Von Reservoir- bis Modelltier –

Nachweis und Charakterisierung von zoonotischen Kuhpockenviren (CPXV)

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Weber S, Jeske K, Ulrich RG et al. **"*In vivo* characterization of a bank vole-derived Cowpox virus isolate in natural hosts and the rat model"** erschienen in *Viruses 2020*, online verfügbar unter doi:10.3390/v12020237

Franke A, Ulrich RG, Weber S, et al. "**Experimental Cowpox Virus (CPXV) Infections of Bank Voles: Exceptional Clinical Resistance and Variable Reservoir Competence**" erschienen in *Viruses* 2017, online verfügbar unter doi:10.3390/v9120391.

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INHALTSVERZEICHNIS

EINLEITUNG

I. EINLEITUNG

Zoonotische Kuhpockenviren (*cowpox virus* = CPXV) sind endemisch in Europa, Nord- und Zentralasien und weisen ein sehr breites Wirtspektrum auf (1, 2). So können sich zum Beispiel Hauskatze (*Felis catus*) (3, 4) und Heimtierratten (*Rattus norvegicus forma domestica*) (5-7), aber auch Zootiere, wie Elefanten (*Elephas maximus*) (8), Alpakas (*Vicungna pacos*) (9) oder Neuweltaffen (10, 11) mit dem Virus infizieren. Von diesen -sogenannten akzidentellen- Wirten kann eine „Spill-over"-Infektion auf den Menschen über Hautläsionen erfolgen (4, 7, 12). Nachdem die Pflichtimpfung gegen Menschenpocken (VARV) mit Beginn der 1980iger Jahre eingestellt wurde, weisen heute mehr als die Hälfte der Menschen keinen Immunschutz gegen VARV oder andere Orthopockenviren wie CPXV auf (13, 14). In Folge dessen steigt die Anzahl humaner Kuhpocken-Infektionen in Europa an (15-17).

Wildlebende Nagetiere gelten als das natürliche Reservoir von CPXV. Bereits Ende der 80iger Jahre wurden in Osteuropa Antikörper bei Gelbzieseln (*Citellus fulvis*) und Gerbilen (*Rhombomys opimus, Meriones libycus,* und *M. meridianus*) mit sehr geringer Prävalenz nachgewiesen (18, 19). In Westeuropa konnten die höchsten Antikörper-Prävalenzen in Rötelmäusen (*Myodes glareolus*), Erdmäusen (*Microtus agrestis*) und Waldmäusen (*Apodemus sylvaticus*) ermittelt werden (20-22). Eine Virusisolation ausgehend von tierischem Material der genannten Spezies war jedoch bis vor kurzem nicht erfolgreich und nur sehr selten konnte virale DNA nachgewiesen werden (23, 24).

Ziel dieser Arbeit war es, Fragen zum zentraleuropäischen Reservoirwirt von CPXV genauer zu eruieren. Dabei wurde das Augenmerk primär auf die Rötelmaus gelegt, da diese Spezies bisher zwar serologisch mit CPXV in Verbindung gebracht wurde (20, 23), aber vor dieser Studie kein CPXV-Isolat aus der Rötelmaus beschrieben werden konnte. Dagegen konnte aus Geweben diverser akzidenteller Wirte (25), zum Beispiel der Ratte (26), oder aus Proben vom Reservoirwirt Feldmaus (*Microtus arvalis*) (27, 28) verschiedene CPXV-Isolate gewonnen werden. Das in dieser Arbeit gewonnene erste CPXV-Isolat aus Rötelmausgewebe (Publikationen I und II) wurde in umfangreichen Infektionsversuchen, sowohl mit Feldmäusen als auch Rötelmäusen als Vertreter der Reservoirwirte (28), wie auch in Ratten, als Modell für einen akzidentellen Wirt (10, 28-30), charakterisiert.

Des Weiteren sollten in dieser Arbeit spezifische Gene identifiziert werden, die einen Einfluss auf die Virulenz von CPXV im Modelltier Ratte haben. In diesem Zusammenhang konnte außerdem gezeigt werden, dass die Identifikation von Virulenzfaktoren des CPXV ausschließlich im Tiermodell möglich ist.

I. Einleitung

LITERATURÜBERSICHT

II. LITERATURÜBERSICHT

1. POCKENVIREN

1.1. Taxonomische Klassifikation von Pockenvieren

Die Familie der *Poxviridae* gehört zu den großen DNA Viren (31) und umfasst zwei Subfamilien (Tabelle 1): Die *Entomopoxvirinae*, die ausschließlich Insekten infizieren und die *Chordopoxvirinae*, welche Wirbeltiere infizieren. Letztere Subfamilie beinhaltet zum jetzigen Zeitpunkt elf Genera, die sich auf Grund von Wirtspektrum, Morphologie, serologischer Kreuzreaktivität und Sequenzähnlichkeit unterscheiden: *Avipoxvirus*, *Capripoxvirus*, *Centapoxvirus*, *Cervidpoxvirus*, *Crocodylidpoxvirus*, *Leporipoxvirus*, *Molluscipoxvirus*, *Orthopoxvirus*, *Parapoxvirus*, *Suipoxvirus* und *Yatapoxvirus*.

Tabelle 1 Systematische Einteilung der Familie *Poxviridae* (nach ICTV, Stand 07.02.2020) (32)

Subfamilie	Genus	Spezies	Wirtsspektrum	Referenz
Entomopoxvirinae	*Alphaentomopoxvirus*	7	Käfer	(33)
	Betaentomopoxvirus	16	Lepidoptera, Orthoptera (Schmetterlinge, Motten, Heuschrecken)	(33)
	Gammaentomopoxvirus	6	Zweiflügler (Fliegen, Moskitos)	(33)
Chordopoxvirinae	*Avipoxvirus*	10	Vögel	(34)
	Capripoxvirus	3	Ziegen, Schafe Rinder, Büffel	(35)
	Centapoxvirus	1	Mensch, Sumpfmaus	(36-38)
	Cervidpoxvirus	1	Hirsch	(39)
	Crocodylidpoxvirus	1	Krokodile	(40)
	Leporipoxvirus	4	Hasenartige, Grauhörnchen	(41)
	Molluscipoxvirus	1	Mensch	(42)
	Orthopoxvirus	10	Säugetiere	
	Parapoxvirus	4	Ziegen, Schafe, Rinder, Mensch	(43)
	Suipoxvirus	1	Schweine	(44)
	Yatapoxvirus	2	Mensch, Primaten	(45)

Das Kuhpockenvirus (CPXV), auf welches in dieser Arbeit vorrangig eingegangen wird, gehört zum Genus der Orthopockenviren (OPV). Wichtige Vertreter dieses Genus sind in Tabelle 2 zusammengefasst.

Tabelle 2 Wichtige Vertreter der Orthopockenviren nach Fenner 1989 (46)

	Wirtsspektrum	Geographische Verbreitung	Referenz
Kamelpockenvirus (Camelpox virus); CMPV	Altwelt- und Neuweltkameliden	Afrika, Asien	(47, 48)
Kuhpockenvirus (Cowpox virus); CPXV	Nagetiere, Katze, Rind, Mensch, Zootiere	Europa, Asien	(1-8, 10-12, 27)
Mäusepockenvirus (Ectromelia virus); ECTV	Maus	Europa, Japan, China	(49)
Affenpockenvirus (Monkeypox virus); MPXV	Affe, Mensch, Zootiere, Präriehund	West-, Zentralafrika	(50-53)
Vaccinia virus; VACV	Mensch, Kaninchen, Wasserbüffel, Rind	Weltweit	(54-57)
Menschenpockenvirus (Variola virus); VARV	Mensch	eradiziert	(58, 59)

1.2. Historischer Hintergrund

Innerhalb der Virusfamilie der Pockenviren ist das Variola Virus (VARV), Erreger der Menschenpocken oder Blattern (*Smallpox*), der bekannteste Vertreter der OPV. Das Virus ist hoch kontagiös, wird durch Tröpfcheninfektion von Mensch-zu-Mensch übertragen und ist ausschließlich humanpathogen (60-62). Ein Faktor, der für die Eradikation der humanen Pocken eine ausschlaggebende Rolle spielte, da VARV keinen tierischen Reservoirwirt aufwies (63).

Man geht heute davon aus, das bereits Pharao Ramses V im Jahre 1157 vor Christus (v. Chr.) an den Menschenpocken erkrankte und verstarb (64). Hinweise drauf geben die pocken-ähnlichen Läsionen seiner Mumie. Interessanterweise finden sich weder in der Bibel noch im Talmud, bedeutende Schriften zur damaligen Zeit, schriftliche Belege für Pocken-Erkrankungen (65).

Erste verlässliche Berichte von Erkrankungen mit Menschenpocken aus China lassen sich bis in das 4. Jahrhundert nach Christus (n. Chr.) zurückverfolgen (64). Zudem gibt es Hinweise, dass VARV bereits im 3. Jahrhundert v. Chr. nach China eingeschleppt wurde (66).

Den Einzug nach Europa fand das Virus voraussichtlich zwischen dem 11. und dem 13. Jahrhundert n. Chr. (67-69). Ab dem 14. Jahrhundert war dieser Erreger als endemisch anzusehen (70). Im 17.-18. Jahrhundert erreichte das Virus mit den europäischen Siedlern den amerikanischen Kontinent (67-69). 1763 wurde dort VARV als biologische Waffe im „French

and Indian War“ (Franzosen- und Indianerkrieg) gegen die indigenen Völker Amerikas eingesetzt. Decken, die zuvor von VARV infizierten Personen genutzt wurden, wurden unter den Ureinwohnern verteilt. Schätzungsweise bis zu 90% der Population der indigenen Bevölkerung wurden durch die Infektion ausgelöscht (71).

Bereits vor Beginn des 18. Jahrhunderts wurde in Asien und Afrika die so genannte Variolation, eine Impftechnik, gegen Menschenpocken durchgeführt (68). Dabei wurde Krustenmaterial von Menschenpocken, die bereits in Abheilung waren, in Wunden an Arm oder Beinen eingebracht. Diese Technik kam mit Lady Mary Wortley Montague 1721 nach England. Nachdem sie ihren Sohn in Konstantinopel 1718 bei dem Arzt Charles Maitland mit VARV inokulieren ließ, wurde auch ihre Tochter 1721 im Beisein von Ärzten des königlichen Hofes inokuliert. Danach breitete sich die Technik der Variolation im gesamten britischen Königreich und kurz darauf auch in Kontinentaleuropa aus, obwohl 2% bis 3% der auf diese Art und Weise inokulierten Personen an Menschenpocken starben (68).

Eine wesentlich sicherere Variante der Impfung gegen VARV wurde 1798 durch den englischen Arzt Edward Jenner eingeführt, der CPXV, ein weniger pathogenes Pockenvirus, anstelle des virulenten VARV zur Prävention gegen humane Infektionen mit VARV einsetzte (68). Er erkannte, dass Melkerinnen, die sich zuvor mit CPXV infiziert hatten, nicht empfänglich für VARV Infektionen waren. 1796 inokulierte er den 8-jährigen Jungen James Phipps mit Material aus einer Kuhpockenläsion der Melkerin Sarah Nelms. Zwei Monate später infizierte er das Kind mit infektiösen VARV-haltigen Pusteln. James blieb gesund. Daraufhin weitete Jenner seine Experimente an Kindern aus, mit demselben Ergebnis (72). Diese neue Art der „Variolation“ wurde Vakzinierung („Vaccination“) genannt, abgleitet vom lateinischen Wort „vacca“: die Kuh. Die Technik verbreitete sich im Folgenden über die ganze Welt. Im 19. Jahrhundert änderte sich das Virus, welches für die Vakzination verwendet wurde von CPXV zu Vaccinia Virus (VACV). Der Grund dafür ist nicht bekannt (71). Der Ursprung von VACV ist nicht abschließend geklärt (73). Da kein natürlicher Wirt für VACV bekannt war, wurde 1939 davon ausgegangen, dass es sich um ein „Laborvirus“ handelt, welches mittels Mutation und Rekombination aus CPXV, VARV und dem „Horsepox“-Virus (Pferdepocken) entstanden ist (73). Edward Jenner selbst vermutet, dass das Horsepox-Virus über Farmarbeiter von Pferden auf die Kühe übertragen wurden, an welchen sich dann später die Melkerinnen infizierten. 1813 schrieb Jenner in einem Brief, dass er das „*Equinating*“, die Vakzination mit dem

Horsepox-Virus, durchführte, ohne dabei Abweichungen in den entstehenden Pocken-Läsionen festzustellen. Er vermutete offenbar, dass CPXV und das Horsepox-Virus den selben Ursprung haben (74). Crookshank ging 1889 davon aus, dass Jenner bereits 1817 das *„Equinating"* der Vakzinierung vorgezogen hat (74). Andere Ärzte wie der Italiener Luigi Sacco oder Jean De Carro in Wien nutzten ebenfalls mit Erfolg das Horsepox-Virus zur Vakzination. Beide konnten zeigen, dass das Horsepox-Virus auch ohne vorher eine Kuh infiziert zu haben vor VARV schütze (73).

1976 kam es in der Mongolei zu einem schweren Ausbruch von Horsepox, bei welchem das bisher einzige Horsepox-Virus Genom (MNR-76) sequenziert werden konnte (75). Das konservierte Viruscore des Horsepox-Virus zeigt phylogenetisch eine enge Verwandtschaft zu VACV. Zudem konnte gezeigt werden, dass das Genom des Horsepox-Virus Gene enthält, die fragmentiert im Genom von VACV vorkommen, ebenso wie vollständige Gene des VACV Genoms fragmentiert im Horsepox-Virusgenom vorkommen (75). Die Autoren gehen davon aus, dass verschiedene Horsepox-Virusstämme den Ursprung für VACV darstellten (75). Um eine deutliche Aussage bezüglich des phylogenetischen Verwandtschaftsverhältnisses zwischen CPXV, VACV und dem Horsepox-Virus treffen zu können, würde man jedoch weitere Horsepox-Virusisolate benötigen (73). Heute scheint das Horsepox-Virus in Europa ausgestorben zu sein (73).

Obwohl die systematische Impfung gegen die Menschenpocken weltweit bereits im 19. Jahrhundert begann, wurde erst mit dem Eradikationsprogramm der Weltgesundheits-organisation (WHO) 1967 ein Durchbruch bei der Ausrottung von VARV erreicht (76). Nach-dem die letzte, natürlich erworbene Infektion 1977 in Somalia aufgetreten war, erklärte die WHO die Menschenpocken 1980 für ausgerottet (71, 77, 78). Die Pflichtimpfung, wurde daraufhin eingestellt. Der letzte Todesfall ereignete sich 1978 bei der britischen medizinischen Fotografin Janet Parker, die sich im Labor mit dem Virus infizierte (71). Heute gilt mehr als die Hälfte der Menschen als ungeimpft gegen VARV (13, 14) und somit bestehen keine kreuz-reaktiven, neutralisierenden Antikörper gegenüber anderen OPV (79). Diese neue Situation kann dazu führen, dass sich vermehrt Menschen mit OPV (anderen als VARV) infizieren (15, 80).

1.3. Morphologie der Orthopocken

Die OPV gehören mit einer Größe von 360 x 270 x 250nm zu den größten bekannten Viren (31) und zeigen in der elektronenmikroskopischen Analyse eine ovale bis ziegelstein-förmige Gestalt mit strukturierter Oberfläche (Abb. 1 A). Im hantelförmigen „Core“ des Virus befindet sich das virale Genom (Abb. 1 B).

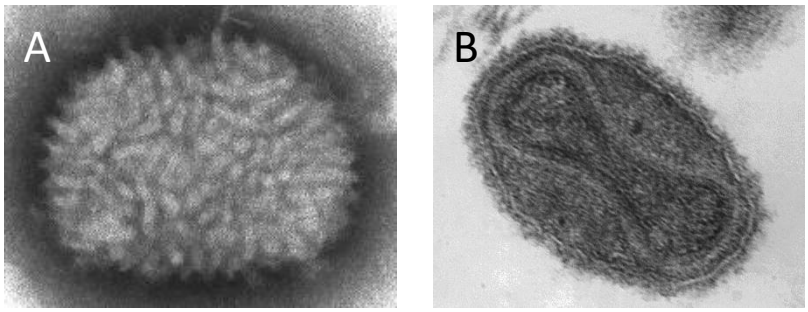

Abbildung 1. Morphologie eines Orthopocken-Virions in elektronenmikroskopischer Aufnahme (A) Oberflächenstruktur von VACV (Aufnahme: CDC/Cynthia Goldsmith; (81)) (B) Querschnitt von VARV. Innerhalb der umhüllenden Membran befindet sich das hantelförmige Virus-Core mit dem viralen Genom (Aufnahme: CDC/ Dr. Fred Murphy; Sylvia Whitfield; (82))

1.3.1. Formen der Virionen

Während einer Pockeninfektion werden insgesamt vier verschiedene Partikelformen beschrieben (83)

(i) intracellular mature virus (IMV, MV);
(ii) intracellular enveloped virus (IEV);
(iii) cell associated enveloped virus (CEV);
(iv) extracellular enveloped virus (EEV, EV).

Hierbei gelten nur IMV und EEV als infektiös und somit essentiell für die Infektion (83). Es wird davon ausgegangen, dass IMVs vor allem die Übertragung zwischen den Wirten vermitteln, während die EEVs für die Ausbreitung innerhalb des Wirtes verantwortlich sind (84). Bei den IMVs umhüllt eine Lipidmembran das bikonkave Kapsid, welches den S-förmigen Genom-Komplex enthält sowie zwei Lateralkörper (Abb. 2). EEVs besitzen eine zusätzliche äußere Hüllmembran, welche von der Wirtszelle abgeleitet wird. Dadurch sind EEVs gut angepasst, um die Infektion außerhalb der Zelle zu verbreiten und zudem relativ resistent gegenüber neutralisierenden Antiköpern (84, 85). Zudem unterscheiden sich die beiden Virionenformen

in den Oberflächenproteinen und nutzen somit verschiedene Mechanismen, um in die Wirtszelle zu gelangen (86).

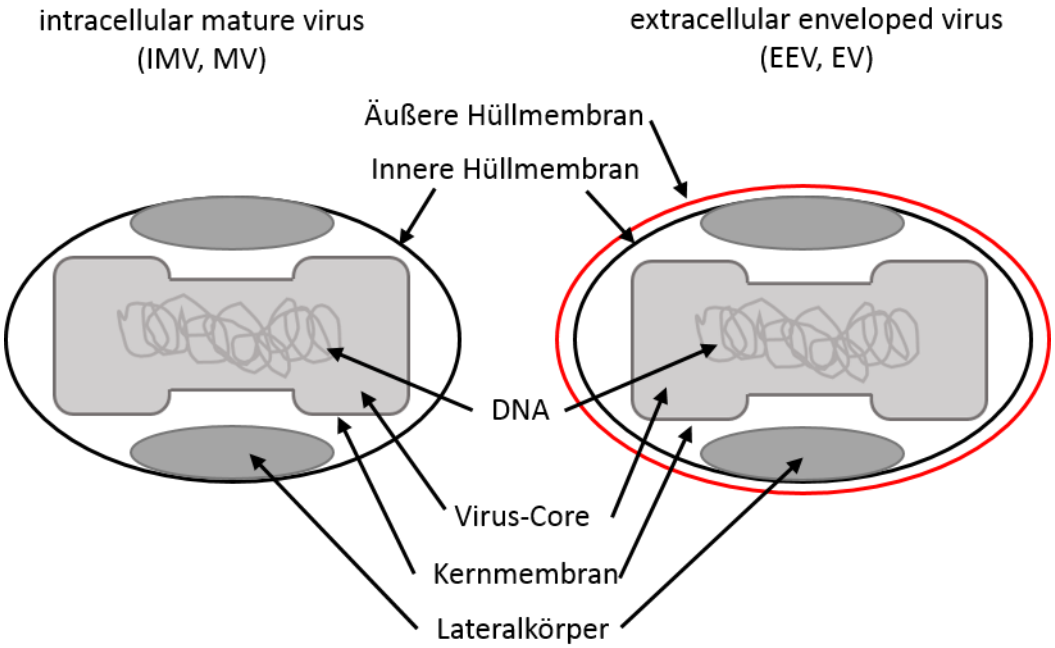

Abbildung 2. Schematischer Aufbau der infektiösen Pockenviruspartikel. Während das *intracellular mature virus* (IMV) von einer Membranhülle umgeben ist, wird das Virus-Core des *extracellular enveloped virus* (EEV) zusätzlich von einer äußeren von der Wirtszelle abgeleiteten Membran umgeben.

1.3.2. Struktur des Pockengenoms (Abb. 3)

OPV besitzen ein lineares, doppelsträngiges DNA-Genom (dsDNA) mit einer Länge von 130 bis 230 Kilobasenpaare (kbp) (87). Die beiden Telomere an den Enden der dsDNA sind durch *Hairpin-Loops* kovalent verbunden und bilden so eine fortlaufende Polynukleotidkette (88). Das Genom kodiert insgesamt für ca. 200 Gene (89). Gene, die Proteine mit Schlüsselfunktionen wie Replikation, Transkription, RNA-Prozessierung aber auch Zelleintritt, Virusassemblierung und Virusfreisetzung kodieren, sind im zentralen Bereich des Genoms angeordnet (90). Dieser zentrale Bereich des Genoms (sog. *Core-Region*) macht ca. 75% der gesamten Sequenz aus und ist am höchsten konserviert (88). Gene in den terminalen Regionen kodieren vorrangig für Proteine, die Einfluss auf die Wirtsspezifität oder Virulenz haben (90). An den

Genomenden befinden sich sogenannte *inverted terminal repeats* („umgekehrte Wiederholungen an den Genomenden“; ITR), die kovalent miteinander verbunden sind und aus 200 bis 500 Basenpaaren (bp) bestehen. ITRs sind invers angeordnete Tandemwiederholungen kurzer DNA-Abschnitten (91).

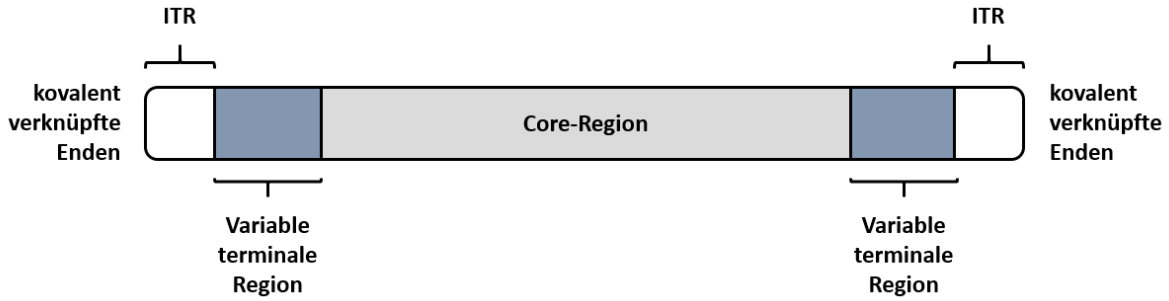

Abbildung 3. Schematische Darstellung des Orthopocken-Genoms. Das dsDNA-Genom der Orthopocken (OPV) ist zwischen 130 und 230 kbp groß und ist an den Enden durch *Hairpin-Loops* kovalent verbunden. An beiden Enden des Genoms befinden sich *inverted terminal repeats* („umgekehrte Wiederholungen an den Genomenden“; ITR).

