dieses dem 8-jährigen Jungen James Phipps. Dieser wurde 6 Wochen später infektiösen VARV haltigen Pusteln ausgesetzt und überlebte. Jenner wiederholte seinen Versuch bei weiteren Kindern aus der Nachbarschaft und bewies damit einen Schutz vor der Erkrankung durch Menschenpocken [6, 9]. Diese neue Technik wurde Vakzinierung (*Vaccination*) genannt, abgeleitet vom lateinischen Begriff für Kuh „Vacca“. Das verwendete Impfvirus wurde folglich als Vaccina virus (VACV) bezeichnet [10]. Wenngleich Edward Jenner nicht der erste und einzige Naturwissenschaftler in dieser Epoche war, der die Technik der Vakzinierung verfolgte, so legte er durch seine strikte wissenschaftliche Dokumentation den Grundstein für die Entwicklung aller heute verwendeten Impfstoffe. Im Jahr 1958 wurde ein weltweites Impfprogramm von der Weltgesundheitsorganisation initiiert, mit dem Ziel Menschenpocken auszurotten. Dieses Ziel wurde im Jahr 1980 erreicht: die WHO erklärte VARV für ausgerottet. Der letzte bekannte natürliche Fall trat in Somalia im Jahr 1977 auf [8].

1.2 TAXONOMIE DER FAMILIE *POXVIRIDAE*

Die Familie *Poxviridae* umfasst zwei Unterfamilien: die Unterfamilie *Chordopoxvirinae* beinhaltet Viren, die Vertebraten infizieren, während Mitglieder der Unterfamilie *Entomopoxvirinae* vorranging Insekten infizieren. Die Unterfamilie *Chordopoxvirinae* beinhaltet gegenwärtig elf Genera, wobei Vertreter dreier Genera als zoonotisch gelten: *Orthopoxvirus*, *Parapoxvirus* und *Yatapoxvirus* (Tabelle 1) [11, 12].

Im Weiteren wird speziell auf die Orthopockenviren (OPV) eingegangen. Es werden insbesondere die Altwelt-OPV näher betrachtet, die Spezies aus Afrika und Eurasien beinhalten. Auf die sogenannten OPV der Neuen Welt, Spezies aus Nordamerika, darunter Racoonpox virus, Volepox virus und Skunkpox virus, wird nicht näher eingegangen.

TABELLE 1 Systematische Einteilung der Familie *Poxviridae* (nach [11])

Unterfamilie	Genus	Bekannte Spezies (Auswahl)
Chordopoxvirinae	*Avipoxvirus*	Geflügelpockenvirus (Fowlpox virus; FWPV)
	Capripoxvirus	Lumpy Skin Disease Virus (LSDV) Schafpockenvirus (SPPV) Ziegenpockenvirus (GTPV)
	Centapoxvirus	Yokapox virus (YKV)
	Cervidpoxvirus	Deerpox virus (DPV)
	Crocodylidpoxvirus	Crocodilepox virus (CRV)
	Leporipoxvirus	Myxoma virus (MYXV)
	Molluscipoxvirus	Molluscum contagiosum virus (MOCV)
	Orthopoxvirus	Variola virus (VARV) Vaccinia virus (VACV) **Kuhpockenvirus (Cowpox virus; CPXV)** Affenpockenvirus (Monkeypox virus, MPXV) Mäusepockenvirus (Ectromelia virus; ECTV)
	Parapoxvirus	Pseudokuhpockenvirus (PCPV) Orfvirus (ORFV) Bovines Papular Stomatitis Virus (BPSV)
	Suipoxvirus	Schweinepockenvirus (SWPV)
	Yatapoxvirus	Tanapockenvirus (TNPV) Yabapockenvirus (YMTV)
Entomopoxvirinae	*A- Entomopoxvirus*	*Anomala cuprea*-Entomopoxvirus
	ß- Entomopoxvirus	*Locusta migratoria*-Entomopoxvirus
	γ- Entomopoxvirus	*Aedes aegypti*-Entomopoxvirus

blau = humanpathogene Pockenviren

1.3 Aufbau der Orthopockenviren

Orthopockenviren zählen zu den größten bekannten Viruspartikeln und sind durch ein komplexes Virion charakterisiert. Sie besitzen eine ovale, ziegelstein-ähnliche Struktur und erreichen in der elektronenmikroskopischen Analyse Ausmaße von 360 × 270 × 250 nm [13]. Insgesamt werden für OPV vier verschiedene Partikelformen beschrieben, wobei jedoch nur zwei als infektiös gelten und somit für die Weiterverbreitung eine Rolle spielen: *intracellular mature virus* (IMV) und *extracellular enveloped virus* (EEV) (Abbildung 1) [14]. Die IMV-Partikel setzen sich neben einem bikonkaven Kapsid, welches das

eng mit Proteinen assoziierte Genom als S-förmigen Komplex enthält, aus zwei Lateralkörpern und einer umhüllenden Lipidmembran zusammen. EEV-Partikel besitzen eine zusätzliche Hüllmembran (Abbildung 1) [15, 16]. Beide Partikelformen sind durch unterschiedliche Oberflächen-Glykoproteine gekennzeichnet [17]. Es wird davon ausgegangen, dass IMV-Partikel hauptsächlich beim Übertragungsprozess des Virus auf den Wirt involviert sind, wohingegen die EEV-Partikel vorrangig an der Ausbreitung innerhalb des Wirtes beteiligt sind [18]. OPV unterscheiden sich signifikant von anderen umhüllten Viren, da sie neben ihrer DNA, den Proteinen und Phospholipiden ebenfalls aus Kohlenhydraten aufgebaut sind (ca. 3 %). Sie besitzen eine hohe Tenazität gegenüber verschiedenen Umwelteinflüssen. Besonders gegenüber Austrocknungsprozessen sind OPV resistent. Darüber hinaus ist VACV selbst bei einer Lagerung von 4°C über 35 Jahre hinweg infektiös. Da OPV nur in geringem Maße Lipide enthalten, sind sie ebenfalls sehr stabil gegenüber pH-Schwankungen. [19]

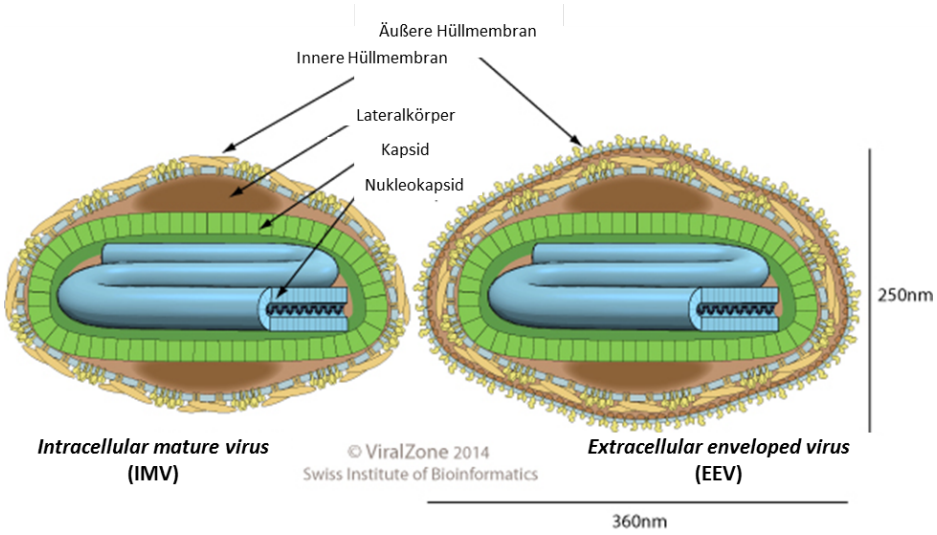

Abbildung 1: Schematischer Aufbau eines Pockenviruspartikels. Bei der *intracellular mature virus* (IMV)-Partikelform ist das bikonkave Kapsid, bestehend aus Genom und zwei Lateralkörpern, von einer Membranhülle umgeben. Wird das Kapsid von 2 Hüllmembranen umschlossen, entsteht die *extracellular enveloped virus* (EEV)-Partikelform (modifiziert nach [20]).

Das Genom von OPV besteht aus einer linearen doppelsträngigen DNA (dsDNA) mit einer Länge von 130-230 Kilobasenpaaren (Abbildung 2) [21]. Von den ca. 200 Genen der Pockenviren sind insgesamt 89 innerhalb der OPV konserviert [22]. Diese gehören vorrangig zum zentralen Bereich des Genoms und kodieren für Proteine, die in Schlüsselfunktionen wie Replikation, Transkription, RNA-Prozessierung, aber auch Zelleintritt, Virusassemblierung sowie Freisetzung von Virionen, eine wichtige Rolle spielen [12]. Die Genomenden bestehen aus sogenannten *inverted terminal repeats* (ITR), welche kovalent miteinander

verbunden sind (Abbildung 2) [21]. Die Gene der terminalen Regionen hingegen sind nicht konserviert und kodieren vorrangig für Proteine, vor allem Virulenzfaktoren, die an der Virus-Wirt-Interaktion beteiligt sind [23, 24].

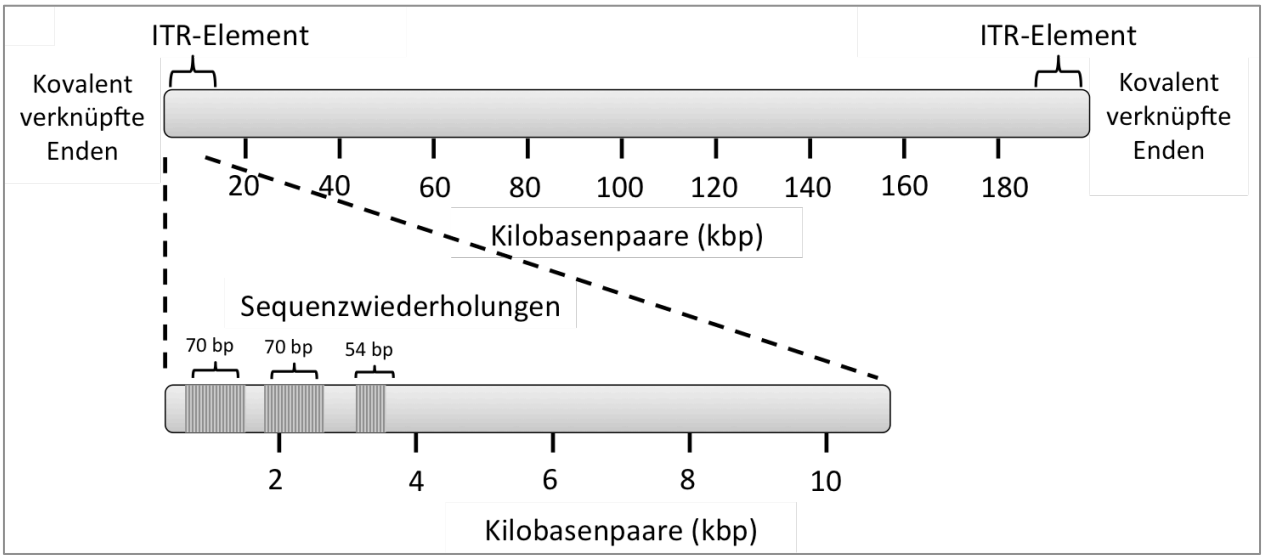

Abbildung 2: Schematischer Aufbau des OPV-Genoms am Beispiel des Vaccinia virus (VACV). Das Genom des VACV besteht aus linearer doppelsträngiger DNA, deren Enden kovalent geschlossen sind. Die Enden wiederum enthalten sogenannte *inverted terminal repeats* (ITR), die aus Sequenzwiederholungen variabler Länge aufgebaut sind.

1.4 Replikation der Orthopockenviren

Die Replikation der OPV ist ein komplexer, weitestgehend konservierter Prozess, der im Zytoplasma der Wirtszelle statt findet, wodurch den OPV eine Sonderstellung innerhalb der DNA-Viren zugeordnet werden kann [15]. Im Folgenden wird der Replikationsprozess von OPV am Beispiel des VACV dargestellt (Abbildung 3). Beide Viruspartikelformen, IMV und EEV, können die Infektion initiieren. Die Bindung der Virionen an die Wirtszellmembran wird durch die Bindung an zelluläre Glykosaminoglykane (GAGs) oder Laminin erreicht [25, 26]. Anschließend werden die Viruspartikel entweder durch Endozytose oder Makropinozytose in die Wirtszelle aufgenommen [25-27]. Der Replikationsprozess verläuft anschließend kaskadenartig und wird in drei Phasen unterteilt: frühe Phase (ca. 20-120 min nach Infektion), mittlere Phase (ca. 100-120 min nach Infektion) und späte Phase (ca. 140 min bis 24 h nach Infektion) [12]. Die frühe Phase beginnt mit dem Eindringen des Viruspartikels in die Wirtszelle; dabei werden im Kapsid des Virions frühe mRNAs gebildet. Die hierfür nötigen Transkriptionsfaktoren sind im Kapsid enthalten. In dieser Phase wird bereits die Hälfte aller viralen Gene transkribiert. Im Anschluss wird das Genom ins

Zytoplasma der Wirtszelle freigesetzt und eine Replikation der Gene der mittleren Phase induziert. Diese Replikation findet in den sogenannten *B-type inclusion bodies* (oder auch Guarnierische Einschlusskörperchen genannt) statt [28]. Hier werden vor allem regulatorische Gene transkribiert, welche wiederum die späte Phase initiieren. Die Gene der späten Phase kodieren überwiegend virale Strukturproteine und Enzyme [29, 30]. Die Viruspartikel werden in den *B-type inclusion bodies* assembliert und anschließend in vom endoplasmatischen Retikulum abgeleiteten Hüllmembranen eingelagert [15]. Die IMV-Partikel werden teilweise noch mit einer weiteren Hüllmembran, abgeleitet vom Golgi-Apparat, umschlossen, wodurch IEV (*intracellular enveloped virion*) entstehen [14]. Bei einigen OPV (CPXV und ECTV) werden die IMV noch zusätzlich in sogenannte *A-type inclusion bodies* eingeschlossen. Dadurch wird eine höhere Stabilität erreicht [18]. Die IEV verlassen die Zelle durch Exozytose, wodurch EEV entstehen. Einige der IEV-Partikel bleiben jedoch an der Wirtsoberfläche haften und bilden die sogenannten CEV (*cell-associated enveloped virion*). Durch Zelllyse werden die IMV-Partikel frei gesetzt [31, 32].

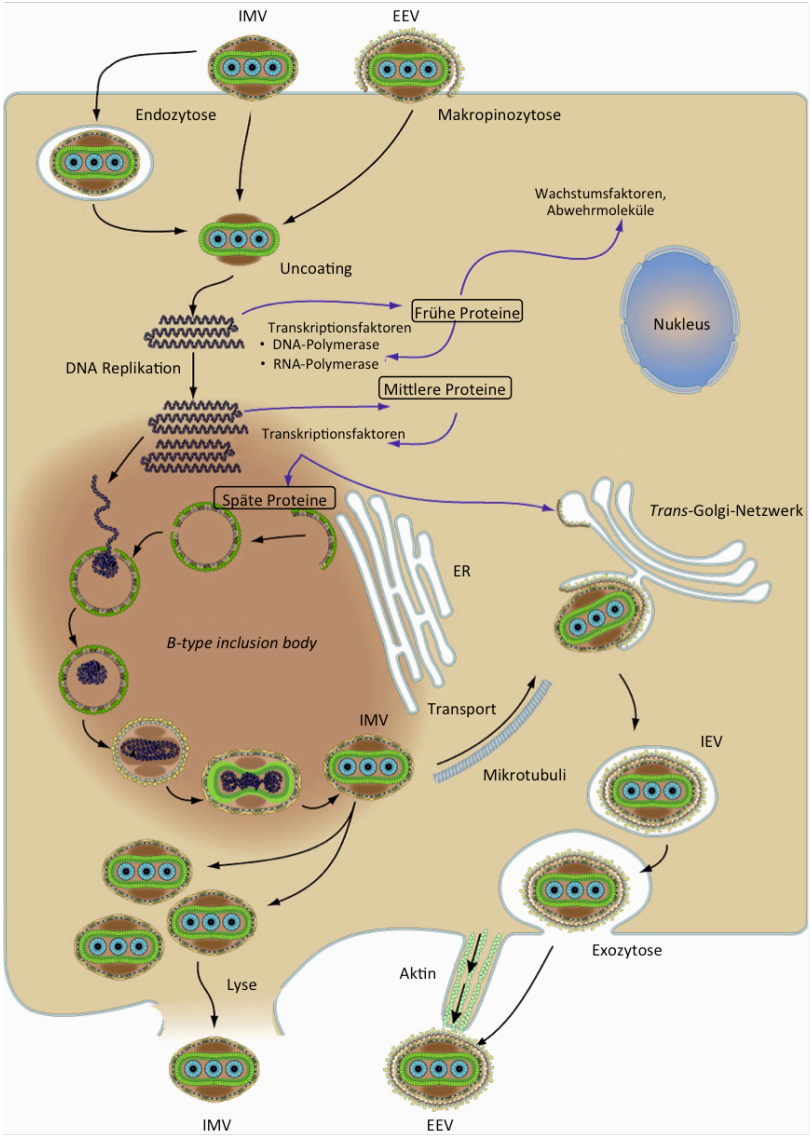

Abbildung 3: Replikationszyklus von Pockenviren. Der Replikationsprozess der Pockenviren läuft kaskadenartig in drei Phasen ab und findet im Zytoplasma der Wirtszelle statt. Nachdem die Pockenviren in die Wirtszelle entweder durch Endozytose oder Makropinozytose eingedrungen sind, bilden sich im Kapsid die frühen mRNAs (frühe Phase), welche die Freisetzung des Virusgenoms in das Zytoplasma initiieren. In sogenannten Virusfabriken (Guarnierische Einschlusskörperchen) werden Gene der mittleren und späten Phase transkribiert. Nach Synthese der Proteine erfolgt die Virusassemblierung und es entstehen IMV-Partikel. Diese werden über Mikrotubuli zum trans-Golgi-Netzwerk transportiert, wo die IMV-Partikel teilweise mit zwei weiteren Membranen umhüllt werden. Die entstandenen IEV-Partikel verschmelzen über ihre äußere Membranhülle mit der Zytoplasmamembran des Wirts, wobei ein Teil der Partikel als CEV-Form an der Zelloberfläche des Wirtes haften bleibt, der andere Teil wird als EEV in die Umgebung freigegeben (modifiziert nach [33]).

1.5 Die Spezies Kuhpockenvirus (CPXV)

Die Spezies Kuhpockenvirus (*cowpox virus*, CPXV) ist bereits seit dem 18. Jahrhundert durch die Impfversuche Edward Jenners bekannt. Heutzutage ist CPXV endemisch in weiten Teilen Europas und Asiens. Innerhalb des Genus *Orthopoxvirus* ist das CPXV durch zwei Besonderheiten gekennzeichnet: es besitzt das längste Genom und das breiteste Wirtsspektrum. Als Reservoirwirt gelten Wühlmäuse, von denen CPXV auf verschiedene akzidentielle Wirtsspezies übertragen werden kann (*spill over*-Infektion)[34].

1.5.1 Klinische Symptome einer CPXV-Infektion

Humane CPXV-Infektionen sind in den meisten Fällen selbst-limitierend und durch lokale Hautläsionen, häufig im Gesicht oder an den Händen, gekennzeichnet. An der Inokulationsstelle bilden sich nach ca. 7-12 Tagen Papeln, die sich zu Vesikeln und später zu hämorrhagischen Pusteln und schwarzen, nekrotischen Krusten entwickeln. Die betroffenen Personen klagen über Grippe-ähnliche Symptome, Übelkeit sowie Muskelschmerzen. Nach ca. 6-8 Wochen haben sich die Patienten von der CPXV-Infektion erholt, die schmerzhaften Krusten trocknen und fallen häufig unter Narbenbildung ab [35, 36]. Bei immunsupprimierten Personen kann es zu einer generalisierten Infektion kommen, die auch letal enden kann [37-39].

CPXV-Infektionen von Tieren verlaufen sehr unterschiedlich. CPXV-Infektionen bei Hauskatzen beginnen z.B. häufig mit einer singulären Primärläsion. Nach ca. 1-2 Wochen entwickeln sich multiple Sekundärläsionen [40, 41]. In seltenen Fällen, wie zum Beispiel bei einer Vorerkrankung mit dem Felinen Immundefizienz-Virus (FIV), kann eine CPXV-Erkrankung auch bei Hauskatzen tödlich verlaufen [42]. Hauskatzen gelten als häufigste CPXV-Infektionsquelle des Menschen [43]. Aber auch *spill over*-Infektionen ausgehend von als Haustieren gehaltenen Ratten wurden in den letzten Jahren, vorrangig aus Mitteleuropa, berichtet [44, 45]. Sogenannte „Schmuseratten“ (*Rattus norvegicus forma domestica*) weisen manchmal Hautläsionen, besonders an den Gliedmaßen, am Schwanz und im Kopfbereich auf, die mit respiratorischen Symptomen einhergehen können [46]. Experimentelle Infektionsstudien zeigen, dass der Krankheitsverlauf in Ratten stark vom verwendeten CPXV-Stamm sowie vom Alter der Tiere abhängig ist [47, 48]. In nicht domestizierten Tieren, wie beispielsweise Zootieren, treten CPXV-Infektionen als schwerwiegende Erkrankung auf und enden häufig tödlich. Fallberichte mit auftretender, ausgeprägter Klinik nach CPXV-Infektion existieren für verschiedenste Spezies wie Elefanten (*Elephas*

maximus) [49-51], Mungos (*Mungos mungo*)[52, 53] oder Neuwelt-Affen (e.g. *Saguinus oedipus; Saimiri sciureus; Callithrix* spp.) [54-56].

CPXV-Infektionen in wildlebenden Nagetierpopulationen verlaufen subklinisch und sind daher bisher wenig untersucht worden. Erste experimentelle CPXV-Infektionsstudien wurden in den 1990er Jahren in Großbritannien durchgeführt. Sowohl Rötelmäuse (*Myodes glaerolus*) als auch Erdmäuse (*Microtus agrestis*) und Waldmäuse (*Apodemus sylvaticus*) waren experimentell für CPXV empfänglich, zeigten jedoch nur minimale klinische Symptome [57].

1.5.2 DIAGNOSTIK

Während der Eradikation der Menschenpocken wurde zum direkten Virusnachweis vorrangig die Methode der Verimpfung der klinischen Proben auf die Chorioallantoismembran (CAM) embryonierter Hühnereier angewendet [58]. Die nach drei Tagen entstandenen Pockenläsionen erlaubten eine Differenzierung zwischen VARV und anderen zoonotischen OPV-Spezies. VARV bildet kleine, opaque weiße Läsionen auf der CAM aus. CPXV entwickelt hingegen große, hämorrhagische Läsionen [59]. Um VARV von anderen Krankheitserregern, wie dem Varizella-Zoster-Virus (einem Herpesvirus), abzugrenzen, wurde zusätzlich die Elektronenmikroskopie eingesetzt. Innerhalb von 30-60 min konnten die zahlreichen Viruspartikel in klinischen Proben anhand ihrer unterschiedlichen Morphologie identifiziert werden [58-60].

Heutzutage erfolgt der direkte Nachweis einer akuten OPV-Infektion hingegen hauptsächlich mittels real-time PCR [59, 61, 62]. Moderne PCR-Ansätze kombinieren dabei eine Genus-spezifische Amplifikation mit einem Spezies-spezifischen Amplifikationsschritt. Bei der im Jahr 2015 von Maksyutov *et al.* entwickelten real-time PCR werden beispielsweise F4L-Primer - spezifisch für OPV - und D8L-Primer - spezifisch für CPXV – eingesetzt, wodurch eine differenzierende Diagnostik möglich wird [63]. Bei positivem CPXV-Ergebnis wird routinemäßig ein Anzuchtversuch des Probenmaterials, beispielsweise auf Verozellen, unternommen [64]. Um CPXV-Isolate im Folgenden zu charakterisieren, werden sie häufig zunächst in der Elektronenmikroskopie hinsichtlich der Bildung von *A-type inclusion bodies* untersucht [65, 66] und das komplette Genom wird mittels *High throughput sequencing* (HTS) bestimmt [67-69]. Indirekte Techniken (z.B. Immunfluoreszenztest, Plaque-Reduktions-Assay, *Enzyme-linked Immunosorbent Assay* (ELISA)) dienen dem serologischen Nachweis einer OPV-Infektion in klinischem Probenmaterial. Die serologischen Ergebnisse werden vor allem für epidemiologische Analysen (z.B. Prävalenzschätzungen) genutzt [64].