1.4. Vorkommen und Beständigkeit in der Umwelt

Das „Virion“ von Pockenviren unterscheidet sich signifikant von anderen behüllten Viren. Neben DNA, Proteinen und Phospholipiden enthält es zu ca. 3% Kohlenhydrate (92). Dadurch zeigen die Partikel in der Umwelt eine sehr hohe Tenazität und bleiben über längere Zeiträume kontagiös. Im Allgemeinen ist ein -aus Wirten oder der Umwelt- isoliertes Virus resistenter gegen Umwelteinflüsse als aus Zellkultur angezogenes Material, ebenso sind zellgebundene Viren stabiler als zellfreies Virus (60). Pockenviren sind sehr resistent gegenüber Austrocknung, was durch ihre Ausscheidung zum Beispiel über Hautkrusten (Sekundäreffloreszenzen der Haut) noch weiter verstärkt wird. Getrocknetes VACV ist zum Beispiel bei einer Lagerung von 4°C auch nach mehr als 35 Wochen noch infektiös, während bei einer Lagerung in Puffer bei -20°C nach 15-Jahren der Virustiter lediglich um drei log-Stufen abnimmt (93). Mit Staub, Decken oder auch Kleidung ist VARV über mehrere Jahre übertragbar (92). Zudem zeigen Pockenviren eine hohe Stabilität gegenüber pH-Werten zwischen 4,5 und 10 und sind auf Grund ihres geringen Lipidanteils weniger sensitiv gegenüber organischen Desinfektionsmitteln (92).

Dagegen sind Pockenviren als behüllte Viren vor allem im zellfreien Zustand sehr sensitiv gegenüber kommerziellen, chemischen Desinfektionsmitteln (92).

1.5. Virusreplikation (Abb. 4)

Der Replikationszyklus der OPV beginnt mit dem Eintritt der Viren in die Zelle und der Freisetzung des Virus-Core in das Zytoplasma. Im Gegensatz zu anderen DNA-Viren findet die Replikation im Zytoplasma statt (87). Dort bilden sich im Verlauf der Replikation die sogenannten Virusfabriken: ein abgegrenzter Bereich innerhalb des Zytoplasmas der Wirtszelle. Da der intrazelluläre Replikationsprozess für VACV am besten beschrieben ist und die essentiellen Schritte der Replikation bei OPV stark konserviert sind, wird im Folgenden die Replikation für das VACV dargestellt (Abb. 4).

1.5.1. Zelleintritt (Abb.4 (1+2))

Die Infektion kann sowohl durch IMV, als auch durch EEV-Partikel initiiert werden. Dabei bewirken Glykosaminoglykane (GAGs) im Zusammenspiel mit anderen Zelltyp-abhängigen Oberflächenmolekülen die Bindung der IMVs an die Zellmembran (94). Diese initiale Interaktion zwischen IMV und Zelle löst eine Signalkaskade und die Ausbildung von Aktin-Fortsätzen aus, wodurch der Viruseintritt begünstig wird (95). Die GAG-unabhängige Bindung wird durch die Interaktion zwischen dem viralen Protein A26 und dem extrazellulären Matrixglykoprotein Laminin vermittelt (94).

Für EEV-Partikel sind bisher keine Bindungsfaktoren bekannt (96). Allerdings zeigen Studien, dass sie auf Grund ihrer zusätzlichen Hülle und EEV-spezifischen Membranproteinen Zellen effizienter infizieren können als IMVs und somit für die schnelle Ausbreitung zwischen den Wirtszellen verantwortlich sind (95).

Unabhängig davon, ob es sich um IMV oder EEV handelt, werden die Viruspartikel entweder durch Makropinozytose oder Plasmamembranfusion in die Wirtszelle aufgenommen (96). Dabei weist gerade die Endozytose entscheidende Vorteile auf: (I) Zelluläre Transportmechanismen befördern das Viruspartikel zu einem Fusionspunkt ohne Zytoskelettbarrieren; (II) ein niedriger pH-Wert und Proteasen aktivieren die Fusion zwischen Vesikel und Virus-Partikel innerhalb der Zelle; (III) es bleiben keine Viruskomponenten auf der Plasmamembran der Zelle zurück, die vom Immunsystem erkannt werden könnten;

(IV) Vesikel verhindern, dass aktive virale Fusionsproteine von Antikörpern erkannt werden. Anschließend wird das Virus-Core über Fusion mit der Vesikelmembran oder mit der Zellmembran im Zytoplasma der Wirtszelle freigesetzt (96).

1.5.2. Genexpression (Abb. 4 (3))

Die anschließende Genexpression erfolgt kaskadenartig in drei Schritten:

Frühe (*early*) Transkription (ca. 20 min nach Infektion)

Beginnend mit dem Eindringen des Viruspartikels in die Zelle, wird im Kapsid des Virions die frühe mRNA transkribiert. Die dafür benötigten Transkriptionsfaktoren sind bereits im Virus-Core enthalten (84). Die frühe mRNA codiert Enzyme und Faktoren für die Synthese der viralen DNA und die Transkription der Gene in der mittleren Phase (97). In dieser Phase wird bereits etwa die Hälfte des viralen Genoms transkribiert (98, 99). Anschließend wird das *Uncoating* des Virus-Cores induziert. Die virale DNA wird freigesetzt, die frühe Transkription herabreguliert und die virale DNA-Replikation beginnt (84).

Mittlere (*intermediate*) Transkription (ca. 100 min nach Infektion)

Die mittlere Phase beginnt im Anschluss an die DNA-Replikation. Es werden Gene transkribiert, die für die Genexpression in der späten Phase benötigt werden. Zusätzlich werden Proteine für DNA-*binding*/*packaging* gebildet (97).

Späte (*late*) Transkription (ca. 120 min nach Infektion)

Die Transkription der späten Gene erfolgt über einen Zeitraum von 48h kontinuierlich, so dass sich große Mengen an viralen Proteinen ansammeln können. Die Gene der späten Phase codieren für die frühen Transkriptionsfaktoren, welche zusammen mit dem neugebildeten Genom, der DNA-abhängigen RNA Polymerase und weiteren Enzymen in Tochter-Virionen verpackt werden (97).

1.5.3. DNA-Replikation

Die DNA-Replikation findet im Cytoplasma in den so genannten B-*type inclusion bodies* oder auch „Virusfabriken“ statt und beginnt nach der frühen Phase der Transkription (97). In den Regionen der ITRs bilden sich ein oder zwei Einzelstrangbrüche (*nicks*), so dass ein 3`-Ende als Primer für die Replikation frei wird (97). Der replizierte DNA-Strang faltet sich zurück und das

restliche Genom wird kopiert. Dabei kommt es an den Genomenden auf Grund der *Hairpin-Loops* zur Ausbildung sogenannter Konkatemere, die bei anhaltender Replikation sehr groß und verzweigt werden können. Nach Beginn der späten Transkriptions-Phase werden die Konkatemere aufgelöst und einheitlich lange Genome entstehen (87, 97).

1.5.4. Morphogenese (Abb. 4 (4-7))

Wie die mittlere und späte Transkription sowie die DNA-Replikation, findet der Zusammenbau der Viruspartikel in den Virusfabriken statt. Als erstes definieren sich halbmondförmige Membranstrukturen (*crescent membranes*) und unreife Virionen (*immature virion*, IV) (97). Die Herkunft dieser Membranen ist noch ungeklärt. Zum einen wird eine *de novo*-Synthese diskutiert, zum anderen, dass sie von bestehenden Membranen zum Beispiel aus dem endoplasmatischen Retikulum abgeleitet sind (83). IVs enthalten bereits virales Genom, sind aber nicht infektiös. Dies ändert sich nach der proteolytischen Prozessierung der Kapsidproteine und der Kondensation des Virus-Cores (85). Aus dem IV wird ein reifes (mature), infektiöses Viruspartikel (97). Die IMVs einiger OPV (auch CPXV und ECTV) bilden Mikrotubuli-abhängige A-*type inclusion bodies* (Typ A Einschlusskörperchen; ATI), die die IMVs in einer Matrix aus Proteinen bis zur Freisetzung schützen (97).

IMVs können entweder über Zelllyse freigesetzt werden oder sie werden von zwei weiteren Membranen aus dem Endosom oder dem trans-Golgi-Apparat umhüllt (*intracellular enveloped virion*) (83). Diese dann dreifach umhüllten Partikel werden über Mikrotubuli zur Plasmamembran transportiert und dort mittels Exozytose durch Fusion der äußeren Membran mit der Zellmembran als EEVs freigesetzt. Einige der IEVs bleiben zell-assoziiert an der Zelloberfläche haften. Sie bilden die CEVs (84). Ebenfalls zur Bildung der EEVs kommt es durch direkte Ausknospung der IMVs an der Plasmamembran (97). Innerhalb eines Wirtes verbreiten sich Pockenviren über CEV- und EEV-Partikel.

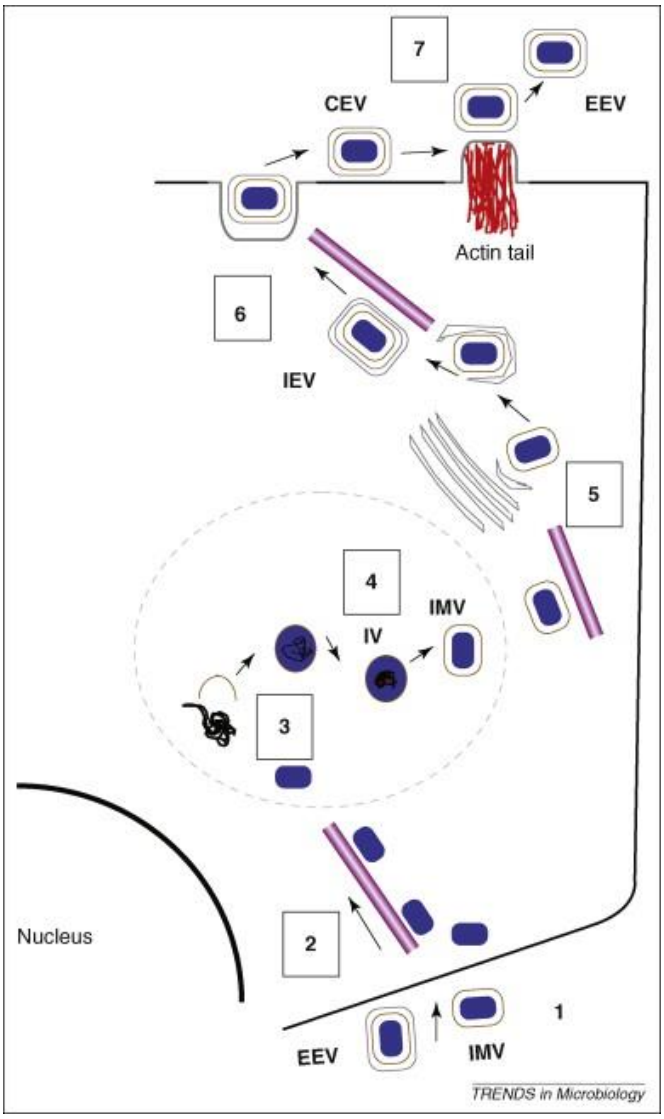

Abbildung 4.Schematische Darstellung des Replikationszyklus am Beispiel des Vaccinia Virus. Nach dem Eintritt des Virus in die Zelle und dem Freisetzten des Virus-Cores in das Zytoplasma (1) wird das Virus-Core weiter in den zentralen Bereich der Zelle transportiert (2). Nach der Transkription der frühen mRNA, wird das Uncoating des Virus-Cores induziert und die virale DNA freigesetzt (3). Im Zytoplasma findet die DNA-Replikation in den Virusfabriken statt und unreife Virionen (IV) werden gebildet (4), die sich dann durch die proteolytische Prozessierung der Kapsidproteine und der Kondensation des Virus-Cores zu infektiösen Virionen (IMV) entwickeln (4). IMVs können entweder über Zelllyse freigesetzt werden oder werden von zwei weiteren Membranen umgeben (5) und als *intracellular enveloped virion* (IVE) zur Zellmembran transportiert (6). Als *cell associated enveloped virus* (CEV) bleiben sie entweder zellassoziiert an der Zelle haften oder werden mittels Exozytose als *extracellular enveloped virus* (EEV) freigesetzt (7). Abbildung: Roberts et al. 2008 (85)

1.6. Verbreitung der Pockenviren

1.6.1. Von Zelle zu Zelle

Für die Übertragung der Pockenviren von Zelle zu Zelle sind vor allem die CEVs und EEVs verantwortlich (83). Zudem sind sie auf Grund verschiedenster immunmodulatorischer Mechanismen in der Lage die wirtseigene Immunantwort zu umgehen (100). Zur Infektion benachbarter Zellen induzieren CEVs die Bildung von Aktinfortsätzen, um die Nachbarzelle zu erreichen. EEV gelangen als freie Viruspartikel in Suspension besser auch zu weiter entfernten Zielzellen (83).

1.6.2. Von Wirt zu Wirt

Verschiedene OPVs nutzen verschiedene Mechanismen, um von Wirt zu Wirt übertragen zu werden. Während VARV und VACV als Aerosole oder als Staub getrockneter Pockenläsionen respiratorisch übertragen werden, verbreitet sich ECTV oder CPXV beim akzidentellen Wirt in der Regel mechanisch über Hautverletzungen (71). Auf Grund ihrer hohen Beständigkeit in der Umwelt sind vor allem IMVs für die Übertragung zwischen zwei Wirten geeignet. Okkludiert in ATIs erhöht deren Polypeptidstruktur die Umweltstabilität der IMVs zusätzlich und unterstützt so die Übertragung (101, 102).

Dagegen sind die Membranen der EEVs / CEVs fragil und können einfach zerstört werden. Sie geben dann die infektiösen IMVs frei (84).

2. DIE SPEZIES KUHPOCKENVIRUS (CPXV)

CPXV gehört zu der am frühsten beschriebenen Spezies innerhalb des Genus OPV. Im 18. Jahrhundert wurde CPXV von Edward Jenner in seinen Publikationen „Inquiry“ und „Further observation on the Variolae Vaccinae“ beschrieben und als effektiver Impfstoff gegen VARV eingesetzt (68). Heute ist CPXV endemisch in Europa und Asien zu finden (103) und zeichnet sich durch das längste Genom (224-228 kbp) und das breite Wirtspektrum innerhalb der OPV aus (1, 2). Zudem lassen sich CPXV von anderen humanpathogenen OPV durch große, eosinophile ATIs im Zytoplasma infizierter Zellen (104) und hämorrhagische Pockenläsionen von 2-4mm Größe auf der Chorioallantoismembran (CAM) embryonierter Hühnereier 72h nach Inokulation abgrenzen (105). Dabei ist der Name „Kuhpocken“ unzutreffend bzw. missverständlich und eher historisch zu erklären, da im 18. Jahrhundert CPXV Infektionen vor allem bei Kühen und Melkerinnen beschrieben wurden (72). Im Jahre 1985 wurde zum ersten Mal von einer humanen CPXV Infektion berichtet, die von einer Katze übertragen wurde (106). Heutzutage sind vor allem Hausratten (*Rattus norvegicus forma domestica*), Katzen (*Felis catus*), Zootiere, aber auch Menschen von einer Infektion betroffen (1). Vor allem junge Menschen zeigen eine erhöhte „Empfänglichkeit“ für CPXV und OPV Infektionen allgemein. Dies lässt sich auf die fehlende Impfung gegen VARV und den somit mangelnden Schutz durch eine kreuzreaktive Immunität zurückführen (13, 15, 80). Zudem gelten Ratten als „neue“ Haustiere und werden vermehrt von jüngeren Menschen als „Schmusetiere“ gehalten (40).

2.1. Klinische Symptome einer Kuhpockeninfektion

2.1.1. CPXV beim Menschen

Die humane CPXV-Infektionen ist im Normalfall eine milde, selbstlimitierende Erkrankung mit lokalen Läsionen vorrangig an den Händen oder im Gesicht (107). Dagegen kann es bei immunsupprimierten Personen zu einer generalisierten Infektion kommen, die auch zum Tode führen kann (108). Die Inkubationszeit beträgt 8-12 Tage (5). Im Gegensatz zu VARV, das als Aerosol übertragen werden kann, findet eine Infektion mit CPXV vorrangig über Verletzungen in der Haut statt (107). An der Inokulationsstelle bilden sich entzündete Maculae, die sich dann innerhalb von sieben bis zwölf Tage weiter zu Papeln und anschließend zu hämorrhagischen bis ulzerierenden Bläschen weiterentwickeln. Anschließend bilden sich schwarze, nekrotische

Krusten, die unter Narbenbildung abfallen. Betroffene Personen klagen über Allgemeinsymptom wie Fieber, Abgeschlagenheit, Übelkeit, Kopf- und Muskelschmerzen, welche aber mit Bildung der Krusten auf den Läsionen verschwinden. Die klinische Erkrankung dauert in der Regel 6-8 Wochen (5, 107).

2.1.2. CPXV bei Haustieren

Die klinische Manifestation einer CPXV-Infektion bei Tieren läuft je nach Spezies und Virus-Stamm sehr unterschiedlich. Infektionen von Kühen wurden bis etwa Mitte der 70er Jahre beobachtet und zeigten sich vor allem durch typische Pockenläsionen an Euterhaut und Maul (109). In den letzten zwei Jahrzehnten wurde nur von einem Fall von CPXV bei Rindern berichtet (110). In Großbritannien wurde serologisch gezeigt, dass CPXV-Infektionen bei Rindern nicht enzootisch vorkommen (111).

Häufiger zu sehen sind CPXV Infektionen heutzutage vor allem bei Katzen (3, 4, 112) und Ratten, die als Haustiere gehalten werden (5-7). Bei den Katzen sind vor allem Freigänger und Mäusefänger betroffen (113), da Nagetiere als natürlicher Reservoirwirt von CPXV gelten (20, 28). Meist tritt an Vorderpfoten, Kopf oder Hals eine Primärläsion nach Infektion über Hautwunden auf. Ein bis zwei Wochen später entwickeln sich multiple Sekundärläsionen, zuerst als kleine erythematöse Maculae später als zirkuläre, ulzerierende Papeln. Diese verkrusten und fallen nach zwei bis drei Wochen ab. Selten stellen sich Fieber, Inappetenz, oft vergesellschaftet mit Bläschenbildung auf der Zunge, und Konjunktivitis ein (113). Vergleichbar zu Menschen mit Immunsuppression kann eine CPXV-Infektion bei Katzen, die mit dem felinen Immundefizienz-Virus (FIV) infiziert sind, tödlich verlaufen (114). Katzen gelten als häufigste Infektionsquelle für humane CPXV Infektionen (114).

Auch als Haustiere gehaltene Ratten können das Virus auf den Menschen übertragen. Ende 2008 und Anfang des Jahres 2009 kam es in Deutschland, den Niederlanden und Nordfrankreich zu „Spill-over"-Infektionen mit CPXV ausgehend von Heimtierratten (5, 26, 115). Bei allen Fällen konnte der identische CPXV-Stamm nachgewiesen werden (115). Die Ratten zeigten krustig bis ulzerierende Hautläsionen vor allem an den Gliedmaßen, dem Schwanz und dem Kopf. Zudem litt ein Teil der Tiere unter respiratorischen Symptomen und Konjunktivitiden (26). Die Infektion verlief bei vielen der betroffenen Ratten tödlich (5, 26). Experimentelle Studien zeigten, dass der klinische Verlauf in Ratten stark vom verwendeten CPXV-Stamm und dem Alter der infizierten Tiere abhängig ist (Abb.5) (29, 30).

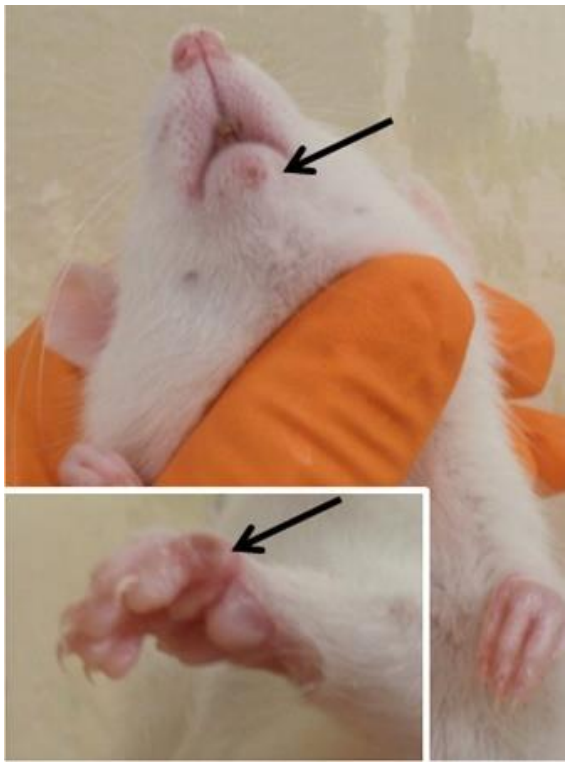

Abbildung 5 Pockenläsionen bei Wistar-Ratten im Tierversuch. Ausbildung von typischen Pockenläsionen an Kinn und Pfote (schwarzer Pfeil) 9 Tage (Kinn) und 7 Tage (Pfote) nach experimenteller Infektion mit CPXV FM2292 (28)

2.1.3. CPXV bei Wild- und Zootieren

CPXV-Infektionen können nicht nur bei Haustieren, sondern auch bei Zootieren beobachtet werden. Bei diesen akzidentiellen Wirten treten CPXV-Infektionen als schwerwiegende, meist letale Erkrankungen auf. Gerade Elefanten gelten als besonders empfänglich und zeigen meist einen letalen Verlauf, so dass die meisten Zooelefanten in Europa heute mit dem modifizierten VACV Stamm Ankara (MVA) vakziniert werden (8, 116). Aber auch Groß- und Kleinkatzen sind mitunter letal betroffen (117). Neuweltkameliden, wie Alpakas (9) und Neuwelt-Affen, wie Lisztaffen (*Saguinus oedipus*) (10) sind ebenfalls empfänglich für CPXV. In insgesamt vier Alpakaherden in Ostdeutschland, die mit dem Virus infiziert waren, und dem Tierpark Bad Liebenstein in Thüringen, der Verluste in seiner Lisztaffen-Gruppe auf Grund von CPXV zu verzeichnen hatte, wurde der Eintrag über wilde Nagetiere diskutiert. In zwei der vier Alpakaherden konnten CPXV-Antikörper mittels Immunfluoreszenztest (IFT) in fünf Brandmäusen (*Apodemus agrarius*; Herde I) und drei Rötelmäusen (Herde II) nachgewiesen werden. Zudem wurde in einer dritten Herde aus einer Feldmaus (*Microtus arvalis*) CPXV mit

einer Sequenzähnlichkeit von mehr als 99% zu CPXV in den Alpakas isoliert (27). Alle getesteten Nagetiere in Bad Liebenstein waren qPCR negativ für CPXV (10).

2.2. Diagnostischer Nachweis

Bei der OPV-Diagnostik ist zwischen den direkten und den indirekten Nachweismethoden zu unterscheiden. Während bei direkten Methoden der Erreger zum Beispiel auf Grund vorhandener DNA oder spezifischer Antigene nachgewiesen wird, nutzen indirekte Methoden den Nachweis von spezifischen Antikörpern.

Die real-time Polymerasekettenreaktion (qPCR) stellt dabei die im Labor am häufigsten eingesetzte Methode dar, da sie OPV-DNA schnell, sensitiv und spezifisch identifiziert (118). Je nach verwendeten Primern und Sonden ist auch eine Speziesdifferenzierende Diagnostik innerhalb der OPV möglich. Während das von Scaramozzino et al. 2007 publizierte qPCR Protokoll nur die Aussage zulässt, ob es sich um OPV handelt und keine weitere Differenzierung zulässt (119), kombinierten zum Beispiel Maksyutov et al. 2015 einen Genus-spezifischen Nachweis mit einer Spezies-spezifischen Amplifikation. Dabei basiert der Nachweis für OPV auf dem konservierten, offenen Leserahmen (*open reading frame*, ORF) F4L, während ein zweites Primerpaar an den ORF D8L, spezifisch für CPXV, bindet (120). Außerdem erlaubt das Verfahren der qPCR eine quantitative Aussage über den Gehalt an viraler Nukleinsäure im Ausgangsmaterial.