1.5.3 WIRTSSPEKTRUM

Bereits im 18. Jahrhundert beschrieb Edward Jenner in seinen Publikationen „*Inquiry*“ und „*Further observations on the Variolae Vaccinae*“, dass CPXV-Infektionen von Milchkühen mit gelegentlichen Übertragungen auf die Hände der Melkerinnen einhergehen [6, 9]. Seit über 40 Jahren sind CPXV-Infektionen von Rindern jedoch eine Seltenheit [64]. Serologische Studien in Kleinsäugern zeigen, dass CPXV-Stämme in Wühlmaus-Populationen zirkulieren (der Begriff „Stamm“ wird im Folgenden für eine der Spezies untergeordnete Einheit genutzt). CPXV-Infektionen scheinen vor allem in Rötelmäusen (*Myodes glaerolus*), Erdmäusen (*Microtus agrestis*), Waldmäusen (*Apodemus sylvaticus*), Feldmäusen (*Microtus arvalis*) sowie in Brandmäusen (*Apodemus agrarius*) verbreitet zu sein [34, 70, 71]. Es wird daher davon ausgegangen, dass Wühlmäuse den Reservoirwirt für CPXV darstellen [34]. Als Reservoirwirt werden Spezies bezeichnet, in deren Populationen ein Pathogen permanent zirkuliert und von denen aus das Pathogen auf akzidentielle Wirte, direkt oder indirekt, übertragen werden kann [72, 73]. Als akzidentielle Wirte wiederum werden Spezies definiert, die auf natürlichem Weg durch ein Pathogen infiziert werden können, jedoch nicht für die Erhaltung des Pathogens notwendig sind [74]. Zu den akzidentiellen Wirten der Spezies CPXV zählen beispielsweise Ratten, Katzen oder auch exotische Zootiere wie Elefanten sowie der Mensch (Abbildung 4) [34, 42].

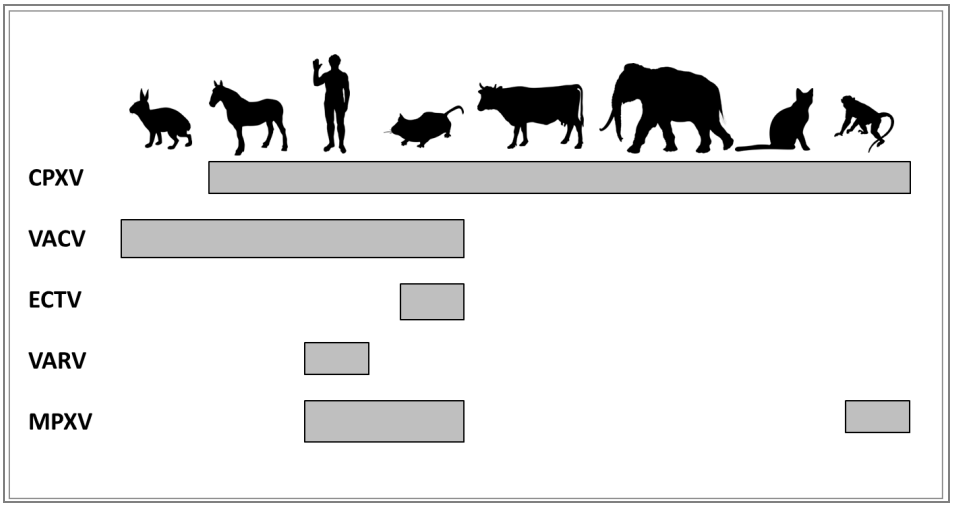

Abbildung 4: Schematische Darstellung der natürlich vorkommenden Wirtspektren von Mitgliedern des Genus *Orthopoxvirus*. Die Spezies Kuhpockenvirus (Cowpox virus, CPXV) kann im Gegensatz zu anderen Mitgliedern des Genus *Orthopoxvirus* verschiedene Wirte auf natürlichem Weg infizieren. Vaccinia virus (VACV) oder MPXV (Monkeypox virus) hingegen besitzen ein wesentlich engeres Wirtsspektrum. Bei ECTV (Ectromelia virus) und VARV (Variola virus) beschränkt sich der Wirt auf nur eine Spezies (ECTV = Maus, VARV = Mensch).

2 ZIELSETZUNG

Kuhpockenviren (CPXV) sind aufgrund ihres sehr breiten Wirtsspektrums und wegen des langen Genoms innerhalb des Genus *Orthopoxvirus* einzigartig. Durch die zahlreichen Genprodukte der variablen Genomenden, welche vorranging in der Virus-Wirt-Interaktion eine Rolle spielen, ist es CPXV möglich, auf natürliche Weise verschiedenste Wirtsspezies zu infizieren. Als Reservoirwirt dienen dabei Wühlmäuse. In diesem Kontext sollten in der vorliegenden Arbeit folgende Aspekte betrachtet werden:

(a) Genetische Variabilität der Spezies CPXV

In Zusammenarbeit mit dem Institut für Mikrobiologie der Bundeswehr wurden insgesamt 20 neue Kuhpockenviren von verschiedenen Säugetierspezies isoliert. Die Genome dieser CPXV-Isolate sollten sequenziert, *de novo* assembliert und umfassend phylogenetisch analysiert werden. Dabei sollte auch der Ursprung der CPXV-Isolate sowie deren Herkunftsort in Zusammenhang gebracht werden. Der CPXV-Stamm Ger2010 MKY, aus einem Neuweltaffen, sollte aufgrund seiner genetischen Besonderheiten näher betrachtet werden.

(b) Rolle des Reservoirwirts

Zunächst sollte ein erstmalig aus der Reservoirwirtsspezies Feldmaus gewonnenes CPXV-Isolat FM2292 eingehend charakterisiert werden. Experimentelle Infektionen in Feldmäusen und Wistar-Ratten waren bereits durchgeführt worden und sollten im Rahmen dieser Arbeit aufgearbeitet und experimentellen Infektionsexperimenten in Rötelmäusen gegenübergestellt werden. Es sollten Rötelmäuse zweier evolutionärer Linien (westliche Linie sowie karpathische Linie) auf verschiedenen Wegen mit unterschiedlichen CPXV-Isolaten infiziert werden. Über einen Zeitraum von bis zu 42 Tagen sollten die Tiere hinsichtlich ihrer Klinik, Virusausscheidung sowie Serologie analysiert werden.

(c) Konsequenz einer CPXV-Infektion beim Pferd

Auf Basis des hier beschriebenen Fallberichtes eines mit CPXV infizierten Fohlens sollte die aktuelle Situation und Gefährdung durch *spill over*-Infektionen besonders in Bezug auf den Menschen dargestellt und diskutiert werden.

3 PUBLIKATIONEN

3.1 CLASSIFICATION OF COWPOX VIRUSES INTO SEVERAL DISTINCT CLADES AND IDENTIFICATION OF A NOVEL LINEAGE

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Classification of Cowpox Viruses into Several Distinct Clades and Identification of a Novel Lineage

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Abstract: Cowpox virus (CPXV) was considered as uniform species within the genus *Orthopoxvirus* (OPV). Previous phylogenetic analysis indicated that CPXV is polyphyletic and isolates may cluster into different clades with two of these clades showing genetic similarities to either variola (VARV) or vaccinia viruses (VACV). Further analyses were initiated to assess both the genetic diversity and the evolutionary background of circulating CPXVs. Here we report the full-length sequences of 20 CPXV strains isolated from different animal species and humans in Germany. A phylogenetic analysis of altogether 83 full-length OPV genomes confirmed the polyphyletic character of the species CPXV and suggested at least four different clades. The German isolates from this study mainly clustered into two CPXV-like clades, and VARV- and VACV-like strains were not observed. A single strain, isolated from a cotton-top tamarin, clustered distantly from all other CPXVs and might represent a novel and unique evolutionary lineage. The classification of CPXV strains into clades roughly followed their geographic origin, with the highest clade diversity so far observed for Germany. Furthermore, we found evidence for recombination between OPV clades without significant disruption of the observed clustering. In conclusion, this analysis markedly expands the number of available CPXV full-length sequences and confirms the co-circulation of several CPXV clades in Germany, and provides the first data about a new evolutionary CPXV lineage.

Keywords: cowpox virus; *Orthopoxvirus*; poxvirus; recombination; phylogeny; genetic diversity; Germany

1. Introduction

The species cowpox virus (CPXV) is a genetically diverse and polyphyletic member of the genus *Orthopoxvirus* (OPV) in the family *Poxviridae* [1]. CPXV is assumed to be the causative agent of cow pox, a zoonotic disease that causes lesions on the udder of dairy cows and the hands of dairymaids. Whether Edward Jenner's effective cross-protective vaccine against human smallpox (variola virus (VARV)) based on a virus belonging to the species CPXV or not is obscure. Later the used protective agent was referred to as vaccinia virus (VACV), whose natural origin has not yet been identified [2]. Differences in the phenotype of lesions on the chorioallantoic membrane of embryonated chicken eggs and the presence or absence of inclusion bodies characterized CPXV and VACV as dissimilar viruses and led to the assignment as species [3] which was later confirmed by restriction fragment length polymorphism

(RFLP) analysis [4]. Sero-surveys showed, that CPXV is endemic in Western Eurasia, and wild rodents, primarily voles, are the reservoir host species [5]. However, the observed biological and experimental host range of CPXV seems to be very broad [6] and spill-over infections to accidental hosts (e.g., rats, cats, cattle, horses, lamas, zoo animals, and humans) are reported regularly, with increasing case numbers for Europe [7]. Recent confirmed zoonotic transmissions of CPXV were mainly caused by direct contact with infected pet rats [8,9], cats [10,11], or zoo animals [12–14]. Although human infections are often mild and self-limiting, immunocompromised patients can develop a systemic and fatal outcome of disease [15–18].

Compared to all other known members of the genus OPV, CPXVs have the largest genome (above 220 kbp) and the most extensive genetic repertoire [19,20]. The central region of the genome is highly conserved and contains genes involved in key functions, such as replication, transcription, and virion-assembly. In contrast, genes located in the terminal genomic regions encode proteins involved in the interaction with the host in order to reduce their anti-viral processes. Therefore, these genes have been described as "virulence genes" [21].

The differentiation of CPXV from other OPV species, such as VACV, monkeypox virus (MPXV), and VARV, was commonly based on phenotypic features like lesions, types of cellular inclusion bodies [22], and RFLP pattern [19]. Nowadays, PCR-based approaches and sequencing of partial genes allows a much more sensitive and robust differentiation for routine diagnostics [23,24]. The advent of high-throughput sequencing approaches resulted in a growing number of available full-length OPV genomes, and phylogenetic analysis indicated, that CPXV is a diverse and polyphyletic group [25–27]. The historically-based unity of CPXV is, therefore, currently under revision and more data from circulating strains is urgently needed.

In order to gain further insights into the evolutionary diversity of CPXV, we determined full-length sequences of 20 CPXV strains that have been isolated over the last seven years from animals and humans in Germany. The provided data and analyses may be used for further re-classification of the genetically versatile species of CPXV.

2. Materials and Methods

2.1. Selection and Isolation of CPXV Strains

During surveillance of the German National Reference Laboratory for Monkeypox (situated within the Friedrich-Loeffler-Institut, Isle of Riems, Germany) several animal-derived samples (taken for routine diagnostics) were subject to CPXV diagnostics. Briefly, DNA was extracted from various organ tissues using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) and OPV specific DNA was detected using a quantitative polymerase chain reaction (qPCR) as described elsewhere [28]. Organ material that scored positive in the qPCR was propagated on Vero76 cells (Collection of Cell Lines in Veterinary Medicine CCLV, Friedrich-Loeffler-Institut). Routine diagnostic analysis at the Bundeswehr Institute of Microbiology led to isolation of four strains: CPXV strain Ger/2007/Vole was derived from the lung tissue of a common vole (*Microtus arvalis*) which had been trapped on a military training ground close to Rottweil (Germany) during a survey to assess the zoonotic potential of rodents. CPXV strains Ger/2015/Human1 and Ger/2015/Human2 were isolated from local lesions on the neck of a veterinary assistant and the head of a farmer, respectively (all samples were taken during routine diagnostics of these cases). In all three cases, virus isolation, genomic DNA extraction and identification as CPXV was performed, as already described for isolate Ger/2014/Human [29]. A summary of isolates used in this study, along with information about the sampling place, year, host, and clinical description can be found in Table 1. Case reports and partial sequences have been described for isolates Ger 2010 MKY [30] and Ger/2014/Human [29].

Table 1. Summary of 20 cowpox virus (CPXV) strains that have been isolated in Germany.

Strain	Sampling Place	Year	Host	Clinical Description
Ger/2007/Vole	Rottweil	2007	Common vole	No clinical signs
Ger/2010/Alpaca	Oberwiesenthal	2010	Alpaca	Diseased
Ger 2010 MKY	Bad Liebenstein	2010	Cotton-top tamarin	Fatal generalization
Ger/2010/Cat	Nordhausen	2010	Cat	Fatal generalization
Ger/2010/Raccoon	Ellrich	2010	Raccoon	Fatal generalization
Ger/2010/Rat	Hannover	2010	Rat	Diseased
Ger/2012/Alpaca	Rositz	2012	Alpaca	Fatal generalization
Ger/2013/Alpaca	Zernitz	2013	Alpaca	Fatal generalization
Ger/2014/Cat1	Bleckede	2014	Cat	Fatal generalization
Ger/2014/Cat2	Nordhausen	2014	Cat	Fatal generalization
Ger/2014/Human	Freiburg	2014	Human	Local lesions
Ger/2015/Cat1	Vogtlandkreis [1]	2015	Cat	Fatal generalization
Ger/2015/Cat2	Rostock	2015	Cat	Local lesions
Ger/2015/Cat3	Vogtlandkreis [1]	2015	Cat	Fatal generalization
Ger/2015/Cat4	Hengelbach	2015	Cat	Local lesions
Ger/2015/Prairie-dog	Dresden	2015	Prairie dog	Local lesions
Ger/2015/Human1	Leipzig	2015	Human	Cervical local lesion
Ger/2015/Human2	Leipzig	2015	Human	Local lesion
Ger/2017/Alpaca1	Brand-Erbisdorf	2017	Alpaca	Fatal generalization
Ger/2017/Alpaca2	Merzdorf	2017	Alpaca	Fatal generalization

[1] Rural district.

2.2. High-Throughput Sequencing

Full-length sequencing of CPXV isolates was conducted as described earlier [27]. In brief, DNA was extracted from infected cell cultures using the High Pure PCR Template Preparation Kit (Roche, Mannheim, Germany) and 0.5–1 µg of DNA was fragmented (mean of 300 bp) using the Covaris M220 ultrasonicator (Covaris, Brighton, UK). Illumina-compatible sequencing libraries were prepared using NEXTflex DNA barcodes (Bioo Scientific, Austin, TX, USA) and SPRIworks Fragment Library Cartridge II (Beckman Coulter, Fullerton, CA, USA) on a SPRI-TE library system (Beckman Coulter). Size exclusion of the library was done manually using Ampure XP magnetic beads (Beckman Coulter) and was controlled on a Bioanalyzer 2100 (Agilent Technologies, Böblingen, Germany) using a high-sensitivity DNA chip and corresponding reagents. A Kapa Library Quantification Kit (Kapa Biosystems, Wilmington, DE, USA) was further used for quantification of the final libraries. Sequencing was performed on an Illumina MiSeq using MiSeq reagent kit, version 2 and version 3 (Illumina, San Diego, CA, USA).

2.3. De Novo Assembly and Genome Annotation

Raw reads were quality trimmed and assembled de novo using the 454 Sequencing System Software (v. 2.8; Roche, Mannheim, Germany), and the resulting contigs were arranged in order to match the CPXV genome. Draft CPXV genomes were further confirmed by reference guided mapping (454 Sequencing System Software) using the "–rst 0" parameter with respect to their repetitive genomic termini. The mean genomic coverage of each full-length CPXV sequence exceeded the minimal acceptable coverage of 20. Full-length CPXV sequences were annotated analogue to the nomenclature of the CPXV Brighton Red reference strain (AF482758) as described elsewhere [27].

2.4. Accession Numbers

Annotated full-length CPXV sequences were uploaded to the European Nucleotide Archive (ENA) and made publicly available under the study accession PRJEB20974.

2.5. Phylogenetic Analysis

A total of 83 full-length OPV sequences, including 63 publicly available and 20 novel sequences from this study, were selected for the phylogenetic analysis. The dataset comprised representative sequences from Old World OPV species camelpox virus (CMLV), CPXV, ectromelia virus (ECTV), MPXV, taterapox virus, VACV, and VARV, as well as New World OPV species, racoonpox virus, skunkpox virus, and volepox virus [1]. Details of the strains used, including accession numbers, can be found in the supplementary material (Table S1). The sequences were initially aligned using the MAFFT plugin (version 7.222; [31]) as incorporated in the Geneious software (version 10.0.9; Biomatters Inc., Aukland, New Zealand, [32]). In order to remove badly aligned or putatively non-homologous regions from the alignment we used the gBlocks program (version 0.91b; [33]) utilizing a minimum block length of five. Subsequently, maximum-likelihood (ML) phylogeny was inferred using IQ-TREE (multicore version 1.5.4; [34]) with options for optimal model selection, considering FreeRate heterogeneity (TVM+R4), and 100,000 ultra-fast bootstrap replicates [35]. Trees were visualized in FigTree (version 1.4.0). Inter- and intra-clade distances were calculated using the uncorrected p-distance with pairwise deletion, as incorporated in MEGA (version 7.0.18; [36]).

Alterations in tree topologies were analyzed by splitting the aforementioned alignment into 28 smaller segments of each 5000 nt and a single segment of 2286 nt. Phylogenetic trees were calculated for each segment as described above and supported by 1000 ultra-fast bootstrap replicates. Based on these trees we subsequently calculated a consensus network [37] using median edge weights and a threshold of 10% as incorporated in SplitsTree4 (version 4.13.1; [38]). In order to further address potential recombination events, a bootscan analysis [39] using the RDP4 program (version 4.85; [40]) was conducted. In detail, potential recombinant sequences were scanned against appropriate reference sequences from each OPV clade over the aforementioned alignment using the Jukes-Cantor substitution model, a sliding window of 5000 nt, a step size of 100 nt and bootstrap support by 100 replicates. The bootstrap cut-off was set to 70%.

2.6. Geographic Analysis

For geographic analysis, the sampling places of 58 (38 public, 20 new) CPXV strains, selected from the aforementioned 83 full-length OPV, were plotted to a map, using ArcGIS Software (version 10.2.2; ESRI, Redlands, CA, USA). A summary of strains used and coordinates can be found in the Supplemental Table S2.

3. Results

3.1. Novel CPXV Isolates

During the years 2010–2017, the German National Reference Laboratory for Monkeypox confirmed a total of 28 CPXV-positive animal cases. From these, 16 CPXV strains could be isolated in Vero76 cell cultures using homogenates of organs of seven cats, five alpacas, a rat, a cotton-top tamarin, a raccoon, and a prairie dog (Table 1). In addition, one isolate was obtained directly from a common vole (*Microtus arvalis*) sampled during rodent screening by the Bundeswehr Institute of Microbiology. Three additional isolates were derived from epidemiologically-independent human cases in Germany. The sampling places of these 20 isolates were mainly located in the Eastern and Southern parts of Germany (Figure 1). From all isolates the full-length genome sequence was determined.

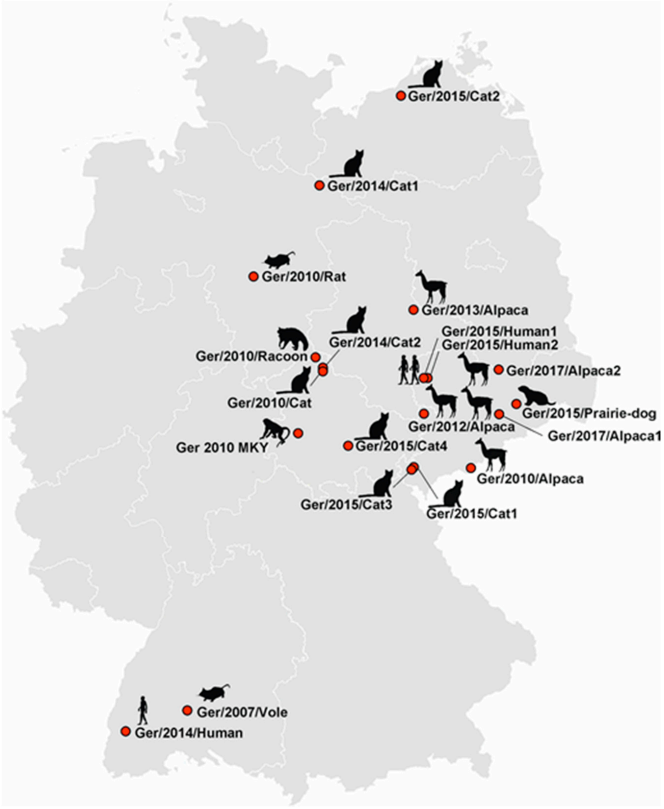

Figure 1. Origin of 20 cowpox virus strains from Germany. Hosts are depicted as black silhouettes accompanied by strain designation.

3.2. CPXV Phylogeny

Together with representative sequences received from public nucleotide archives, a total of 83 full-length OPV sequences were aligned. Discarding nucleotide positions not present in all strains 142,286 nt were used for analyzing the phylogenetic relationship. The resulting phylogenetic tree clearly separated New- and Old-World OPV species into two sister groups (Figure 2A). The phylogenetic distance between both groups (15.4%) was rather high in comparison to the distance observed within them (New-World OPV species: 8.3%, Old-World OPV species: 2.0%), respectively. Regarding the Old-World OPV group, virus strains belonging to the species ECTV, MPXV, VACV, VARV, and CMLV clearly formed distinct clades (Figure 2B). Taterapox (TATV) appeared as a single branch. In contrast, CPXV strains were polyphyletic and did not form a single phylogenetic group. We identified in our analysis four different CPXV clades, which we tentatively named CPXV-like 1, CPXV-like 2, VARV-like, and VACV-like clades. Three of the novel CPXV isolates described here grouped into the CPXV-like 2 clade (15%), and 16 belonged to the CPXV-like 1 clade (80%). The strains CPXV HumLit08/1, CPXV Germany_1998_2, and CPXV Ger 2010 MKY appeared as single branches (Figure 2B, indicated by an asterisk). CPXV Ger 2010 MKY from a cotton-top tamarin is separated from all other clades, and its closest phylogenetic neighbors are virus strains belonging to the species ECTV. We did not observe any relation between the hosts of the analyzed CPXV strains and their phylogenetic clustering into

the clades. Interestingly, the common vole isolate CPXV Ger/2007/Vole was grouped together with CPXV FM2292, another CPXV strain originating from a common vole. The overall nucleotide sequence identity of both vole isolates was about 99% based on the described alignment.

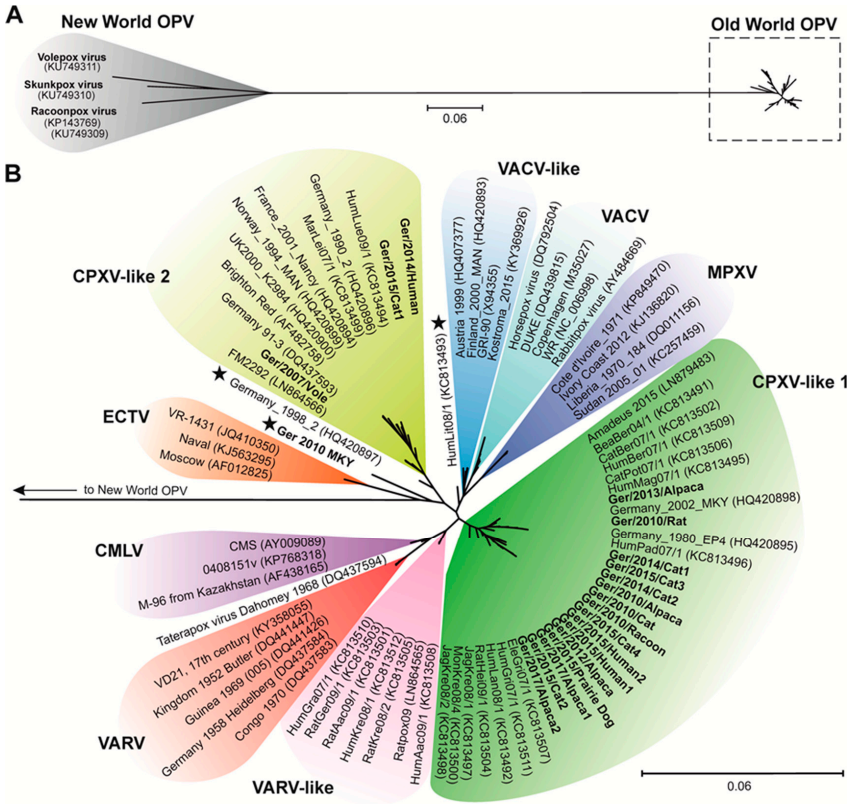

Figure 2. Phylogeny of Orthopoxviruses (OPVs). (**A**) New-World (highlighted grey) and Old-World OPVs (dotted box) were clearly separated by the unrooted phylogenetic tree. (**B**) Enlarged unrooted phylogenetic tree of Old-World OPVs defined the species ectromelia virus (ECTV), monkeypox virus (MPXV), vaccinia virus (VACV), and variola virus (VARV) as monophyletic clades. Cowpox virus (CPXV) strains were polyphyletic and grouped into four different clades (CPXV-like 1, CPXV-like 2, VACV-like, VARV-like). Single-branch CPXV isolates are indicated by a black asterisk. Isolates sequenced in this study appear in bold face. Scales represent substitutions per position. All species and clade segregating branches are supported by bootstrap values of at least 80% (see Supplementary Figure S1).

3.3. CPXV Consensus Network and Bootscan Analysis

A phylogenetic consensus network from the 83 sequences was created to analyze the three CPXV strains appearing as single branch in the phylogenetic tree in more detail. This consensus network is a combination of 29 phylogenetic trees showing incompatibilities between them. The defined four CPXV clades were confirmed and clearly distinguishable from each other. The CPXV strains HumLit08/1, CPXV Germany_1998_2, and CPXV Ger 2010 MKY again appeared as single branches. Nevertheless, the CPXV isolate Germany_1998_2 contained parts from CPXV-like 1 and the CPXV-like 2 clade

showed by the edges of the network. In contrast, the CPXV isolate HumLit08/1 showed edges of the network from the CPXV-clade 1, VARV-like and VACV-like, whereas the new CPXV strain Ger 2010 MKY seemed to be positioned separately (Figure 3).

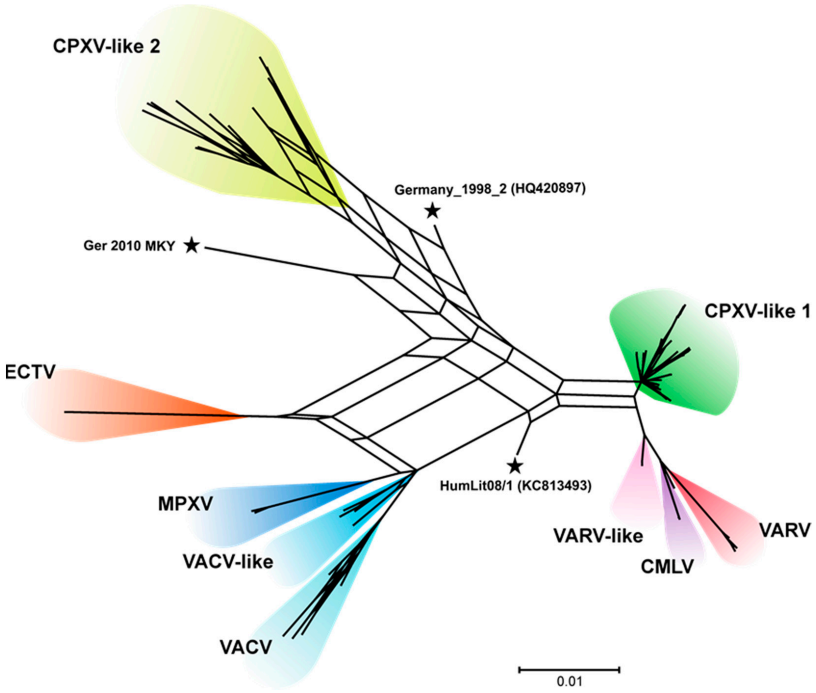

Figure 3. A consensus network calculated from 29 sub-genomic segments of the Orthopoxviruses (OPV) alignment indicates recombination between the clades. The genome alignment of 83 OPV was split into 28 segments of each 5000 nt and a single segment of 2286 nt and phylogenetic trees were constructed for each segment. The trees were further combined into a consensus network with SplitsTree software, visualizing incompatibilities between them. Splits that are conserved within all trees will produce unique bifurcating trees (coloured), while splits that are present in only some of the trees (at least 10%) will result in box-like structures. Box-like structures are therefore created by strains that consist of genomic segments that cluster into different genetic groups. The variable grouping of genomic segments in a single strain might be interpreted as a result of recombination. The established OPV clades were clearly separated, while isolates that appeared as single branches in the conventional phylogenetic analyses (black asterisks) were again grouped between them. Box-like structures between the clades possibly indicate recombination events during their evolution. The scale represents substitutions per position and the New-World OPVs are hidden in the illustration.

These edges, which may be indicators of recombination events, were further analysed by a so-called bootscan analysis. As already indicated by the consensus network, the CPXV strain Germany 1998_2 showed genomic regions which either clustered in the CPXV-like 1 or the CPXV-like 2 clade. The genomic sequence of the CPXV isolate HumLit08/1 grouped within the VACV- or the VARV-like clade. The new strain CPXV Ger 2010 MKY in contrast showed less recombination and can, therefore, be regarded as a CPXV strain establishing a novel CPXV lineage separated from the defined CPXV clades (Figure 4).

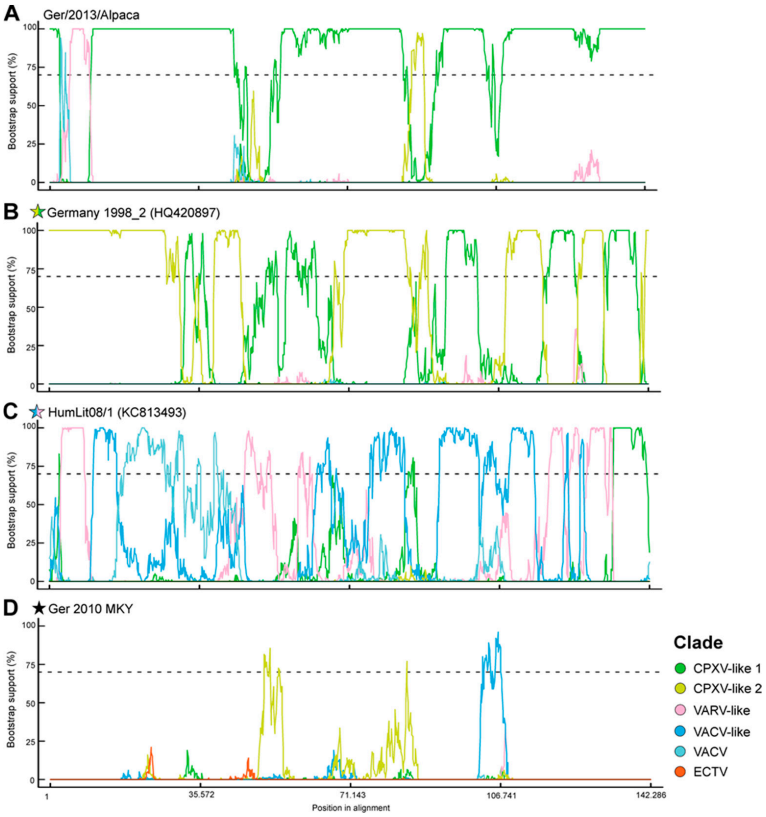

Figure 4. Bootscan analysis performed with RDP4 software reveals potential recombination events between cowpox virus (CPXV) clades. (**A**) The exemplary chosen CPXV isolate Ger/2013/Alpaca (CPXV-like 1) shows only minor influences of recombination. (**B**) Genomic regions of Germany 1998_2 cluster either within the CPXV-like 1 or 2 clade. (**C**) A more complex mosaic-like pattern was observed for the sequence of HumLit08/1 that comprises genomic segments related to VACV-like, VACV, and VARV-like clades. (**D**) In contrast to that, the CPXV isolate Ger 2010 MKY is phylogenetically distant from all of the established clades, resulting in only a few significant groupings.

3.4. CPXV Geographic Distribution

The sampling places of all CPXV strains included in this study (37 publicly available and 20 strains described here) were plotted onto a map of Europe in order to analyse a potential correlation to their phylogeny (Figure 5). The CPXV isolates mainly originated from Germany and the neighbouring countries, Austria and France, as well as Great Britain, Norway, Finland, Lithuania, and Russia. In Central Europe, the observed CPXV isolates were present in all defined phylogenetic clades. In contrast, CPXV strains from Great Britain and Norway only grouped within the CPXV-like 1 clade. Up to now, the CPXV-like 2 clade seems to be restricted to Germany. The VACV-like clade was limited to far eastern parts of Europe and to a single case in Austria. Single-branch CPXV strains that did not cluster in any of the defined clades were found in Germany and Lithuania. Therefore, co-circulation of at least three different CPXV clades within a geographic region was verified, as well as the occurrence of viruses with mixed sequences as a result of recombination events.

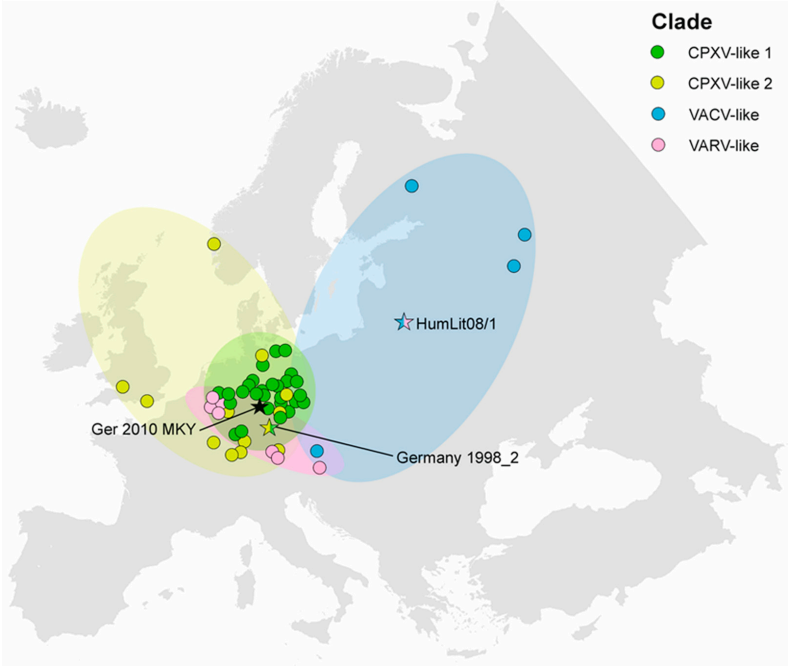

Figure 5. Sampling places of cowpox virus (CPXV) strains from different phylogenetic clades in Europe. The clade CPXV-like 2 seems to be restricted to the Northern and Western parts of Europe, while VACV-like strains are present in Eastern Europe. CPXV-like 1 has so far only been observed within Germany. In Central Europe all phylogenetic CPXV clades, including the VARV-like clade and a putative new clade that is represented by isolate Ger 2010 MKY (black asterik), seems to be co-circulating. The potential recombinant isolates Germany 1998_2 and HumLit08/1 are highlighted by multicolored asteriks according to their genomic composition.

4. Discussion

CPXV is considered as a potential re-emerging zoonotic pathogen in Europe and the annual number of human cases might be rising because the number of immunised humans (against VARV) decreases [41]. To date, numerous CPXV infections of multiple different host species have been recognized all over Western Eurasia, however, less is known about the genetic diversity of circulating clades. Previous studies showed that the species CPXV is indeed a relatively diverse group within the genus OPV and therefore seems to exist in different phylogenetic clusters [20,25,26]. Although the full spectrum of variability is only accessible by full-length genome sequences, the number of them is currently limited. In order to expand the available data, we isolated CPXV strains from different animal species, as well as humans, from Germany and determined their full-length genome sequences by high-throughput sequencing.

4.1. Phylogenetic Analysis

An alignment comprising most of the core genome region was used for phylogenetic and recombination analysis. Our results confirmed that the species CPXV is polyphyletic. There are two clades, which are clearly related to either VARV (VARV-like clade) or VACV (VACV-like clade), in accordance to previous analysis [20,25,26]. In addition, we confirmed two separated clades that

have previously been designated as CPXV-like 1 and 2 [25]. Their grouping and number is fluctuating between the current publications, but seems to be dependent on the number of analysed strains and genomic region. Dabrowski et al. identified two clades based on phylogenetic analysis of a set of highly-conserved genes within either *Poxviruses*, *Chordopoxviruses*, or *Orthopoxviruses* [26]. In an analysis of the core genome region, including intergenic regions, at least three CPXV-like clades have been described [27,42]. In contrast to that, Carrol et al. identified four different CPXV-like groups on basis of only nine CPXV isolates. However, two of these groups consisted of only a single member (Ger 91-3 and Germany 1998_2) [25].

In our analysis, including the currently broadest spectrum of 58 CPXV strains, two CPXV-like clades were identified, that mostly reflected previous groupings. However, the strains Germany 1998_2, HumLit08/1 and Ger 2010 MKY were not assigned to any certain clade, since they clustered in relative remote positions. This might indicate that the actual diversity within CPXV is much higher than reflected by previous analysis and more clades might need to be defined. Another explanation for these single branch strains might be the influence of recombination events that result in conflicting phylogenetic signals. In order to study the latter hypothesis, we addressed the phylogenetic arrangement in smaller segments of the genome and compared them in a consensus network. This type of analysis has been successfully used for detection of recombination events within other large double-stranded DNA viruses, such as human herpesvirus 1 [43]. The overall grouping into the defined clades was confirmed by this analysis, however, a certain degree of phylogenetic instability was observed between the established clades that may indicate recombination among them, especially the three strains that were not assigned into the clade system, grouped separately as a result of incompatibility between their subgenomic phylogeny. Further analysis of CPXV strain Germany 1998_2 showed that genome segments clustered with either the CPXV-like 1 or the CPXV-like 2 clade, whereas for the CPXV isolate HumLit08/1 similarities to both, the VARV-like and VACV-like clade, were observed. Recombination within OPV has, so far, been described in vivo [44,45] and in vitro [46–49], and might explain the observed mosaic genomes. However, a retrospective analysis of recombination is challenging when only limited numbers of sequences are available and the underlying phylogeny is not characterized in detail. Another limitation for this type of analysis is the fact that our alignment mainly comprises the core genome region, neglecting the more variable terminal regions, which are more frequently involved in recombination [20,50]. Recombination events among OPV might be rare, but they have to be considered in future species classification attempts, since they might violate phylogenetic analysis.

In contrast to that, the CPXV isolate Ger 2010 MKY did not display any significant recombination events with other CPXV clades. Considering the results of the recombination analysis and the remote phylogenetic position, it is very likely that Ger 2010 MKY is the prototypic member of a novel CPXV-clade, tentatively designated as "CPXV-like 3". Ger 2010 MKY has been isolated during an outbreak in at least four captive cotton-top tamarins and has already been described as rather low pathogenic for the model species Wistar rats [30]. The unique phylogenetic position of Ger 2010 MKY was initially observed based on phylogenetic analysis of the vaccinia virus homolog F1L. Based on this separated phylogenetic position we, furthermore, investigated the phenotype of the A-type inclusion bodies (V^+), the gene repertoire and the molecular weight of the predicted *atip* gene (150 kD), which were all typical for classical CPXV strains rather than ECTV (data not shown). Further analyses are needed in order to identify additional strains related to this novel lineage and to clarify its (rather accidental) relation to New World Monkeys. Whether this lineage represents a novel species among the OPV is, at the moment, not addressable, but could be topic of a revised nomenclature and classification of the OPVs that is urgently needed.

4.2. Geographic Distribution of CPXV Clades

4.2.1. CPXV Situation in Germany

The origin of CPXV isolates from this study was mainly from the eastern and southern parts of Germany. This, however does not reflect a real CPXV distribution, which is better reflected by the German Animal Disease Reporting System (TSN 3.0, Germany). A total of 97 clinical cases of CPXV-infected animals were reported since 2007 in Germany (date: 20/02/2017) and all German federal states were affected, with the highest case numbers reported in Bavaria (Southwest Germany, 24/97 cases). Therefore, the spatial density of CPXV strains sampling places in this study has to be separated from the actual CPXV scenario in Germany. In addition, the bias caused by international travel and animal transport on spatio-temporal patterns has to be considered, as shown for several human CPXV cases in South and West Germany, and Northern France during 2008, 2009, and 2011 (see Figure 5). The strains from these cases were genetically uniform (VARV-like clade) and attributed to infected pet rats, which probably originated from a breeder in the Czech Republic [9,51–53].

Interestingly, we observed a co-circulation of CPXV-like 1 and CPXV-like 2 clades within Germany. Both clades seem to be present simultaneously in the same region as indicated by isolates Ger/2015/Cat1 (CPXV-like 2) and Ger/2015/Cat3 (CPXV-like 1). These CPXV isolates were derived from infected cats in the autumn of 2015, but are genetically different, as determined by phylogenetic analysis. Since CPXV infections in cats are believed to occur due to transmission from wild rodents during hunting, both isolates may represent adaptations to different species of small mammals that are present in the same area. In contrast, all strains isolated from alpaca-associated cases clustered solely into the CPXV-like 1 clade, although they originated from different parts of Eastern Germany. Whether these New World Camelids are more susceptible to the CPXV-like 1 clade, the spectrum of wild rodents in their direct contact is limited or the CPXV-like 1 clade is more abundant needs to be addressed in further studies. This is especially interesting since the number of New World Camelids held as livestock is growing in Germany and CPXV infections were reported repeatedly [54].

4.2.2. CPXV Situation in Europe

All defined CPXV clades are present in Europe. Until now, in Great Britain only CPXV strains from the CPXV-like 2 clade were found, and a single Norwegian isolate also belongs to the CPXV-like 2 clade. Whether this clade is predominantly found in northern parts of Europe could only be confirmed by analysing more CPXV strains from these regions. In addition, the VACV-clade was only detected in the Eastern part of Europe, as well as in Austria. Again, a lack of appropriate numbers of isolates may account for that phenomenon. As stated above, central Europe seems to be a melting pot with co-circulation of CPXV-like 1, CPXV-like 2, as well as VARV-like clades. Novel zoonotic CPXV-like clades or OPV species might be present in other parts of Europe and Western Eurasia, as currently shown for the Akhmeta virus, which has been isolated from humans and cows in Georgia [55].

5. Conclusions

Over the last years, an increasing number of CPXV infections of humans and animals have been reported in Europe but still little is known about the genetic diversity and geographic distribution of the different CPXV clades. Here, we could show, that phylogenetic analyses based on full-length genome sequences allows a robust classification and differentiation of OPV species, as wells as different CPXV clades. Furthermore, the CPXV strain Ger 2010 MKY was identified as phylogenetically different from any other described CPXV strain and might represent the first member of a novel CPXV clade. It also indicates, that the diversity within the existing CPXV is currently underestimated and novel lineages or recombinants might emerge in geographic areas with co-circulating clades. This is of special interest, since CPXV possess a zoonotic potential and is currently considered as growing human health threat, and new CPXV strains might pose even higher risks concerning the transmission to and adaption within the human host. In the future, the identification and characterization of further CPXV strains

from European countries are necessary to confirm the current phylogenetic picture and to address the question of a common ancestor of all OPV species.

Supplementary Materials: The following are available online at www.mdpi.com/199-4915/9/6/142/s1, Table S1: Sixty-three full-length OPV sequences, included in the phylogenetic analysis; Table S2: Sampling coordinates of 58 CPXV strains; Figure S1: Phylogeny of *Orthopoxviruses*, rooted in New-World species.

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3.2 Out of the Reservoir: Phenotypic and Genotypic Characterization of a Novel Cowpox Virus Isolated from a Common Vole

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Out of the Reservoir: Phenotypic and Genotypic Characterization of a Novel Cowpox Virus Isolated from a Common Vole

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ABSTRACT

The incidence of human cowpox virus (CPXV) infections has increased significantly in recent years. Serological surveys have suggested wild rodents as the main CPXV reservoir. We characterized a CPXV isolated during a large-scale screening from a feral common vole. A comparison of the full-length DNA sequence of this CPXV strain with a highly virulent pet rat CPXV isolate showed a sequence identity of 96%, including a large additional open reading frame (ORF) of about 6,000 nucleotides which is absent in the reference CPXV strain Brighton Red. Electron microscopy analysis demonstrated that the vole isolate, in contrast to the rat strain, forms A-type inclusion (ATI) bodies with incorporated virions, consistent with the presence of complete *ati* and *p4c* genes. Experimental infections showed that the vole CPXV strain caused only mild clinical symptoms in its natural host, while all rats developed severe respiratory symptoms followed by a systemic rash. In contrast, common voles infected with a high dose of the rat CPXV showed severe signs of respiratory disease but no skin lesions, whereas infection with a low dose led to virus excretion with only mild clinical signs. We concluded that the common vole is susceptible to infection with different CPXV strains. The spectrum ranges from well-adapted viruses causing limited clinical symptoms to highly virulent strains causing severe respiratory symptoms. In addition, the low pathogenicity of the vole isolate in its eponymous host suggests a role of common voles as a major CPXV reservoir, and future research will focus on the correlation between viral genotype and phenotype/pathotype in accidental and reservoir species.

IMPORTANCE

We report on the first detection and isolation of CPXV from a putative reservoir host, which enables comparative analyses to understand the infection cycle of these zoonotic orthopox viruses and the relevant genes involved. *In vitro* studies, including whole-genome sequencing as well as *in vivo* experiments using the Wistar rat model and the vole reservoir host allowed us to establish links between genomic sequences and the *in vivo* properties (virulence) of the novel vole isolate in comparison to those of a recent zoonotic CPXV isolated from pet rats in 2009. Furthermore, the role of genes present only in a reservoir isolate can now be further analyzed. These studies therefore allow unique insights and conclusions about the role of the rodent reservoir in CPXV epidemiology and transmission and about the zoonotic threat that these viruses represent.

Cowpox virus (CPXV), a member of the genus *Orthopoxvirus* (OPV) in the *Poxviridae* family, is suspected to be widespread in Western Eurasian rodents, particularly vole species (1, 2). From the presumed reservoir hosts, spill-over infections to accidental hosts are regularly observed (3). The accidental hosts include domestic cats and also exotic animals in zoos, such as large felids and elephants, which regularly develop severe disease (3). As CPXV is a zoonotic virus, humans in direct contact with infected accidental hosts are at risk of infection, while direct infection of humans from presumed reservoir hosts has never been reported. Pet rat-associated human CPXV infections resulted in an epidemiologically linked infection cluster in France and Germany in 2009 (4–6). Interestingly, these human cases resulted from a single CPXV strain that was spread by the infected pet rats and most likely was also responsible for an infection of a woman in France 2 years after the original episode (7). While disease symptoms and virological data are well documented for human infections and infections of domestic and standard laboratory animals, data on the reservoir host consist primarily of serological surveys, with the exception of experimental infection studies in bank voles (*Myodes glareolus*), field voles (*Microtus agrestis*), and wood mice (*Apodemus sylvaticus*) (8, 9). Using a CPXV strain isolated from a cat, only very mild clinical signs were recorded in the experimentally infected rodent hosts, and CPXV could be reisolated from the inoculation sites only (8). Nevertheless, epidemiological surveys based on the serology of different vole populations in Europe demonstrated repeated CPXV infection cycles (10–13). However, CPXV was never

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recovered from the presumed reservoir hosts in Central, Northern, or Western Europe although the presence of viral genomes was verified (14). Indeed, the only reported vole reservoir sample from which CPXV was isolated originated from a Russian root vole (15), but that virus was never characterized in any detail. In addition, whereas laboratory breeds of selected mouse strains are used for CPXV inhibitor research (16) and whereas rats are used for assessment of viral pathogenicity (17, 18), the nature of CPXV infection in common voles has never been documented.

Here, we describe, to our knowledge, for the first time the *in vitro* and *in vivo* characterization of a CPXV strain isolated from a reservoir host, the common vole (*Microtus arvalis*). Whole-genome sequence analysis of the reservoir-derived CPXV allowed sequence comparisons to a rat isolate and the reference strain Brighton Red (BR). Infection experiments using Wistar rats as a surrogate animal model and common voles as the original reservoir host were performed to evaluate differences in pathogenicity levels of virus strains as well as similarities or differences of clinical symptoms. These experiments revealed various pathogenic potentials among the CPXV strains and suggest mild disease but efficient spread of the reservoir isolate and an increase in virulence after a host switch.