Eine weitere Nachweismöglichkeit ist die Virusisolation in Zellkultur, eine sensitive Methode, wenn auch langsamer als die PCR. Die Anzucht kann auf verschiedenen etablierten Zelllinien wie Vero- oder HeLa-Zellen erfolgen. Einen nachweisbaren zytopathischen Effekt (CPE) findet man in der Regal nach 24-48h (118). Eine weitere Charakterisierung kann mittels Elektronenmikroskopie, vor allem mit Blick auf ATIs als spezifisch für humanpathogene OPV (1, 104, 116), erfolgen. Gerade während der Eradikation der Menschenpocken wurde die Elektronenmikroskopie ab den 1950er Jahren als Standardmethode eingesetzt. Auf Grund ihrer Größe und ihrer Form sind Pockenviren gut zu identifizieren. Zwar können sie elektronenmikroskopisch nicht auf Genus-Level unterschieden werden, doch die Abgrenzung zu wichtigen Differentialdiagnosen, wie Windpocken, ist gut möglich (118).

Bereits 1937 wurden embryonierte Hühnereier zur Pockendiagnostik eingesetzt (118). Dabei wurde die CAM mit Material aus Pockenläsionen beimpft. Nach zwei bis vier Tagen erlaubten die entstandenen Pockenläsionen eine Differenzierung zwischen VARV und anderen zoonotischen OPV. Während VARV kleine, weiße, opaque Läsionen auf der CAM ausbilden, entwickeln sich bei Inokulation mit CPXV große, hämorrhagische Läsionen (121). Das *High throughput sequencing* (HTS) zur Bestimmung des Genoms (25, 122, 123) ist die spezifischste Methode, um bis auf den Spezieslevel zu einer Diagnose zu gelangen, gelingt aber am besten mit hoch positivem Material.

Als indirekte Methoden zur OPV Diagnostik kommen zum Beispiel Immunfluoreszenztest, Plaque-Reduktions-Assay und *Enzyme-linked Immunosorbent Assay* (ELISA) in Frage. Diese werden vor allem für retrospektive Studien und in Kombination auch bei Screening-Untersuchungen genutzt (23, 121).

3. WIRTSSPEKTRUM UND GENETISCHE VARIABILITÄT

3.1. Definition: Was ist ein Wirt?

3.1.1. Akzidenteller Wirt

Als akzidentellen Wirt beschreibt Ashford, 2003, einen Wirt, der mit einem Pathogen infiziert werden kann, aber nicht zum Erhalt des Pathogens innerhalb einer Population beiträgt (124). Solche Zufallswirte stellen -in Bezug auf CPXV-Infektionen- zum Beispiel Katzen und Ratten, aber auch der Mensch, da. Sie erkranken an der Infektion, sind aber nur in einem sehr begrenzten Rahmen an der Verbreitung des Virus beteiligt. Zudem kann sich das Virus nicht eigenständig in der Population halten (124). Ein Versuch mit Katzen zeigte, dass eine Übertragung von Tier zu Tier im experimentellen Setting nicht möglich war. Dabei wurden Katzen mit einem aus einer Katze gewonnenen Isolat inokuliert. 24 Stunden später wurden serologisch negative Tiere dazu gesetzt und für 21 Tage serologisch auf Antikörper untersucht. Während die inokulierten Tiere milde klinische Symptome zeigten und serokonvertierten, zeigten die Sentineltiere weder Krankheitssymptome noch konnten Antikörper nachgewiesen werden (125).

3.1.2. Reservoir Wirt

Das Oxford English Dictionary (OED) definiert einen Reservoirwirt als „a population, which is chronically infested with the causative agent of a disease and can infect other populations" („Eine Population, welche chronisch mit einem Krankheitserreger einer Krankheit befallen ist und andere Populationen infizieren kann") (126).

Haydon et al. ergänzt diese Definition, indem sie nicht nur von einer Population, sondern von mehreren, epidemiologisch verbundenen Populationen oder Umgebungen spricht, die ein Pathogen permanent erhalten können. Dabei legt er sich nicht fest, ob es sich innerhalb einer Population um nur eine Spezies handelt, da auch Vektoren als Zielpopulation in Betracht gezogen werden müssen. Die absolute Größe eines Reservoirs ist somit nicht genauer definiert (127).

Gerade die Einbindung der Umgebung in die Definition eines Reservoirs ist für CPXV und Pocken allgemein interessant, da sie in Form von IMVs und gebunden in ATIs eine hohe Beständigkeit in der Umwelt aufweisen (100-102).

3.2. Rötelmaus und Feldmaus als Reservoirwirte

Wildlebende Nagetiere, insbesondere Wühlmäuse, gelten als das natürliche Reservoir von CPXV. Während bereits Ende der 80iger Jahre in Osteuropa Antikörper in Zieseln und Gerbilen mit sehr geringer Prävalenz nachgewiesen werden konnten (18, 19), wurden in Westeuropa die höchsten Antikörper-Prävalenzen bei Rötelmäusen (*Myodes glareolus*), Erdmäusen (*Microtus agrestis*) und Waldmäusen (*Apodemus sylvaticus*) ermittelt (22). Die Virusisolation war jedoch bis vor kurzen nicht erfolgreich und nur selten konnte DNA in Feld- und Rötelmäusen nachgewiesen werden (23, 24).

1997 publizierte Bennett et al. Versuche mit Rötelmäusen, Erdmäusen und Waldmäusen (20). Das dabei verwendete CPXV Isolat L97 wurde aus Proben einer Katze isoliert und bereits in Katzen charakterisiert (125, 128). Die Mäuse wurden intradermal in ein Bein inokuliert und entwickelten in Folge dessen eine lokale Schwellung, die sich innerhalb von 14 Tagen zurückbildete. Bennett et al. zeigten, dass Rötelmäuse zwar serokonvertierten, aber kein Virus isoliert werden konnte, mit Ausnahme von zwei Tieren (einmal in der Nasenscheidewand, einmal in der Leber), die mit sehr hoher Dosis inokuliert worden waren. Dasselbe Ergebnis zeigte sich bei Erdmäusen und Waldmäusen. Infektionsversuche mit ECTV in Rötelmäusen zeigten weder eine Serokonversion noch klinische Symptomatik bei den inokulierten Tieren (20). Bennett et al. konnten damit zeigen, dass Rötelmäuse zwar empfänglich für CPXV-Infektionen sind und somit als Reservoirwirt in Frage kommen könnten, allerdings konnten sie keine Aussage bezüglich der Übertragung des Virus treffen (20).

Weitere Studien die Prävalenz von CPXV in Rötelmäusen und Waldmäusen betreffend wurden von 1995-1998 in England durchgeführt (103, 129). Über mehrere Jahre wurden Fänge durchgeführt und Serumproben mittels IFT ausgewertet. Die meisten Rötel- und Waldmäuse wurden jeweils im Spätsommer und Frühherbst gefangen. In diesen Monaten war auch die Seroprävalenz innerhalb der Population am höchsten (103, 129). Passend dazu wurden in diesen Monaten auch am häufigsten Fälle von CPXV-Infektionen bei Menschen und Katzen gemeldet (129). Es wurde geschlussfolgert, dass Rötelmäuse ein bedeutenderes CPXV-Reservoir in Großbritannien darstellen als Waldmäuse (129). Ferner konnten experimentelle Infektionen mit Waldmäusen zeigen, dass diese weniger empfänglich für eine CPXV-Infektion sind als Rötelmäuse (20).

Interessanterweise wurde bei Hazel et al. 2000 ein höherer Prozentsatz an Serokonversionen männlicher Tieren ermittelt, so dass die Autoren davon ausgehen, dass männliche Rötelmäuse wichtiger in ihrer Funktion als CPXV-Reservoir sein könnten als weibliche Tiere (129). Dem widersprechen „Space-Time Interaction" Studien, welche von Carslake et al. 2006 durchgeführt wurden und zeigen, dass weibliche Rötelmäuse mit höherer Wahrscheinlichkeit für die Übertragung von CPXV in der Rötelmaus-Population verantwortlich sind (130). Bei Waldmäusen dagegen scheinen männliche Tiere CPXV häufiger zu übertragen (130). Dieser Unterschied zwischen den beiden Spezies scheint auch dafür verantwortlich zu sein, dass eine Übertragung zwischen Rötel- und Waldmäusen als sehr unwahrscheinlich eingeschätzt wird (130).

Auf Populationsebene zeigten Untersuchungen über mehrere Jahre bei Erdmäusen den Zusammenhang zwischen Infektionsrate und Wirtsdichte (131). Die Daten zeigen eine Saisonalität für den Transmissionskoeffizienten und das Verhältnis von Wirtskontaktrate und Wirtsdichte. Diese Saisonalität wird spekuliert ergibt sich z. B. aus einem veränderten Sozialverhalten (131).

2015 wurde von Hoffmann et al. eine Studie zur Empfänglichkeit von Feldmäusen (M*icrotus arvalis*) durchgeführt, bei der neben CPXV RatPox09 auch ein aus der Feldmaus gewonnenes CPXV-Isolat („FM2292") in Feldmaus und Wistar Ratte als Modell-Tier charakterisiert wurde. Diese Studie konnte zeigen, dass CPXV mittels Tröpfcheninfektion innerhalb einer Population, unabhängig ob Reservoir oder akzidenteller Wirt, übertragen werden kann. Sowohl bei inokulierten Feldmäusen als auch bei den Ratten konnte die Ausscheidung von infektiösem Virus gezeigt werden. Dabei zeigte das Reservoir „Feldmaus" mit Ausnahme eines Tieres einen subklinischen Infektionsverlauf, während der akzidentelle Wirt „Ratte" prominente Symptome und Pockenläsionen zeigte. Dies demonstriert, dass verschiedene CPXV-Isolate ein variables Virulenzpotential besitzen und die Virulenz einzelner Isolate zudem auch wirtsabhängig ist (28).

Eine ähnliche Studie mit Rötelmäusen war bis zum jetzigen Zeitpunkt nicht möglich, da kein Isolat aus der Rötelmaus zur Verfügung stand.

3.3. CPXV als polyphyletische Spezies

Bei CPXV handelt es sich um eine polyphyletische Spezies (25). Während sich bei einer monophyletischen Spezies der Ursprung auf einen gemeinsamen Vorfahren zurückführen lässt, ist dies bei einer polyphyletischen Spezies nicht möglich (132).

Bereits im Jahr 2011 gab es phylogenetische Studien, die darauf hinwiesen, dass es sich bei CPXV vermutlich um eine polyphyletische Spezies handelt. Die von Carroll et al. verwendeten 12 CPXV-Isolate ließen sich dabei in zwei Kladen einteilen: „Cowpox-like“ und „Vaccinia-like“ (105). 2013 wurde eine neue dritte Klade als „VARV-like“ definiert. Diese setzt sich vorrangig aus CPXV-Isolaten zusammen, die aus Ratten isoliert werden konnten, unter anderem das in Ratten hochpathogene Isolat „RatPox09“. Ebenso lassen sich dort Isolate einordnen, die aus Menschen gewonnen werden konnten, die sich an Ratten infiziert hatten (122). Dazu sortierten sich 2017 weitere 20 Isolate, die von Franke et al. berichtet worden sind. Es konnten vier verschiedene CPXV-Kladen identifiziert werden, welche als CPXV-like 1, CPXV-like 2, VARV-like und VACV-like definiert wurden. Die größte Klade machte dabei CPXV-like 1 aus, in welcher vorrangig Isolate aus akzidentellen Wirten versammelt sind, während sich in der Klade CPXV-like 2 auch zwei Isolate aus der Reservoirspezies Feldmaus finden lassen, unter anderem CPXV FM2292. Ebenfalls in diese Klade ordnet sich der CPXV-Laborstamm Brighton Red (BR) ein (Abb. 6) (25).

Interessanterweise konnten drei CPXV-Isolate in keine der CPXV-spezifischen Kladen ein-geordnet werden. Sie werden als *single branch* im phylogenetischen Baum geführt (Abb. 6). Bei einem dieser Isolate handelt es sich um das 2010 aus einem Lisztaffen gewonnene CPXV Isolat „Ger2010MKY“ (25).

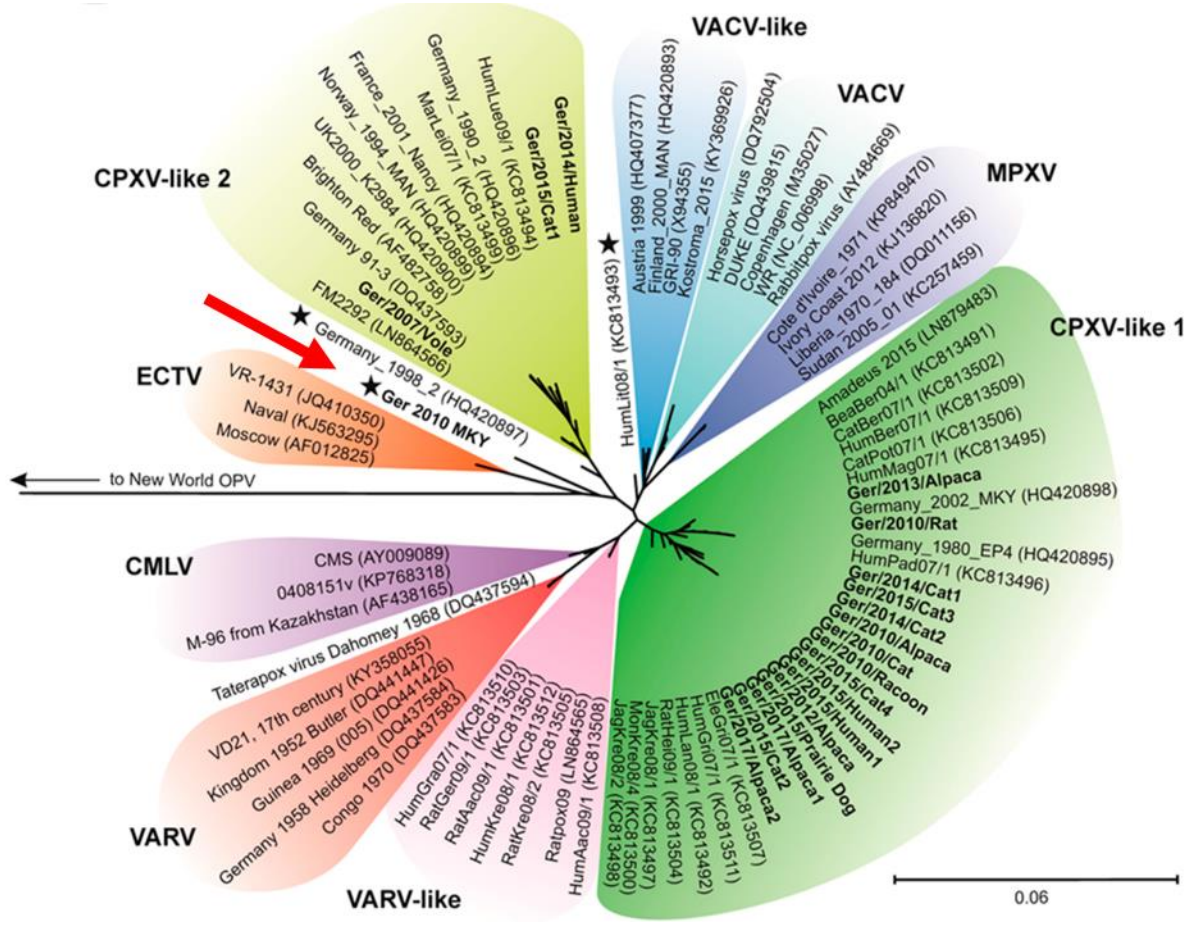

Abbildung 6. Phylogenie der Orthopockenviren (OPV) modifiziert nach Franke et al. 2017 (25). Der Phylogenetische Baum der OPV stellt die Spezies *Ectromelia virus* (ECTV), Affenpockenvirus engl. *Monkeypox virus* (MPXV), *Variola Virus* (VARV) und Kamelpockenvirus engl. *Camelpox virus* (CMPV) als monophyletische Gruppen dar, während die Spezies Kuhpockenvirus *Cowpox virus* polyphyletisch in vier Kladen (CPXV-like 1, CPXV-like 2, VAC-like und VARV-like) aufgeteilt wird. Der rote Pfeil markiert die Position des CPXV-Isolates Ger2010MKY als *single branch* im phylogenetischen Baum.

ZIELSETZUNG

III. ZIELSETZUNG

Obwohl bereits Studien zum CPXV Reservoir durchgeführt worden sind, ist speziell die Rolle der Rötelmaus als Reservoirtier weiterhin ungeklärt.

(a) Rolle der Rötelmaus als Reservoirwirt

Im Rahmen eines großen Reservoir-Screenings im Verbundprojekt „*Population dynamics of rodent hosts of zoonotic disease: interaction of climate, landuse and biodiversity*“ sollte Organmaterial (Nasenscheidewand) gefangener Reservoirwirte, vorrangig Rötelmäuse, auf OPV-DNA untersucht und bei DNA-positiven Proben eine Virusisolation unternommen werden. Reservoirwirt-originäre CPXV-Isolate sollten im Folgenden die Möglichkeit eröffnen, vergleichende *in vitro*-Studien auf Rötelmaus- und Feldmaus-Zelllinien (133) und der CAM durchzuführen.

(b) Reservoir Spezies-spezifische phylogenetische Abstammung innerhalb der CPXV

Anschließend sollten neue Reservoirwirt-originäre CPXV-Isolate *in vivo* charakterisiert werden. Experimentelle Infektionen mit Rötel- und Feldmäusen als Vertreter des Virusreservoirs sollten vergleichend zum Infektionsmodell Ratte hinsichtlich Symptomatik, Virusausscheidung und Serologie analysiert werden.

Die Ergebnisse von Infektionsstudien mit Rötelmäusen, die mit CPXV-Isolaten aus der Feldmaus inokuliert wurden -und auch von akzidentellen Wirten- sollten dann vergleichend ausgewertet werden. Die Hypothese einer „spezies-spezifischen“ polyphyletischen Gruppierung von CPXV-Kladen wurde schließlich mittels der Infektionsstudien evaluiert.

(c) Virulenzfaktoren des CPXV im Rattenmodell

Abschließend sollte sich diese Arbeit der Frage nach Virulenzfaktoren und deren Auswirkungen auf das Modelltier Ratte widmen. Dazu wurden der CPXV-Laborstamm Brighton Red (BR) und das in Ratten hochpathogene CPXV-Isolat RatPox09 phylogenetisch verglichen und Gene in RatPox09 identifiziert, die im Genom von BR fehlen. Mittels dieser Gene wurden BR-Mutanten im Sinne des „gain of function“ Prinzips erzeugt, die *in vitro* und *in vivo* charakterisiert wurden.

PUBLIKATIONEN

IV. PUBLIKATION I

Molecular Detection and Characterization of the First Cowpox Virus Isolate Derived from a Bank Vole

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Molecular Detection and Characterization of the First Cowpox Virus Isolate Derived from a Bank Vole

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Abstract: *Cowpox virus* (CPXV) is a zoonotic *orthopoxvirus* (OPV) that infects a wide range of mammals. CPXV-specific DNA and antibodies were detected in different vole species, such as common voles (*Microtus arvalis*) and bank voles (*Myodes glareolus*). Therefore, voles are the putative main reservoir host of CPXV. However, CPXV was up to now only isolated from common voles. Here we report the detection and isolation of a bank vole-derived CPXV strain (GerMygEK 938/17) resulting from a large-scale screening of bank voles collected in Thuringia, Germany, during 2017 and 2018. Phylogenetic analysis using the complete viral genome sequence indicated a high similarity of the novel strain to CPXV clade 3 and to OPV "Abatino" but also to *Ectromelia virus* (ECTV) strains. Phenotypic characterization of CPXV GerMygEK 938/17 using inoculation of embryonated chicken eggs displayed hemorrhagic pock lesions on the chorioallantoic membrane that are typical for CPXV but not for ECTV. CPXV GerMygEK 938/17 replicated in vole-derived kidney cell lines but at lower level than on Vero76 cell line. In conclusion, the first bank vole-derived CPXV isolate provides new insights into the genetic variability of CPXV in the putative reservoir host and is a valuable tool for further studies about CPXV-host interaction and molecular evolution of OPV.

Keywords: bank vole; *Myodes glareolus*; cowpox virus; orthopoxvirus; Germany

1. Introduction

Members of the genus *Orthopoxvirus* (OPV) belong to the subfamily *Chordopoxvirinae* within the family *Poxviridae*. The relatively large enveloped virions are generally brick-shaped and contain a double-stranded linear DNA genome. The viral genome consists of a unique region that is flanked by inverted terminal repeats (ITR). While the core genome, encoding proteins that are essential for the viral DNA replication machinery as well as structural and regulatory factors, is highly conserved, the flanking and ITR regions encode host response modulating proteins that vary between different OPV species [1]. Currently, the genus *Orthopoxvirus* comprises ten species [2]. These virus species differ drastically in their host range: Although the infection with *Variola virus* (VARV), the eradicated causative agent of smallpox, was limited to humans, some other OPV do cross species barriers. For example, *Monkeypox virus* (MPXV), *Vaccinia virus* (VACV), and *Cowpox virus* (CPXV) have a wide

host range and can cause spillover infections in multiple non-reservoir species [3,4]. Zoonotic CPXV is endemic in Eurasia and believed to be a "rodent-borne" virus. CPXV-related disease has been reported from 27 host species, including humans, cats, livestock, and zoo animals [5,6]. Most of these mammals need to be considered accidental dead-end rather than reservoir hosts, as maintenance of CPXV in these species does not occur. The broad host range of CPXV is thought to be mediated by a large number of genes, resulting in the most multitudinous genetic repertoire of all known OPV [7,8]. CPXV spillover infections from animals to non-vaccinated persons usually result in local skin lesions but rarely cause generalized and fatal disease in immunocompromised patients [9,10]. Since the eradication of smallpox and the subsequent cessation of the vaccination in the 1980s, the susceptibility of the human population for OPV spillover infections is increasing and, therefore, the risk of OPV adaption in humans [11].

Cowpox viruses are well characterized and known for a long time with references back to Edward Jenner, but their classification within the genus *Orthopoxvirus* is a matter of ongoing debate. The definition of *Cowpox virus* as a single species was historically based on host specificity and phenotypic properties, e.g., formation of hemorrhagic pocks on infected chorioallantoic membranes (CAM) of eggs and electron microscopy-mediated identification of A-type inclusion bodies (ATI) [12–14]. Genome characterization was initially done by restriction fragment length polymorphism (RFLP) [15] and currently by high-throughput sequencing (HTS) of whole genomes [16,17]. Recent phylogenetic investigations using full-length genomes demonstrated that "*Cowpox virus*" is rather a polyphyletic group than a single species [16,18].

Furthermore, to date the natural reservoir of CPXV has not been clearly identified. Rodents, especially the common vole (*Microtus arvalis*), the field vole (*Microtus agrestis*), and the bank vole (*Myodes glareolus*), are thought to act as natural reservoir hosts of CPXV [19,20]. Voles belong to the order Rodentia, family Cricetidae, subfamily Arvicolinae that is further divided into several tribes including tribe *Myodini* with genus *Myodes* (including the bank vole) and tribe *Arvicolini* with genus *Microtus* (including common vole and field vole) [21]. In contrast, mice and rats belong to the same order, Rodentia, but to a different family, Muridae. The bank vole is one of the most abundant rodent species in Europe, detected mostly in forest habitats. It is found in most parts of the Western Palearctic region from Spain and Great Britain in the west up to Siberia in the east [22]. Infections with CPXV or other OPV were confirmed in these vole species using serological and PCR analyses [5,19,20,23–33] (Fischer et al., submitted). Furthermore, OPV-reactive antibodies were detected in other rodent species like wood mouse (*Apodemus sylvaticus*), yellow-necked mouse (*Apodemus flavicollis*), striped field mouse (*Apodemus agrarius*), and even in shrews like the common shrew (*Sorex araneus*) [16,20,29,31,32,34–36]. CPXV isolates originating from natural reservoirs are rare, currently only isolates originating from common voles have been described [16,20,37]. Animal experiments proved the reservoir competence of common voles; inoculation with a common vole-derived CPXV strain resulted in an asymptomatic infection with virus shedding [37]. In contrast, bank voles seem to be resistant to experimental infection with strains derived from common vole, rat, cat, and human, questioning the reservoir competence of bank voles [38]. Currently no bank vole-derived CPXV isolate was reported, which might be used for an experimental proof of the reservoir competence of this vole species.