MATERIALS AND METHODS

Viruses. CPXV strain FM2292 was isolated from the liver of a female common vole, *Microtus arvalis* (KS11/2292), collected during a large rodent screening project of the network Rodent-borne Pathogens on a grassland site in the federal state of Baden-Wuerttemberg, Germany, in October 2011 (19). The trapping of rodents was coordinated by the Julius Kühn-Institut, Münster, Germany, and approved by the responsible authority, the Regierungspräsidium Stuttgart (Landwirtschaft, ländlicher Raum, Veterinär- und Lebensmittelwesen, permit number BW 35-9185.82/0261). The screening of the rodent samples was performed according to the following protocol. DNA was extracted using a BioSprint 96 instrument and a MagAttract Virus Mini M48 kit (both, Qiagen, Hilden, Germany) from liver samples of individual rodents, and an orthopoxvirus (OPV)-specific quantitative PCR (qPCR) assay was applied (20). The CPXV rat isolate (RatPox09) was obtained from a diseased pet rat, which had infected two humans in southern Germany in 2009, and had already been characterized by experimental infection of Wistar rats (17). CPXV strain BR was used as the reference strain. All CPXV strains were propagated on Vero76 cells (Collection of Cell Lines in Veterinary Medicine [CCLV], Friedrich-Loeffler-Institut, Greifswald-Insel Riems, Germany) and amplified to stock titers of approximately 10^7 50% tissue culture infective doses ($TCID_{50}$) ml^{-1}.

NGS. For next-generation sequencing (NGS), viral DNA was extracted using a High Pure PCR template preparation kit (Roche, Mannheim, Germany) according to the manufacturer's instructions. Subsequently, 0.5 to 1 μg of DNA was fragmented to approximately 300 bp using a Covaris M220 ultrasonicator (Covaris, Brighton, United Kingdom). For library preparation, the fragmented DNA was prepared using NEXTflex DNA bar codes compatible with Illumina systems (Bioo Scientific, Austin, TX, USA) and SPRIworks Fragment Library Cartridge II (Beckman Coulter, Fullerton, CA, USA) on a SPRI-TE library system (Beckman Coulter). As the size selection step was skipped during automated library preparation, upper- and lower-size exclusion of the library was done manually with Ampure XP magnetic beads (Beckman Coulter). The quality of the library was checked on a Bioanalyzer 2100 (Agilent Technologies, Böblingen, Germany) using a high-sensitivity DNA chip and corresponding reagents. Quantity was determined via qPCR with a Kapa Library Quantification kit (Kapa Biosystems, Wilmington, MA, USA). Sequencing was performed on an Illumina MiSeq using a MiSeq reagent kit, version 2 (Illumina, San Diego, CA, USA).

Data analysis. For *de novo* assembly and reference mapping, the Genome Sequencer software suite (version 2.8; Roche, Mannheim, Germany) was used. Assembled contigs were connected according to information in the 454ContigGraph.txt file. For reference mapping against the assembled genome, the -rst 0 parameter (repeat score threshold) was used; therefore, reads were mapped to both inverted terminal repeats rather than to just one.

Genome annotation and comparison. Generated full-genomic sequences were annotated using the nomenclature of CPXV reference strain BR deposited in the NCBI RefSeq database (www.ncbi.nlm.nih.gov/refseq/; complete genome, GenBank accession number AF482758). Open reading frames (ORFs) not annotated in BR were numbered as follows: *gCPXV0XXX* for forward and reverse genes and pCPXV0XXX for proteins. Nomenclature was chosen in relation to BR nomenclature with additional prefixes to distinguish both. Genomic sequences were aligned in Geneious (version 8.0.5) (21) using the MAFFT algorithm (22). A similarity plot of the aligned sequences was generated using the plotcon tool (EMBOSS, version 6.3.1) (23). Parameters were winsize 180, scorefile EDNAFULL, and graph data. Data were normalized to a range of 0 to 1 and plotted in R (version 3.1.1) (24).

Phylogenetic analysis. For determination of the CPXV clade, available full-length genomes of OPVs in GenBank were aligned in Geneious (version 8.1.3) using the MAFFT algorithm (22), and the coding region of vaccinia virus (VACV) strain Copenhagen (VACV-Cop C23L-B29R) (GenBank accession number M35027) as described by Carroll et al. (25) was selected. Phylogenetic analysis was done using IQ-Tree (version 1.2.2) (26) with the best-fitting model and 5,000 ultrafast bootstraps.

Virus growth kinetics. Overnight cultures of Vero, HeLa, rat lung, and bank vole kidney cells (27) were infected with CPXV FM2292, CPXV RatPox09, or BR using multiplicities of infection (MOIs) of 0.01 or 3. After an incubation period of 60 min at 37°C, the cells were washed three times with phosphate-buffered saline (PBS). Afterwards, 1 ml of fresh culture medium (Dulbecco's modified Eagle's medium [DMEM]) was added to each well. In total, samples were obtained at five different time points after infection (0 h, 6 h, 12 h, 24 h, and 48 h). Two technical replicates were done, and the whole experiment was performed in duplicate ($n = 4$ per time point). Virus titers were determined by endpoint dilution and calculated as $TCID_{50}$ per milliliter.

Electron microscopy. For morphological examinations of ultrathin sections, Hep-2 cell cultures (CCLV, Friedrich-Loeffler-Institut) were infected with strain BR, RatPox09, or FM2292 at an MOI of 0.1. Cells were fixed at 36 h postinfection (p.i.) for 60 min with 2.5% glutaraldehyde buffered in 0.1 M Na-cacodylate, pH 7.2 (300 mosmol; Merck, Darmstadt, Germany). Cells were then scraped off the plate and pelleted by low-speed centrifugation and embedded in low-melting-point (LMP) agarose (Biozym, Oldendorf, Germany). Small pieces were postfixed in 1.0% OsO_4(aq) (Polysciences Europe, Eppelheim, Germany) and stained with uranyl acetate. After stepwise dehydration in ethanol, cells were cleared in propylene oxide, embedded in Glycid ether 100 (Serva, Heidelberg, Germany), and polymerized at 59°C for 4 days. Ultrathin sections of epoxy resin-embedded material, counterstained with uranyl acetate and lead salts, were examined with a 120-kV transmission electron microscope (FEI Tecnai Spirit G2; Eindhoven, The Netherlands).

Animal experiments. Mixed-sex Wistar rats at 5 to 6 weeks of age (outbred; Charles River, Sulzfeld, Germany) and 3- to 4-month-old mixed-sex common voles (outbred; Federal Environment Agency, Berlin, Germany) were housed in groups of 2 to 4 animals (rats) or separately (common voles) in standard laboratory rodent cages. All animal experiments were approved by the Ministry of Agriculture of Mecklenburg-Vorpommern, Germany (reference numbers LALLF M-V/TSD/7221.3-2.1.-005/09 and LALLF MV 7221.3-1.1-020/13).

The experimental design complied with the experiments published previously (17, 18). Eleven rats and 9 common voles were infected oronasally with CPXV strain FM2292 in two groups using titers of 10^4 and 10^6 $TCID_{50}$/animal, respectively. Additionally, 10 (5 per dose group) com-

TABLE 1 Comparison of the genomes of CPXV RatPox09 and FM2292 with the genome of the reference strain Brighton Red[a]

Gene group and name	Strain profile[b]		Description
	RatPox09	FM2292	
Genes absent in Brighton Red			
gCPXV0002	+	+	NMDA receptor-like protein[c]
gCPXV0003	+	+	CrmE homologues protein[d]
gCPXV0030	+	+	Putative 7-transmembrane G protein-coupled receptor-like protein 7tGP
gCPXV0284	+	+	Kelch-like protein similar to VACV D7L
gCPXV0285	−	+	Unknown function
Genes present in Brighton Red			
CPXV001/CPXV229	−	−	Unknown function
CPXV002/CPXV228	−	−	Unknown function
CPXV007/CPXV224	−	−	Ankyrin repeat-containing protein
CPXV192	−	−	Unknown function
CPXV004	−	−	Unknown function
CPXV216	−	−	Similar to VACV Western Reserve 204.5
CPXV051A	−	+	Unknown function
CPXV152A	−	+	Unknown function

[a] For strains RatPox09 and FM2292, the genomes are characterized as follows (in respective order): genome sizes of 228,162 and 227,639 bp, ITRs of 7.6 and 7.2 kb, and core genomes of 212,800 and 213,300 bp. RatPox09 has 289 ORFs, and FM2292 has 294 ORFs; of these, 280 ORFs are shared.
[b] Plus and minus signs indicate presence and absence, respectively, of the gene.
[c] *N*-methyl-D-aspartate receptor-like protein.
[d] Cytokine response modifier E homologous protein.

mon voles were inoculated oronasally with CPXV strain RatPox09 (10^4 or 10^6 $TCID_{50}$/animal). Body temperature, weight, and general health status of all animals were checked daily over a period of 30 days, and every other day oropharyngeal swabs (Bakteriette; EM-TE Vertrieb, Hamburg, Germany) were taken. The animals were either humanely killed for autopsy on days 5 or 30 p.i. or when severe signs of disease were apparent. All animals were dissected for collection of organ specimens. Blood was drawn from freshly deceased animals, and peritoneal lavage samples were taken from common voles at 30 days p.i. In addition, inoculation of BR using titers of 10^4 or 10^6 $TCID_{50}$/animal was performed using Wistar rats (12 animals). Swab samples were taken every other day, and the experiment was finalized at 24 days p.i.

Oropharyngeal swab samples were resuspended in 2 ml of cell culture medium supplemented with antibiotics (enrofloxacin, 1 mg/ml; gentamicin, 0.05 mg/ml; lincomycin, 1 mg/ml). Samples of organ tissue were transferred to reaction tubes containing 1 ml of DMEM supplemented with 10% fetal bovine serum (FBS), antibiotics (1% penicillin-streptomycin [PenStrep]), and stainless steel beads (diameter, 5 mm). The samples were subsequently homogenized (TissueLyser II; Qiagen, Hilden, Germany). DNA from all swab and organ samples was extracted using a BioSprint 96 instrument and a MagAttract Virus Mini M48 kit (both, Qiagen) and then quantified by real-time PCR using an OPV DNA-specific protocol (20). In addition, endpoint dilution assays using the Spearman-Kärber algorithm were done for each swab and organ sample. To detect virus-specific antibodies in sera of infected animals, serum samples were incubated for 30 min at 56°C. Subsequently, CPXV virus-infected Hep2 cells (fixed with methanol-acetone at 1:1 and incubated with Tris-buffered saline plus Tween [TBS-T] for 30 min) were incubated for 1 h at room temperature with a 1:200 or 1:500 dilution of serum. After three washing steps with PBS, commercial anti-mouse and anti-rat conjugates as secondary antibodies (both, Life Technologies) were used. Evans Blue (Sigma-Aldrich, Deisenhofen, Germany) was added to stain the cytoplasm of the infected cells.

Nucleotide sequence accession numbers. The complete coding sequences of CPXV FM2292 and RatPox09, with the exception of the terminal loop regions, were deposited in the DDBJ/ENA/GenBank under accession numbers LN864566 and LN864565, respectively.

RESULTS

Isolation of a CPXV from a common vole. During the large-scale screening of rodent tissues, one liver sample of a feral common vole scored positive for the CPXV genome by our diagnostic qPCR. The female common vole was collected during a screening program of rodents in the federal state of Baden-Wuerttemberg in Germany in 2011 (19). Virus isolation using Vero76 cells finally resulted, in the fourth passage, in a high-titer stock (10^7 $TCID_{50}$ ml^{-1}) of the vole isolate named FM2292.

Whole-genome analysis of CPXV from a common vole. The complete coding sequences of CPXV FM2292 (DDBJ/ENA/GenBank accession number LN864566) and RatPox09 (DDBJ/ENA/GenBank accession number LN864565), with the exception of the terminal loop regions, were generated by NGS. The obtained sequences comprise 227,639 bp for FM2292 and 228,162 bp for RatPox09 (Table 1). The inverted terminal repeat regions (ITRs) are approximately 7.2 kbp and 7.6 kbp in length, respectively, which result in core genome regions of 213,300 bp (FM2292) and 212,800 bp (RatPox09) (Table 1). The nucleotide sequence of FM2292 had the highest identity (98%) to a human isolate from 1998 in Germany (GenBank accession number HQ420897), while CPXV isolates FM2292 and RatPox09 have an overall identity of 96%. Both isolates have a nucleotide sequence identity of approximately 92% to the reference CPXV strain BR. Figure 1 shows a similarity plot of all three genomes over a window size of 180 nucleotides (nt). Clearly, and as expected, the core genome parts tend to be more conserved than the 5′ and 3′ ends of the genome (Fig. 1).

Phylogenetic analysis showed that RatPox09 clusters within the variola-like clade while FM2292 is part of the CPXV-like clade 2. BR belongs to the CPXV-like clade 3 (Fig. 2), according to Dabrowski et al. (28) and Carroll et al. (25). Detailed analyses of the complete coding sequences were performed for CPXV strains

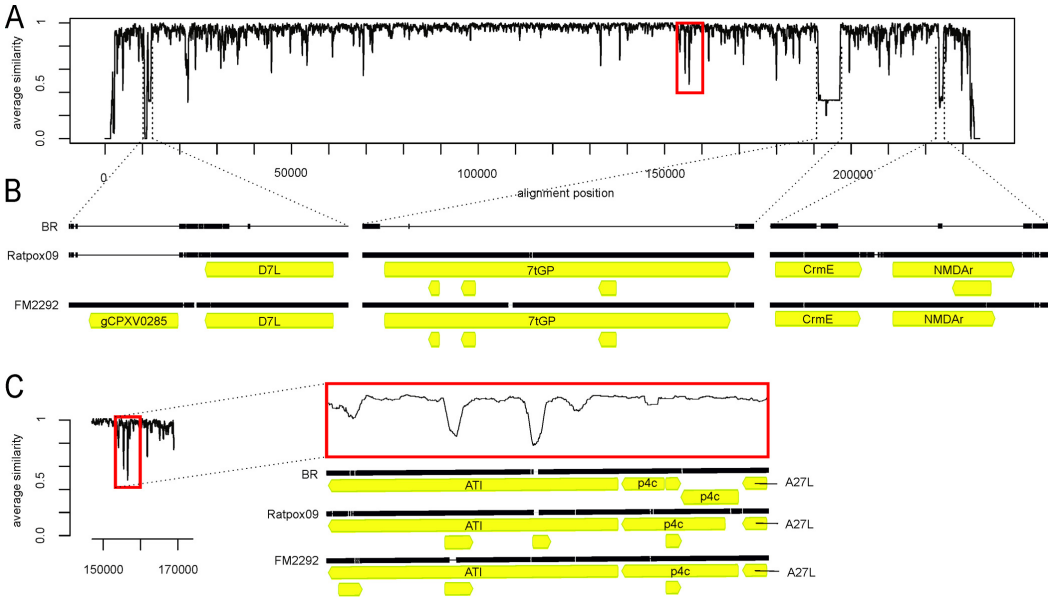

FIG 1 Nucleotide sequence comparison of RatPox09, FM2292, and Brighton Red (BR). (A) Normalized similarity plot of Brighton Red, FM2292, and RatPox09. A value of 1 indicates an identity of 100%, while a value of 0 indicates no conservation at all. Regions of special interest are enlarged. (B) Alignments of genes coding for CPXV0285, D7L-like protein, 7-transmembrane G protein-coupled receptor-like protein (7tGP), CrmE (CPXV0002) protein, and the NMDA receptor-like protein (NMDAr). (C) Alignment of the genes encoding the A-type inclusion protein (ATI), the A26L protein (p4c), and A27L protein.

BR, RatPox09, and FM2292 as these viruses were further characterized in the experimental rat model.

RatPox09 and FM2292 share 280 open reading frames (ORFs), out of a total of 289 ORFs for RatPox09 and 294 ORFs for FM2292 (Table 1). The major differences between the three CPXV isolates are (i) a deletion of approximately 660 bp in BR and RatPox09 from position 10600 to position 11200 relative to FM2292 and (ii) three deletions of approximately 770 bp, 5,800 bp, and 1,000 bp in BR relative to the other two strains at positions 11500 to 12300, 191200 to 197000, and 223500 to 224600, respectively. Hence, one complete ORF (*gCPXV0285*) in BR and RatPox09 and four additional ORFs (*gCPXV0284*, *gCPXV0030*, *gCPXV0002*, and *gCPXV0003*) in BR are absent when compared to the FM2292 genome (Fig. 1B and Table 1). Within the similarity plot (Fig. 1A), these events are clearly indicated by peaks with values below 0.3 at the respective positions.

ORF *gCPXV0030* encodes a putative 7-transmembrane G protein-coupled receptor-like protein (7tGP), a receptor-like protein of unknown function with one predicted C-terminal transmembrane domain, which is encoded only in two other CPXV virus strains, namely, Ger91-3 (GenBank accession number DQ437593; CPXV-like 2 clade) and FRA2001 (GenBank accession number HQ420894; CPXV-like 3 clade).

Only FM2292 and Ger91-3 (both CPXV-like 2) harbor the *gCPXV0285* gene, but there is no known or predicted function for the encoded protein. pCPXV0284 is a kelch-like protein that shares homologies with VACV D7L, which has an unknown function in CPXV. Putative proteins pCPXV0002 and pCPXV0003 are homologues of the OPV cytokine response modifier CrmE and the *N*-methyl-D-aspartate (NMDA) receptor-like proteins, respectively.

The genome of BR harbors 233 annotated ORFs. We did not resolve the sequence of the repeats in the terminal loops and were therefore unable to confirm the presence of *CPXV001*/*CPXV229* (identical ORFs within the ITR) in both rodent CPXV genomes and of *CPXV002*/*CPXV228* in RatPox09 (Table 1). These unresolved tandem repeat regions represent approximately 1,500 bp that are missing in the 3′ and 5′ terminal loop ends of RatPox09 and FM2292 genomes compared to the BR sequence. The most striking feature of the RatPox09 and FM2292 sequences is the absence of the *CPXV004*, *CPXV007*/*CPXV224*, *CPXV192*, and *CPXV216* ORFs that are present in the genome of BR (Table 1). In addition, the *CPXV051A* and *CPXV152A* ORFs, which are present in the BR and FM2292 genomes, are absent in the RatPox09 genome (Table 1). These ORFs were identified as incomplete in the two rodent-derived isolates due to missing start codons within the sequences. These findings are, therefore, not reflected by any peaks as shown in Fig. 1. While the *CPXV216*-encoded protein is similar to the uncharacterized protein of VACV strain Western Reserve 204.5, the *CPXV007*/*CPXV224* ORFs are predicted to encode ankyrin repeat-containing proteins. No putative functions have been assigned to ORFs *CPXV004*, *CPXV051A*, *CPXV192*, and *CPXV152A*. Additionally, various deletions/insertions and substitutions were found in noncoding regions or led to extended or truncated ORFs, for example, in the p4c-coding region, which

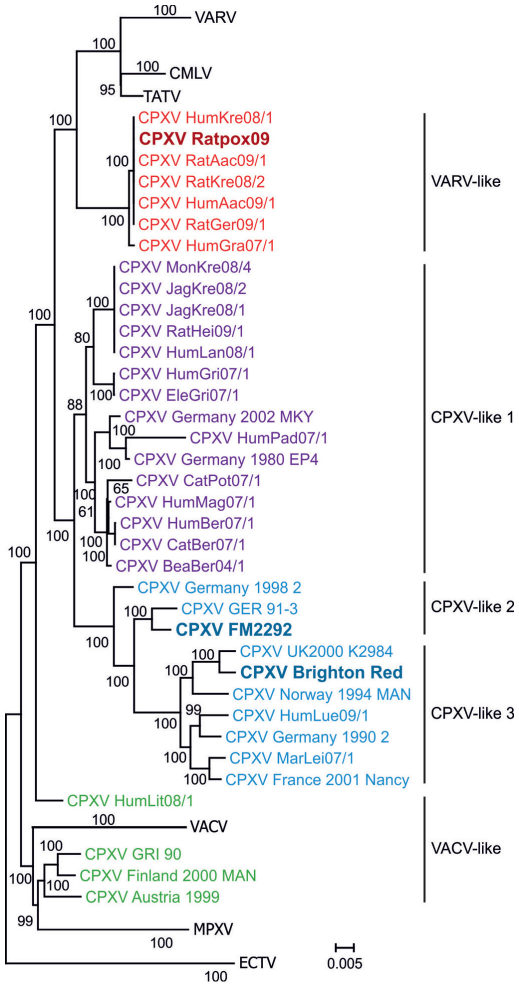

FIG 2 Phylogenetic analysis of whole-genome sequences of orthopoxviruses. CPXV clades (25, 28) are displayed in different colors. RatPox09, FM2292, and BR are highlighted in bold. The variola virus (VARV), camelpox virus (CMLV), taterapox virus (TATV), vaccinia virus (VACV), ectromelia virus (ECTV), and monkeypox virus (MPXV) clusters are presented as collapsed clades and include available whole-genome sequences in GenBank.

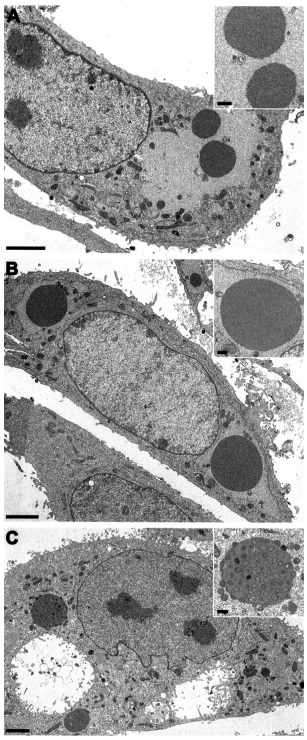

FIG 3 A-type inclusions (ATIs) produced by the three CPXVs. Electron microscopy images of thin sections of Vero76 cells infected with Brighton Red (A), RatPox09 (B), and FM2292 (C) at 36 h postinfection are shown. ATIs were seen in all experiments; however, the V^+ phenotype could be observed only for FM2292 (C). Scale bars: 2.5 μm and 500 nm (inset).

will be presented in detail below. Major differences are additionally summarized in Table 1.

***In vitro* characterization of RatPox09 and FM2292.** Presence or absence of A-type inclusion (ATI) bodies was one of the first properties used to distinguish CPXV from VACV (29). ATI bodies are proteinaceous intracellular aggregates that appear late in infection with some OPVs (30). Three strain-specific phenotypes of ATIs are described: inclusions that have virions embedded within the ATI matrix (V^+), those that lack virions within or on the surface of the ATI matrix (V^-), and those in which virions are attached to the ATI periphery but are absent from the ATI matrix ($V^{+/}$) (31, 32). The ATI phenotypes of CPXV FM2292, RatPox09, and BR were evaluated by electron microscopy. Like CPXV BR, RatPox09 produces the ATI V^- phenotype, where the inclusion bodies lack virus particles incorporated within or located on the surface of the structure (Fig. 3A and B). In contrast, the common vole isolate FM2292 was clearly characterized by having an ATI V^+ phenotype, with numerous virions embedded within the inclusion bodies (Fig. 3C).

It was shown that three proteins, the homologues of the VACV A25L, A26L, and A27L, are necessary for formation of V^+ ATIs. The ATI matrix is formed by multiple copies of the A25L polypeptide, which corresponds to the CPXV158 protein (30). A26L, also named p4c or CPXV159 protein, is required to direct intracellular mature virions (IMVs) into ATIs (33); the protein has a bridging function between the A25L matrix protein and IMVs containing the membrane-associated A27L (CPXV162 protein) (34). In addition, the capability to embed mature virions in the ATIs was recently shown to influence the virulence of CPXV (35). We showed that the RatPox09 p4c amino acid sequence (A26;

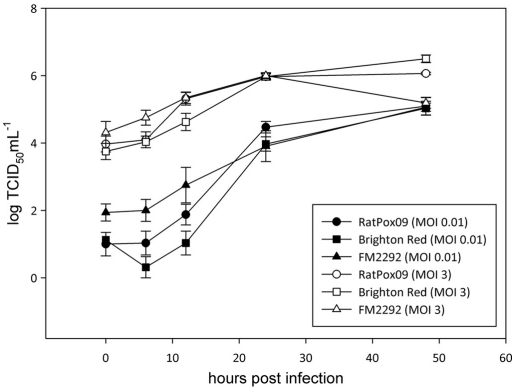

FIG 4 Comparison of replication characteristics of FM2292, RatPox09, and Brighton Red. Vero76 cells were infected with different CPXV strains at an MOI of 0.01 or 3. Infected cells were harvested at several time points postinfection. Virus titers were determined by endpoint dilution assays. The means and standard deviations of two independent experiments are shown, including two technical replicates ($n = 4$).

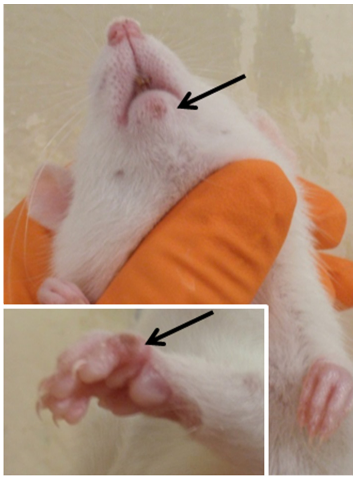

FIG 5 Typical pox-like lesions of a Wistar rat. Wistar rats infected with FM2292 at both doses developed pox-like lesions on the nose, ears, paws, and tail. Images were taken at 9 days p.i. (mouth) and 11 days p.i. (paws).