Here, we describe a qPCR-based CPXV-screening of bank voles collected in Thuringia, Germany, to gain new insights into the role of bank voles as potential reservoir of CPXV. A bank vole-derived CPXV strain was isolated, sequenced, and further characterized in vitro.

2. Materials and Methods

2.1. Rodent Trapping

During spring, summer, and fall of the years 2017 and 2018, bank voles were collected by snap-trapping at 21 forest locations within Thuringia ("Thüringer Becken"), Germany (Figure 1 and Table S1) [39]. All procedures involving animals were conducted according to relevant legislation and by permission of the responsible authority in Thuringia (permit 22-2684-04-15-105/16, 13/04/2017).

All collected voles were frozen at −20 °C until necropsy. During dissection, species, body size, weight, and sex were recorded. Nasal septum and kidney tissues were taken and stored at −20 °C until nucleic acid extraction.

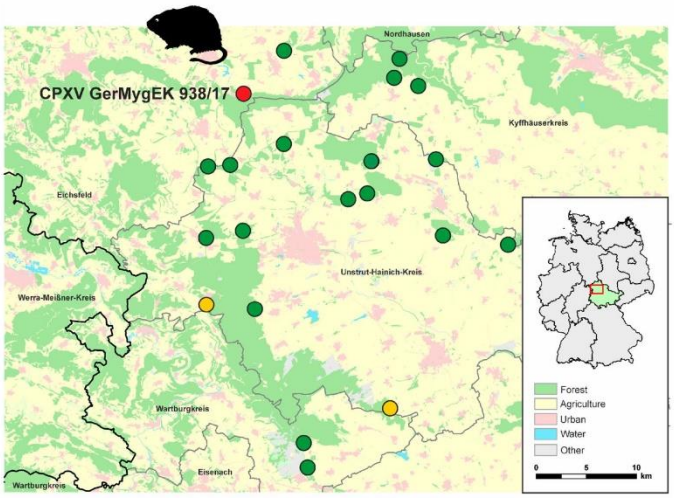

Figure 1. Bank vole trapping locations in Thuringia, Germany, during spring, summer, and fall, 2017 and 2018 (circles). The inset map of Germany shows Thuringia highlighted in green and the trapping area "Thüringer Becken" marked by a red frame. Yellow dots mark locations where *Cowpox virus* (CPXV)-DNA positive voles were sampled, and a red dot marks the trapping position of the bank vole from which the CPXV strain GerMygEK 938/17 was isolated. Green dots represent locations where only negative bank voles were sampled.

For molecular confirmation of the rodent species, DNA was extracted from kidney tissue (Tissue DNA Kit, Roboklon, Berlin, Germany). Subsequently, a cytochrome *b* specific PCR was performed [40], PCR products were sequenced and compared to GenBank entries using Nucleotide Basic Local Alignment Search Tool (BLASTn)-based analysis.

2.2. OPV DNA Screening

OPV DNA screening was based on nasal septum samples, as the nasal septum has been shown to be better suitable for OPV detection than other internal organs [37]. Nose septum samples were transferred into reaction tubes with 1 mL Eagle's minimal essential medium (MEM; Biochrom GmbH, Berlin, Germany) supplemented with 10% fetal calf serum (FCS, Biochrom GmbH), antibiotics (1% penicillin-streptomycin, Biochrom GmbH) and stainless steel beads (5 mm in diameter, TIS Wälzkörpertechnologie GmbH, Gauting, Germany) for mechanic homogenization (TissueLyser II; Qiagen, Hilden, Germany). DNA extraction was done semi-automatically in a BioSprint 96 instrument (Qiagen) using the NucleoMag VET kit (Macherey-Nagel, Düren, Germany). The isolated DNA was analyzed using a quantitative polymerase chain reaction (qPCR) assay (QuantiTect Multiplex PCR NoROX Kit, Qiagen) targeting a 146 nucleotide (nt) region of the 14-kD protein-encoding (*A27L*) gene of CPXV [41].

2.3. Cell Lines and Virus Isolation

Virus isolation was performed with all CPXV DNA-positive nasal septum samples. Hence, overnight cultures of Vero76 cells (Collection of Cell Lines in Veterinary Medicine CCLV,

CCLV-RIE 0228, Friedrich-Loeffler-Institut, Greifswald-Insel Riems, Germany), were inoculated with 100 µL of the homogenized tissue material and kept at 37 °C under a 5% CO_2 atmosphere.

Vero76 cells were grown and maintained in MEM supplemented with 10% FCS containing antibiotics (1% Enrofloxacin; Bayer, Leverkusen, Germany; 0.2% Amphotericin/Gentamicin; Thermo Fisher Scientific Inc., Schwerte, Germany; 0.5% Linomycin; WDT, Garbsen, Germany).

Inoculated cells were passaged until a cytopathic effect (CPE) was observed, and virus was collected in Tris-EDTA buffer. For virus detection and characterization, a qPCR [20] and whole genome sequencing was performed. The obtained virus isolate was designated GerMygEK 938/17 indicating the country of origin, Germany; the animal species, *Myodes glareolus;* the trapping location, Eichsfelder Kessel; the individual number (938) and the year of trapping, 2017.

2.4. Sequencing, Genome Assembly, and Annotation of the CPXV Isolate

Viral DNA was extracted from CPXV positive cell culture using the MasterPure™. Complete DNA and RNA Purification Kit (Lucigen Simplifying Genomics, Middleton, WI, USA) according to the manufacturer's instruction. The DNA preparation was submitted to Eurofins GATC Biotech (Konstanz, Germany) for HTS. In total, 5 million paired-end reads with a read length of 150 base pairs (bp) were obtained using the Illumina HiSeq 4000 platform. The reads were quality and adapter trimmed using the 454 Sequencing System Software (version 3.0; Roche, Mannheim, Germany) along with appropriate Illumina specific adapter sequences. Subsequently, the trimmed reads were mapped to a non-redundant version (one copy of the ITR deleted) of the genome of CPXV strain Ger2010MKY (lineage tentatively named "CPXV-like 3" [16]; LT896721.1) using Bowtie2 (version 2.3.4.3; [42]), and only mapped reads were further used for de novo assembly using 454 Sequencing System Software (version 3.0; Roche). The resulting contigs were subsequently used as reference sequences for an additional round of mapping and de novo assembly. The iterative process was repeated five times, and the resulting contigs were then arranged to resemble the entire CPXV genome. The resulting full-length CPXV sequence was annotated analogous to the nomenclature of the CPXV Brighton Red (BR) reference strain (AF482758) as described elsewhere [37] as well as to the closely related CPXV strain Ger 2010 MKY (LT896721) [43]. Additional tentative open reading frames (ORF) were numbered as follows: gCPXV0XXX for genes and pCPXV0XXX for proteins [37].

2.5. Phylogenetic Analysis

An alignment of representative OPV complete genomes (Supplementary Table S1) was constructed using MAFFT within Geneious (version 11.1.5; https://www.geneious.com; Biomatters Limited, Auckland, New Zealand). Poorly aligned regions were removed by using trimAl (version 1.2; [44]) as implemented in Phylemon (version 2.0; [45]). A phylogenetic tree was constructed using IQ-TREE (version 1.6.10; [46]), visualized using FigTree (version 1.4.3; [47]) and clades were compressed using MEGA (version 7.0.14; [48]). The similarity plot was constructed with a sequence alignment of CPXV GerMygEK 938/17 with reference strains CPXV Ger2010MKY (LT896721), OPV Abatino (MH816996) and ECTV Moscow (NC_004105) aligned with MAFFT within Geneious. The complete alignment and selected gene alignments were used under RStudio (version 1.1.463, [49]) showing the identity of the sequences. In addition, potential recombinant sequences were parsed using the exact algorithms established before [16]. In brief, the analysis was based on the Jukes–Cantor substitution model using a sliding window of 5000 nt, a step size of 100 nt, and bootstrap support by 100 replicates.

2.6. Phenotypic Analysis Using Chorioallantoic Membrane Culture

Embryonated chicken eggs, obtained from the Friedrich-Loeffler-Institut, Insel Riems, were incubated at 37 °C and a relative humidity of 50% in an incubator for 10 to 13 days. Inoculation onto the chorioallantoic membrane (CAM) was performed as described in [50]. In brief, a hole was abraded in the eggshell using a grinder (Dremel, Racine, WI, USA), and CAMs were inoculated with 10^5 tissue culture infectious dose 50 ($TCID_{50}$) of virus in 100 µL phosphate-buffered saline (PBS) or

with the same volume of PBS as mock control. Inoculated eggs were sealed with paraffin wax and incubated at 37 °C for 72 h without moving. CAMs were harvested after chilling all eggs for at least 2 h at 4 °C, washed at least three times with PBS, and photographed immediately. CAM were inoculated with ECTV strain US#4619, CPXV strain RatPox09 or the novel isolate CPXV GerMygEK 938/17.

2.7. Virus Replication Kinetics in Different Cell Lines

To analyze the replication kinetics, the novel bank vole-derived strain GerMygEK 938/17, the common vole-derived strain FM2292 [37], and the commonly used laboratory strain Brighton Red (BR) [12] were tested in overnight cell culture. A multiplicity of infection (MOI) of 0.01 and 3 was used in (A) a bank vole-derived kidney cell line (BVK168, CCLV-RIE 1313; [51]) and (B) a common vole-derived kidney cell line (FMN-R, CCLV-RIE 1102; [52]) as well as on (C) Vero76 cells as a reference cell-line for CPXV growth. After 60 min at 37 °C, the inoculated cell cultures were washed three times with PBS, and fresh MEM supplemented with 10% FCS was added. Samples were collected at six time points post inoculation (0, 6, 12, 24, 48, and 72 h post inoculation (hpi)) including two biological replicates. Virus titers were determined by endpoint dilution assay and calculated as $TCID_{50}$ mL^{-1} using the Spearman–Kärber algorithm [53,54].

2.8. Data Availability

The annotated full-length genome sequence of the bank vole-derived CPXV strain GerMygEK 938/17 and sample information was uploaded to the European Nucleotide Archive (ENA) and made publicly available under project accession PRJEB32300.

3. Results

3.1. Rodent Trapping and Detection of OPV DNA

A total of 533 bank voles were collected during 2017 and 2018 at 21 sites in Thuringia (Figure 1); for 509 carcasses nasal septum samples were available. OPV DNA was detected by qPCR in nasal septum samples of five out of 509 (0.98%) bank voles. Virus DNA-positive bank voles originated from three trapping areas and were captured in spring (n = 4, two females and two males) or summer (n = 1, male), in 2017. In spring, 2017, three of these positive voles originated from the same trapping area: Eichsfelder-Kessel. The C_q-values of the individual samples varied between 21 and 38 (see Supplementary Table S2).

3.2. Virus Isolation, Sequence Determination, and Genome Characterization

Virus isolation in Vero76 cells was successful for one out of the five OPV DNA-positive bank vole septum samples. After three passages, the novel CPXV strain GerMygEK 938/17 showed a titer of 10^6 $TCID_{50}$ mL^{-1} on Vero76 cells. HTS resulted in the determination of the complete genome sequence of CPXV GerMygEK 938/17 with 220,822 bp. Two contigs resulted from the assembly. One contig (206,524 nt) resembled the unique core of the genome, and the second contig resembled the terminal tandem repeat (7190 nt). Contig junctions were confirmed by manual inspection of the overlapping reads between the contigs. The mean sequence depth of the unique region was 750 and that of the terminal repeat region 1525.

3.3. Phylogenetic Analysis

Comparative analysis of the full-length sequence of CPXV GerMygEK 938/17 showed the highest nucleotide sequence identity (99.2%) to a virus isolated from a cotton-top tamarin (*Saguinus oedipus*) in Germany 2010 (CPXV Ger2010MKY, GenBank accession number LT896721) [43] (Figure 2a). CPXV GerMygEK 938/17 and CPXV Ger2010MKY (CPXV MKY) sequences form a separate cluster provisionally named "CPXV-like 3" clade [16] in close proximity to the OPV isolate Abatino from Italy (MH816996) pairwise identity 93.8%, which was referred to as ECTV-like [55,56] and ECTV

strains (pairwise identity ECTV strain Moscow 85.3%). Similarity plot analysis showed areas of high sequence variation between CPXV GerMygEK 938/17 and OPV Abatino (Figure 2b), while comparison to CPXV MKY indicated only small variations (Figure 2c). Analysis of the CPXV GerMygEK 938/17 sequence using a bootscan analysis revealed a similar pattern like the one established for CPXV Ger2010MKY [16] (data available upon request). We interpret CPXV GerMygEK 938/17 as second member of the separated lineage "CPXV-like 3".

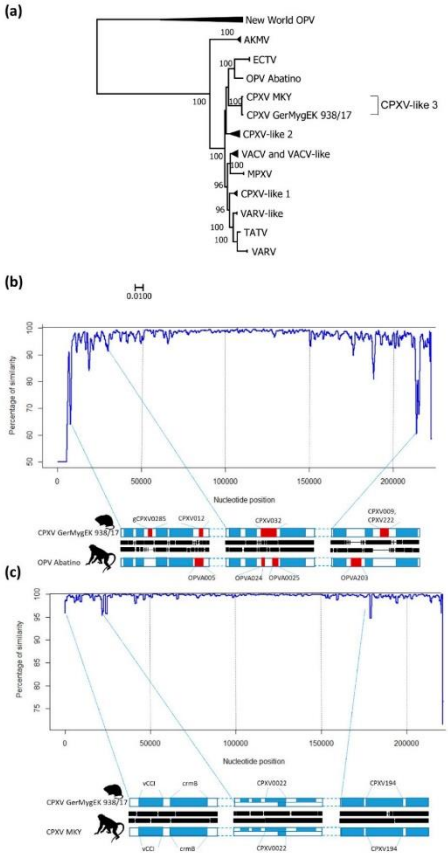

Figure 2. Phylogenetic analysis and sequence similarity analyses of CPXV GerMygEK 938/17. (**a**) Phylogenetic tree using New World orthopoxviruses as outgroup. Clades where named after Franke et al., 2017 [16]. Only bootstrap supporting values over 70 are given at the supported nodes. Black triangles indicate compressed branches. (**b**) Similarity plot showing the sequence identity between CPXV GerMygEK 938/17 (query sequence, black line) to the reference sequences OPV Abatino (MH816996, blue line) along their full genomes. Three regions exhibiting prominent differences are detailed below as a zoom-in visualization. Gaps between black lines indicate gaps in the DNA sequence. (**c**) Similarity plot showing the sequence identity between CPXV GerMygEK 938/17 (query sequence, black line) to the reference sequences CPXV MKY (LT896721, blue line) along their full genomes. Alignment details of three genomic parts are depicted below. Open reading frames (ORFs) that are conserved in both genomes are colored in blue, while ORFs that are present in either one of the two genomes are colored in red. White areas represent intergenic regions.

3.4. Phenotypic Characterization on the CAM

The CAM of chicken eggs showed CPXV-specific hemorrhagic pocks after inoculation with CPXV GerMygEK 938/17 comparable to the CPXV RatPox09 control (VARV-like clade) inoculation (Figure 3a,c). In contrast, white pock lesions on the CAM were detected after inoculation using ECTV (Figure 3b).

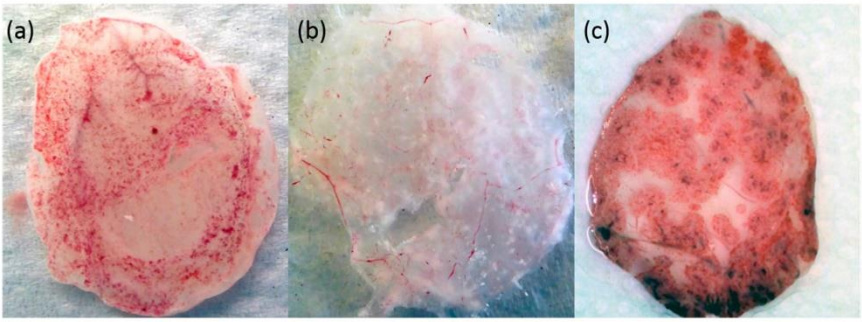

Figure 3. Lesions detected on chorioallantoic membranes (CAM) after inoculation with CPXV GerMygEK 938/17 (**a**), *Ectromelia virus* US#4619 (**b**) and RatPox09 (**c**). Lesions were photographed after 72 h incubation at 37 °C.

3.5. Virus Replication Kinetics in Different Cell Lines

Isolates CPXV GerMygEK 938/17, CPXV FM2292, and BR were compared using single and multi-step growth kinetics in Vero76 and two vole cell lines (see Supplementary Figure S1). All three virus strains showed some level of replication independent of the cell line used. The highest virus titers regardless of the isolates were detectable on Vero76 cells. Here, the replication kinetics of all three CPXV strains were similar. While, the novel strain GerMygEK 938/17 clearly displayed an impaired replication capacity on both vole-derived cell lines, the virus is generally able to establish replication cycles, as passaging of the virus in these cells is possible (data available upon request). However, titers of CPXV GerMygEK 938/17 achieved on vole-derived cell lines at 48 h were in the range of the inoculated titers (see Supplementary Figure S1). Interestingly, the lowest titers were achieved on BVK168 cells derived from bank vole kidney tissue for all used viruses.

4. Discussion

We detected CPXV-specific DNA in five out of 509 tested bank voles (0.98%) from Thuringia, Germany. The nasal septum sample from one of these DNA-positive bank voles was successfully used to isolate a novel CPXV strain. To our knowledge, this is the first description of a bank vole-derived CPXV isolate.

The here observed CPXV DNA prevalence of 0.98% in bank voles is comparable to other studies that detected 0.19 up to 1.33% [31] (Fischer et al., submitted). OPV infections in bank voles—as detected by antibodies and/or DNA—were reported in different Eurasian countries: Belgium [28], Buryatia [31], England [19,23–27], Hungary [32], Norway [29,30], and Finland [31,33]. In line with our data, it seems that active CPXV infections in bank voles are rare. Therefore, virus persistence in endemic regions might be mediated by a high stability outside the host, as described for other OPV [34], rather than by a high number or proportion of infected voles. Unlike the common vole, which may be subject to infection with different CPXV strains resulting in seroconversion, virus shedding, and clinical signs, bank voles seem to be resistant, showing neither virus shedding nor clinical symptoms and exhibiting a low rate of seroconversion [37,38,57]. Thus, it is not clear whether bank voles are solitary maintenance/reservoir hosts for CPXV, or if they are only the reservoir for a special type of CPXV. In vitro testing of CPXV

GerMygEK 938/17 as well as CPXV FM2292, a common vole isolate, resulted in infection of bank and common vole-derived cell lines regardless of the used CPXV-isolate. Interestingly, viral titers generated from infection of Vero76 cells were comparable and of highest level. Using bank vole and common vole-derived kidney cell lines, CPXV GerMygEK 938/17 exhibited at least tenfold-reduced viral titers at 24 h and at later time points. Probably interferon-related responses are responsible for this effect. In addition, vole cells of other tissue origins might be able to assist CPXV replication more potently.

Infection of CAM using the new bank vole CPXV strain GerMygEK 938/17 resulted in hemorrhagic pock formation. This was described as CPXV-specific phenotype in contrast to non-hemorrhagic "white" pocks seen after ECTV inoculation of CAM cultures [12–14].

Interestingly the CPXV GerMygEK 938/17 sequence clustered close to ECTV, a virus causing white pocks lesions and not able to infect bank voles even with high titer inoculation [58]. ECTV itself has so far only been detected in laboratory colonies of house mice (*Mus musculus*) except for the human strain ERP [59]. In comparison to CPXV, ECTV comprises a shortened genome and a reduced number of genes, whereby many of the lost genes are associated with host interaction [60,61]. It seems likely that ECTV originated from a CPXV-like ancestor virus and adapted to house mice together with the loss of viral genes. The loss of hemorrhagic phenotype-related genes resulted in the white pox lesion phenotype on CAM [62].

The bank vole-derived isolate clustered closest to strain CPXV Ger2010MKY isolated from a cotton-top tamarin from a zoo in Thuringia [43]. Therefore, it seems likely that cotton-top tamarin infection was mediated by bank voles. While rodents, including bank voles, were trapped in the surroundings of the zoo, none were tested positive for CPXV [43]. This might be explained by the low number of tested rodents (n = 23) compared to the current study. The closest branch to CPXV GerMygEK 938/17 and CPXV Ger2010MKY consists of OPV Abatino isolate from an Italian Tonkean macaque (*Macaca tonkeana*). This strain is very similar to another OPV-derived sequence from an Italian cat [55,56,63]. During the Italian macaque outbreak, trapped rodents were tested negative for OPV-reactive antibodies [55].

Overall, it seems reasonable to assume that bank voles might also be the source of the CPXV spillover cases in Italy. However, this would need further testing as during the outbreak in Italy no bank voles were trapped, and it is also important to include higher numbers of bank voles in future monitoring studies since the expected prevalence of viremic bank voles is low.

The observed genetic distance of bank vole-derived CPXV GerMygEK 938/17 to common vole-derived isolates CPXV FM2292 and CPXV Ger/2007/vole, both from Baden-Wuerttemberg (CPXV-like 2 clade), and CPXV FMEimka from Saxony (CPXV-like 1 clade), implies that at least three different vole-associated CPXV lineages exist in Germany [16,20]. This finding may suggest that CPXV in general is maintained by multiple small mammal species, mainly voles, rather than by one specific host [4,16,18]. Moreover, highlighted by the fact that bank voles were not susceptible for productive infection with a common vole-derived CPXV FM2292 strain [38], but replicating virus CPXV GerMygEK 938/17 could be isolated, vole species-dependent CPXVs clades might be the reason for high seroprevalences detected in bank voles throughout Europe. For a definite answer, experimental inoculations of different vole species using different CPXV-isolates would be necessary.

Currently, CPXV isolates, especially isolates from voles and other wild rodents are out-numbered by isolates from accidental hosts like cats, alpacas, or zoo animals. Therefore, a Europe-wide screening of rodent populations for CPXV-infected individuals would help to better understand CPXV-reservoir host relationships and the role of voles for CPXV transmission. In addition, virus isolation combined with whole-genome sequencing is essential to gain deeper insights into CPXV phylogeny, distribution of CPXV clades, as well as OPV evolution in general.

5. Conclusions

Here we provide the first description and in vitro characterization of a bank vole-derived CPXV-isolate. Interestingly, the analyzed whole-genome sequence revealed a clustering of this isolate

next to ECTV, the orthopox virus of mice, also phenotypically both viruses differ drastically in the CAM system. With the first direct evidence of replication competent CPXV in bank voles and together with the reports about seropositive bank voles, bank voles have also to be considered as a reservoir species for CPXV. Due to limited sample numbers and the localized screening, the presented results are biased. Nevertheless, bank voles have a very wide distribution area in Eurasia and the detection of related sequences in Italy suggests a possibly similar wide distribution of bank vole-derived CPXVs. We therefore would like to encourage the monitoring of further bank vole samples as well as other small mammals in order to improve the data basis about the occurrence, genetic variability, and reservoir hosts of CPXV.

Supplementary Materials: The following supplementary figure and tables are available online at http://www.mdpi.com/1999-4915/11/11/1075/s1. Figure S1: Virus replication kinetics of vole derived-CPXV on different cell lines. Table S1: Individual information on the bank voles (*M. glareolus*) tested positive for orthopoxvirus (OPV) DNA. Table S2: List of orthopoxvirus (OPV) sequences and classification into several clades.