CPXV159 protein) results in a 59-amino-acid (aa) truncation at the N terminus, which likely is responsible for the observed V^- phenotype; in contrast, FM2292 harbors a full-length *p4c* gene (Fig. 1C). *CPXV162* corresponding to VACV *A27L* (Fig. 1C) and *CPXV159* corresponding to VACV *A25L* (96 to 99% identity) show differences in length and sequence, especially within the repeat regions, but the translational initiation and stop sequences of the two ORFs are identical to each other.

CPXV FM2292 was further characterized in terms of its *in vitro* growth characteristics and compared to RatPox09 and the BR reference strain. In two independent experiments using technical duplicates as internal controls, growth kinetics of the CPXV strains were determined in Vero76 cells at different MOIs. Generally speaking, the three CPXV isolates replicated similarly in cultured cells, irrespective of whether we used an MOI of 0.01 or 3 (Fig. 4). At 48 h p.i. titers of $\sim 10^4$ $TCID_{50}$ ml^{-1} (MOI of 0.01) or $\sim 10^5$ (MOI of 3) were determined. Using an MOI of 3, only the FM2292 virus reached a lower titer at 48 h p.i. ($\sim 10^{4.1}$ $TCID_{50}$ ml^{-1}) than the 24-h value (10^5 $TCID_{50}$ ml^{-1}). In addition, viral growth kinetics were evaluated using HeLa, bank vole kidney, and rat lung cells; however, the shapes of the growth curves for the different viruses were virtually identical in all of the cell types investigated (data not shown).

***In vivo* characterization.** Inoculation of BR into Wistar rats was performed to compare the *in vivo* data of CPXV FM2292 and RatPox09 with those of an experimentally well-defined virus isolate (see, for example, reference 36). For the determination of the *in vivo* characteristics of CPXV FM2292, we infected common voles and Wistar rats. In addition, we also infected common voles with RatPox09 in order to test this zoonotic isolate in a reservoir host species.

After oronasal infection with FM2292 or RatPox09, the first clinical signs were respiratory in nature and included dyspnea, sneezing, and nasal discharge at 4 to 6 days p.i. in all animal groups, i.e., regardless of whether voles or Wistar rats were infected. An exception was the common vole group that had been infected with the low-titer preparation of FM2292, where obvious clinical signs were not observed. In addition, Wistar rats inoculated with strain BR exhibited no or only very mild clinical symptoms. In contrast, common voles inoculated with the high-titer preparation of RatPox09 showed the earliest onset of respiratory symptoms at 4 days p.i. and the most severe course of disease in general. Respiratory signs vanished quickly in common voles infected with high-titer FM2292 and low-titer RatPox09, whereas the average duration of clinical disease in Wistar rats was 16 days in the low-titer FM2292 group and 16 days in the high-titer FM2292 group. In addition to the respiratory symptoms, Wistar rats also developed pox-like rashes on noses, ears, paws, and tails (Fig. 5) at approximately 14 days p.i., which began to heal around 20 days p.i. Interestingly, CPXV infection in common voles was not associated with any obvious skin lesions.

Overall, Wistar rats showed more prominent symptoms after FM2292 infection than the common voles in which subclinical disease was recorded, with the notable exception of one animal in the FM2292 high-dose group. Nevertheless, all Wistar rats survived the infection and recovered. In contrast, 4 of the 5 common voles infected with high-titer RatPox09 virus had to be euthanized between 4 and 8 days p.i. as their health deteriorated dramatically. Two additional common voles, one in the high-titer CPXV FM2292 group and one in the low-titer CPXV RatPox09 group, succumbed to infection. Body temperatures of the individual animals correlated with subnormal temperatures of terminally diseased voles, while such correlation was not detected with the milder symptoms (data not shown). Body weight data demonstrated a clear increase for Wistar rats, while common voles had relatively steady weights throughout the experiment (Fig. 6). Interestingly, body weight increases in rats inoculated with high doses of FM2292 were delayed compared to increases in rats inoculated with the low dose.

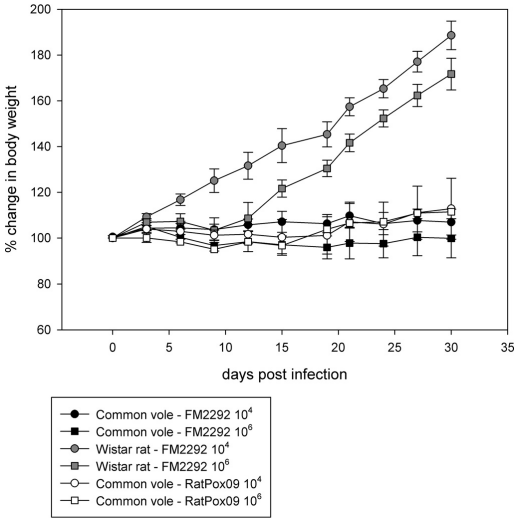

FIG 6 Body weight evolution of common voles and Wistar rats inoculated with FM2292 and RatPox09, respectively. The starting weight was set as 100%, and values are the means with standard deviations.

Oropharyngeal shedding was detected in all animal groups (Fig. 7). Common voles infected with FM2292 showed nearly the same virus shedding pattern as the Wistar rats. Generally, the first excretion of virus was recorded between 4 and 8 days p.i. The largest amount of virus was shed on day 8 p.i. in common voles ($10^{1.375}$ $TCID_{50}$ ml^{-1}) (Fig. 7A) and on day 10 p.i. in Wistar rats (10^{3} $TCID_{50}$ ml^{-1}) (Fig. 7B). Between 10 and 16 days p.i., small amounts of virus were excreted from individual animals ($<10^{1.5}$ $TCID_{50}$ ml^{-1}) (Fig. 7A and B). Only one common vole, inoculated with a high dose of FM2292, had to be euthanized on day 8 p.i. as a consequence of its deteriorating health (Fig. 7A, inset). Viral shedding in this particular animal was 10^{4} $TCID_{50}$ ml^{-1}, while all other animals shed less than $10^{1.5}$ $TCID_{50}$ ml^{-1} of virus.

Viral shedding following RatPox09 infection of the common voles was detected between 2 and 4 days p.i. (Fig. 7C). Generally, animals in the high-dose group shed more virus than those in the low-dose group (Fig. 7C). On day 6 p.i., single voles shed up to $10^{4.5}$ $TCID_{50}$ ml^{-1} (high-dose group) or $10^{3.6}$ $TCID_{50}$ ml^{-1} (low-dose group) of virus. Due to progressive clinical signs, these animals had to be euthanized between 4 and 8 days p.i. (Fig. 7C, inset). The remaining common voles excreted virus at maximal titers from day 4 p.i. until day 8 p.i., and virus titers reached up to $10^{4.56}$ $TCID_{50}$ ml^{-1}. After 12 days p.i., no virus shedding was detected in any of the animals (Fig. 7C). Only Wistar rats inoculated with the high-dose BR preparation shed virus from 5 to 10 days p.i., when small amounts of excreted virus were detected (Fig. 7D).

Virus distribution in various organs was determined by real-time PCR and also by endpoint dilution assay (Tables 2 and 3). All voles that succumbed to CPXV infection scored positive for virus in turbinates (6/6 animals) (Table 2) and rhinarium (5/5 animals) (Table 2). Turbinate samples taken from Wistar rats euthanized at 5 days p.i. were also positive (Table 3). The turbinates showed the highest viral load of all tissues analyzed. Common voles infected with RatPox09 had viral loads of up to 10^{6} $TCID_{50}$ ml^{-1} in this organ, while virus titers in the turbinates of Wistar rats infected with FM2292 reached 10^{4} $TCID_{50}$ ml^{-1}, and BR-infected Wistar rats shed virus at mean titers of $10^{3.875}$ $TCID_{50}$ ml^{-1}. Furthermore, virus was detected in several organ samples of voles that succumbed to the infection (Table 2).

In single FM2292-infected Wistar rats necropsied on day 30 p.i., the skin lesions still contained virus with loads up to $10^{3.875}$ $TCID_{50}$ ml^{-1}, while BR-infected Wistar rats did not display skin lesions, and only the turbinate sample of one animal scored positive ($10^{2.375}$ $TCID_{50}$ ml^{-1}) at 24 days p.i. Likewise, one turbinate sample of a common vole infected with RatPox09 also still contained infectious virus ($10^{1.375}$ $TCID_{50}$ ml^{-1}) at day 30 p.i. Other organs of voles and rats scored positive only when animals succumbed or were examined at 5 days p.i. (Tables 2 and 3). Blood samples from common voles that had succumbed to infection or animals dissected on day 5 p.i. were negative for viral DNA. Antibodies against CPXV were detected in all but one of the individual serum samples obtained, which originated from a common vole in the low-titer CPXV FM2292-infected group (data not shown).

DISCUSSION

The number of human CPXV infections is growing, possibly due to the cessation of smallpox vaccination since its official eradication was made public in 1978 (37). Above all, animal caretakers and veterinarians having contact with infected accidental hosts are at risk of infection with CPXV. Accidental hosts, except humans, become initially infected after direct or indirect contact with infected reservoir host species (3). Despite the presumed continued presence of CPXV in the reservoir hosts, successful virus isolation from wild rodents is extremely rare. In the present study, we characterized CPXV FM2292, which we isolated recently from a rodent reservoir, specifically, a female common vole (*Microtus arvalis*). Using NGS, we generated a whole-genome sequence of FM2292 and compared it to that of RatPox09, a pet rat strain from 2009, and reference strain BR. The FM2292 and RatPox09 strains have a sequence identity of 96%. Both RatPox09 and FM2292 have sequence identities of approximately 92% to the reference strain BR. A first analysis of whole-genome sequences identified four genes that are present in RatPox09 and FM2292 but absent in strain BR, namely, *gCPXV0284*, *gCPXV0002*, *gCPXV0030*, and *gCPXV0003*. As we detected differences in the virulence of RatPox09 and FM2292 compared to that of BR, these four gene products might represent bona fide virulence genes. All genes have homologues in other OPVs and encode the kelch-like protein D7L, the cytokine response modifier CrmE, the putative 7-transmembrane G protein-coupled receptor-like protein 7tGP, and the NMDA receptor-like protein. For example, a contribution to virulence was demonstrated for BTB/kelch proteins (where BTB is broad-complex, tramtrack, and bric a brac) other than D7L (38–43) and for CrmE in the case of VACV (44, 45).

The annotation of the genome of FM2292 revealed that this strain is equipped with additional ORFs compared to the standard BR genome and even to that of the RatPox09 isolate. Clearly, the annotation used here accounts for the increased number of ORFs relative to the number in BR. However, it is clear that FM2292 absolutely and relatively specifies more ORFs (294 in FM2292 versus 289 in RatPox09). The fact that shorter genomes are iso-

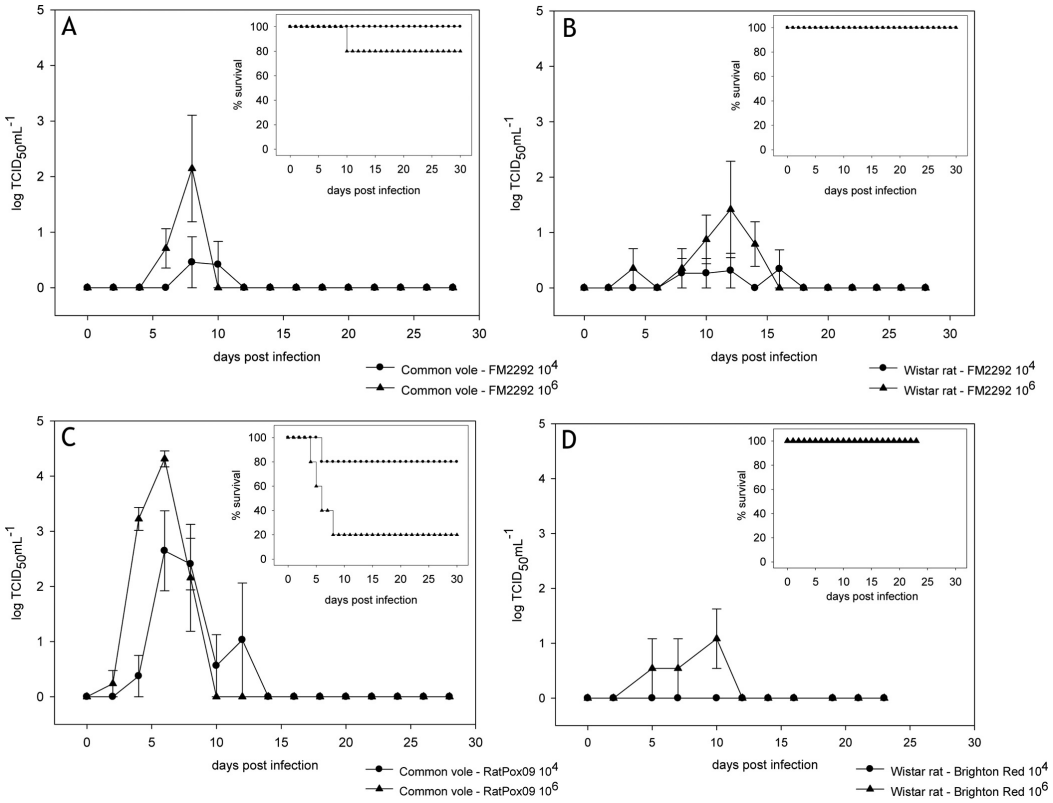

FIG 7 Oropharyngeal viral shedding patterns of common voles and Wistar rats. Shedding patterns are shown for common voles inoculated with FM2292 (A) or RatPox09 (C) and for Wistar rats infected with FM2292 (B) or BR (D). Values are the means with standard deviations.

lated from accidental host species supports the hypothesis of gene loss as a result of adaptation to a new host and may even confirm the hypothesis that CPXV-like genomes represent the phylogenetically oldest representatives of this group of viruses (46, 47). Certainly, the functions of the newly annotated ORFs have to be analyzed in more detail.

The ATI phenotypes of the viruses studied here were explored in more detail, and our analyses revealed that both rodent-derived CPXV strains produced ATI bodies, but only in the case of FM2292 were virions present in the ATI matrix (V^+). In contrast, RatPox09 produced ATIs with no virions in either the ATI matrix or on its surface (V^-). Most natural CPXV strains produce V^+ or V^- ATIs as the dominant but stable phenotype. Recently, the first poxvirus isolate, CPXV-No-H2, was described that produced $V^{+/}$ ATI bodies as a stable phenotype (48). Nevertheless, all other CPXVs isolated from Fennoscandia represent the V^+ ATI phenotype and, hence, encode full-length *ati* and *p4c* genes (49). Since RatPox09, unlike FM2292, was isolated from rats and not a reservoir host, it is tempting to speculate that it may have lost the ability to form V^+ ATIs. Kastenmayer and coworkers (35) constructed and characterized a *p4c* deletion mutant of the GER1991_3 CPXV strain. Mice inoculated intranasally with the mutant lost slightly more weight than mice infected with the parental wild-type virus, but the difference did not reach statistical significance. Consistent with this finding, RatPox09 seemed more virulent than FM2292 in our study, which may at least in part be caused by the *p4c* mutation and the inability of its encoded protein to incorporate virions into ATIs. However, final testing of this hypothesis will require the production of isogenic RatPox09 and FM2292 mutants, experiments that are under way.

From analyzing the growth kinetics of FM2292 in comparison to those of BR and RatPox09, we deduced that replication *in vitro* is not influenced by the differences between the viruses in the number of genes they carry. Whether reservoir hosts, which according to serological data include mainly vole species (the common vole *Microtus arvalis*, the bank vole *Myodes glareolus*, and the field vole *Microtus agrestis*) and wood mice (*Apodemus sylvaticus*) (12), are truly clinically affected by CPXV infection is unknown, but it was suggested that overt infection is unlikely (8, 9). The comparative experimental infection of captivity-bred common voles and regular Wistar rats described here is the first controlled experimental infection with different CPXV strains in reservoir

TABLE 2 qPCR and virus titration results for the infection experiments with CPXV isolates FM2292 and RatPox09 in common voles

Virus and tissue type[a]	Virus distribution by time point and method					
	5 dpi[b]		30 dpi		Death[e]	
	PCR[c]	Endpoint dilution[d]	PCR	Endpoint dilution	PCR	Endpoint dilution
FM2292						
Turbinates	2/3	2/3	3/5	0/5	1/1	1/1
Trachea	0/3	0/3	0/5	0/5	0/1	0/1
Lung	0/3	0/3	0/5	0/5	1/1	0/1
Esophagus	0/3	0/3	0/5	0/5	1/1	0/1
Stomach	1/3	0/3	0/5	0/5	1/1	0/1
Liver	1/3	0/3	0/5	0/5	1/1	1/1
Kidney	0/3	0/3	0/5	0/5	1/0	0/1
Bladder	0/3	0/3			1/1	1/1
Gonads	0/3	0/3			1/1	0/1
Spleen	2/3	0/3	0/5	0/5	1/1	0/1
Thymus	0/3	0/3			1/1	0/1
Skin	0/3	0/3	0/5	0/5	0/1	0/1
Myocardium	0/3	0/3			0/1	0/1
Brain	0/3	0/3			0/1	0/1
Rhinarium	2/3	2/3	0/5	0/5	1/1	1/1
RatPox09						
Turbinates	2/2	2/2	1/3	1/3	5/5	5/5
Trachea	0/2	1/2	0/3	0/3	5/5	4/5
Lung	0/2	0/2	0/3	0/3	2/5	2/5
Esophagus	0/2	0/2	0/3	0/3	3/5	5/5
Stomach	0/2	0/2	0/3	0/3	4/5	3/5
Liver	0/2	0/2	0/3	0/3	5/5	1/5
Kidney	0/2	0/2	0/3	0/3	1/5	0/5
Bladder	0/2	0/2			1/5	2/5
Gonads	0/2	0/2			1/5	0/5
Spleen	0/2	0/2	0/3	0/3	4/5	1/5
Thymus	0/2	0/2			2/5	1/5
Skin	0/2	0/2	0/3	0/3	4/5	3/5
Myocardium	0/2	0/2			0/5	2/5
Brain	0/2	0/2			2/5	2/5
Rhinarium	0/2	0/2	0/3	0/3	4/4	4/4

[a] CPXV infection in common voles was not associated with any obvious skin lesions.
[b] dpi, days postinfection.
[c] Determined by qPCR as the number of positive samples (quantification cycle value of <38)/total number of samples tested.
[d] Number of samples testing positive for CPXV by endpoint dilution assay/total number of samples tested.
[e] Succumbed to infection or had to be euthanized.

hosts instead of an accidental host. CPXV FM2292 isolated from the liver of a common vole was used to inoculate common voles and Wistar rats as a surrogate model and compared to an already characterized virus isolated from a pet rat in 2009 (17). Based on data evaluated after experimental infection of Wistar rats using RatPox09 (17), low- and high-dose infections via the oronasal route were assessed.

Unexpectedly, common voles exhibited clear signs of respiratory disease rather than skin lesions, irrespective of the virus strain used. This might suggest that CPXV could also be transmitted via droplet infection within the reservoir host population and also to accidental hosts. In contrast, Wistar rats inoculated with the reservoir host-derived FM2292 isolate developed severe and characteristic pox lesions, primarily on noses, ears, paws, and tails, but recovered after infection. In addition, body weight increase was delayed in a dose-dependent fashion in Wistar rats inoculated with FM2292. Therefore, FM2292 seems to be less virulent than RatPox09 in the surrogate model, which is also reflected by smaller amounts of virus shed. This is evidenced by the observation that rats inoculated with the high dose of RatPox09 shed up to $10^{4.5}$ $TCID_{50}$ ml^{-1} for up to 12 days (17). Likewise, organ samples of animals in this group yielded higher titers, $>10^{6}$ $TCID_{50}$ ml^{-1} (17), than those of FM2292-infected rats. Similarly, RatPox09 caused higher mortality and more robust shedding in the common vole. The rat-adapted CPXV strain from 2009 has, however, gained virulence in the new host, probably as a result of consecutive animal passages that have occurred primarily in rat breeding farms. However, one out of five low-dose-infected common vole individuals did not show seroconversion, which might indicate that an infectious dose of 10^{4} $TCID_{50}$ ml^{-1} approaches the minimal infectious dose for that species. As common voles inoculated were older than the Wistar rats, body weight increases were not detected, but diseased voles did not show a prominent drop in weight.

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3.3 Experimental Cowpox Virus (CPXV) Infections of Bank Voles: Exceptional Clinical Resistance and Variable Reservoir Competence

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Experimental Cowpox Virus (CPXV) Infections of Bank Voles: Exceptional Clinical Resistance and Variable Reservoir Competence

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Abstract: Cowpox virus (CPXV) is a zoonotic virus and endemic in wild rodent populations in Eurasia. Serological surveys in Europe have reported high prevalence in different vole and mouse species. Here, we report on experimental CPXV infections of bank voles (*Myodes glareolus*) from different evolutionary lineages with a spectrum of CPXV strains. All bank voles, independently of lineage, sex and age, were resistant to clinical signs following CPXV inoculation, and no virus shedding was detected in nasal or buccal swabs. In-contact control animals became only rarely infected. However, depending on the CPXV strain used, inoculated animals seroconverted and viral DNA could be detected preferentially in the upper respiratory tract. The highest antibody titers and virus DNA loads in the lungs were detected after inoculation with two strains from Britain and Finland. We conclude from our experiments that the role of bank voles as an efficient and exclusive CPXV reservoir seems questionable, and that CPXV may be maintained in most regions by other hosts, including other vole species. Further investigations are needed to identify factors that allow and modulate CPXV maintenance in bank voles and other potential reservoirs, which may also influence spill-over infections to accidental hosts.

Keywords: Cowpox virus; reservoir; host; voles

1. Introduction

Over the past 15 years, many new viruses and known viruses have (re-)emerged and are frequently causing zoonotic diseases [1,2]. The viruses are either transmitted to humans from non-human vertebrates (vertebrate-borne diseases) or by arthropods (vector-borne diseases). Cowpox virus (CPXV) is a zoonotic pathogen known to circulate among rodents in Europe [1]. Human CPXV infections are relatively rare [3–9] and CPXV usually causes a self-limiting disease in humans, predominantly lesions on hands or face [3–9]. However, in immunocompromised patients, CPXV infections can readily generalize and result in severe and sometimes lethal infections [4,5].

The species *Cowpox virus* belongs to the genus *Orthopoxvirus* (OPV), subfamily *Chordopoxvirinae*, family *Poxviridae*. CPXV is endemic in Europe and Northern and Central Asia [10,11]. Many mammal species are known to be susceptible to CPXV infection, among them cats [6,12], rats [7–9], alpacas [13], elephants [14], and primates such as cotton-top tamarins [15]. Cats seem to be the main source of

human CPXV infections, although wild rodents, primarily voles, are believed to be the definitive reservoir hosts for the virus [10,11].

Investigations on wild rodents as potential reservoir hosts of CPXV started in England in the 1980s. Until now, serological surveys indicating CPXV infections in wild rodents have been reported for several countries of Eurasia: The United Kingdom [16–18], Belgium [19], Finland [20,21], Norway [22], Germany [21,23], Turkmenia [24], Vietnam [25], Georgia [26] and Hungary [27]. Here, voles (bank vole, *Myodes glareolus* [18,19,21,27], field vole, *Microtus agrestis* [17,21]), and murine rodents such as the striped field mouse, *Apodemus agrarius* [21], wood mouse, *Apodemus sylvaticus* [19,22], and Norway rat (*Rattus norvegicus* [22]), tested positive for CPXV-specific antibodies. Bank voles were shown to reach maximum seroprevalence of 71% in Hungary [27], 64% in Belgium [19], and 72% in the UK [18].

In the UK, different field studies indicated the circulation of CPXV in rodents and demonstrated peaks of infections in bank voles and wood mice, although interspecies transmission was negligible [28]. Correlations of CPXV infection and vole survival [29] or interactions of CPXV and other microparasites in simultaneously infected voles were observed [30]. First experimental infections in the late 1990s revealed that young bank voles (three to five weeks old) developed antibodies between 10 and 14 days post infection (dpi) independently of the inoculation route (CPXV strain L97; intradermal, subcutaneous or oronasal) [31]. In addition, Feore et al. reported that CPXV infections of bank voles reduced fecundity by increasing the time to first litter [32].

However, CPXV has not yet been isolated from vole or mice species other than the common vole (*Microtus arvalis*) [33]. As isolation is one of the criteria supporting the identification of a species being a natural reservoir of a certain pathogen (according to [34]), the role of bank voles in central Europe for CPXV epidemiology is doubtful. Post-glacial colonization of Europe by bank voles from different refuges resulted in the establishment of different evolutionary lineages, with the Western, Eastern and Carpathian lineages in Central Europe [35,36]. The experimental inoculation of the supposed reservoir species resulting in infection and shedding is a criterion that needs to be met for a natural reservoir definition [34]. Our recent infection experiments showed susceptibility of common voles to oronasal CPXV infection, which also resulted in respiratory symptoms and virus excretion [33]. We, therefore, decided to perform similar experimental inoculations with bank voles of different evolutionary lineages and age groups to further determine their potential as putative CPXV reservoir species. The CPXV isolates used here originated from different geographical origin, from accidental hosts (human, rat or cat), and also from one reservoir host species, the common vole. With the polyphyletic nature of the species *Cowpox virus* in mind, members of four CPXV clades (according to [37]) were used. In addition, CPXV was applied by different inoculation routes.

2. Materials and Methods

2.1. Viruses

CPXV strains of different origins (summarized in Table 1) were propagated on Vero76 cells (Collection of Cell Lines in Veterinary Medicine (CCLV), Friedrich-Loeffler-Institut, Greifswald-Insel Riems, Germany).

2.2. Animals

Outbred bank voles (*Myodes glareolus*) originated from in house-breeding and were kept under standardized conditions: type III cages; 22 °C; 12/12 h light cycle, ≈60% humidity; water and rodent pellets ad libitum as the diet. The specific pathogen-free status with regard to CPXV of the breeding colonies was controlled on a regular basis by serological assays. The breeding colonies originated from voles of the Western evolutionary lineage, provided by the Federal Environmental Agency in Berlin, Germany, and voles of the Carpathian evolutionary lineage, provided by Jagiellonian University Krakow, Poland. PCR amplification and sequencing of the partial cytochrome *b* gene following a standard protocol [42] confirmed the different evolutionary lineages (data not shown).

Table 1. Characterization of CPXV strains used for experimental infections.

Isolate (Accession Number)	Host	Origin	Genetic Clade [37]	Reference
Brighton Red (AF482758)	Human	UK, Northern Europe	CPXV-like 2	[38]
RatPox09 (LN864565)	Pet rat	Germany, Central Europe	VARV-like	[39]
Ger 91/3 (DQ437593)	Human	Germany, Central Europe	CPXV-like 2	[40]
Ger/2007/Vole (LT896722)	Common vole	Germany, Central Europe	CPXV-like 2	[37]
FM2292 (LN864566)	Common vole	Germany, Central Europe	CPXV-like 2	[33]
Ger/2010/Cat (LT896729)	Cat	Germany, Central Europe	CPXV-like 1	[37]
FIN_MAN_2000 (HQ420893)	Human	Finland, Northern Europe	VACV-like	[41]

CPXV, cowpox virus; VACV, vaccinia virus, VARV, variola virus.

2.3. Infection Experiments and Sampling

The animal experiments were evaluated by the responsible ethics committee of the State Office for Agriculture, Food Safety and Fishery in Mecklenburg-Western Pomerania (LALFF M-V) and governmental approval was obtained (registration number 7221.3-1.1-020/13, 27 May 2013). The design of all experiments is summarized in Table 2. Initially, we inoculated bank voles of the Western lineage with seven CPXV strains originating from different host species (Tables 1 and 2, experiment #1). The voles were of mixed ages (3 to 4 months or 1-year-old) and mixed sex. Virus was given intranasally at 10^5 $TCID_{50}$/animal. Body temperature, weight, and general health status were checked daily over a period of 21 days. In addition, nasal swabs were taken every other day until 21 dpi by applying a wetted swab onto the rhinarium of the individual vole. Some animals were euthanized for autopsy on 5 dpi or 21 dpi, when different organ samples (rhinarium and nasal epithelia, skin, liver, lung, spleen, trachea) and blood were collected individually.

Further experiments were done to examine the influence of the application route, the age and origin of the voles (Table 2, experiments #2–#5). As indicated, in-contact animals were grouped together with CPXV-inoculated animals in some experiments. After 24 h of separation, contact voles were caged together with CPXV-inoculated animals to determine transmission potential. All nasal swabs were directly suspended in 2 mL cell culture medium (mixture of equal volumes of Eagle MEM (Hanks' balanced salts solution) and Eagle MEM (Earle's balanced salts solution), 2 mM L-Glutamine, nonessential amino acids, adjusted to 850 mg/L $NaHCO_3$, 120 mg/L sodium pyruvate, 10% fetal bovine serum (FBS), pH 7.2; suited for closed tissue culture vessels and incubation under 2.5% CO_2 atmosphere and supplemented with antibiotics: 1% enroflaxin (Bayer, Leverkusen, Germany), 0.5% lincomycin (WDT, Garbsen, Germany) and 0.2% amphotericin/gentamicin (Gibco Life technologies, Carlsbad, Germany). The organ samples were placed into reaction tubes of 1 mL cell culture medium (see above) supplemented with 1% penicillin-streptomycin and a stainless-steel bead (5 mm diameter).

2.4. Analysis of the Samples

Viral DNA loads of all samples were determined by quantitative PCR (qPCR) using OPV-specific primers [43]. Organ tissues were homogenized (TissueLyser II; Qiagen; Hilden, Germany). DNA extraction was done semi-automatically by the BioSprint 96 instrument (Qiagen) using the NucleoMag VET kit (Macherey-Nagel, Düren, Germany). The sera were analyzed by an indirect immunofluorescence (IF) assay to detect OPV-specific antibodies. In brief, serum samples were first inactivated for 30 min at 56 °C. Subsequently, CPXV-infected Hep2 cells (fixed with methanol-acetone at 1:1 and incubated with Tris-buffered saline plus Tween (TBS-T) for 30 min) were incubated for 1 h at room temperature with different serum dilutions (1:20, 1:40, 1:80, 1:160, 1:320). After three washing steps with PBS, a commercial anti-mouse secondary antibody conjugated to Alexa488 (Thermo Fisher Scientific, Waltham, MA, USA) was applied. The cells were visualized under a fluorescence microscope. The titer was taken as reciprocal of the greatest serum dilution, which showed positive detection and those animals with reaction at titers of $\geq$1:40 were considered positive.

Table 2. Design of the animal experiments.

Experiment	Voles			Cowpox Virus				
No./Objective	**Lineage**	**Age**	**Number of Animals per Group**	**Strain**	**Application Route**	**Dose of Inoculum/Animal**	**Duration of the Experiment**	**Link with Results**
# 1 susceptibility to different strains	Western lineage	Up to 1 year	9 or 11	Brighton Red Ger/2010/Cat FM2292 Ger/2007/Vole Ger 91/3 RatPox09 FIN_MAN_2000	Intranasal	10^5 $TCID_{50}$	21 dpi (4/9 or 5/11 animals euthanized for autopsy on 5 dpi)	Tables 3 and 4 and Table S1
# 2 comparison of different application routes	Western lineage	≈1 year	6 and 2 contacts	RatPox09	Intranasal Subcutaneous	$10^{5.5}$ $TCID_{50}$	14 dpi	Table 5 and Table S2
# 3 comparison of different vole lineages	Western lineage	4 weeks	6 and 3 contacts	RatPox09	Intranasal	10^6 $TCID_{50}$	14 dpi	Table S3
	Carpathian lineage	4 weeks	6 and 3 contacts	RatPox09	Intranasal	10^6 $TCID_{50}$	14 dpi	
# 4 comparison of different application routes	Western Lineage	<3 months	6 and 3 contacts	FM2292 RatPox09	Footpad method	10^6 $TCID_{50}$	28 dpi	Table 6 and Table S4
	Carpathian lineage	<3 months	6 and 3 contacts	FM2292 RatPox09				
# 5 reaction on multiple antigen contact	Mixed	<3 months	6	Brighton Red FM2292 RatPox09	Intranasal (Booster on 21 dpi)	$10^{5.5}$ $TCID_{50}$	42 dpi	Table 7 and Table S5

dpi, days post inoculation; $TCID_{50}$, Tissue Culture InfectiousDose 50.

2.5. Statistics

Results were statistically evaluated per groups by ANOVA using SPSS (IBM, Ehningen, Germany). The Tukey HSD test ($p = 0.05$) was performed (SPSS) to determine whether results were significantly different between groups.

3. Results

3.1. CPXV Infection of Bank Voles of the Western Evolutionary Lineage with Different CPXV Strains Induced no Clinical Signs (Experiment #1)

The initial infection experiment (Table 2, experiment #1) did not result in clinical signs when any of the Western lineage bank voles were inoculated intranasally with different CPXV strains. In addition, body weight and body temperature were stable for all animals for the duration of the observation period (data not shown). Most animals developed antibodies, but with varying titers (Table 3, Table S1). Inoculation with the reference CPXV strain Brighton Red or the CPXV isolate FIN_MAN_2000 induced anti-CPXV antibodies in all animals and resulted in the highest antibody titers (up to 1:320, Table S1). In contrast, in the group inoculated with the common vole-derived CPXV strain Ger/2007/vole, only one individual developed antibodies with a low titer of 1:20 (Table S1). Statistical evaluation of antibody titers revealed significant differences of the seropositivity in animals inoculated with Brighton Red compared to Ger 91/3 and Ger/2007/Vole (Table 3). In addition, antibody titers in animals inoculated with FIN_MAN_2000 differed significantly from those in voles inoculated with RatPox09, Ger 91/3 and Ger/2007/Vole (Table 3). The other group comparisons showed no significant differences ($p > 0.05$).

The distribution of virus DNA in different organs was tested by qPCR and the results are summarized in Table 4. On five dpi, viral DNA was detected in the rhinarium and in the trachea in nearly all animal groups (except the voles inoculated with CPXV RatPox09). In addition, in two animals inoculated with CPXV Brighton Red or FIN_MAN_2000, respectively, the lungs also scored positive for viral DNA. Besides the respiratory tract, CPXV DNA could also be found in the skin (1 x CPXV Brighton Red, 1 x CPXV Ger 91/3). Organ samples from autopsy at 21 dpi were all negative (data not shown). Furthermore, no viral shedding was detected in the CPXV-inoculated animals over a period of 21 days (data not shown).

Table 3. Seroconversion rate of CPXV-inoculated bank voles at 21 dpi.

CPXV Strain	Tukey HSD †	Positive Antibody Titers #
Brighton Red		100%
FIN_MAN_2000	*	100%
RatPox09	* * * *	40%
Ger 91/3		67%
Ger/2007/Vole		0%
FM2292		60%
Ger/2010/Cat		67%

† Post-hoc-test between the serological reactivity at different dilutions (Table S1) of the different groups for $\alpha < 0.05$;
# Antibody titers of ≥1:40 were considered positive.

Table 4. Viral DNA detection in different organs at 5 dpi.

CPXV Strain	No. Positive/Total No. of Tested Voles *					
	Rhinarium	Trachea	Liver	Spleen	Lung	Skin
Brighton Red	4/5 (1)	4/5 (2)	0/5	0/5	4/10 (4)	1/5 (1)
FIN_MAN_2000	5/5 (1)	3/5 (1)	0/5	0/5	4/10 (0)	0/5
RatPox09	0/4	0/4	0/4	0/4	08	0/4
Ger 91/3	1/4 (1)	2/4 (1)	0/4	0/4	0/8	1/4 (0)
Ger/2007/Vole	1/4 (0)	2/4 (0)	0/4	0/4	0/8	0/4
FM2292	2/4 (0)	2/4 (1)	0/4	0/4	0/8	0/4
Ger/2010/Cat	2/4 (0)	2/4 (1)	0/4	0/4	0/8	0/4

* Cq values of less than 36 were considered positive. Two lung localisations per animal were analysed. Numbers in brackets refer to Cq values below 30, which is considered as positive for replicating virus.

3.2. Intranasal Inoculation of Western Lineage Bank Voles with RatPox09 Induced a Stronger Antibody Response than Subcutaneous Inoculation (Experiment #2)

The second experiment (Table 2) was limited to the CPXV strain RatPox09 in order to compare the outcome with our previous studies using the same strain in both rats [39] and common voles [33].

Two different application routes (intranasal, as used in experiment #1, and subcutaneous) were tested. Bank voles were checked daily and nasal swabs were taken over a 14-day period. None of the inoculated animals showed any clinical signs and all swab samples as well as all organ samples scored negative by qPCR. Serum antibodies were detected with the IF test (Table 5), with titers up to 1:320 (Table S2). Generally, voles inoculated intranasally developed significantly higher antibody titers than voles inoculated subcutaneously ($p = 0.05$; Table 5 and Table S2). One contact animal, which was housed together with the intranasally inoculated animals, also produced high titers of OPV-specific antibodies (Table S2).

Table 5. Seroconversion rate of CPXV-inoculated bank voles inoculated with CPXV RatPox09 via different routes, and in contact animals at 14 dpi.

CPXV RatPox09		Tukey HSD †	Positive Antibody Titers #
Subcutanous	Inoculated	*	50%
	Contact animals		0%
Intranasal	Inoculated		83%
	Contact animals		50%

† Post-hoc-test between serological reactivity at different dilutions (Table S2) groups for $p < 0.05$; # Antibody titers of ≥40 were considered positive.

3.3. The Evolutionary Bank Vole Lineage Had no Influence on the Results of Intranasal Inoculation with RatPox09 (Experiment #3)

In order to evaluate potential influences of the evolutionary lineage, young voles at an age of four weeks from the Western and Carpathian lineage were inoculated intranasally with CPXV RatPox09 (Table 2, experiment #3). None of the animals excreted virus over a period of 14 dpi as proven by negative swab samples (data not shown). There was also no viral DNA detected in the organ samples (data not shown), and the voles produced only low amounts of antibodies with a titer of up to 1:80 (Table S3) that did not differ significantly between the two vole lineages. In addition, none of the contact animals showed any signs of infection or seroconversion. Interestingly, with the restriction that the results were generated in two independent experiments (experiment #2 and experiment #3),

younger bank voles exhibited significant (ANOVA analysis, p value of 0.00029) lower seroconversion rates compared to adult bank voles (greater than four weeks of age).

3.4. Footpad Inoculation Induced No Clinical Signs, but a Strong Antibody Response, Independent of CPXV Strain and Bank Vole Evolutionary Lineage (Experiment #4)

Next, we investigated the footpad inoculation route, which is widely used in *Vaccinia virus* (VACV) trials. Bank voles from both lineages (all younger than three months) were inoculated via footpad with either CPXV RatPox09 or CPXV FM2292 (Table 2, experiment #4). However, neither viral shedding in the nasal swabs nor viral DNA in the organ samples could be detected (data not shown); however, CPXV-specific antibodies were detected in almost all inoculated animals with titers reaching 1:320 for single animals in each group (Table S4), irrespective of the lineage origin of the individual (data not shown). In addition, one contact animal, housed together with CPXV FM2292-inoculated voles, seroconverted with a high antibody titer (Table S4). Nevertheless, seroreactivity did not differ significantly (ANOVA analysis) between animals inoculated with either virus strain after footpad inoculation (Table 6).

Table 6. Seroconversion rate of bank voles inoculated via the footpad method with either CPXV RatPox09 or CPXV FM2292 (28 dpi); details see Table S4.

Footpad Inoculation		Positive Antibody Titers [#]
CPXV RatPox09	Inoculated	91.7%
	Contact animals	0%
CPXV FM2292	Inoculated	91.7%
	Contact animals	16.7%

[#] Antibody titers of ≥1:40 were considered positive.

3.5. Repeated Inoculations with Different CPXV Strains Resulted in Subclinical Infection with a Strong Antibody Response (Experiment #5)

Finally, a possible booster effect of repeated inoculations was investigated. Therefore, bank voles were inoculated intranasally with either CPXV Brighton Red, CPXV FM2292 or CPXV RatPox09. These strains were selected for this experiment as Brighton Red and FM2292 belong to the same clade CPXV-like 2 (Table 1), while FM2292 and RatPox09 were both isolated from a rodent origin sample. At 21 dpi the initial inoculation was followed by a second intranasal application of the same virus strain (Table 2, experiment #5). Swabs were analyzed over a period of 42 days, but no viral DNA could be detected (data not shown). Half of the CPXV RatPox09-inoculated voles seroconverted. In contrast, all animals inoculated with CPXV Brighton Red or CPXV FM2292 developed OPV-specific antibodies reaching higher levels (Table S5). Statistical evaluation revealed significant differences only between the groups inoculated with CPXV Brighton Red and CPXV RatPox09 (Table 7).

Table 7. Seroconversion rate of bank voles repeatedly inoculated with the same CPXV strain at 42 dpi.

Intranasal Inoculation with Booster	Tukey HSD [†]	Positive Antibody Titers [#]
CPXV Brighton Red		100%
CPXV FM2292	*	100%
CPXV RatPox09		50%

[†] Post-hoc-test between serological reactivity at different dilutions (Table S5) groups for p <0.05; # Antibody titers of ≥40 were considered positive.

4. Discussion

It has been reported that wild rodents are the reservoir hosts for CPXV [10,11,28], and we recently demonstrated the susceptibility of common voles for CPXV by experimental infection [33]. However, bank voles evidently are also affected as shown by serological and molecular surveys in Eurasia [16–23,25–27]. In addition, recent PCR investigations indicated CPXV infections in bank voles mainly of the Western lineage, but also in a single animal of the Eastern lineage ([21]; Fischer, Drewes, Ulrich et al., unpublished data). Nevertheless, in these cases, the genome load was very low and CPXV could not be isolated. Still, little is known about the pathogenesis of CPXV infections in potential reservoir hosts including the bank vole. We therefore conducted a series of experimental infections of bank voles to investigate the susceptibility to CPXV infection in this potential reservoir host, and compared the data to those from recent experiments with common voles [33].

Bank voles from two different evolutionary origins (Western and Carpathian lineage) were infected with various CPXV strains originating from either accidental or natural host species and belonging to different genetic clades (Table 1). Different inoculation routes were used ranging from intranasal to subcutaneous and footpad applications. The experimental layout was limited by the animal numbers available at any one time and, therefore, resulted in several independent experiments. In addition, the available animals were outbred, which may also account for variability between the individual experiments. However, we contend that general patterns of infections are deducible from our experiments and a clear picture emerged concerning the clinical outcome.

4.1. Bank Voles Are Resistant to CPXV-Induced Clinical Signs

Of note, not a single bank vole in the experiments conducted here exhibited any clinical symptoms, although wild rodents have been reported to exhibit clinical signs [24,33,44]; therefore, the asymptomatic course of infection observed here is exceptional. The outcome was independent of the CPXV strain used, the age, the sex, the inoculation route and the bank vole lineage. Our observations support the results of experimental infection of British vole species performed by Bennett et al., which also resulted in subclinical infection [31]. In conclusion, bank voles seem to be one of the most resistant species for CPVX-induced clinical signs.

4.2. CPXV Replication and Shedding Is Very Limited in Bank Voles

Viral shedding was not detected by nasal and buccal swab testing (irrespective of age, sex, virus strain, inoculation route and host lineage), and transmission as evidenced by seroconversion occurred in only 2 out of 18 contact animals (sum of contact animals from all five experiments performed here). These findings contradict previous studies with a different vole species in which we showed that experimentally infected common voles (*Microtus arvalis*) were clinically affected and excreted virus between 4 dpi and 14 dpi via respiratory secretions [33]. Sensitivity of the diagnostic tests were demonstrated in the previous study and therefore did not contribute to low score genome detection. The possible transmission route between individual bank voles remains elusive, and respiratory transmission seems unlikely. Shedding via urine and feces was reported from experimentally infected rats [45], and might be a limited source of infectious virus also for naïve bank voles in the used experimental setup. It cannot be excluded either that shedding below the detection limit might be sufficient to infect in-contact animals, albeit irregularly. As CPXV has high tenacity [46], contaminated materials for example grass or hay, may function as fomites and might be the epidemiological connection resulting in maintenance of the pathogen in their environment.

It is worth noting that not all inoculated animals seroconverted and only some bank voles had titers above 1:80. Even sequential inoculation did not result in seroconversion of all individuals, which indicates that the adaptive immune system of bank voles may not be necessary to control CPXV infection. This is especially true for individuals inoculated with virus strains originating from central Europe (e.g., CPXV RatPox09 or CPXV/2007/Vole). However, there were some differences

detected in bank voles infected with isolates from either Great Britain or Finland (CPXV Brighton Red or CPXV FIN_MAN_2000) compared to the other viral strains used: positive genome loads in the lungs (at least for Brighton Red considered as replicating virus) and 100% seroconversion rates that also were concomitant with higher titers. Strikingly, these virus isolates originate from locations were bank voles, but not common voles, are present. The CPXV-specific seroconversion, therefore, suggests that bank voles constitute as a possible reservoir host. Generally, common voles have a large geographic range extending from Spain across much of Western, Central and Eastern Europe all the way to the Middle East and central Russia [47]. In contrast, common voles are not found in most parts of southern Europe, Fennoscandia, Northern Russia, Iceland and the British isles (apart from the Orkney-islands) [47].

One might speculate from our observation (strains from UK and Finland induce 100% seroconversion in bank voles, that are endemic in UK and Finland) that CPXV strains might be better adapted to a certain main reservoir host in a given geographic region. As a possible consequence, virus strains of Central European origin are mainly adapted to the common vole as reservoir host, while virus strains in Britain or Fennoscandia are more adapted to bank voles. Consequently, in order to obtain virus isolates originating from reservoir host species, common voles should be sampled in Central Europe, while bank voles/field voles and wood mice are species to be sampled in UK and Fennoscandia, respectively. Seroprevalence and molecular survey data indicate CPXV infections are also occurring in bank voles in Central Europe (Belgium [19], Hungary [27]; Kinnunen 2011; our unpuplished data). However, these studies categorized sera as "positive" if titers were 1:20 or higher and could be the result of inefficient replication without efficient transmission. The low genome copy numbers in the very few PCR-positive bank voles in those field studies also support this and are in line with the here reported course of experimental infection. From the comparative data presented here, a robust "cut-off" value of at least 1:40 for scoring sera as reactive against OPV is suggested and may change the number of seropositive animals in field studies.

4.3. Bank Voles as CPXV Reservoir Host

From the results of our extensive infection experiments we conclude that, on the basis of the basic minimal definition of a reservoir host as "being a host that transmits, but is not diseased", bank voles could indeed present a reservoir host of CPXV, although with inefficient transmission to other voles. However, the more precise definition of Haydon et al. [48], defining a reservoir species as "one or more epidemiologically connected populations or environments in which the pathogen can be permanently maintained and from which infection is transmitted to the defined target population", is more challenging. Following this definition, the role of bank voles as a general reservoir host for CPXV is questionable since the maintenance of the pathogen in a population is a prerequisite for a reservoir host species. Our studies indicated only limited transmission to contact animals, ergo facilitating limited maintenance. In contrast to a one host species–one virus association as specified, e.g., for hantaviruses [49], CPXV strains might be therefore maintained by multiple species reservoirs. In our opinion, there is generally no unique reservoir host of CPXV rather than a favorite vole species taking the part of the reservoir within a given geographic region.

The competence of a certain vole species to act as reservoir is dependent on host factors as was shown for the cycling of *Borrelia* ssp. in voles and ticks [50]. Additional factors, including co-infections with bacteria or parasites and a general immunosuppression, might be additive, which will have to be tested in future experimental setups. Turner et al. analyzed interactions between microparasite species in field voles and demonstrated that 79% of CPXV-infected animals were co-infected with either *Bartonella* spp., *Anaplasma* spp. or *Babesia* spp. [29]. Furthermore, stress and fecundity are also most likely important key factors playing a role in the kinetics of viral replication in voles. Regarding seroconversions and some hematological parameters, studies of Beldomenico et al. showed in field voles that a poor body condition significantly increased the probability of CPXV-infection, especially for males [30].

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In general, experimental infections of reservoir host species are a prerequisite for the dynamic modeling of infectious (zoonotic) diseases. Virus tropism obviously differs between natural reservoirs and accidental host species, and we posit that reservoir studies as conducted here are fundamental. Future studies will have to focus onto the identification of viral genetic markers involved in the interaction of the reservoir host species and their "matching" CPXV strains. In addition, bank vole host factors influencing the level of CPXV replication will have to be analyzed in more detail, and we propose to particularly evaluate the contribution of co-infection and immunosuppression. Finally, the factors preventing bank voles from clinical signs after CPXV infection should be studied, including the role of innate immunity.

Supplementary Materials: Supplementary tables are available online at www.mdpi.com/1999-4915/9/12/391/s1.