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Conflicts of Interest: The authors declare no conflict of interest.

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IV. PUBLIKATION II

In Vivo Characterization of a Bank Vole-Derived Cowpox Virus Isolate in Natural Hosts and the Rat Model

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Abstract: Cowpox virus (CPXV) belongs to the genus *Orthopoxvirus* in the *Poxviridae* family and is endemic in western Eurasia. Based on seroprevalence studies in different voles from continental Europe and UK, voles are suspected to be the major reservoir host. Recently, a CPXV was isolated from a bank vole (*Myodes glareolus*) in Germany that showed a high genetic similarity to another isolate originating from a Cotton-top tamarin (*Saguinus oedipus*). Here we characterize this first bank vole-derived CPXV isolate in comparison to the related tamarin-derived isolate. Both isolates grouped genetically within the provisionally called CPXV-like 3 clade. Previous phylogenetic analysis indicated that CPXV is polyphyletic and CPXV-like 3 clade represents probably a different species if categorized by the rules used for other orthopoxviruses. Experimental infection studies with bank voles, common voles (*Microtus arvalis*) and Wistar rats showed very clear differences. The bank vole isolate was avirulent in both common voles and Wistar rats with seroconversion seen only in the rats. In contrast, inoculated bank voles exhibited viral shedding and seroconversion for both tested CPXV isolates. In addition, bank voles infected with the tamarin-derived isolate experienced a marked weight loss. Our findings allow for the conclusion that CPXV isolates might differ in their replication capacity in different vole species and rats depending on their original host. Moreover, the results indicate host-specific differences concerning CPXV-specific virulence. Further experiments are needed to identify individual virulence and host factors involved in the susceptibility and outcome of CPXV-infections in the different reservoir hosts.

Keywords: cowpox virus (CPXV) *Orthopoxvirus*; bank vole (*Myodes glareolus*), common vole (*Microtus arvalis*), reservoir host; animal model

1. Introduction

The zoonotic Cowpox virus (CPXV), currently a single species within the genus *Orthopoxvirus*, is endemic in Europe and northern and central Asia [1,2]. Many mammals are known to be susceptible to CPXV infection and disease, like cats (*Felis catus*) [3,4], pet rats (*Rattus norvegicus forma domestica*) [5–7], alpacas (*Vicugna pacos*) [8], Asian elephants (*Elephas maximus*) [9], and primates [10,11]. Maintenance of viral infections within these species is not achieved and therefore these

species represent accidental hosts for the virus [12]. Spill-over infections from these accidental hosts to humans happen frequently, mostly through micro skin lesions like scratches or abrasions. The resulting human infections are generally characterized by a mild and self-limiting illness with typical pox lesions at the site of virus entry [3,6,7,13]. However, the virus can also cause systemic and fatal disease in immuno-compromised patients [14,15]. Routine vaccination against smallpox—which supposedly provides protection against CPXV-induced disease—ended in the 1970s. Today, more than half of the world's human population is unvaccinated [16,17], due to the cessation of smallpox vaccination, and the number of human CPXV infections have increased [18].

In contrast to an accidental host, a reservoir could be defined as "a population which is chronically infested with the causative agent of a disease and can infect other populations" [19]. A reservoir host in particular could be "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" [20].

For CPXV, wild rodents are thought to be reservoir hosts in different parts of Eurasia. In Turkmenistan and Georgia, the virus was detected in a very low prevalence in wild ground squirrels (yellow suslicks; *Citellus fulvis*) and gerbils (*Rhombomys opimus*, *Meriones libycus,* and *M. meridianus*) [21,22]. In Western Europe, the highest prevalence of orthopoxvirus (OPV) reactive antibodies was detected in bank voles (*Myodes glareolus*), field voles (*Microtus agrestis*), and wood mice (*Apodemus sylvaticus*). However, no virus isolation was successful until recently [23,24], and the DNA detection rate in common voles (*Microtus arvalis*) and bank voles was very low [25,26].

In 2015, the first molecular detection and isolation of CPXV from a putative reservoir host, the common vole, was described by our group [27]. In vitro and in vivo studies with the common vole-derived isolate CPXV FM2292 (phylogenetically classified as CPXV-like 2, [28]) confirmed the reservoir competence of this vole species. Virus shedding was detectable in all inoculated animals and viral titers in swab samples reached up to 10^4 tissue culture infectious doses 50% ($TCID_{50}$) mL^{-1}. The animals had to be euthanized because of their deteriorating health. Norway rats (*Rattus norvegicus*), strain Wistar, representing an established animal model for CPXV [29,30], were also intranasally inoculated with CPXV FM2292. They exhibited even more prominent respiratory clinical signs than common voles [27]. In addition, experimental studies with the other putative reservoir host, the bank vole, were performed [31]. However, independent of the application route or the CPXV isolate used (different phylogenetic clades and host origin) or the bank vole evolutionary lineage, viral shedding was not detectable. Consequently, the reservoir competence of bank voles for CPXV was put into question. Nevertheless, a bank vole-derived isolate for experimental proof was not available at this time.

That changed in 2017 and 2018, when bank voles were trapped in Thuringia, Germany and 509 nasal septa were screened for OPV-DNA via quantitative PCR (qPCR) [32]. Five samples tested positive for OPV-DNA, and one bank vole-derived virus isolate (CPXV GerMygEK 938/17) could be obtained. Phylogenetic analyses demonstrated a nucleotide sequence identity of 99.2% to another German CPXV isolate, Ger2010MKY (CPXV-like 3, [28]), and a clustering together with Ectromelia virus (ECTV), and a separation from other CPXV isolates derived from either common voles or accidental host species. Viral replication on the chorioallantoic membrane (CAM) resulted in hemorrhagic pocks typical for CPXV [33], whereas ECTV develops non-hemorrhagic pocks on the CAM [34]. The taxonomical species definition for other orthopox viruses conceded the presumption that the different clades "CPXV-like 1", "CPXV-like 2", and "CPXV-like 3" would be better classified as different species within the genus *Orthopoxvirus*.

The closest relative CPXV strain Ger2010MKY was isolated during a fatal outbreak among cotton-top tamarins (*Saguinus oedipus*) in a Thuringian animal park in 2010 [10]. All infected cotton-top tamarins died peracutely, while common marmosets (*Callithrix jacchus*) in the same husbandry remained healthy, but scored qPCR positive in swab samples [10]. Whether bank voles or other rodents were involved in the history of the diseased cotton-top tamarins in 2010 remained unclear. The isolate CPXV Ger2010MKY was tested in Wistar rats for assessment of viral pathogenicity according to established protocols [29,30]. Rats were inoculated intranasally using 10^4 or 10^5 $TCID_{50}$

mL^{-1}. All collected buccal swabs were OPV DNA negative and only one animal out of four per dosage group seroconverted [10].

Here we describe the first in vivo characterization of the bank vole-derived CPXV strain "GerMygEK 938/17". Comparative infection experiments were performed using (i) bank voles, as the putative original reservoir host, (ii) common voles as an additional potential reservoir host, and (iii) Wistar rats as a surrogate animal model. The closely related Ger2010MKY isolate was also included to prove if strains of the same Ectromelia-like clade—provisionally named "CPXV-like 3"—behave similarly [28].

These experiments revealed for the first time the detection of viral shedding by CPXV-infected bank voles, while the Wistar rats only seroconverted and the common voles exhibited no indication of detectable viral replication.

2. Materials and Methods

2.1. Viruses and Cell Lines

CPXV strain GerMygEK 938/17 was isolated from the nasal septum of a bank vole collected during the project "Population dynamics of rodent hosts of zoonotic disease: interaction of climate, landuse and biodiversity" on a forest plot in the federal state of Thuringia, Germany, in spring 2017 [32]. CPXV Ger2010MKY has been isolated from cutaneous lesions of cotton-top tamarins housed in a animal park in Thuringia, Germany and has been already characterized by experimental inoculation of Wistar rats before [10]. As reference strains and controls for in vitro characterization, the CPXV laboratory reference strain Brighton Red and the CPXV isolate RatPox09, as representative of a highly virulent strain in Wistar rats [27] were used.

African green monkey (*Chlorocebus* spec.) cells (Vero76, Collection of Cell Lines in Veterinary Medicine CCLV, Friedrich-Loeffler-Institut, Greifswald-Insel Riems, Germany), were grown and maintained in Eagle's minimal essential medium (MEM; Biochrom GmbH, Berlin, Germany) supplemented with 10% fetal calf serum (FCS, Biochrom GmbH, Berlin, Germany) and kept under a 5% CO_2 atmosphere at 37 °C.

All CPXV strains were cultivated and titrated on Vero76 cells, and stock titers of approximately 10^7 $TCID_{50}$ mL^{-1} were achieved.

2.2. In Vitro Characterization: Virus Growth Kinetics

Vero76 cells from overnight cultures were inoculated with CPXV GerMygEK 938/17 or CPXV Ger2010MKY at a multiplicity of infection (MOI) of 0.01 or 3. For reference, we used a virus strain characterized as highly virulent in Wistar rats, namely CPXV RatPox09, and laboratory strain CPXV Brighton Red. After inoculation, cells were incubated at 37°C for 60 min under a 5% CO_2 atmosphere and were washed afterwards three times with phosphate-buffered saline (PBS). Subsequently, fresh cell culture medium (MEM supplemented with 10% FCS) was added. The first samples were collected immediately afterwards (time point 0h), and further samples from five different time points (6h, 12h, 24, 48, and 72h) were obtained (four biological replicates per sample). Virus titers were determined as technical duplicates by endpoint dilution assay on Vero76 and calculated as $TCID_{50}$ mL^{-1} using the Spearman-Kärber algorithm [35,36]. The detection limit of our test is $10^{1.625}$ $TCID_{50}$ mL^{-1}. Altogether, for each biological replicate we got two technical replicates and therefore overall eight values for each virus.

2.3. In Vivo Characterization: Animal Experiments and Analysis of Samples

2.3.1. Animals

The animal experiments were approved by the Ministry of Agriculture of Mecklenburg-Western Pomerania, Germany, under reference number LALLF M-V 7221.3-2-004/18, 15/03/2018.

Bank voles originated from our in-house breeding. Wistar rats were purchased from Charles River Laboratories (Sulzfeld, Germany) and common voles originated from the Julius Kühn-Institute.

They were housed in standard laboratory rodent cages in groups of 2 to 4 animals (rats and bank voles) or individually (common vole) under standardized conditions (22°C; 12/12h light cycle). As diet, rodent pellets (SSniff Spezialdiäten GmbH, Soest, Germany) and water ad libitum were fed. For acclimatization, rodents were housed under these conditions for one week prior to inoculation.

All animals were outbred animals, of mixed sex and mixed age except the Wistar rats, which were all six weeks of age.

2.3.2. Infection Experiments and Sampling

The design of the animal experiments is shown in Table 1. Ten rats, six bank voles, and six common voles were inoculated with the new isolate CPXV GerMygEK 938/17. Because CPXV Ger2010MKY had been already tested in Wistar rats [10], only six bank voles and six common voles were inoculated with this isolate. All CPXV strains were applied oronasally with a dosage of $10^{5.5}$ $TCID_{50}$/animal after a short isoflurane induced anesthesia. The general health status was checked daily over a period of 28 days. In addition, body weight measurement and buccal swab (Copan, Brescia, Italy) sampling (under isoflurane anesthesia) was performed every other day until day 21 post inoculation.

The animals were euthanized 28 days post inoculation (dpi) and serum, peritoneal lavage and an organ panel (turbinate, skin, liver, lung, spleen, and trachea) were collected.

Table 1. Design of the animal experiments.

Cowpox Virus (CPXV)			Animals			Experiment Design			
Phylogenetic Lineage	Strain	Origin	Species	Number of Animals per Group	Application Route	Dose of Inoculum/ Animal	Duration of the Experiment	Nasal/buccal Swabs	Study
CPXV-like 3	GerMygEK 938/17	Bank vole	Bank vole	5*	oronasal	$10^{5.5}$ $TCID_{50}$	28 dpi	Every 2nd day	This study
			Common vole	6	oronasal	$10^{5.5}$ $TCID_{50}$	28 dpi	Every 2nd day	This study
			Wistar rat	10	oronasal	$10^{5.5}$ $TCID_{50}$	28 dpi	Every 2nd day	This study
CPXV-like 3	Ger2010MKY	Cotton-top tamarin	Bank vole	6	oronasal	$10^{5.5}$ $TCID_{50}$	28 dpi	Every 2nd day	This study
			Common vole	6	oronasal	$10^{5.5}$ $TCID_{50}$	28 dpi	Every 2nd day	This study
			Wistar rat	4	oronasal	10^{4}/10^{6} $TCID_{50}$	28 dpi	Every 2nd day	[10]

dpi, days post inoculation; $TCID_{50}$, Tissue Culture Infectious Dose 50 %. *One additional animal died unrelated during anesthesia.

2.3.3. Determination of Viral Genome Loads and Infectious Virus Titers from Buccal Swabs and Organ Samples

Buccal swab samples were resuspended in 2 mL cell culture medium (MEM) and antibiotics were added (enrofloxacin, 1%; Bayer, Leverkusen, Germany; amphotericin/gentamicin, 0.2%; Thermo Fisher Scientific Inc, Schwerte, Germany; lincomycin, 0.5%; WDT, Garbsen, Germany). The organ samples were transferred into 1 mL cell culture medium supplemented with 10% FCS and antibiotics (1% penicillin-streptomycin, Biochrom GmbH, Berlin, Germany). For mechanic homogenization (TissueLyser II; Qiagen, Hilden, Germany) all reaction tubes contained stainless steel beads (5 mm diameter; TIS Wälzkörpertechnologie GmbH, Gauting, Germany).

Viral DNA from all buccal swabs and tissue samples was extracted by using the BioSprint 96 instrument (Qiagen, Hilden, Germany) and the NucleoMag VET kit (Macherey-Nagel, Berlin, Germany). OPV-specific DNA was detected by qPCR as described previously [37].

In addition, an endpoint-dilution assay was used for virus titration of the samples, and the respective data are calculated as $TCID_{50}$ mL^{-1} using the Spearman-Kärber algorithm [35,36].

2.3.4. Serology

Sera as well as peritoneal lavage samples were analyzed for OPV-reactive antibodies by an indirect immunofluorescence test as previously described [27]. In brief, CPXV-infected Hep2 cells (CCLV, Friedrich-Loeffler-Institut) were fixed with methanol-acetone (1:1) and incubated for 30 min at 50°C with Tris-buffered saline plus Tween (Sigma, St. Louis, MO, USA). In the following step, the cells were incubated for 1h at room temperature with a dilution series (1:20, 1:40, 1:80, 1:160, 1:320, 1:640, and 1:1280) of the inactivated (30 min at 56 °C) serum samples. After washing three times with PBS, a commercial anti-mouse or in case of the rat samples a commercial anti-rat secondary antibody conjugate (Thermo Fisher Scientific Inc, Schwerte, Germany) was applied. The cells were visualized under a fluorescence microscope (Leica DMI3000 B, Leica Wetzlar, Germany). Animals with titers of 1:40 or higher were considered positive. The titer was taken as the reciprocal of the greatest serum dilution, which showed a positive detection. Peritoneal lavage samples were handled just as the sera.

2.3.5. Figures

GraphPad PRISM (GraphPad Software, La Jolla, CA, USA) was used for generating illustrations.

3. Results

3.1. In Vitro Characterization: Virus Growth Kinetics

CPXV GerMygEK 938/17 and CPXV Ger2010MKY were characterized on Vero76 cells in terms of their in vitro growth kinetics and compared to RatPox09, a strain highly virulent for Wistar rats, and the laboratory reference strain Brighton Red. In four independent experiments with technical duplicates as internal controls, growth kinetics of the CPXV strains were determined using different MOIs. In general, all four tested CPXV strains replicated similarly in cell culture independently from the used MOI. At 72 h post inoculation (hpi) titers of ~10^5 $TCID_{50}$ mL^{-1} (MOI 0.01) or ~10^6 $TCID_{50}$ mL^{-1} (MOI 3) were determined (Figure 1).

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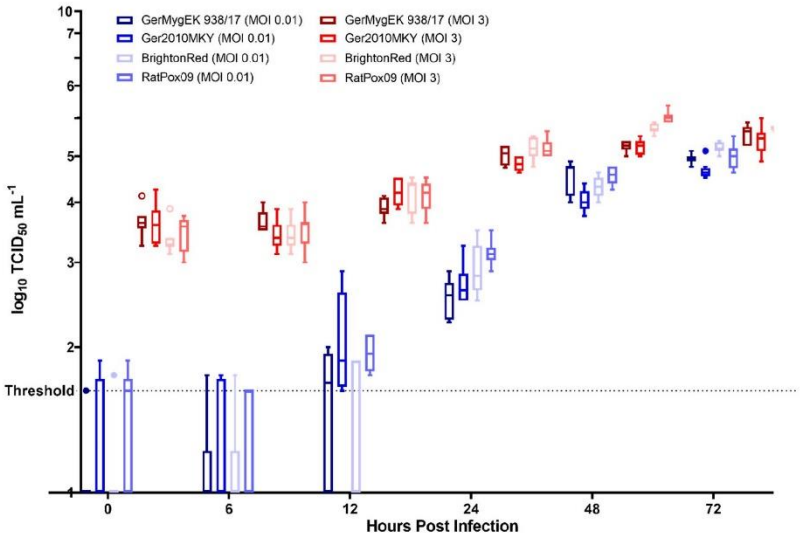

Figure 1. Comparison of replication characteristics of GerMygEK 938/17, Ger2010MKY, Brighton Red, and RatPox09. Vero76 cells were inoculated using multiplicities of infection (MOI)s of 0.01 and 3. Viral titers were determined by endpoint dilution assays. The mean and the standard deviations of four independent experiments, including two technical replicates per sample, are presented. The detection limit of our test was defined at $10^{1.625}$ $TCID_{50}$ mL^{-1} (threshold).

3.2. In Vivo Characterization: Animal Experiments and Sample Analyses

For the determination of the in vivo characteristics of CPXV GerMygEK 938/17, we inoculated bank voles, common voles, and Wistar rats. In addition, bank voles and common voles were inoculated with CPXV Ger2010MKY.

3.2.1. Morbidity and Mortality: Weight Loss in Bank Voles in the CPXV Ger2010MKY Group

Oronasal inoculation with the novel bank vole-derived isolate CPXV GerMygEK 938/17 did not result in any clinical signs regardless of the inoculated species. In addition, body weights were stable for all animals for the duration of the observation period (Figure 2A). One bank vole died during inoculation (unrelated death due to anesthesia).

Following CPXV Ger2010MKY inoculation, all bank voles showed weight loss, two bank voles with up to more than 25% of their initial weight (Figure 2B), and these two animals had to be euthanized at day 12 post infection. Finally, a survival rate of 75% was recorded for the CPXV Ger2010MKY infection in bank voles (Figure 3). Obvious clinical signs were not recorded in the other bank voles.

In contrast, body weight data from common voles demonstrated weight loss in males and weight gain in females regardless of the inoculated virus (data not shown).

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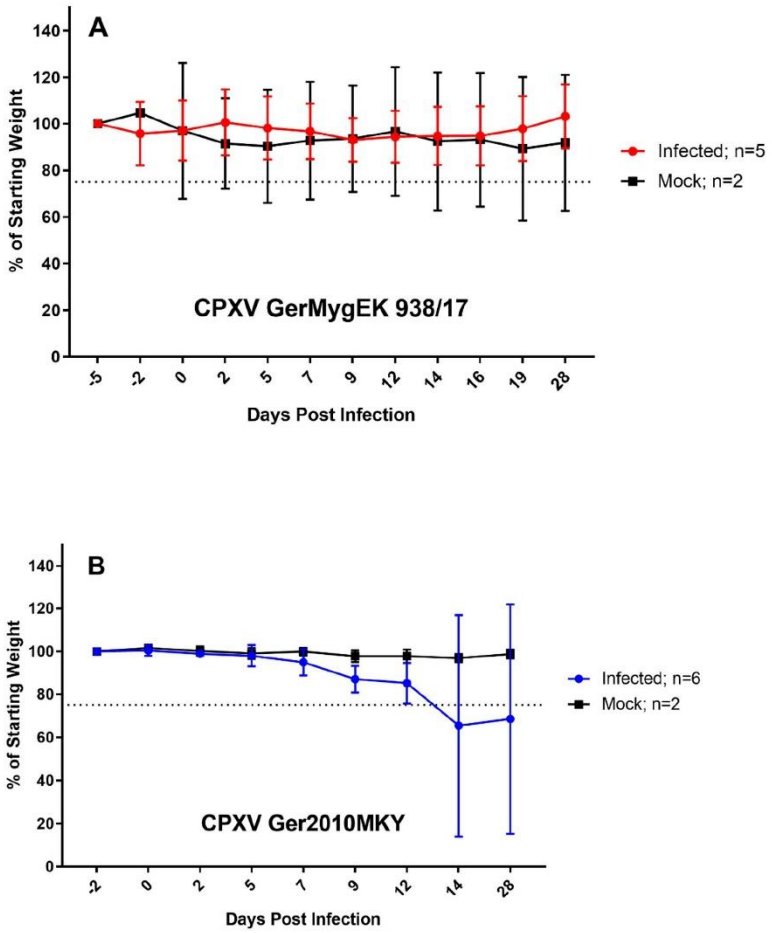

Figure 2. Body weight development of bank voles inoculated with (**A**) Cowpox virus (CPXV) GerMygEK 938/17 (bank vole-derived) or (**B**) CPXV Ger2010MKY (cotton-top tamarin-derived). The starting weight was set as 100%, and all values are means with standard deviations. Weight loss of more than 25% of initial weight (dotted line) was defined as termination criterion.

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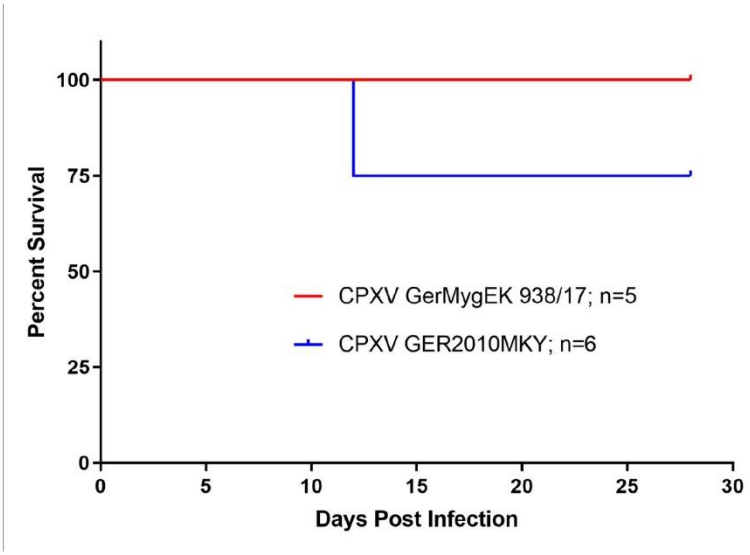

Figure 3. Bank vole survival rate over the time in two experiments. Bank voles were inoculated with $10^{5.5}$ $TCID_{50}$ per animal of CPXV GerMygEK 938/17 or Ger2010MKY, respectively.

3.2.2. Virus shedding: infected bank voles excrete CPXV

Viral DNA could be detected in buccal swabs in each CPXV GerMygEK 938/17-infected bank vole and in four of ten Wistar rats via qPCR between 2 and 14 dpi. Interestingly, the infectious virus could be verified from those swab samples in only three individual bank voles. Between 5 and 7 dpi rather low titers of virus were excreted from bank voles ($\geq 10^{1.625}$ $TCID_{50}$ mL^{-1}), while the highest virus titer was shed on day 9 post infection ($10^{2.6875}$ $TCID_{50}$ mL^{-1}) (Figure 4).

Individual buccal swabs from bank voles inoculated with CPXV Ger2010MKY were qPCR-positive between 2 and 14 dpi. Four bank voles also shed infectious virus: low titers ($\geq 10^{1.625}$ mL^{-1}) were excreted between 7 and 12 dpi. The highest level of virus was excreted at day 12 post infection ($10^{2.75}$ $TCID_{50}$ mL^{-1}) (Figure 4). Swabs of both bank voles that had to be euthanized due to >25% weight loss were tested positive for CPXV DNA (cycle quantification (Cq) values 29 and 31), but no virus could be isolated prior to euthanasia.

All swab samples taken from individual common voles tested negative for CPXV DNA regardless of the inoculated virus isolate.

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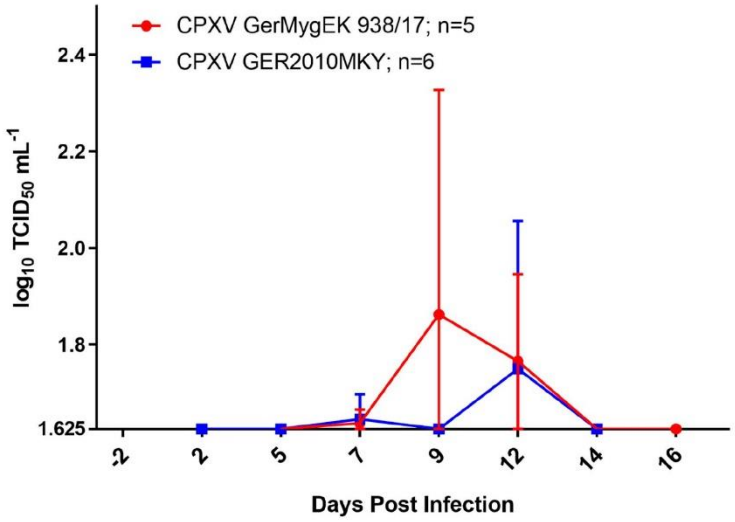

Figure 4. Viral shedding patterns of bank voles inoculated with CPXV GerMygEK 938/17 (red; $n = 5$) or CPXV Ger2010MKY (blue; $n = 6$) determined for buccal swabs. Values are the means with standard deviations. The detection limit of our test was defined at $10^{1.625}$ $TCID_{50}$ mL^{-1}.

3.2.3. Viral load in organ samples: CPXV positive turbinate sample of a bank vole at 28 dpi

The distribution of OPV DNA in different organs sampled at 28 dpi was tested by qPCR. Organ samples from rodents inoculated with CPXV GerMygEK 938/17 revealed no viral DNA regardless of the animal species (bank vole, common vole, and Wistar rat). In contrast, after infection with CPXV Ger2010MKY euthanized bank voles (12 dpi) scored positive for infectious virus in turbinates (both $10^{3.25}$ $TCID_{50}$ mL^{-1}), and for viral DNA in the trachea (Cq values: 34 and 36), the skin (Cq values: 35 and 36), and the lung (Cq value: 32) of one bank vole. Organ samples from all other bank voles (28 dpi) were negative for OPV DNA except of one turbinate sample of a single animal. The CPXV titer of this sample reached $10^{3.75}$ $TCID_{50}$ mL^{-1} (Table 2).

Table 2. Viral DNA detection and virus titers in different organs.

CPXV Strain	Species	Dissection		Tissue					
				Turbinate	**Trachea**	Lung	Liver	Spleen	Skin
GerMygEK 938/17	Bank vole	28 dpi		0/5*	0/5	0/5	0/5	0/5	0/5
	Common vole	28 dpi		0/6	0/6	0/6	0/6	0/6	0/6
	Wistar rat	28 dpi		0/10	0/10	0/10	0/10	0/10	0/10
Ger2010MKY	Bank vole	12 dpi#		2/2	2/2	1/2	0/2	0/2	2/2
			Cq value ($TCID_{50}$ mL^{-1})	24.8 ($10^{3.25}$) 26.3 ($10^{3.25}$)	34.2 36.1	32.2			35.1 36
		28 dpi		1/4	0/4	0/4	0/4	0/4	0/4
			Cq value ($TCID_{50}$ mL^{-1})	22.9 ($10^{3.75}$)					
	Common vole	28 dpi		0/6	0/6	0/6	0/6	0/6	0/6

Cowpox virus, CPXV; dpi, days post inoculation; $TCID_{50}$, Tissue Culture Infectious Dose 50 %. * Number of animals tested viral genome positive per total tested animals. # Two animals were euthanized at 12 dpi because of weight loss of more than 25%.

3.2.4. Serology: CPXV-Specific Seroconversion in Bank Voles with High Titers

Serum samples were obtained from every individual common vole and Wistar rat. Due to the sensitivity to anesthesia, we were able to achieve serum samples from eight of eleven individual bank voles (Table 3). In parallel, from all voles peritoneal lavage samples were collected.

Bank voles and Wistar rats inoculated with CPXV GerMygEK938/17 developed CPXV-specific antibodies with titers up to 1:640 (bank vole) or 1:1280 (Wistar rats), while no seroconversion was detectable in any of the inoculated common voles (Table 3). The sensitivity of the antibody detection in lavage samples was much lower—only in one of the bank vole samples antibodies were detected at a dilution of 1:20.

Four bank voles infected with CPXV Ger2010MKY developed high CPXV-specific antibody titers up to 1:1280. From the remaining two bank voles, only peritoneal lavage was analyzed for antibodies and only one lavage sample reacted with an antibody titer of 1:20, while again no seroconversion was detectable in any of the inoculated common voles.

Overall, six of eight lavage samples from voles that showed seroconversion in the corresponding serum sample were reactive against CPXV with a low titer of 1:20 (Table 3). At the 1:40 dilution, only two CPXV-positive samples out of eight were observed (Table 3).

Table 3. Seroconversion of CPXV-inoculated rodents at 28 days post inoculation.

CPXV Strain	Species	Number of Animals per Group	Number of Serum/Lavage Samples	Dilution							
				1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560
				seroreactive		seropositive					
GerMygEK 938/17	Bank vole	5*	Serum 4 Lavage 5	4/4 1/5#	4/4 0/5#	4/4	4/4	3/4	2/4	0/4	0/4
	Common vole	6	Serum 6 Lavage 6	0/6 0/6	0/6 0/6						
	Wistar rat	10	Serum 10	10/10	10/10	10/10	10/10	10/10	10/10	6/10	0/10
Ger2010MKY	Bank vole	6	Serum 4 Lavage 6	4/4 5/6##	4/4 2/6##	4/4	4/4	4/4	2/4	1/4	0/4
	Common vole	6	Serum 6 Lavage 6	0/6 0/6	0/6 0/6						

Cowpox virus, CPXV; *One additional animal died unrelated during anesthesia. # Peritoneal lavage of all animals was tested. Only one bank vole sample, already positive in the serum, tested positive. ## Peritoneal lavage of all animals was tested. Lavage of both bank voles without parallel serum sample reacted with a titer of 1:20.

4. Discussion

Wild rodents, in particular voles (subfamily Arvicolinae), are presumed reservoir animals for CPXV maintenance, however, virus isolations from vole samples are extremely rare and most CPXV-isolates of today were generated from accidental host tissue samples. Human infections mostly happened because of the contact to diseased accidental hosts like cats or pet rats, while infections from a reservoir host to humans were not reported [38].

In 2015, the susceptibility of common voles for CPXV infection was demonstrated by experimental inoculations [27]. However, the role and importance of bank voles as reservoir hosts for CPXV is still a matter of debate. Our recent studies from 2017 have justified doubts on the susceptibility of bank voles as reservoirs for CPXV, because independent of the CPXV-isolate or infection route used, virus shedding was never detectable and only very few bank voles showed antibody titers above 1:80 [31]. However, a bank vole-derived isolate for experimental inoculation was not available at this time.

In 2018, we successfully isolated the first bank vole-derived CPXV strain, GerMygEK 938/17 [32]. This new isolate provided the possibility to test the susceptibility of bank voles by experimental infection with a bank vole-derived strain. Nevertheless, growth kinetics of CPXV GerMygEK 938/17 on Vero76 cells in comparison to those of other CPXV strains like Brighton Red, RatPox09, or the very closely related CPXV strain Ger2010MKY allowed no proposition concerning the in vivo virulence. While all four tested CPXV strains showed a very similar outcome in the replication kinetics on Vero76 cells, the results of the animal experiments differed markedly [31]. Along with a previous study, we concluded that in vitro assays could not predict in vivo properties or virulence differences [39]. The same was observed in a recent study for the new CPXV strain GerMygEK 938/17 in comparison to Brighton Red and CPXV FM2292 on different cell lines (bank vole- and common vole-derived kidney cells and Vero76 cells) [32].

The here described animal experiments are to our knowledge the first comparing the infection of the most likely reservoir hosts, in particular bank vole and common vole with a bank vole-derived CPXV isolate and a very closely related CPXV strain originating from a cotton-top tamarin. As summarized in Table 4, bank voles inoculated with the bank vole-derived CPXV isolate GerMygEK 938/17 showed virus shedding from oropharyngeal secretions and a seroconversion rate of 100%. Interestingly, some bank voles inoculated with the most related strain CPXV Ger2010MKY even lost body weight of more than 25% and had to be euthanized.

Interestingly, not a single GerMygEK 938/17 inoculated bank vole showed any obvious clinical signs. However, animals from the wild are known to mask signs of disease as well as possible e.g., in the presence of human observers. This has to be taken into consideration for the evaluation of the absence of any clinical signs. It can be also speculated that sequence variation of the two related CPXV-like 3 strains in previously identified relevant genes like *vCCI*, *CrmB*, *CPXV0022*, and *CPXV194* are the reason of differences in virulence [32], and further studies will be necessary for clarification.

It needs to be highlighted that inoculated common voles exhibited neither virus shedding nor seroconversion. This is in clear contrast to the experimental infections of common voles with other CPXV strains, e.g., isolated from this vole species [27] (Table 4). The weight loss of the male animals might be explained due to their birth in the wild and the increased stress for these animals in captivity. The physical closeness to adult female voles without the possibility to mate has to be taken into consideration as well [40].

Wistar rats inoculated with the bank vole-derived isolate CPXV GerMygEK 938/17 exhibited no clinical signs, but all animals seroconverted. In former experiments, with the closely related cotton-top tamarin isolate CPXV Ger2010MKY, only two out of eight rats in the two different dose groups seroconverted [10]. In addition, the slight genetic differences might play a role.

The outcome of all these animal experiments further substantiates the recent hypothesis of reservoir species–specific phylogenetic lineages within the CPXV classification [31]. As depicted in Table 4, different CPXV strains display clearly different behavior in the different corresponding presumed reservoir animals and the Wistar rat animal model. While a common vole-derived CPXV isolate (CPXV-like 2 clade) leads to respiratory signs and prominent virus shedding in both the

original reservoir animal and an animal model (Wistar rats) for an accidental host [27], the inoculated bank voles revealed no clinics, no virus shedding and a low titer seroconversion rate of 60% [31]. Vice versa, common voles inoculated with the bank vole-derived CPXV isolate (CPXV-like 3 clade) exhibited neither virus shedding nor seroconversion. Interestingly, in all inoculation experiments with different vole species and a series of different CPXV isolates, we could never observe any poxvirus lesions. This is only seen in accidental hosts and the rat model used here [3–11,29,30].

The basis of resistance or susceptibility of a mammal species for CPXV infection is determined by host characteristics [41,42] as well as viral factors [43]. An example is the outcome of ECTV infection in laboratory mice: resistance and susceptibility are controlled by genetic factors of the host and are associated with multiple immune response mechanisms [44]. However even C57/BL6 mice, resistant to infection, respond serologically to ECTV inoculation [45].

To further support the hypothesis of a reservoir host specificity or preference of diverse CPXV clades, we will need more animal experiments with CPXV-like 3 isolates both in bank voles and in common voles. Furthermore, more detailed studies reflecting the genetic differences between CPXV-like 2 clade and CPXV-like 3 clade and the immune response (innate and adaptive) of the different hosts versus the different CPXV.

An additional conclusion from both the phylogenetic data and the in vivo characterization is that bank voles possibly also mediated the emergence of the CPXV Ger2010MKY isolate in the cotton-top tamarin holding in Thuringia, Germany, 2010 [10], although the low number of trapped rodents a few months later tested negative for CPXV.

In addition, Wistar rats as the accidental host model are susceptible for an infection with clear clinical signs including respiratory signs and virus shedding when inoculated with viruses of the common vole-related CPXV-like 2 clade, but are much more resistant to the bank vole-related isolates of CPXV-like 3 clade or ECTV [46]. This is also matching the observation that CPXV strains from the bank-vole related clade were only in very few cases isolated from accidental hosts [10].

A possible explanation for the differences between both CPXV clades could be the fact that the gene *CPXV0030* is missing in the genome of CPXV-like 3 isolates. We were recently able to demonstrate the impact of gene *CPXV0030* on the virulence in Wistar rats [47]. The gene encodes the CPXV 7-transmembrane G protein-coupled receptor-like protein (g7tGP) [48]. Further experiments with knock-out/knock-in mutants are conceivable just as infection experiments with isolates of CPXV-like 1 lineage, where the *g7tGP* gene is also missing.

In summary, the experimental inoculation of bank voles with isolates of CPXV-like 3 clade provided very important pieces of the puzzle about competent reservoir species of CPXV in central Europe. In addition to the sequence data-based polyphyletic character of the "species" CPXV [49], the "clades" of CPXV might also rely on different reservoir host preferences and adaptations.

Table 4. Comparison of different CPXV Strains and Reservoirs.

Cowpox Virus (CPXV)			Animals Inoculated			Results of Animal Experiments				Study
	Phylogenetic Lineage	Strain / Origin	Species	Dose of Inoculum/ Animal	Clinical Signs	Virus Shedding	Viral DNA in Organs (28 dpi)	Seroconversion	Mortality	
Common vole reservoir	CPXV-like 2	FM2292 / Common vole	Common vole	10^4 $TCID_{50}$	none	2/3	none	66%	0%	[27]
				10^6 $TCID_{50}$	respiratory	3/3	1/3 (nasal septum)	100%	33%	[27]
			Bank vole	10^5 $TCID_{50}$	none	none	none	60%	0%	[31]
			Wistar rat	10^4 $TCID_{50}$	Respiratory Pox-like lesions	4/4	none	100%	0%	[27]
				10^6 $TCID_{50}$	Respiratory Pox-like lesions	3/3	none	100%	0%	[27]
Bank vole reservoir	CPXV-like 3	GerMygEK 938/17 / Bank vole	Bank vole	$10^{5.5}$ $TCID_{50}$	none	5/5	none	100%	0%	This study
			Common vole	$10^{5.5}$ $TCID_{50}$	none	none	none	0%	0%	This study
			Wistar rat	$10^{5.5}$ $TCID_{50}$	none	none	none	100%	0%	This study
		Ger2010MKY / Cotton-top tamarin	Bank vole	$10^{5.5}$ $TCID_{50}$	Weight loss up to 25%	6/6	1/6 (nasal septum)	83%	33%	This study
			Common vole	$10^{5.5}$ $TCID_{50}$	none	none	none	0%	0%	This study
			Wistar rat	10^4 $TCID_{50}$	none	none	none	25%	0%	[10]
				10^6 $TCID_{50}$	none	none	none	25%	0%	[10]

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

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IV. PUBLIKATION III

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 $\geq$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].

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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IV. PUBLIKATION IV

What a Difference a Gene Makes:
Identification of Virulence Factors of Cowpox Virus

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PATHOGENESIS AND IMMUNITY

What a Difference a Gene Makes: Identification of Virulence Factors of Cowpox Virus

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ABSTRACT Cowpox virus (CPXV) is a zoonotic orthopoxvirus (OPV) that causes spillover infections from its animal hosts to humans. In 2009, several human CPXV cases occurred through transmission from pet rats. An isolate from a diseased rat, RatPox09, exhibited significantly increased virulence in Wistar rats and caused high mortality compared to that caused by the mildly virulent laboratory strain Brighton Red (BR). The RatPox09 genome encodes four genes which are absent in the BR genome. We hypothesized that their gene products could be major factors influencing the high virulence of RatPox09. To address this hypothesis, we employed several BR-RatPox09 chimeric viruses. Using Red-mediated mutagenesis, we generated BR-based knock-in mutants with single or multiple insertions of the respective RatPox09 genes. High-throughput sequencing was used to verify the genomic integrity of all recombinant viruses, and transcriptomic analyses confirmed that the expression profiles of the genes that were adjacent to the modified ones were unaltered. While the *in vitro* growth kinetics were comparable to those of BR and RatPox09, we discovered that a knock-in BR mutant containing the four RatPox09-specific genes was as virulent as the RatPox09 isolate, causing death in over 75% of infected Wistar rats. Unexpectedly, the insertion of *gCPXV0030* (*g7tGP*) alone into the BR genome resulted in significantly higher clinical scores and lower survival rates matching the rate for rats infected with RatPox09. The insertion of *gCPXV0284*, encoding the BTB (broad-complex, tramtrack, and bric-à-brac) domain protein D7L, also increased the virulence of BR, while the other two open reading frames failed to rescue virulence independently. In summary, our results confirmed our hypothesis that a relatively small set of four genes can contribute significantly to CPXV virulence in the natural rat animal model.

IMPORTANCE With the cessation of vaccination against smallpox and its assumed cross-protectivity against other OPV infections, waning immunity could open up new niches for related poxviruses. Therefore, the identification of virulence mechanisms in CPXV is of general interest. Here, we aimed to identify virulence markers in an experimental rodent CPXV infection model using bacterial artificial chromosome (BAC)-based virus recombineering. We focused our work on the recent zoonotic CPXV isolate RatPox09, which is highly pathogenic in Wistar rats, unlike the avirulent BR reference strain. In several animal studies, we were able to identify a novel set of CPXV virulence genes. Two of the identified virulence genes, encoding a putative BTB/POZ protein (CPXVD7L) and a B22R-family protein (CPXV7tGP), respectively, have not yet been described to be involved in CPXV virulence. Our results also show that single genes can significantly affect virulence, thus facilitating adaptation to other hosts.

KEYWORDS cowpox virus, pathogenesis, virulence factors, zoonotic infections

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Cowpox virus (CPXV) is a model orthopoxvirus (OPV) for several reasons. First, CPXV has the broadest host range of all known orthopoxviruses and is capable of productively infecting various species, including humans (1–4). Second, rising numbers of CPXV infections in humans and animals have been reported over the last few years and have resulted in CPXV being considered a (re)emerging virus with increasing epizootic and zoonotic potential (5–11). It is important to note that the name "cowpox" is a misnomer, since wild rodents, specifically, voles, represent the main reservoir host for the virus (12, 13). However, spillover infections from accidental hosts, including cats and pet rats, to humans occur frequently, mostly through skin lesions (14–19). The resulting human infections are generally self-limiting with typical pox lesions at the site of virus entry. However, the virus can also cause systemic and fatal disease in immunocompromised patients (20, 21). CPXV possesses the largest genome and the most complete genetic repertoire of all known OPVs (22–25), and the remarkable set of host range and immunomodulatory genes, in particular, might allow the virus to evolve and increase its virulence (21, 22).

It is becoming increasingly clear that CPXV is polyphyletic with several distinct clades (12, 26, 27). The frequent isolation of new strains and accessibility to whole-genome sequencing data (currently, more than 80 strains are deposited in GenBank) have increased the complexity of the CPXV phylogenetic tree and also demonstrate the close genetic relationship to human smallpox virus (13, 28, 29). However, despite the impressive number of field isolates sequenced, only rarely were CPXV strains compared with each other regarding their pathogenicity in experimental animal models. Interestingly, different CPXV strains resulted in quite variable mortality rates ranging from 0% to 100% in mice and rats, despite similar growth kinetics *in vitro*, and increased virulence did not correlate with stronger cytopathic effects (CPE) in cell culture (30, 31). It seems, therefore, that the variation of *in vivo* virulence is a direct effect of genetic differences among the strains, and it is tempting to speculate that the presence or absence of single virulence factors is responsible for different disease outcomes. However, such factors can be reliably identified only using *in vivo* studies, since even three-dimensional (3D) skin cultures that should represent a more natural model of infection failed to recapitulate the differences in virulence detected in the animal model (32).

CPXV Brighton Red (BR) is a commonly used laboratory strain that was originally isolated in 1937 from the finger of a cowman and maintained by serial passage, first in guinea pigs and rabbits and later in cell culture (33, 34). Despite the clear genomic adaptations to cell culture conditions that laboratory strains of many viruses usually undergo, CPXV BR is still considered a CPXV reference strain.

In 2009, a new strain was isolated from a diseased pet rat, which had bitten and infected two girls (13, 35). The isolate was named RatPox09 and exhibited *in vitro* viral growth characteristics similar to those of the reference strain, CPXV BR (31, 32). *In vivo* experiments using Wistar rats and pet rats demonstrated, however, that animals infected with RatPox09 developed severe and often fatal disease, whereas the infection of mice or rats with CPXV BR resulted in no or very mild clinical symptoms (31, 35, 36). RatPox09 is an ideal example of a CPXV strain which adapted after spillover infection to rats and causes severe clinical disease. The complete genomic sequence of RatPox09 was determined by high-throughput sequencing and compared with that of BR, which revealed a nucleotide sequence identity of 92% (31). Inspection of the sequences revealed four unique open reading frames (ORFs) which are present in the RatPox09 genome but absent in the BR genome (31). These genes were named *gCPXV0002*, *gCPXV0003*, *gCPXV0284*, and *gCPXV0030* after their relative position in the genome (31), but information on the encoded gene products is still scarce. *gCPXV0002* shares 99% nucleotide sequence identity with the CPXV gene encoding the *N*-methyl-D-aspartate (NMDA) receptor-like protein, and we refer to this gene as *gNMDAr* here. The function of the protein in CPXV infection is unknown (12), but the NMDAr protein sequence has 98% amino acid identity with vaccinia virus (VACV) Golgi apparatus antiapoptotic protein (vGAAP) (37). GAAPs are highly conserved and resident in the Golgi apparatus.

They inhibit apoptosis induced by intrinsic and extrinsic stimuli and, in addition, form cation-selective ion channels that regulate Ca^{2+} levels and fluxes that participate in cell adhesion and migration (37, 38). NMDAr has a high amino acid sequence identity with different lifeguard (Lfg) proteins in cattle (78% identity), mice (74% identity), and rats (74% identity). Members of the Lfg family are widely distributed in eukaryotes, and some of them have been reported to play a cytoprotective role during apoptosis (39). In the VACV infection model of mice, the loss of vGAAP was associated with increased virulence (37). *gCPXV0003* has 99% identity with the VACV gene encoding the cytokine response modifier E (CrmE), a known virulence factor of VACV (40). CrmE is a secreted molecule, belongs to the tumor necrosis factor (TNF) receptor superfamily, and blocks the binding of TNF to high-affinity TNF receptors on the cell surface (40). *gCPXV0284* shares 92% nucleotide sequence identity with the gene that encodes D7L, a broad-complex, tramtrack, and bric-à-brac (BTB) domain protein of CPXV strain GRI-90 (D7L). However, the function of the D7L protein is unknown (25). *gCPXV0030* has 99% nucleotide sequence identity with the gene encoding the CPXV 7-transmembrane G protein-coupled receptor-like protein (*7tGP*) (12). Despite its misleading name, multiple structure analysis tools predict only one transmembrane domain and two poxvirus B22R-like domains between amino acids (aa) 10 and 777 as well as aa 786 and 1053 (41). B22 proteins are conserved in representatives of most poxviruses, which may indicate a long evolutionary history and host adaptation. In fact, some poxviruses contain multiple copies of genes encoding these proteins (42). Monkeypox B22 proteins directly inhibit T cells by rendering them unresponsive to stimulation by the T cell receptor and major histocompatibility complex (MHC)-dependent antigen presentation or to MHC-independent stimulation (43). Deletion of the B22 homolog in ectromelia virus (ECTV) resulted in a significant reduction of virulence in the relevant mouse model (42). In addition, two ORFs, *gCPXV158* (*gATI*) and *gCPXV159* (*gp4c*), were markedly different in the RatPox09 and BR genomes (31). The amino acid sequence of RatPox09 p4c (A26) has a 59-aa truncation at the N terminus, which likely is responsible for its failure to direct intracellular mature virions (IMVs) in acidophilic-type inclusion bodies (ATI) (31), while the BR p4c gene is disrupted due to a premature stop codon introduced by a frameshift mutation (44). Furthermore, CPXV BR and RatPox09 ATI-encoding genes show modifications in length and sequence, particularly in the repeat regions, even though the translational initiation and stop codons of these two ORFs are identical. The overall nucleotide sequence identity of CPXV BR and RatPox09 ATI-encoding genes is 87%. Both ATI and p4c are vital for incorporation of IMVs into A-type inclusion bodies (44–46). It is assumed that ATIs are proteinaceous matrices which shield infectious particles after they are released into the environment (47). Moreover, genome comparison also revealed many single nucleotide polymorphisms (SNPs) that were scattered all over the genome of RatPox09 (31). The impact of these SNPs on the coding potential of the virus is currently unclear.