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3.4 FATAL COWPOX VIRUS INFECTION IN AN ABORTED FOAL

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CASE STUDY

Fatal Cowpox Virus Infection in an Aborted Foal

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Abstract

The article describes the isolation of a cowpox virus (CPXV) isolate originating from a horse. The skin of a foal, aborted in the third trimester, displayed numerous cutaneous papules. The histological examination showed A-type inclusion bodies within the lesion, typical for CPXV infections. This suspicion was confirmed by real-time PCR where various organs were analyzed. From skin samples, virus isolation was successfully performed. Afterwards, the whole genome of this new isolate ''CPXV Amadeus'' was sequenced by next-generation technology. Phylogenetic analysis clearly showed that ''CPXV Amadeus'' belongs to the ''CPXV-like 1'' clade. To our opinion, the study provides important additional information on rare accidental CPXV infections. From the natural hosts, the voles, species such as rats, cats, or different zoo animals are occasionally infected, but until now only two horse cases are described. In addition, there are new insights toward congenital CPXV infections.

Key Words: Cowpox virus—Virology—Zoonosis.

The species *Cowpox virus* (CPXV) belongs to the genus *Orthopoxvirus* (OPV) and the family *Poxviridae*. The definitive reservoir hosts for CPXV are wild rodents and voles. But spill-over infections can occur in other species, including rats, cats, various zoo animals, and primates, including humans and are, therefore, named ''accidental hosts.'' Domestic cats and pet rats are now the most common accidental hosts. However, infections in other domestic animals such as dogs and horses are rare (Vorou et al. 2008; Essbauer et al. 2010).

There are only two reported cases in horses. One case involved a 7-year-old CPXV-infected-Arabian horse in Zwickau (Germany) with typical signs including pox lesions distributed throughout the body surface (Pfeffer et al. 1999). There was a second CPXV case documented in Germany in 2001. The animal was a premature foal with weakness, low body temperature, and gasping respiration. However, the animal did not display any typical pox lesions (Ellenberger et al. 2005). In addition to these CPXV cases, there was also one *Horsepox virus* (HPXV) case documented in Mongolian horses in 1976 (Tulman et al. 2006).

In this case report, we describe a CPXV infection of a pregnant warm blood mare that resulted in an abortion. The female foal was aborted within the third trimester (weight 19.5 kg) at the end of February 2015. Macroscopic evaluation revealed multifocal and occasionally coalescing brown-reddish papules with a maximum diameter of 1.2 cm distributed throughout the entire body surface including the mucocutaneous junctions and the oral cavity. There were also multifocal central hemorrhages present, and there were several larger lesions covered by crusts (Fig. 1A).

We collected tissues from various organs including the placenta, lung, liver, spleen, thymus, and skin for further pathological, virological, and molecular investigations. The tissue samples for histopathology were fixed in 4% formalin and embedded in paraffin. The tissue sections were cut 5 μm thick, dewaxed, and stained with hematoxylin and eosin following standard protocols. The microscopic examination of the epidermis revealed multifocal thickened regions with swollen or degenerated keratinocytes and multifocal hemorrhages. The basal epidermal layers and follicular epithelia contained numerous cytoplasmic eosinophilic inclusion bodies (Fig. 1B, C). These A-type inclusions or Downie bodies are typical of CPXV infections.

DNA was extracted from all organs and tested by real-time PCR using a protocol that can differentiate between CPXV and other OPV (Maksyutov et al. 2015). The Cq value (Cq = cycle of quantification) is the cycle in which fluorescence increased above background. The Cq values measured were less than 22 in all organs. The low Cq values indicate there is high CPXV

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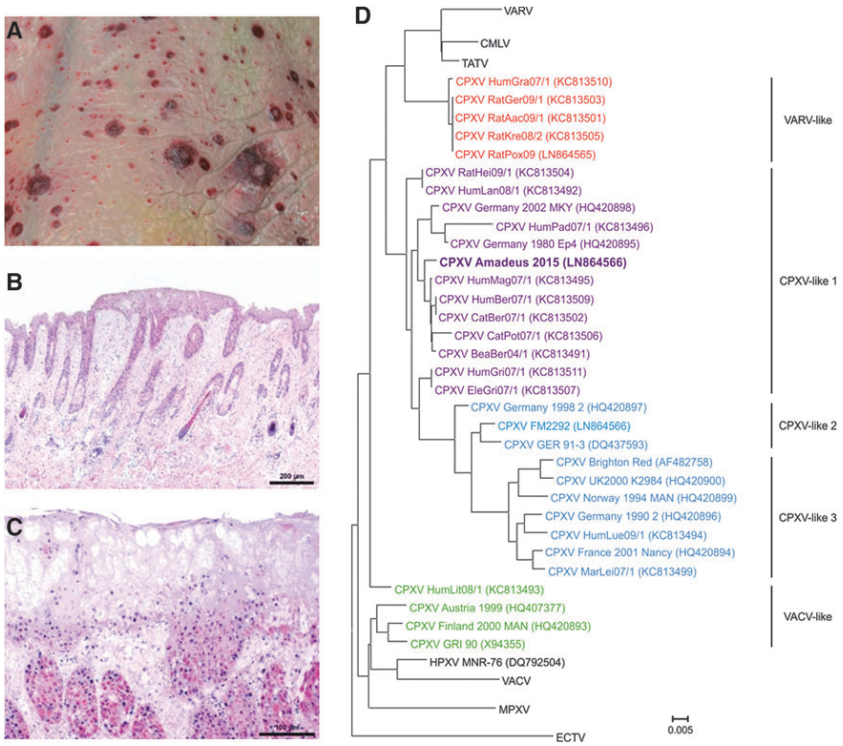

FIG. 1. **(A)** Skin from the aborted foal displaying cutaneous papules of variable size. Numerous lesions show hemorrhage and several larger lesions are covered by crusts. **(B)** Histopathological picture of hematoxylin- and eosin-stained skin sections with epidermal, plaque-like proliferation. **(C)** Degeneration, necrosis, and abundant cytoplasmic and eosinophilic A-type inclusion bodies. **(D)** Phylogenetic analysis was performed with whole genome sequences and showed that CPXV Amadeus 2015 belongs to the ''CPXV-like 1'' clade. CPXV, cowpox virus.

genome copy number. The highest viral load was detected in a skin sample, which had a Cq value of 12.

The DNA extracted from skin was analyzed using *de novo* sequencing of the complete genome by next-generation sequencing with a MiSeq reagent kit v2 and the Illumina MiSeq (Illumina, San Diego, CA). This analysis generated a complete genome consisting of 222,069 bp. A phylogenetic analysis was performed for all full-length genome sequences using IQ-Tree (v. 1.2.2) using a best fit model (TVM+I+G4). The novel isolate was a CPXV clustering within the ''CPXV-like clade 1.'' The sequence is clearly distinguished from the HPXV isolate MNR-76 (DQ792504), which clustered with *Vaccina virus* (VACV) (Fig. 1D).

Virus was isolated from skin using the African green monkey liver cell line Vero76 (Collection of Cell Lines in Veterinary Medicine CCLV; Friedrich-Loeffler-Institut, Greifswald-Insel Riems, Germany). The isolate was named ''CPXV Amadeus 2015'' (LN879483).

Interestingly, the mare did not display any clinical signs during pregnancy. However, there were OPV antibodies detectable in the serum by immunofluorescence assay. The serum was collected 10 weeks after abortion (titer ≥ 1:500). The epitheliocoreal placenta of horses is impermeable to macromolecules such as immunoglobulins (Furukawa et al. 2014). Therefore, the fetus was not protected by maternal antibodies.

Our findings indicate this is a very rare case of an equine CPXV infection resulting in a spontaneous abortion. There is another report of a foal born 29 days prematurely that showed clinical signs of a CPXV infection. The foal was euthanized on the sixth day of life. The foal did not display any cutaneous pox lesions, and the case was clearly associated with streptococcal septicemia. Therefore, the authors assumed the foal was infected orally after birth (Ellenberger et al. 2005). The first case of a stillbirth caused by CPXV was described in 2001 for an Asian elephant housed in a zoo. The stillborn calf was mature and showed generalized pox lesions. There were no clinical signs observed in the mother or the other elephants of the group (Wisser et al. 2001).

In this case report, we describe for the first time a congenital CPXV infection in a horse that was lethal. Interestingly,

lesions of this generalized infection appear in different stages in infected animals (Schmiedeknecht et al. 2010). This result conflicts with the smallpox phenotype observed in humans. Other poxvirus infections such as congenital *Ectromelia virus* (ECTV) infections are endemic in farms of silver foxes and minks in the Czech Republic. The adult animals did not show clinical signs (Mahnel et al. 1993). In addition, fetal VACV infections have been reported previously, and this rare complication of smallpox vaccination has resulted in fetal or neonatal death (Cono et al. 2003).

Previous studies have demonstrated that voles are the definitive reservoir of CPXV. Thus, it is possible that the pregnant mare grazed or received supplemental feeding that contacted hay or straw contaminated by urine, feces, or exhaled droplets from CPXV-infected voles.

The phylogenetic analysis showed the new isolate grouped with other CPXV strains found in a specific area of Berlin (Dabrowski et al. 2013). Additional information regarding the exact location of infection would allow a direct relationship between these CPXV strains to be determined. It is currently unclear whether CPXV infections in horses are frequently caused by contaminated hay and straw. Furthermore, it is unknown why the infection in this specific case resulted in an abortion with severe clinical signs and typical poxvirus lesions in the fetus. Thus, further studies of CPXV seroprevalence in horses may improve our understanding of CPXV epidemiology in spill-over hosts such as the horse.

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Author Disclosure Statement

No competing financial interests exist.

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4 EIGENANTEIL

(I)

Franke A*, Pfaff F*, Jenckel M, Hoffmann B, Höper D, Antwerpen M, Meyer H, Beer M, Hoffmann D: **Classification of cowpox viruses into several distinct clades and identification of a novel lineage**. *Viruses* 2017, 9(6): e142

Franke, A.*:	Isolation und Anzucht von Virusstämmen, Vorbereitung von Nukleinsäuren zur Sequenzierung, finale taxonomische Klassifizierung, Interpretation aller Daten sowie Erstellung der Publikation
Pfaff, F.*:	Annotation und Formatierung der fertigen Sequenzen für die Veröffentlichung in den INSDC-Datenbanken; Phylogenetische Analysen: Evaluierung und Optimierung der verschiedenen Methoden zur phylogenetischen Analyse, Auswahl geeigneter Referenzsequenzen, Maximum-Likelihood-Phylogenie basierend auf Sequenzen der core-Region, Rekombinationsanalyse mittels Consensus Networks und Bootscan kleinerer Alignmentsegmente, finale taxonomische Klassifizierung; Geografisches Mapping; Interpretation aller Daten sowie Erstellung der Publikation inkl. Abbildungen
Jenckel, M:	Sequenzdatenauswertung: *de novo*-Assemblierung und *polishing* der kompletten Genomsequenzen
Hoffmann, B.:	Konzeption der Studie, Beschaffung von geeignetem Probenmaterial und Mitarbeit an der Publikation
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Meyer, H.:	Bereitstellung von Komplettgenomsequenzen und Mitarbeit an der Publikation
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(II)

Hoffmann D*, Franke A*, Jenckel M*, Tamosiunaite A, Schluckebier J, Granzow H, Hoffmann B, Fischer S, Ulrich RG, Höper D, Goller K, Osterrieder N, Beer M: **Out of the reservoir: phenotypic and genotypic characterization of a novel cowpox virus isolated from a common vole**. *J Virol* 2015, 89(21): 10959-10969.

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Franke, A.*:	Aufarbeitung aller Zellkultur- und Tierversuchsproben, Analyse der Ergebnisse und Interpretation, Erstellung der Publikation
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Osterrieder, N.:	Interpretation aller Ergebnisse und Mitarbeit an der Publikation
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(III)

Franke A, Ulrich RG, Weber S, Osterrieder N, Keller M, Hoffmann D, Beer M: **Experimental cowpox virus (CPXV) infections of bank voles: exceptional clinical resistance and variable reservoir competence**. *Viruses* 2017, 9(12):e391.

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Ulrich, R.G.:	Interpretation der Daten und Mitarbeit an der Publikation
Weber, S.:	Statistische Auswertung der Ergebnisse
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(IV)

Franke A, Kershaw O, Jenckel M, König L, Beer M, Hoffmann B, Hoffmann D: **Fatal cowpox virus infection in an aborted foal**. *Vector Borne Zoonotic Dis* 2016, 16(6), 431-433.

Franke, A.:	Anzucht und Untersuchung des neuen CPXV-Isolats, Analyse und Interpretation der Daten, Erstellung der Publikation
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Jenckel, M.:	Next-Generation-Sequenzierung des Genoms des CPXV-Isolats und *de novo*-Assemblierung
König, L.:	Durchführung und Beurteilung der Pathologie des Fohlens
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Obige Angaben werden bestätigt:

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Promovierende Annika Franke

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Betreuer 1, PD Dr. Rainer G. Ulrich

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Betreuer 2, Prof. Dr. Martin Beer

5 Ergebnisse und Diskussion

5.1 Genetische Variabilität der Spezies Kuhpockenvirus (CPXV)

Phylogenetische Studien von Carroll *et al.* Aus dem Jahr 2011 gaben erste Hinweise, dass die Spezies CPXV polyphyletisch ist. Im Gegensatz zu einer monophyletischen Spezies lässt sich bei einer polyphyletischen Spezies der Ursprung nicht auf einen gemeinsamen Vorfahren zurückzuführen. Vielmehr scheinen mehrere Vorfahren nebeneinander zu existieren [75]. Basierend auf der kodierenden Region der CPXV-Genome (Genprodukte C23L-B29R, Gesamtlänge von 145.177 bp nach Entfernen der durch das Alignment erhaltenen Lücken) teilte das von Carroll *et al.* verwendete Alignment die verschiedenen CPXV-Isolate in zwei Kladen, „Cowpox-like“ und „Vaccinia-like“, ein. Die Cowpox-like Klade wiederum kann in 4 monophyletische Kladen (Cowpox-like 1-4) unterteilt werden. Diese Studien beschränkten sich bis dato jedoch auf lediglich 12 vollständige CPXV-Genome [69]. Im Jahr 2013 wurden durch Dabrowski *et al.* 22 weitere Volllängensequenzen deutscher CPXV-Isolate bestimmt und phylogenetisch analysiert. Der polyphyletische Charakter der Spezies CPXV wurde bestätigt. Zusätzlich zu den bereits definierten Kladen wurde eine weitere, neue Klade als „VARV-like“ definiert, in der vorrangig CPXV-Isolate gruppiert sind, die aus Ratten stammen oder aus Menschen, die sich von den Ratten infiziert hatten [68].

Um einen noch genaueren Einblick in die Phylogenie der Spezies CPXV zu erhalten, wurden 20 weitere CPXV-Isolate aus Deutschland vollständig sequenziert, *de novo* assembliert und analysiert (Publikation 1). Diese CPXV-Stämme wurden von verschiedenen akzidentiellen Wirten isoliert: Hauskatze, Alpaka, Präriehund, Waschbär und Neuweltaffe. Drei Isolate konnten aus humanen Proben gewonnen werden. Zudem wurde ein CPXV-Stamm aus einer Feldmaus, einem der putativen Reservoirwirte, isoliert (Publikation 1, Tabelle 1). Insgesamt konnten so 83 OPV-Genome, darunter repräsentative Vertreter der Altwelt- und Neuwelt-OPV, für die phylogenetische Untersuchung verwendet werden. Hierbei wurde der größte gemeinsame Genombereich (142.286 Nukleotide, ca. 65 % des Gesamtgenoms) genutzt.

Mit Hilfe der neuen und umfassenden Untersuchungen wurden vier verschiedene CPXV-Kladen identifiziert, welche als CPXV-like 1, CPXV-like 2 sowie VARV-like und VACV-like definiert werden konnten. Die neu gewonnenen CPXV-Isolate dieser Studie gruppierten sich vorrangig in die CPXV-like 1 Klade (80 %). Einige wenige CPXV-Stämme ordneten sich in die CPXV-like 2 Klade (15 %) ein.

Interessanterweise gab es drei CPXV-Stämme, die keiner spezifischen Klade zugeordnet wurden, sondern als sogenannter *single branch* im phylogenetischen Baum auftraten. Neben zwei humanen CPXV-Isolaten, CPXV HumLit08/1 und CPXV Germany_1998_2, traf dies auch auf den CPXV-Stamm Ger 2010 MKY zu, der aus einem Neuweltaffen (Lisztaffe, *Saguinus oedipus*) isoliert worden war. Die Bootscan-Analyse zeigte außerdem potenzielle Rekombinationsereignisse der beiden humanen CPXV-Isolate an. Das neue CPXV-Isolat Ger 2010 MKY eröffnet hingegen eine neue, weitere CPXV-Klade, da nur minimale Rekombinationsereignisse nachgewiesen werden konnten (Publikation 1, Abbildung 4). Im Ergebnis unserer Studien muss daher jetzt von mindestens 5 CPXV-Kladen ausgegangen werden.

Das im Vergleich zu allen anderen Vertretern des Genus *Orthopoxvirus* lange Genom der Spezies CPXV ist ein Hinweis darauf, dass vermutlich ein CPXV-ähnliches Virus die Ursprungsformen aller OPV darstellt. Bereits Ende der 90er Jahre wurde nach eingehender Genomanalyse des CPXV-Stammes GRI-90 vermutet, dass die Spezies CPXV ein möglicher Vorläufer der Spezies VARV und VACV ist [76]. Kürzlich konnte diese Theorie durch Analysen auf Basis des Hämagglutinin-Gens bestätigt werden. VARV und VACV als „jüngere“ Viren sind demnach durch Mutationen und insbesondere Deletionen entstanden [77]. Die Spezies CMLV, TATV und VARV scheinen ebenfalls von einem gemeinsamen CPXV-Vorläufer, abzustammen [1], was auch die in vorliegender Arbeit durchgeführten phylogenetischen Analysen aller OPV-Gesamtgenome bestätigten (Publikation 1, Abbildung 2). Im Gegensatz zu OPV haben sowohl CMLV als auch TATV und VARV ein sehr enges Wirtsspektrum. Diese Anpassung an jeweils einen Wirt scheint in allen drei Fällen mit der Verkürzung des Genoms einherzugehen.

Interessanterweise ist das Genom des in Publikation 4 beschriebenen CPXV-Isolats aus einem Fohlenabort, „CPXV Amadeus 2015“, phylogenetisch deutlich vom bisher einzigen Pferdepockenvirus (horsepox virus, HPXV) MNR-76 abgegrenzt. HPXV gruppiert sich in eine gemeinsame Klade mit VACV ein (Publikation 4, Abbildung 1 und Publikation 1, Abbildung 2). Gesamtgenomanalysen bestätigen, dass HPXV MNR-76 ein VACV-ähnliches Virus mit zusätzlichen, CPXV-ähnlichen Sequenzen ist [78]. Somit ist die Bezeichnung „Pferdepockenvirus“ eine nur die jeweils betroffene Wirtsspezies beschreibende Nomenklatur, ähnlich wie bei Kaninchenpockenvirus (Rabbitpox virus) oder Büffelpockenvirus (Buffalopox virus), während die Erreger tatsächlich der Spezies CPXV bzw. VACV zuzurechnen sind.

Vorliegende phylogenetische Arbeiten deuten an, dass der Speziesbegriff im Hinblick auf CPXV zukünftig dringend überarbeitet werden sollte. Dies deckt sich mit den Forderungen von Mauldin *et al.* [67]. Die aktuelle Definition einer Spezies durch das Internationale Komitee zur Taxonomie von Viren

(*International Committee on Taxonomy of Viruses*, ICTV) lautet: *„a species is monophyletic group of viruses whose properties can be distinguished from those of other species by multiple criteria*“ [11, 79]. Der durch vorliegende Analysen bestätigte polyphyletische Charakter der Spezies CPXV entspricht dieser Definition jedoch nicht. Weitere Merkmale einer Spezies müssten somit in der aktuellen Definition ergänzt werden [80] bzw. sollte die neue taxonomische Einteilung auf Basis der jetzt vorliegenden genetischen Daten erfolgen.

5.2 Virus-Wirt-Interaktionen: Pathogenität in Reservoir- und akzidentiellen Wirten

5.2.1 Genotypische Charakterisierung von CPXV-Stämmen aus dem Reservoirwirt

CPXV kommt natürlicherweise in Wühlmauspopulationen in Europa vor. Die Seroprävalenz ist besonders hoch bei Rötelmäusen sowie Erd- und Feldmäusen. Neben den Wühlmäusen findet man CPXV auch bei Langschwanzmäusen, vorrangig in Brandmäusen [71]. Dennoch konnte von einem Reservoirwirt bisher kein CPXV-Isolat gewonnen und untersucht werden. Im Rahmen eines vom BMBF über die Nationale Forschungsplattform für Zoonosen geförderten Projektes (Netzwerk „Nagetier-übertragene Pathogene“) wurden deshalb verschiedene Kleinsäuger mittels spezifischer quantitativer PCR auf eine akute CPXV-Infektion hin untersucht. Von insgesamt über 2000 Nagetier-Leberproben waren lediglich 4 Proben in der qPCR positiv (persönliche Mitteilung R. G. Ulrich, D. Hoffmann und S. Fischer). Aus der Leberprobe einer Feldmaus konnte schließlich ein CPXV-Isolat in Verozellen gewonnen werden, welches im Folgenden als CPXV FM2292 bezeichnet wird. Dieses neue CPXV-Isolat FM2292 wurde sowohl *in vitro* als auch *in vivo* untersucht und mit einem aus einer Ratte gewonnenen CPXV-Stamm „RatPox09“ und dem in der Pockenforschung häufig genutzten Referenzstamm „CPXV Brighton Red“ verglichen. In der vergleichenden Analyse der Gesamtgenomsequenz weist CPXV FM2292 im Gegensatz zum Laborstamm Brighton Red in den nicht-konservierten Enden zusätzliche *open reading frames* (ORFs) auf, die ebenfalls im Genom des Rattenisolates CPXV RatPox09 vorkommen. Diese zusätzlichen ORFs sind: *gCPXV0284*, *gCPXV00030*, *gCPXV002* und *gCPXV0003*. Interessanterweise besitzt das Genom von CPXV FM2292 einen weiteren zusätzlichen ORF, *gCPXV0285*, der in keinem der beiden zum Vergleich hinzugezogenen CPXV-Stämme vorzufinden ist (Publikation 2, Tabelle 1 und Abbildung 1). Die Sequenzidentität von CPXV FM2292 und CPXV RatPox09 bezogen auf das Gesamtgenom liegt bei 96 %. Zum Referenzstamm CPXV Brighton Red besitzen CPXV FM2292 und CPXV RatPox09 eine Sequenzidentität von 92 %. Da in Publikation 1 ein weiteres, von einer Feldmaus stammendes CPXV-Isolat beschrieben wurde, CPXV

Ger/2007/Vole, lassen sich nun auch erste Vergleiche von CPXV-Isolaten des Reservoirwirtes ziehen. Die Sequenzidentität von CPXV FM2292 und CPXV Ger/2007/Vole liegt bei 99 %, sie gehören zur Klade CPXV-like 2 (Publikation 1, Abbildung 2). Der zuvor beim CPXV-Isolat FM2292 identifizierte ORF *gCPXV0285* konnte ebenfalls im Genom von CPXV Ger/2007/Vole nachgewiesen werden. Infolgedessen wurden alle bisher in GenBank veröffentlichten CPXV-Gesamtgenome untersucht: 32 von insgesamt 67 CPXV-Isolaten besitzen in ihrem Genom den ORF *gCPXV0285*. Auffällig ist, dass alle ORF *gCPXV0285* positiven CPXV-Isolate aus Deutschland bzw. Großbritannien stammen.