We hypothesized that the additional full-length gene products of RatPox09 rather than individual SNPs would significantly contribute to the increased virulence observed in previous infection studies using Wistar rats (31, 35), in which the virulence would simulate the real-world situation previously observed in pet rats in the field (48).

In order to test our hypothesis, we inserted individual RatPox09 genes as well as combinations thereof into the CPXV BR genome by Red mutagenesis of BR cloned into a bacterial artificial chromosome (BAC). To address the impact of the minor sequence polymorphisms that are spread all over the genome, we also generated BR-RatPox09 chimeric viruses with large genomic segments exchanged between the two strains. This allowed us to screen for potential additional virulence factors in an unbiased fashion. To exclude the possibility of any secondary-site mutations introduced during the mutagenesis, the genomic integrity of all viruses was verified by whole-genome sequencing and comprehensive transcriptomic analyses. Finally, animal experiments conducted in infected rats allowed the analysis of the generated hybrid viruses and comparison with the parental BR and RatPox09 viruses.

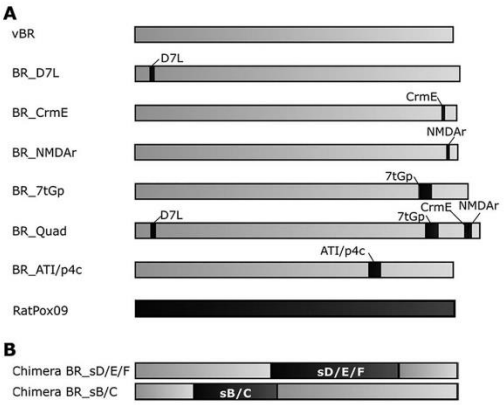

FIG 1 Schematic illustration of newly generated BAC knock-in mutants (A) and the generated chimeric viruses (B). Gray boxes, BR sequences; black boxes, RatPox09 sequences.

RESULTS

Generation of CPXV BR knock-in mutants. First, we generated BR-based mutant viruses that harbored the unique RatPox09 ORFs (*gD7L*, *gCrmE*, *gNMDAr*, *g7tGP*) either individually or as multiple gene knock-ins at their original loci (Fig. 1A). Mutant viruses were constructed using the full-length pBRF BAC clone. Moreover, a CPXV BR mutant virus harboring both RatPox09 *gATI* and *gp4c* was generated. After Red recombination, all final BACs were verified by restriction fragment length polymorphism (RFLP) analysis and Sanger sequencing of the modified genomic loci (data not shown). Virus mutants were reconstituted on Vero cells from the modified BAC clones, and the genomic integrity of the viruses was verified by whole-genome sequencing (data are available by request). Apart from the desired modifications, no secondary mutations or rearrangements were present in the genomes of the final mutant viruses (data not shown). Furthermore, transcriptomic analysis confirmed all of the mRNA transcripts of the inserted sequences and unchanged transcription levels of the respective adjacent genes (Fig. 2).

Generation of CPXV BR-RatPox09 chimeric viruses. In order to identify additional virulence genes in RatPox09, we swapped large genomic segments of the low-virulence strain BR with the corresponding regions of RatPox09. For this purpose, we divided the RatPox09 genome into seven segments of 20 to 40 kb in size and named the fragments A to G. To allow homologous recombination, the sequence of each segment was chosen such that it contained at least 800 bp of overlapping, identical sequences between the two strains. In order to generate the chimeras, we first deleted the respective genome regions in pBRF by Red mutagenesis. This step was necessary to avoid homologous recombination events during reinsertion of the segments. We confirmed successful deletion from pBRF of the segments in question by RFLP analysis and sequencing (data not shown). In a second step, we amplified the RatPox09-derived regions of interest by high-fidelity PCR and again used Red recombineering to reinsert segments at the deletion sites of pBRF. Using this strategy, we generated chimeric viruses containing segments B and C or segments D, E, and F in combination (chimeras BR_sB/C and BR_sD/E/F, respectively) (Fig. 1B).

***In vitro* characterization of the mutants and chimeras does not reveal growth differences.** All CPXV chimeras or CPXV knock-in mutants were compared to the respective reference strains, BR and RatPox09, by single and multistep growth kinetics in cell culture. In four independent experiments using technical duplicates as internal controls, growth kinetics were determined in Vero 76 cells using a multiplicity of infection (MOI) of 0.01 or an MOI of 3.

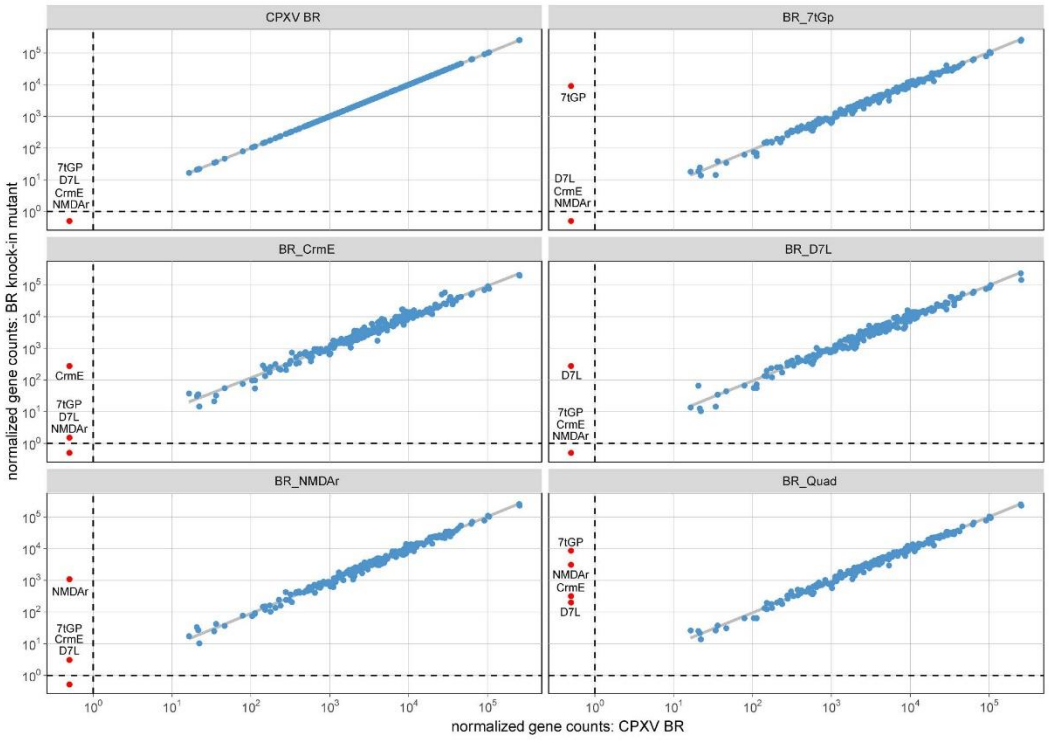

FIG 2 Transcriptomic analysis. The normalized gene count for CPXV BR is compared to the normalized gene count for the knock-in mutant CPXVs.

BR and RatPox09 exhibited similar viral titers with no significant differences ($P > 0.05$ in all cases). The differences in the viral titers detected from any of the tested knock-in mutants or chimeric viruses did not exceed 1 order of magnitude (data not shown). At 24 h postinfection (hpi), titers of ~10^5 50% tissue culture infective doses ($TCID_{50}$) ml^{-1} (MOI, 0.01) or ~10^6 (MOI, 3) were determined, regardless of whether the cells were infected with BR, RatPox09, or any of the mutants or chimeras. There were also no significant differences between the titers at 48 hpi, regardless of whether an MOI of 0.01 or an MOI of 3 was used (data not shown).

***In vivo* characterization of mutants and chimeras.** In order to explore any gain of function of the different mutants and chimeras *in vivo*, we inoculated Wistar rats and used the experimentally well-defined virus isolates BR, BAC-derived BR (vBR), and RatPox09 as controls.

Clinical signs: high clinical scores for rats infected with BR_7tGP and BR_D7L/CrmE/NMDAr/7tGP. After intranasal infection, the first clinical signs were respiratory in nature and included a prominent nasal discharge at 3 to 7 days postinfection (dpi), regardless of the virus used for infection. The higher clinical scores presented in Fig. 3 over this time span are attributed to respiratory symptoms. In addition, pox-like lesions on the skin and mucous surfaces were observed, typically at later time points during the infection (>8 dpi). As expected, the BR strain caused no or only mild symptoms of nasal discharge, decreased activity, and rough fur but no skin lesions, resulting in clinical scores ranging from 1 to 2 throughout the animal experiment (Fig. 3A and B). As seen in earlier studies (35, 49), RatPox09 caused severe symptoms that included inflated stomachs, moderate to severe respiratory distress, loss of body weight, and pox

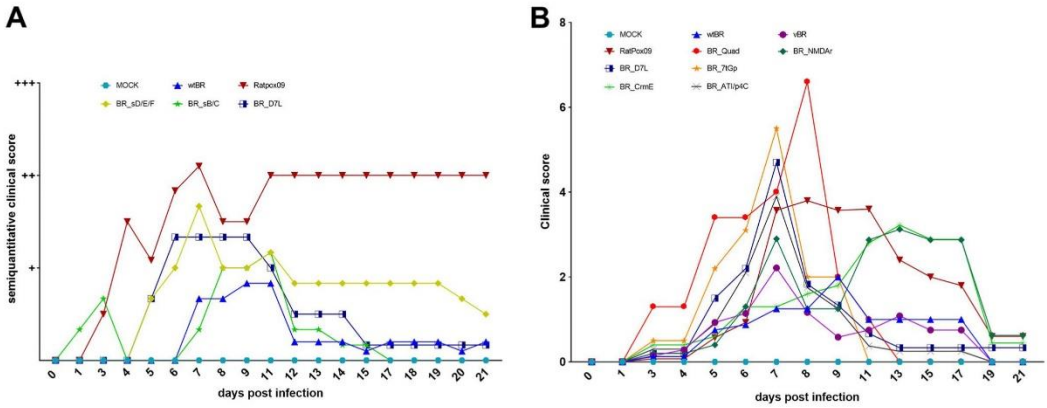

FIG 3 Rat CPXV infection clinical score over time. Rats infected with $10^{5.5}$ $TCID_{50}$ per animal were clinically evaluated semiquantitatively on a daily basis in experimental setup 1 (A), while in experimental setup 2, evaluation by a quantitative clinical score system was used (B).

lesions on the skin (>8 dpi). Clinical scores (Table 1) reached a peak of approximately 4 at 8 dpi (Fig. 3B).

In particular, infections with the BR_7tGP virus and the BR_D7L/CrmE/NMDAr/7tGP virus (referred to as the BR_Quad virus from here on) resulted in clinical scores at earlier time points even higher than those achieved by infection with reference strain RatPox09 (Fig. 3B). This observation was also correlated to increased mortality (see "Mortality: BR_7tGP and BR_Quad are highly virulent and lethal" below). Nevertheless, animals that recovered from infection with BR_Quad or BR_7tGP did not exhibit skin lesions.

The scores induced by the BR knock-in mutants BR_ATI/p4C and BR_D7L exhibited a peak at about 7 dpi, which clustered with those induced by RatPox09, while the scores induced by BR_NMDAr were almost similar to those induced by RatPox09 at later time points (11 dpi to 17 dpi). Reflecting the delayed onset of respiratory symptoms, only a minority of animals developed skin lesions.

All tested recombinant viruses caused severe symptoms, as evidenced by the accumulation of high clinical scores (Fig. 3B). However, the clinical scores induced by chimeric viruses in the Wistar rat model did not exceed the clinical scores induced by RatPox09 infection (Fig. 3A). In order to analyze the kinetics of the clinical signs induced by the recombinant knock-in viruses, we calculated the start, the time point of the peak, and the duration of the observed clinical signs as well as the peak scores and the scores at the beginning and the end of the symptomatic period (Tables 2 and 3). While the duration of clinical signs was not significantly different between the groups, the maximum values of the clinical scores induced by the different viruses exhibited significant differences when comparisons between BR and RatPox09 and between BR and BR_Quad were made (Table 4).

Mortality: BR_7tGP and BR_Quad are highly virulent and lethal. Two mutant viruses led to a dramatic reduction in the survival of infected Wistar rats, namely, BR_7tGP and BR_Quad, where survival rates of only 20% to 35% were recorded. These rates were comparable to those observed after RatPox09 infection. Interestingly, the mortality in the BR_Quad-infected group was the highest, and only 20% of the inoculated animals survived. However, the differences in survival rates between the groups inoculated with the mutant and RatPox09 were not significant ($P \geq 0.05$). Nevertheless, RatPox09 and BR_Quad resulted in survival rates significantly lower than those achieved with the vBR control ($P \leq 0.05$) in all instances (Table 4). All other chimeras and mutant viruses caused mortalities that matched the mortality after

TABLE 1 Clinical scores used to evaluate CPXV-infected rats

Clinical parameter	No. of points
Body temp	
Above 36°C	0
35–36°C	1
34–35°C	2
Below 34°C	3
Activity	
Normal	0
Moderate	1
Inactive	2
Nasal discharge	
Absent	0
Doubtful	1
Clearly present	2
Body wt loss	
Absent	0
>10%	1
>20%	2
Respiratory distress	
Normal breathing	0
Light	1
Moderate	2
Severe	3
Wheezing	
Absent	0
Present	1
Pox lesions	
Absent	0
Present	1
Inflated stomach	
Absent	0
Present	1
Rough fur	
Absent	0
Present	1

infection with BR (approximately 72 to 100% survival) (Fig. 4). Since the Wistar rats used in the different experiments were outbred and therefore not genetically identical, a certain level of variation of survival rates between the experiments was to be expected and also recorded. The survival rates of animals infected with BR varied from 75% in the first experimental setup to 100% in the second one. Similarly, rats infected with RatPox09 had a survival rate of 29% in experimental setup 1 and 36% in the second setup. Most importantly, however, virus mutants that caused low survival rates similar to or even lower than those caused by RatPox09, namely, BR_7tGP and BR_Quad (also containing the gene for 7tGP), always produced clinical scores that matched the highest clinical scores evaluated (Fig. 3B).

Finally, one mock-infected animal was euthanized due to malocclusion of the dentition. The virus genome was not detectable in either buccal swab or organ tissue samples in that case (Fig. 4A).

Virus shedding: no enhanced shedding of recombinant CPXV. Oropharyngeal shedding was detected in all groups (Fig. 5). Generally, the first excretion of virus was recorded at between 3 and 5 dpi, and most rats showed positive buccal swab samples between 5 and 7 dpi (Tables 2 and 3). Generally, animals infected with RatPox09 shed more virus than the animals in all other groups ($10^{3.1}$ $TCID_{50}$/ml at 7 dpi). Interestingly,

TABLE 2 Median parameters describing CPXV shedding and kinetics of swab specimens from the different groups

Parameter	Units	Value for swabs from rats infected with[a]:								
		vBR (n = 13)	RatPox09 (n = 14)	BR_Quad (n = 8)	BR_NMDAr (n = 7)	BR_D7L (n = 10)	BR_7tGP (n = 10)	BR_CrmE (n = 10)	BR_ATI/p4c (n = 9)
T_{max}	Days	7 (5, 9)	7 (7, 9.5)	7 (5, 7)	6 (5, 9)	5 (5, 7.5)	5 (5, 5)	9 (6.5, 9)	7 (5, 9)
T_{start}	dpi	5 (5, 5)	5 (5, 7)	5 (4.5, 7)	5 (5, 5)	5 (5, 5)	4 (3, 5)	5 (3, 7)	5 (3.5, 5)
T_{end}	dpi	7 (5, 11)	8 (7, 11)	7 (7, 7)	9 (7, 10.5)	7 (7, 11)	5 (5, 7)	11 (10.5, 11)	7 (7, 9)
Duration	Days	3 (1, 5)	3 (3, 5)	1 (1, 3.5)	5 (3, 7)	3 (3, 7)	2.5 (1:3)	5 (4.75, 7)	4 (3, 5)
V_{max}	Log_{10} $TCID_{50}$/ml	2.313 (1.781, 3.031)	3.75 (2.875, 3.906)	1.813 (1.625, 2.125)	2.125 (1.906, 3.5)	2.5 (2.094, 2.938)	1.625 (1.625, 1.875)	2.625 (2.219, 3.156)	2.25 (2, 2.75)
V_{start}	Log_{10} $TCID_{50}$/ml	1.75 (1.75, 2.25)	2.188 (1.875, 3.25)	1.625 (1.625, 1.823)	1.875 (1.656, 2)	2.25 (1.875, 2.5)	1.625 (1.625, 1.875)	2 (1.875, 2.156)	1.875 (1.625, 2.094)
V_{end}	Log_{10} $TCID_{50}$/ml	2.25 (1.75, 2.594)	3.5 (2.719, 3.875)	1.75 (1.625, 2.031)	1.813 (1.75, 1.969)	1.75 (1.75, 1.906)	1.625 (1.625, 1.875)	1.875 (1.625, 2.313)	2 (1.781, 2.406)

[a]The data represent the median (the interval between the first and the third quartiles) calculated from the individual parameters. *n*, number of swabs.

TABLE 3 Median parameters describing CPXV kinetics of clinical scoring values for animals in the different groups

Parameter	Units	Value for rats infected with[a]:							
		vBR (n = 14)	RatPox09 (n = 14)	BR_Quad (n = 10)	BR_NMDAr (n = 10)	BR_D7L (n = 9)	BR_7tGP (n = 10)	BR_CrmE (n = 10)	BR_ATI/p4c (n = 10)
T_{max}	Days	7 (6.25, 11)	8 (7, 9)	8 (8, 8)	11 (7, 11)	7 (7, 7)	7 (7, 7)	13 (11, 13)	7.5 (7, 8)
T_{start}	dpi	5 (4.25, 6.75)	6 (5, 7)	3 (3, 5)	6 (5.25, 7)	5 (5, 5)	4 (3, 5)	5.5 (3, 6)	5.5 (3.5, 6)
T_{end}	dpi	17 (8, 17)	8.5 (7.25, 11)	8 (8, 8)	19 (17, 21)	8 (7, 11)	7 (7, 9)	17 (17, 20)	8 (7, 10.5)
CS_{max}	CS	2 (1.25, 2)	4.5 (4, 7)	7.5 (7, 8)	4 (3.25, 4.75)	3 (3, 9)	5.5 (3, 8)	3.5 (2, 4.75)	3 (2.25, 3)
CS_{start}	CS	1 (1, 1)	2 (1, 3)	2.5 (2, 4.5)	1 (1, 1)	2 (1, 2)	1.5 (1, 2)	1 (1, 1)	2 (1, 2)
CS_{end}	CS	1 (1, 2)	4.5 (3.25, 7)	7.5 (7, 8)	1 (1, 1)	2 (1, 9)	5.5 (2, 8)	2 (2, 2)	2.5 (1, 3)
S_{max}	Days	21 (21, 21)	8.5 (7.25, 21)	8 (8, 8)	21 (21, 21)	21 (7, 21)	7 (7, 21)	21 (21, 21)	21 (21, 21)
S_{end}	CS	0 (0, 0)	4.5 (1, 7)	7.5 (7, 8)	1 (0.25, 1)	1 (0, 9)	5.5 (0, 8)	0 (0, 1)	0 (0, 0)

[a]The data represent the median (the interval between the first and the third quartiles) calculated from the individual parameters. *n*, number of rats.

TABLE 4 Statistical evaluation of median parameters for the vBR-inoculated group in comparison to those for single- and multiple-knock-in mutants

Parameter	Unit of measurement	*P* value for vBR vs[a]:						
		RatPox09	BR_Quad	BR_NMDAr	BR_D7L	BR_7tGP	BR_CrmE	BR_ATI/p4c
T_{max}	d	0.1031	>0.9999	>0.9999	>0.9999	0.0161	0.501	>0.9999
T_{start}	dpi	0.1258	>0.9999	>0.9999	>0.9999	0.0498	>0.9999	>0.9999
T_{end}	dpi	0.0905	>0.9999	0.7744	0.4634	0.0297	0.0019	>0.9999
Duration of shedding	dpi	>0.9999	0.9723	0.2018	0.1243	0.7422	0.0186	>0.9999
V_{max}	log_{10} $TCID_{50}$/ml	<0.0001	0.0291	>0.9999	>0.9999	0.0061	>0.9999	>0.9999
V_{start}	log_{10} $TCID_{50}$/ml	<0.0001	>0.9999	>0.9999	0.4794	>0.9999	>0.9999	>0.9999
V_{end}	log_{10} $TCID_{50}$/ml	<0.0001	0.1663	0.1857	0.8106	0.0159	>0.9999	>0.9999
T_{max}	d	>0.9999	>0.9999	0.8033	>0.9999	>0.9999	<0.0001	>0.9999
T_{start}	dpi	>0.9999	0.0434	>0.9999	>0.9999	0.0915	0.8233	>0.9999
T_{end}	dpi	>0.9999	0.0878	0.2042	>0.9999	0.035	0.0878	0.5555
CS_{max}	CS	0.0257	0.0015	0.5054	0.0489	0.0445	>0.9999	>0.9999
CS_{start}	CS	0.0285	<0.0001	>0.9999	0.8623	0.6542	>0.9999	0.6542
CS_{end}	CS	0.1765	0.0052	>0.9999	0.4073	0.151	>0.9999	>0.9999
S_{max}	d	0.0348	0.0072	>0.9999	0.705	0.0783	>0.9999	>0.9999
S_{end}	CS	0.1236	0.0056	>0.9999	0.272	0.2038	>0.9999	>0.9999

$P < 0.05$	$P < 0.01$	$P < 0.001$	$P < 0.0001$

[a]Each cell contains the *P* value. The *P* values are color coded in the key at the bottom.

albeit the mutant viruses BR_7tGP and BR_Quad induced excessive mortality rates of up to 80% (Fig. 4), viral shedding was within the range of that achieved with BR, with virus titers being between $10^{0.74}$ and $10^{1.5}$ $TCID_{50}$/ml (at 5 to 7 dpi) (Fig. 5). This was confirmed by the statistical evaluation of shedding kinetics (Table 5): comparison of viral excretion titers (determined as the time of peak shedding [T_{max}], the time at the start of the shedding period [T_{start}], and the time at the end of virus shedding [T_{end}]) of RatPox09 with those of BR, BR_Quad, and BR-7tGP displayed highly significant differences.

Viral load in organ samples. The distribution of viral DNA in various organs was determined by quantitative PCR. All rats that succumbed to CPXV infection scored positive for virus DNA in turbinate samples, regardless of which virus mutant or chimera was used for inoculation. Turbinate samples taken from Wistar rats euthanized at 5 dpi were positive as well. Other organs scored positive only when animals succumbed or were examined at 5 dpi (data not shown).