Tabelle 2: CPXV-Gesamtgenome, in denen der ORF *gCPXV00285* vorkommt

CPXV-Isolat	Herkunft (Spezies/Land)	GenBank Accession #	ORF-Länge (Nukleotide)
CPXV Amadeus 2015	Pferd/Deutschland	LN879483	576
CPXV BeaBer04/1	Biber/Deutschland	KC813491	576
CPXV CatBer07/1	Katze/Deutschland	KC813502	576
CPXV CatPot07/1	Katze/Deutschland	KC813506	576
CPXV FM2292	Feldmaus/Deutschland	LN864566	573
CPXV Ger/2007/Vole	Feldmaus/Deutschland	LT896722	573
CPXV Ger/2010/Alpaca	Alpaka/Deutschland	LT896718	576
CPXV Ger/2010/Cat	Katze/Deutschland	LT896729	576
CPXV Ger/2010/Racoon	Waschbär/Deutschland	LT896730	576
CPXV Ger/2010/Rat	Ratte/Deutschland	LT896728	576
CPXV Ger 2010 MKY	Lisztaffe/Deutschland	LT896721	576
CPXV Ger/2012/Alpaca	Alpaka/Deutschland	LT896726	576
CPXV Ger/2013/Alpaca	Alpaka/Deutschland	LT896719	576
CPXV Ger/2014/Cat1	Katze/Deutschland	LT896723	576
CPXV Ger/2014/Cat2	Katze/Deutschland	LT896725	576
CPXV Ger/2015/Cat2	Katze/Deutschland	LT896727	576
CPXV Ger/2015/Cat3	Katze/Deutschland	LT896733	576
CPXV Ger/2015/Cat4	Katze/Deutschland	LT896731	576
CPXV Ger/2015/Prairie Dog	Präriehund/Deutschland	PRJEB20974	576
CPXV Ger/2017/Alpaca1	Alpaka/Deutschland	PRJEB20974	576
CPXV Ger/2017/Alpaca2	Alpaka/Deutschland	LT896732	576
CPXV Germany 91-3	Mensch/Deutschland	DQ437593	576

Tabelle 2 (Fortsetzung): CPXV-Gesamtgenome, in denen der ORF *gCPXV00285* vorkommt

CPXV-Isolat	Herkunft (Spezies/Land)	GenBank Accession #	ORF-Länge (Nukleotide)
CPXV Ger/2015/Prairie Dog	Präriehund/Deutschland	PRJEB20974	576
CPXV Ger/2017/Alpaca1	Alpaka/Deutschland	PRJEB20974	576
CPXV Ger/2017/Alpaca2	Alpaka/Deutschland	LT896732	576
CPXV Germany 91-3	Mensch/Deutschland	DQ437593	576
CPXV Germany_1980_EP4	Elefant/Deutschland	HQ420895	576
CPXV Germany_1998_2	Mensch/Deutschland	HQ420897	576
CPXV Germany_2002_MKY	Weißbüschelaffe/Deutschland	HQ420898	576
CPXV HumGri07/1	Mensch/Deutschland	KC813511	576
CPXV HumLan08/1	Mensch/Deutschland	KC813492	576
CPXV HumMag07/1	Mensch/Deutschland	KC813495	576
CPXV JagKre08/1	Jaguarundi/Deutschland	KC813497	576
CPXV JagKre08/2	Jaguarundi/Deutschland	KC813498	576
CPXV RatHei09/1	Ratte/Deutschland	KC813504	576
CPXV_K779	Katze/GB: England	KY549146	576
CPXV_K780	Katze/GB: England	KY549147	576

Weiterhin wurde der Phänotyp der *A-type inclusion bodies* (ATI) in der Zellkultur vergleichend für CPXV FM2292, CPXV RatPox09 und CPXV Brighton Red analysiert (Publikation 2, Abbildung 3). Das aus der Feldmaus stammende CPXV-Isolat FM2292 besitzt im Gegensatz zu CPXV RatPox09 und dem Referenzstamm CPXV Brighton Red den Phänotyp V^+: Virionen sind in den Einschlusskörperchen eingebettet. Tiefergehende Analysen mit dem in Publikation 2 beschriebenen zweiten Isolat aus einer Feldmaus, CPXV Ger/2007/Vole zeigen, dass der gleiche V^+-Phänotyp vorliegt (Daten nicht veröffentlicht). Während die Bildung von *A-type inclusion bodies* des Typs V^+ mit einer hohen Umweltstabilität assoziiert ist, wird bei der V^--Variante eine Virulenzsteigerung im Mausmodell gesehen [81]. Daraus lässt sich die Hypothese ableiten, dass primär respiratorisch übertragene Orthopocken wie VARV oder MPXV ohne ATI-Expression eine gesteigerte Transmissionseffizienz mit gesteigerter Virulenz erreichen [66, 81, 82]. Entsprechend sind weniger virulente Viren auf eine hohe Tenazität in der Umwelt angewiesen. Die hier gezeigte Assoziation des V^+-Phänotyps bei Isolaten aus den Reservoirwirtsspezies stützt diese Hypothese.

5.2.2 Experimentelle CPXV-Infektionen von Reservoirwirtsspezies

Derzeit existieren verschiedene, oftmals widersprechende Definitionen eines Reservoirwirts. Einerseits wird beschrieben, dass ein Reservoir nur eine Spezies beinhalten kann. Andererseits wird vorgeschlagen, dass auch ein ökologisches System als Reservoir fungieren kann [72]. Oftmals wird ein Reservoir auch als eine Spezies mit subklinischem Krankheitsverlauf beschrieben [83]. Eine hohe Seroprävalenz innerhalb einer Population ist ein weiteres Indiz dafür, dass entsprechende Infektionen stattfinden. Dies kann jedoch auch eine Spezies betreffen, die als akzidentieller Wirt fungiert [72, 74]. Bei einer Reservoirwirtspezies muss eine Übertragung des Erregers nachgewiesen werden, ob direkt oder indirekt, spielt dabei eine untergeordnete Rolle [72].

Serologische Untersuchungen von Nagetieren deuten darauf hin, dass Wühlmäuse der wahrscheinliche Reservoirwirt von CPXVs sind. Da es bis auf sehr wenige, ältere Studien in Großbritannien jedoch keine experimentellen Daten zu Wühlmaus-Infektionen gibt, sollten sowohl Feldmäuse als auch Rötelmäuse unter definierten Bedingungen experimentell infiziert werden (Publikation 2; Publikation 3). Drei bis vier Monate alte Feldmäuse wurden im Rahmen dieser Arbeit intranasal mit CPXV FM2292 oder CPXV RatPox09 infiziert und über einen Zeitraum von 28 Tagen untersucht (Publikation 2). Dabei wurden beide CPXV-Stämme mit jeweils zwei unterschiedlichen Infektionsdosen verabreicht (10^4 und 10^6 $TCID_{50}$ pro Tier). Erste klinische Symptome traten vier Tage nach Infektion auf und betrafen vorrangig den Respirationstrakt: Niesen, Ausfluss von Nasensekret und Dyspnoe. Der Krankheitsverlauf verlief bei den mit CPXV FM2292 infizierten Feldmäusen in der Gruppe 10^4 $TCID_{50}$ pro Tier subklinisch, zwischen Tag 5 und Tag 12 nach Infektion konnte Virusausscheidung nachgewiesen werden (Publikation 2, Abbildung 7A). Bei den mit CPXV RatPox09 infizierten Feldmäusen entwickelte sich eine stark ausgeprägte Klinik, einige Tiere mussten aufgrund ihres schlechten körperlichen Zustands euthanasiert werden. Bei der hohen CPXV RatPox09 Dosis (10^6 $TCID_{50}$ pro Tier) wurden insgesamt 4 von 5 infizierten Feldmäuse vorzeitig aus dem Experiment genommen. Die Virusausscheidung fand bereits ab Tag 3 nach Infektion statt und dauerte länger als bei den mit CPXV FM2292 infizierten Tieren (Publikation 2, Abbildung 7C). Zum Vergleich wurden 6-Wochen alte Wistar-Ratten analog mit CPXV Brighton Red bzw. FM2292 infiziert. Generell zeigten die mit CPXV FM2292 infizierten Tiere eine stärker ausgeprägte Klinik als die mit CPXV Brighton Red infizierten Tiere. Neben respiratorischen Symptomen entwickelten die Wistar-Ratten auch Pockenläsionen der Haut, vorrangig am Schwanz sowie am Maul und an den Krallen (Publikation 2, Abbildung 5).

Im Gegensatz zu den Feldmäusen zeigten Rötelmäuse nach CPXV-Infektion keinerlei klinische Symptomatik. Zudem konnte keine Virusausscheidung nachgewiesen werden (Publikation 3). Insgesamt wurden hierzu 5 verschiedene Infektionsexperimente mit Rötelmäusen durchgeführt (Publikation 3, Tabelle 2). Dabei wurden unterschiedliche Parameter, die einen möglichen Einfluss auf die Virus-Wirt-Interaktion haben könnten, betrachtet. Zu diesen Parametern zählte neben dem Alter, dem Geschlecht und dem evolutionären Ursprung der Rötelmäuse auch die Untersuchung der gewählten Inokulationsmethoden sowie der CPXV-Stämme (Publikation 3, Tabelle 2).

Generell zeigten sowohl Rötelmäuse der westlichen als auch der östlichen evolutionären Linie unabhängig von der gewählten Inokulationsmethode oder des CPXV-Stammes einen subklinischen Infektionsverlauf, welcher auch von Bennett *et al.* für Rötelmäuse beschrieben wurde [57]. Darüber hinaus konnte bei keiner infizierten Rötelmaus Virusausscheidung über Nasensekrete nachgewiesen werden. Jedoch war ein leichter Unterschied in den Serokonversationsraten der Rötelmäuse in Abhängigkeit von der gewählten Inokulationsroute erkennbar, der sich wie folgt darstellen lässt: intranasal > Footpad-inokuliert > subkutan (Publikation 3, Tabelle 5, Tabelle 6). Interessanterweise konnten bei zwei Kontakttieren (24 h nach Infektion zugesetzte nicht infizierte Rötelmäuse) OPV-spezifische Antikörper nachgewiesen werden. Es stellt sich somit die Frage, warum in intranasal infizierten Rötelmäusen die höchsten anti-OPV-Antikörpertiter im Serum nachgewiesen wurden, obwohl keine nasale Virusausscheidung messbar war, und durch welchen Übertragungssweg sich die beiden Kontakttiere infizieren konnten.

Die hohen anti-OPV-Antikörpertiter von Rötelmäusen nach intranasaler CPXV-Infektion deuten darauf hin, dass eine Virusreplikation erfolgt sein muss. In Publikation 2 konnte für die experimentellen Feldmaus-Infektionen gezeigt werden, dass eine Virusausscheidung über Nasensekrete erfolgte, womit eine Tröpfchen-basierte Übertragung wahrscheinlich ist. Die anti-OPV-Antikörpertiter in den beschriebenen Rötelmaus-Experimenten lassen die Interpretation zu, dass auch für Rötelmäuse die intranasale Applikation dem natürlichen Übertragungsweg entspricht. Dennoch stellt sich die Frage, warum keine intranasale Virusausscheidung nachgewiesen werden konnte. Einerseits könnte die Viruslast unter dem Detektionslimit der hier verwendeten qPCR liegen, was aufgrund der hohen analytischen Sensitivität der qPCR allerdings sehr unwahrscheinlich ist. Andererseits könnte der natürliche Übertragungsweg indirekt, z.B. über mit CPXV kontaminiertem Heu oder Einstreu, stattfinden. In der vorliegenden Arbeit wurden Urin und Kot jedoch nicht untersucht; dies sollte aber in zukünftigen Studien in Betracht gezogen werden. Kürzlich konnte durch Peres *et al.* im Falle von natürlichen

Infektionen mit VACV bei wilden Nagetieren gezeigt werden, dass dieser Erreger durch Kot und Urin ausgeschieden wird [84]. Dies könnte ebenfalls eine Erklärung dafür sein, dass zwei naive Kontakttiere zu Versuchsende spezifische anti-OPV-Antikörper entwickelt hatten. Eine 100%ige Serokonversationsrate der Kontakttiere wurde jedoch wahrscheinlich aufgrund des wöchentlich stattfindenden Wechsels des Einstreumaterials nicht erreicht.

5.2.3 Die Spezies CPXV als Zoonose-Erreger: Infektionen von akzidentiellen Wirten

CPXV-bedingte Erkrankungen des Menschen kommen selten vor, trotzdem wird die Spezies CPXV derzeit zu den Erregern der *emerging diseases* in Europa gezählt [85, 86]. In den letzten 20 Jahren wurden vereinzelt humane CPXV-Infektionen, häufig bei jungen Menschen, berichtet [39, 87, 88]. Diese humanen Fallberichte haben vermutlich folgende Gründe: (1) Einhergehend mit der Einstellung des Impfprogrammes gegen VARV Ende der 1980er Jahre, ist der Schutz gegen andere Vertreter des Genus *Orthopoxvirus* nicht mehr gegeben [89, 90]. (2) Gleichzeitig steigt die Anzahl der Menschen, die eine Immunsuppression aufweisen und daher gefährdet sind, im Falle einer Infektion schwer zu erkranken. (3) Auch die Infektionen bei Haus- und Nutztieren nehmen zu, was wiederum mehr Kontaktinfektionen ermöglicht. Diese akzidentiellen Wirte, besonders Nutz- und Haustiere, aber auch Zootiere, stellen eine CPXV-Infektionsquelle für den Menschen dar [34, 89].

Das steigende Bewusstsein für eine artgerechte Haltung, z.B. Freilandhaltung, führt zu zunehmenden Kontaktmöglichkeiten von akzidentiellen Wirten zu CPXV-Reservoirwirtsspezies bzw. deren kontaminierter Umgebung. Beispielhaft hierzu wird im Folgenden der Fall eines CPXV-infizierten Fohlens näher betrachtet.

In Europa sind CPXV-Infektionen von Pferden heute sehr selten. In der jüngeren Literatur wurden lediglich zwei CPXV-Erkrankungen beschrieben: (a) ein 7-Jahre altes Arabisches Pferd entwickelte multiple Hautläsionen [91] und (b) ein Fohlen ohne erkennbare Läsionen verstarb aufgrund seiner schlechten körperlichen Verfassung (Unterernährung, Unterkühlung, Atemnot) [92]. In vorliegendem Fallbericht wurde nun eine dritte CPXV-Erkrankung einer tragenden Warmblut-Stute beschrieben (Publikation 4). Die Stute erlitt eine Fehlgeburt im dritten Trimester. Die Haut des Fohlens war durch zahlreiche Läsionen unterschiedlicher Größe gekennzeichnet (Publikation 4, Abbildung 1A). Durch die histopathologische Untersuchung wurden die für eine CPXV-Infektion charakteristischen *A-type inclusion bodies* sichtbar (Publikation 4, Abbildungen 1B und 1C). Diverse fetale Gewebe und Organe (Plazenta, Lunge, Leber, Milz, Thymus, Haut) wurden im Folgenden molekularbiologisch untersucht. Eine für CPXV

entwickelte spezifische qPCR bestätigte eine CPXV-Infektion. Die höchste Viruslast wurde in der Haut des Fohlens nachgewiesen (Cq 12). Ausgehend von der stark qPCR-positiven Haut konnte Virus isoliert werden. Das Genom des als „CPXV Amadeus 2015“ bezeichneten Isolats wurde vollständig sequenziert und *de novo* assembliert. Interessanterweise zeigte die Mutterstute keinerlei Symptome einer CPXV-Erkrankung vor der Fehlgeburt. Zehn Wochen nach der Fehlgeburt lag der anti-OPV-Antikörper-Titer der Stute bei 1:500. Dieser Wert ist somit der serologische Beweis einer Infektion mit einem OPV. Die Ansteckungsquelle lässt sich nur mutmaßen, am wahrscheinlichsten erscheint jedoch eine CPXV-Übertragung durch von Nagetieren kontaminiertes Stroh und Heu. Zukünftig wären daher Seroprävalenz-Studien sinnvoll, um einen Einblick in die CPXV-Epidemiologie bei Pferden, als akzidentiellem Wirt, zu bekommen. Gegebenenfalls könnten Pferde infolgedessen ebenfalls, wie beispielsweise Elefanten in Zoos, gegen CPXV immunisiert werden, um so fatalen Folgen wie im vorliegenden Fall (Publikation 4) vorzubeugen. Eventuell stellt sich auch heraus, dass CPXV-Infektionen von Pferden sehr selten sind, sodass keine präventiven Maßnahmen nötig sind.

Der vorliegende Fallbericht zeigt deutlich, dass auch Pferde als potenzielle Überträger-Spezies des CPXV auf den Menschen fungieren könnten. Als eine weitere Überträgerspezies zeigten sich in Deutschland überraschenderweise auch Alpakas (*Vicugna pacos*), eine südamerikanische domestizierte Kamelart. In verschiedenen Alpaka-Herden wurden in den letzten Jahren CPXV-Infektionen beobachtet, die teilweise letal endeten [93]. Besonders der Einsatz von Alpakas als Therapietiere oder zu Erholungszwecken, wie Trekking-Ausflügen, lässt eine erhöhte Übertragungsgefahr auf den Menschen befürchten. Als die Spezies mit der höchsten Übertragungswahrscheinlichkeit des CPXV auf den Menschen muss jedoch die Hauskatze (*Felis silvestris catus*) angesehen werden [43]. Besonders sogenannte „Freigänger“ infizieren sich mit CPXV und stellen dann wiederum eine Infektionsgefahr für den Menschen dar. Die Seroprävalenz deutscher Hauskatzen variiert zwischen 2 % und 31 % [43, 94]. Im Hinblick auf die zunehmende Zahl humaner CPXV-Infektionsfälle wird auch der Bedarf an antiviralen Therapeutika deutlich. Derzeit sind die Behandlungsmöglichkeiten bei einer CPXV-Erkrankung limitiert. Bezogen auf vorhandene antivirale Agenzien besitzt lediglich Brincidofovir eine europäische Zulassung als „Orphan Drug“ zur Behandlung von VARV Infektionen [95].

6 ZUSAMMENFASSUNG

Die Spezies Kuhpockenvirus (CPXV), ein Mitglied des Genus *Orthopoxvirus*, ist endemisch in weiten Teilen Europas und Asien verbreitet. CPXV besitzt ein sehr breites Wirtsspektrum und zählt zu den zoonotischen Erregern. Phylogenetische Analysen deuten darauf hin, dass CPXV polyphyletisch ist. Die bisher definierten Kladen wurden in vorliegender Arbeit bestätigt. Die 20 neu gewonnenen CPXV-Stämme verschiedenster Wirtsspezies gruppieren vorrangig in die CPXV-like 1 und CPXV-like 2 Kladen. Ein CPXV-Stamm, isoliert von einem Neuweltaffen, erscheint jedoch als *single branch* und lässt sich keiner bisher bekannten Klade zuordnen.

Gegenwärtig ist über die Rolle der Wühlmäuse, die als Reservoirwirt der Kuhpockenviren betrachtet werden, wenig bekannt. In vorliegender Arbeit sollte deshalb das aus einer Feldmaus (*Microtus arvalis*) stammende CPXV-Isolat FM2292 eingehend charakterisiert werden. CPXV FM2292 weist das bisher längste CPXV-Genom auf, das in die CPXV-like 1 Klade clustert. Neben der Sequenzanalyse sollten vergleichende experimentelle Infektionsstudien in Feldmäusen und Wistar-Ratten durchgeführt werden. Der Krankheitsverlauf nach intranasaler CPXV FM2292-Infektion bei Feldmäusen verlief subklinisch; Wistar-Ratten hingegen zeigten ausgeprägte klinische Symptome. Im Gegensatz dazu verursachte die Infektion mit einem aus einer Schmuseratte isolierten CPXV-Stamm bei den Feldmäusen eine starke Klinik. Daraus lässt sich schließen, dass Feldmäuse gegenüber Wühlmaus-assoziierten Stämmen, wie CPXV FM2292, eine Adaptation entwickelt haben. Die nachgewiesene Virusausscheidung weist auf eine Tröpfchen-basierte Übertragung hin.

Neben Feldmäusen sollten auch Rötelmäuse im Tierversuch näher betrachtet werden. Unabhängig vom eingesetzten CPXV-Stamm resultierten die experimentellen CPXV-Infektionsstudien in Rötelmäusen (*Myodes glaerolus*) in subklinischen Verläufen. Eine nasale Virusausscheidung konnte nicht detektiert werden, was im starken Kontrast zu den Ergebnissen aus experimentell infizierten Feldmäusen steht. Dennoch deuteten die Serokonversionsraten der Rötelmäuse darauf hin, dass eine Replikation im Wirt stattgefunden hat. Zudem entwickelten zwei Kontakttiere ebenfalls OPV-spezifische Antikörper, was auf eine Übertragung des Virus schließen lässt.

Wühlmäuse als Reservoirwirt dienen der Übertragung des Kuhpockenvirus auf akzidentielle Wirtsspezies wie Hauskatzen oder Nutztiere. Der in dieser Arbeit betrachtete Fallbericht eines mit CPXV-infizierten Fohlens zeigt, dass CPXV-Infektionen bei akzidentiellen Wirten mit stark ausgeprägter Klinik verbunden

sein können. Zudem wird die wachsende Gefahr der Übertragung von CPXV auf Nutztiere und Menschen deutlich.

Zusammenfassend unterstreicht die in der vorliegenden Arbeit verwendete phylogenetische Betrachtung die genetische Variabilität der Spezies CPXV. Zudem konnten neue Erkenntnisse zu Feldmäusen und Rötelmäusen als Reservoirwirtsspezies gewonnen werden. CPXV-Infektionen von Wühlmäusen verlaufen subklinisch, im Gegensatz zu dem hier ebenfalls beschriebenen, lethal endenden CPXV-Infektionsverlauf eines akzidentiellen Wirts, eines abortierten Fohlens.

7 SUMMARY

The species cowpox virus (CPXV), a member of the genus *Orthopoxvirus* (OPV), is endemic in parts of Europe and Asia. CPXV has got a broad host range and belongs to the zoonotic diseases. Phylogenetic analyses revealed that the species *cowpox virus* (CPXV) is polyphyletic. Previously defined clades were confirmed in the present study; the majority of the 20 new CPXV strains clustered in CPXV-like 1 and CPXV-like 2 clades. However, one CPXV strain, isolated from a New-world monkey, appears as single branch and has an unique phylogenetically position which is clearly separated from all other known clades.

Currently only little is known about the role of voles as reservoir host of CPXV. To overcome this, the CPXV strain FM2292, which was isolated from a common vole (*Microtus arvalis*), was analyzed in detail. CPXV FM2292 is characterized by the longest CPXV genome within the CPXV-like 1 clade. Comparative experimental infections of common voles and Wistar rats were performed. Generally, CPXV FM2292 infection in common voles caused subclinical disease, whereas Wistar rats developed respiratory clinical signs and dermal lesions. In contrast, common voles exhibited prominent nasal discharge after intranasal infection with a pet rat derived CPXV strain. Taken together vole-derived strains like CPXV FM2292 induced subclinical infection reflecting most probably a virus-host adaption in accordance to reservoir host conditions. However, virus shedding was generally obtained independently from the CPXV strain used for infection.

Moreover, experimental infection studies using bank voles (*Myodes glaerolus*) were performed. All CPXV-infections of bank voles resulted in subclinical diseases. No virus shedding was detected, which is in strong contrast to the results obtained from experimental infections using common voles. Nevertheless, the rates of seroconversions suggest that virus replicated within the host. Interestingly, two contact animals developed specific antibodies, also demonstrating transmission processes within the voles.

CPXV can be transmitted from vole reservoirs to accidental species like cats or farmed animals. The case report of the present study analyzed a foal infected with CPXV. This case clearly shows that CPXV infections of accidental host species might result in severe or lethal disease. In addition this case draws attention towards the increasing danger of CPXV transmission on farmed animals and humans.

Taken together, the genetic variability of CPXV is confirmed by the phylogenetic analysis done in the present study. In addition new insights of common and bank voles as reservoir species of CPXV could be obtained. Generally, CPXV infections of voles resulted in subclinical diseases, whereas the infection of a foal, an accidental reservoir species, resulted in an abortion of the foal and seroconversion of the mare.

8 LITERATUR

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EIGENSTÄNDIGKEITSERKLÄRUNG

Hiermit erkläre ich, dass diese Arbeit bisher von mir weder an der Mathematisch-Naturwissenschaftlichen Fakultät der Universität Greifswald noch einer anderen wissenschaftlichen Einrichtung zum Zwecke der Promotion eingereicht wurde.

Ferner erkläre ich, dass ich diese Arbeit selbstständig verfasst und keine anderen als die darin angegebenen Hilfsmittel und Hilfen benutzt und keine Textabschnitte eines Dritten ohne Kennzeichnung übernommen habe.

PUBLIKATIONEN UND TAGUNGSBEITRÄGE

PUBLIKATIONEN, DIE BESTANDTEIL DIESER ARBEIT SIND

Franke A*, Pfaff F*, Jenckel M, Hoffmann B, Höper D, Antwerpen M, Meyer H, Beer M, Hoffmann D: **Classification of cowpox viruses into several distinct clades and identification of a novel lineage**. *Viruses* 2017, 9(6): e142

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POSTERBEITRÄGE UND VORTRÄGE

Posterbeiträge

10/2014 Hoffmann D, Schluckebier J, Franke A, Ulrich RG, Jenckel, M, Hoffmann B, Beer M.
"**Isolation and characterization of a cowpox virus derived from its supposed natural rodent reservoir host"**
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03/2015 Franke A, Hoffmann D, Ulrich RG, Jenckel M, Hoffmann B, Beer M.
"Cowpox virus: virulence studies in different animal species"
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06/2015 Franke A, Hoffmann D, Ulrich RG, Jenckel M, Hoffmann B, Beer M.
"Out of the reservoir: Phenotypic and genotypic characterization of a novel cowpox virus isolated from a common vole"
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06/2016 Franke A, Hoffmann D, Tamošiūnaitė A, Jenckel M, Osterrieder N, Beer M.
Cowpox virus-host interactions: Determination of defined virulence factors
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"**Out of the reservoir: CPXV-infections of bank voles (*Myodes glareolus*)"**
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