Serology. Blood serum samples from all rats that succumbed to infection or animals dissected on day 5 postinfection (p.i.) were negative for antibodies, while antibodies against the mutants or chimeras were detectable in all serum samples obtained on 21 or 28 dpi (data not shown).

DISCUSSION

With the eradication and subsequent cessation of vaccination against smallpox, zoonotic OPV infections are on the rise due to waning immunity in the human population, and an increasing number of humans are becoming susceptible to poxvirus infections every year. Such infections include those caused by monkeypox virus (MPXV) in Central/West Africa, CPXV in Europe, and VACV in Brazil and India (50–54). Molecular characterization of OPVs isolated from different geographic locations or hosts is therefore of high importance for understanding their geographic distribution, variability, and evolution, as well as for monitoring the emergence of atypical OPV strains with enhanced virulence.

It has been documented that CPXV strains vary significantly regarding their virulence in rodent models, with mortality rates ranging from 0 to 100% (31, 35, 36, 49). Our studies and those of other laboratories have shown that these *in vivo* differences

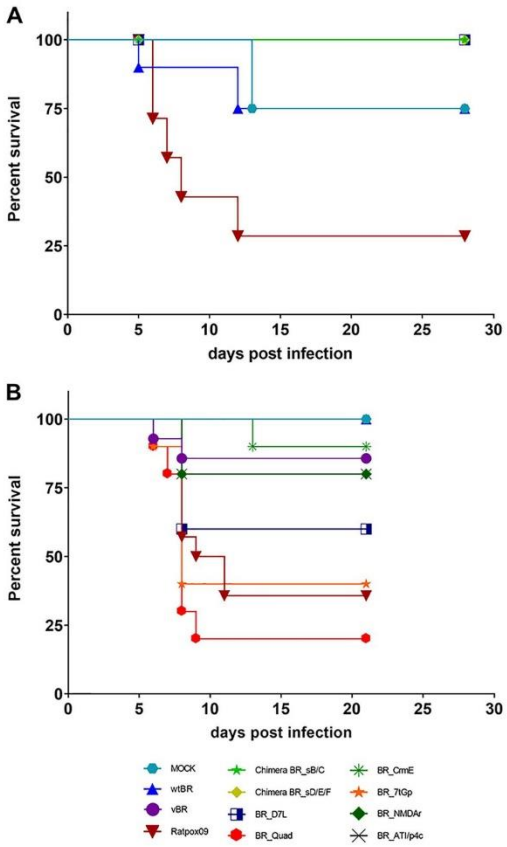

FIG 4 Rat survival over time in two experiments. Rats were infected with $10^{5.5}$ $TCID_{50}$ per animal of wtBR, RatPox09, chimeric viruses, and the D7L knock-in virus (A), and in the final two experiments, rats were infected with $10^{5.5}$ $TCID_{50}$ per animal of wtBR, vBR, RatPox09, BR_ sF, and the knock-in mutants (B).

cannot be recapitulated by *in vitro* assays that focus exclusively on cytopathic effects in cell culture. Similarly, a three-dimensional (3D) skin model as a possible replacement for animal experiments for the identification of CPXV virulence factors failed to address the virulence of individual mutants: The histopathological and immunohistochemical studies showed that the 3D model reflected the development of pox lesions in normal skin very well, but it did not allow the differentiation between virulent and avirulent CPXV strains (32). Two lessons could be learned from that study. First, *in vivo* experiments still present the gold standard for the identification of CPXV genes that control virulence. Second, and more importantly, virulence factors appear to have important functions that impact the viral replication cycle only in complex and diverse multicellular organisms. These functions include diversions of host defense mechanisms, particularly evasion of the immune system, and, hence, can currently be identified only *in vivo*. In addition, the role in countering host defenses exerted by certain proteins should be studied in relevant animal host models to avoid incompatibilities, as was demonstrated for CPXV studies in laboratory mice and subsequent studies using ECTV in the mouse footpad model (42, 55). Therefore, a matching pair of virus and host species should be evaluated. This goes hand in hand with the claim of biological congruence between the

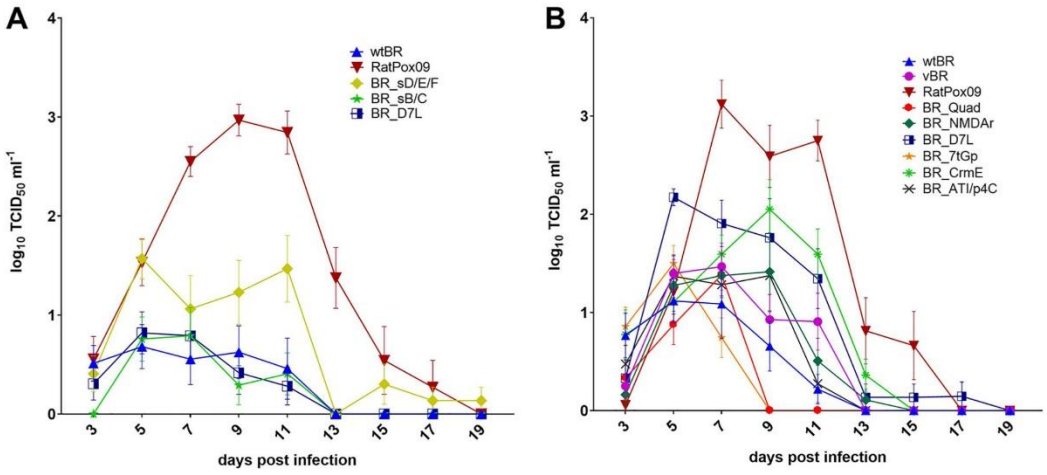

FIG 5 Viral shedding patterns of infected Wistar rats. In the initial two experiments, rats were infected with $10^{5.5}$ $TCID_{50}$ per animal of wtBR, RatPox09, chimeric viruses, and the D7L knock-in virus (A), and in the two follow-up experiments, rats were infected with $10^{5.5}$ $TCID_{50}$ per animal of wtBR, vBR, RatPox09, and the knock-in mutants (B).

properties of the test population and those of the target population as a central part of the construct validity proposed for the explanatory value of experimental findings (56). We contend that the use of the established CPXV-Wistar rat model provides a unique possibility to study pathogen determinants in a spillover animal model which closely mimics the situation in the field. However, this is connected to further challenges, like the variation of the noninbred animals themselves, but this, again, is likely a scenario that is much closer to that in the real world and reflects the field situation better than any inbred, and thus somewhat artificial, mouse model.

TABLE 5 Statistical evaluation of median parameters for the RatPox09-inoculated group in comparison to those for the single- and multiple-knock-in mutants

Parameter	Unit of measurement	*P* value for RatPox09 vs[a]:						
		vBR	BR_Quad	BR_NMDAr	BR_D7L	BR_7tGP	BR_CrmE	BR_ATI/p4c
T_{max}	d	0.1031	0.0368	0.3159	0.0154	<0.0001	>0.9999	0.1742
T_{start}	dpi	0.1258	0.4713	0.0205	0.0135	<0.0001	0.0306	0.0368
T_{end}	dpi	0.0749	0.0035	0.9975	0.9976	<0.0001	0.5913	0.221
Duration of shedding	dpi	>0.9999	0.0815	>0.9999	>0.9999	0.044	0.3426	>0.9999
V_{max}	log_{10} $TCID_{50}$/ml	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
V_{start}	log_{10} $TCID_{50}$/ml	<0.0001	<0.0001	0.0061	0.0575	<0.0001	0.0055	<0.0001
V_{end}	log_{10} $TCID_{50}$/ml	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
T_{max}	d	>0.9999	>0.9999	>0.9999	0.3947	0.3543	0.0012	0.5911
T_{start}	dpi	>0.9999	0.0434	>0.9999	>0.9999	0.0915	0.8233	>0.9999
T_{end}	dpi	0.7766	0.5537	0.0094	>0.9999	0.3333	0.0033	0.977
CS_{max}	CS	0.0257	>0.9999	>0.9999	>0.9999	>0.9999	0.35	>0.9999
CS_{start}	CS	0.0285	0.0096	0.2829	>0.9999	>0.9999	0.073	>0.9999
CS_{end}	CS	0.1339	0.5945	0.3806	0.9999	0.9996	0.1473	0.789
S_{max}	d	0.0348	>0.9999	0.163	>0.9999	>0.9999	0.0222	0.163
S_{end}	CS	0.1236	>0.9999	>0.9999	>0.9999	>0.9999	0.1144	0.6792

$P < 0.05$	$P < 0.01$	$P < 0.001$	$P < 0.0001$

[a]Each cell contains the *P* value. The *P* values are color coded in the key at the bottom.

In the study presented here, we show that recombineering of avirulent CPXV BR represents a workable platform to identify novel virulence genes of CPXV field isolates in the Wistar rat model. We employed two different approaches in our study. (i) *En passant* Red recombination was used to introduce six specific RatPox09 genes of interest into the BR genome. (ii) Furthermore, since whole-genome sequencing revealed small genetic mutations of unknown impact scattered all over the RatPox09 genome, we used an unbiased approach to generate BR-RatPox09 chimeric viruses in which combinations of multiple genome segments from RatPox09 replaced the authentic BR sequences. The introduced mutations were verified by whole-genome sequencing and further by complete transcriptomic analysis of the different recombinant viruses to ensure that the knock-in genes and neighboring genes were correctly transcribed. In each of the animal experiments, groups of rats infected with wild-type BR (wtBR), vBR, and RatPox09 served as controls and defined the virulence threshold ranging from low to high.

From our *in vivo* data, we can conclude that the gene encoding the protein 7tGP is responsible for a significant increase in the virulence of an avirulent CPXV laboratory strain. Reintroduction of the gene into the BR genome resulted in virulence levels that matched those of the highly virulent field isolate RatPox09. Moreover, the so-called quadruple mutant BR_Quad (BR_D7L/CrmE/NMDAr/7tGP), whose genome also encodes the 7tGP gene, caused significantly lower survival rates in infected rats than vBR or wtBR ($P \leq 0.05$, analysis of variance [ANOVA]). The protein encoded by the *g7tGP* gene has some similarity to poxviral proteins of the B21R/B22R family (41). Previous reports have suggested that the B21/B22R proteins of MPXV and CPXV render human T cells unresponsive to stimulation both by MHC-dependent antigen presentation and by MHC-independent stimulation (43, 55). However, the detailed functions of the B21/B22R protein MPXV197 and its CPXV homologue (CPXV219) have not yet been elucidated (43, 55). It is tempting to hypothesize that CPXV 7tGP might have an immunomodulatory function similar to that which is pivotal for the significantly increased virulence of BR_7tGP, and we are currently further investigating this hypothesis.

Nevertheless, the notion that a single gene in RatPox09 might be sufficient to transform a CPXV strain from a low-virulence to a high-virulence phenotype was unexpected. However, viral shedding, detected by the collection of buccal swab samples from animals infected with BR_7tGP, did not reach the levels seen in rats infected with RatPox09. The induction of clinical disease and lethal infection is therefore linked to CPXV 7tGP, while shedding in oral secretions is probably not. This leads to one speculative explanation: the mortality caused by 7tGP-positive orthopoxviruses could be related to less functional T cells in infected animals. In addition, the quadruple mutant BR_Quad also was not excreted as RatPox09 was, and thus, the insertion of *gD7L*, *gCrmE*, *gNMDAr*, and *g7tGP* increased the virulence significantly but not the excretion levels. The *in vivo*-detected viral replicative fitness of BR_7tGP and BR_Quad was clearly similar to that of RatPox09 (e.g., as measured by the organ viral load in the diseased rats).

Therefore, CPXV 7tGP is most likely a relevant virulence factor in our *in vivo* model. It is responsible for the observed highly virulent phenotype of CPXV in Wistar rats, consisting of (i) a prominent viral replication fitness in the animal, while (ii) excretion is impaired in this model. This is contrary to the findings for other animal viruses, where *in vivo* increases in virulence often lead to prominent viral shedding (57). The increased virulence and shedding are therefore related to the effects of multiple genes and, possibly, epistatic interactions between these genes.

The individual insertion of the two remaining unique RatPox09 genes, *gCrmE* and *gNMDAr*, resulted in only a nonstatistically significant increased virulence of BR. However, the rate of mortality in rats infected with BR_CrmE or BR_NMDAr was higher than that in rats infected with BR. The respective clinical scores were in the intermediate range, with an obvious and peculiar shift of the peak scores at late time points of the animal experiments. Homologues of both proteins in VACV have been the focus of

studies in the past: VACV CrmE is a soluble tumor necrosis factor alpha (TNF-α) receptor with both soluble and membrane viral tumor necrosis factor alpha receptor (vTNFR) activity and was shown to bind to rat, mouse, and human TNF-α and to protect against human, but not mouse or rat, TNF-mediated cytolysis *in vitro* (40, 58, 59). As expected, CPXV BR with RatPox09 CrmE inserted induced stronger symptoms than wtBR, a finding that is in full agreement with previous publications describing the effects of VACV infection. Deletion of *gCrmE* in the VACV strain USSR in an intranasal mouse infection model resulted in marked attenuation of the virus, whereas overexpression of the protein led to increased virulence (59). Nevertheless, all recorded symptoms were of a more general nature: severe clinical signs, such as pox skin lesions, were not detected in animals infected with wild-type or mutant viruses. Furthermore, the NMDAr homolog in VACV, viral GAAP (vGAAP; 98% similarity with RatPox09 NMDAr), and a human orthologue, human GAAP (hGAAP), were shown to protect cells from apoptosis derived from both intrinsic and extrinsic pathways (37).

The single insertion of the RatPox09 gene for D7L increased the virulence of BR to higher levels in one animal experiment, whereas BR_D7L did not cause mortality in the second experimental setup. As mentioned previously, we believe that the outbred status of animals could explain these differences. Depending on the function of the protein under investigation, the different genetic background could potentially influence the impact of certain virulence genes but not others. By the use of independently repeated experiments, this drawback was minimized. Generally, the use of outbred animals is admissible in the context of dominant factors to be evaluated. Nevertheless, we are currently exploring the function of D7L in CPXV virulence. The protein contains a predicted, N-terminally located BTB (broad-complex, tramtrack, and bric-à-brac)/POZ (for poxvirus and zinc finger) domain between amino acids 31 and 131 (41). BTB domain-encoding genes are found among eukaryotic and poxvirus genomes (60), and the domain itself functions as a docking station for protein-protein interactions (61). Unlike many poxviral BTB proteins, the BTB-only protein D7L misses a C-terminally located kelch domain, a protein interaction domain that mediates the proteasomal degradation of cellular substrates by ubiquitin ligase complexes. Mammalian BTB proteins were found to have diverse functions, from playing a part in embryogenesis to fulfilling important roles in the development of specialized lymphocyte effector cells. In many cases, they function as transcriptional suppressors (62–67).

Interestingly, none of the tested BR-RatPox09 chimeric viruses exhibited any significant increase in virulence above the BR baseline virulence, as measured by mortality (Fig. 4). We swapped the sequences of the genes for ATI and p4c in BR for the RatPox09 ATI and p4c gene sequences. However, BR_ATI/p4c failed to reach virulence levels comparable to those of RatPox09 (Fig. 3B and 4B).

In conclusion, proteins with putative immunomodulatory functions are potent virulence genes in many different viruses, including CPXV. Here, we identified CPXV *7tGP* especially to be a novel relevant factor modifying virulence in the well-established Wistar rat model. Interestingly, virulence was not linked to the levels of virus excretion. Our study also underlines the value of BAC mutagenesis when comparing highly adapted standard lab strains like BR—in the case of CPXV—to rodent-borne isolates. The targeted engineering of mutant viruses is easy and fast and, most importantly, has a low error rate, as confirmed by whole-genome sequencing. The seamless introduction of almost any desired modification, e.g., whole genes or even large fragments, allows the straightforward screening and identification of relevant virulence markers and fitness-related genes by *in vivo* animal model studies. Future studies will surely benefit from this versatile platform.

MATERIALS AND METHODS

Cell lines and viruses. African green monkey cells (Vero 76 cells; Collection of Cell Lines in Veterinary Medicine, Friedrich-Loeffler-Institut, Greifswald-Insel Riems, Germany) were grown and maintained in Eagle's minimal essential medium (MEM; Biochrom, Berlin, Germany) supplemented with 10% fetal calf serum (FCS; Biochrom) and kept at 37°C under a 5% CO_2 atmosphere. Chicken embryo cells (CECs) were prepared fresh from 10-day-old specific-pathogen-free (SPF) White Leghorn chicken embryos (Valo;

BioMedia GmbH, Osterholz-Scharmbeck, Germany) by a standard protocol (68). Cells were cultured in MEM containing 10% FCS and 100 U/ml penicillin (Fisher Scientific, Schwerte, Germany), 100 U/ml streptomycin (AppliChem GmbH, Darmstadt, Germany) and kept at 37°C under a 5% CO_2 atmosphere. CPXV strain Brighton Red (strain AF482758, kindly provided by Philippa Beard, University of Edinburgh, Edinburgh, UK), was cloned as a bacterial artificial chromosome (BAC) and termed pBRF (69). The CPXV rat isolate (RatPox09) was obtained in 2009 from a diseased pet rat in southern Germany. All CPXVs used in this study were propagated and titrated on Vero 76 cells or CECs. Fowlpox virus (FWPV; Nobilis-PD strain, kindly provided by D. Lüschow, Freie Universität Berlin, Berlin, Germany) was grown on CECs and used as the helper virus for the reconstitution of CPXVs. Virus stocks were prepared as described by Xu et al. (70).

Plasmid, mutant, and chimeric virus construction. The sequences of RatPox09 genes *gCPXV0002* (*gNMDAr*), *gCPXV0003* (*gCrmE*), *gCPXV0284* (*gD7L*), and *gCPXV0030* (*g7tGP*) and the combination of *CPXV158* (*gATI*) and *CPXV159* (*gp4c*), including the predicted promoter sequences, were amplified from CPXV RatPox09 viral DNA using PrimeSTAR GXL polymerase (TaKaRa, Clontech Laboratories, Inc., USA). The *g7tGP* sequence was cloned into the SacI and PstI unique restriction sites of the pUC19 plasmid (New England Biolabs, Frankfurt am Main, Germany). A kanamycin resistance cassette and an adjoining I-SceI site were amplified by PCR and inserted into unique restriction sites of the corresponding plasmid. The *gD7L*, *gCrmE*, and *gNMDAr* sequences were cloned into the BamHI and KpnI sites of the pEP-kanS2 high-copy-number plasmid containing an *aphAI*-I-SceI cassette (71, 72). In the case of *gATI-gp4c*, an SbfI restriction site upstream and a HindIII site downstream of the gene were used. Duplicate sequences for removal of the *aphAI* cassette were added to the transfer construct through 5-prime extensions of the primers. The transfer constructs were then used to insert the sequences of interest into the desired target sequences present in pBRF using a two-step Red recombination as described earlier (71, 72).

Red recombination was also used for the generation of chimeric viruses. For deletion of the segments of interest from pBRF and recovery of the segments of interest from RatPox09, PCR primers were designed to amplify the *aphAI* cassette from recombinant plasmid pEP-kanS2. Besides the marker cassette, the PCR fragments contained at each end 40 bp of sequences that were homologous to the target locus in the CPXV sequence. LongAmp *Taq* DNA polymerase (New England Biolabs) was used for this purpose. Plasmid and BAC DNAs were extracted by alkaline lysis and verified by restriction fragment length polymorphism (RFLP) analysis. PCR products overlapping the modified loci were confirmed by agarose gel electrophoresis, purified using a GF-1 AmbiClean nucleic acid extraction kit (Vivantis Technologies, Subang Jaya, Malaysia), and sequenced to verify the correct modification of the genome.

Reconstitution of infectious virus from BAC DNA. For virus reconstitution, Vero cells seeded in 6-well plates (1×10^6 cells per well) were transfected with 2 μg of purified plasmid or BAC DNA and 4 μl the FuGENE HD transfection reagent (Promega, Mannheim, Germany) according to the manufacturer's instructions. Chimeric viruses were generated by cotransfection of 1 μg of pBRF deletion mutant DNA diluted in 2 μl TE (Tris-EDTA) buffer together with 1 μg of RatPox09 segment-of-interest DNA diluted in 2 μl TE buffer. Transfected cultures were infected with 500 PFU of FWPV at 2 h after transfection. Since the BAC DNA used for virus reconstitution encodes the green fluorescent protein (GFP) under a late 4b FWPV promoter (69), virus reconstitution was monitored by examining GFP expression using an Axiovert S100 fluorescence microscope (Carl Zeiss, Jena, Germany). Newly reconstituted virus was than passaged not less than four times, in order to remove the helper virus. Later, mini-F vector sequences present in pBRF were removed and the thymidine kinase (TK) gene was repaired by transfection with a TK-containing plasmid.

High-throughput sequencing of full-length CPXV genomes. Full-genome sequencing of CPXV isolates was conducted as previously described (31). In brief, DNA was extracted from infected cell cultures using a High Pure PCR template preparation kit (Roche, Mannheim, Germany), and 0.5 to 1 μg of DNA was fragmented to approximately 300 bp using a Covaris M220 ultrasonicator (Covaris, Brighton, UK). Illumina-compatible sequencing libraries were prepared using NEXTflex DNA barcodes (Bioo Scientific, Austin, TX, USA) and SPRI works 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 instrument (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 sequencer using the MiSeq reagent kit, versions 2 and 3 (Illumina, San Diego, CA, USA).

***De novo* assembly and genome annotation of full-length CPXV genomes.** Raw reads were quality trimmed and assembled *de novo* using 454 sequencing system software (version 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 analogously to the nomenclature of the CPXV BR reference strain (strain AF482758) as described elsewhere (31).

Transcript expression analyses of CPXV knock-in mutants. Total RNA was extracted from Vero cells infected with CPXV BR or CPXV knock-in mutants using the TRIzol reagent (Life Technologies, Carlsbad, CA, USA) in combination with an Agencourt RNAdvance tissue kit (Beckman Coulter, Krefeld, Germany) on a KingFisher 96 Flex magnetic particle processor (Thermo Fisher Scientific Inc., Schwerte, Germany). During extraction, DNA was digested on-bead using an RNase-free DNase set (Qiagen, Hilden, Germany). Subsequently, the polyadenylated RNA fraction was extracted from a mixture of 5 μg total

TABLE 6 Design of animal experiments[a]

Expt	Group	No. of animals/group	Virus strain	Duration of experiment (dpi)	Tissues sampled at autopsy
1	Inoculated	8	Chimera BR_sB/C, chimera BR_sD/E/F, or BR_D7L	28[b]	Turbinates, trachea, lung, liver, spleen, skin, bladder
	Control	4	wtBR or RatPox09	28[b]	Turbinates, trachea, lung, liver, spleen, skin, bladder
2A	Inoculated	10	BR_CrmE or BR_NMDAr	21	Turbinates, trachea, lung, liver, spleen, skin
	Control	8	wtBR, vBR, or RatPox09	21	Turbinates, trachea, lung, liver, spleen, skin
2B	Inoculated	10	BR_D7L, BR_7tGP, BR_ATI/p4c, or BR_ Quad	21	Turbinates, trachea, lung, liver, spleen, skin
	Control	6	vBR or RatPox09	21	Turbinates, trachea, lung, liver, spleen, skin

[a]All animals were inoculated with the virus at a dose of $10^{5.5}$ 50% tissue culture infective doses ($TCID_{50}$) intranasally when they were 6 weeks of age, and buccal swab specimens were collected every 2 days. dpi, days postinoculation.
[b]Two animals from both the inoculated and the control groups were euthanized and autopsied at 5 dpi.

RNA and a supplemented internal control (ERCC ExFold RNA spike-in mix 1; Invitrogen, Carlsbad, CA, USA) using a Dynabeads mRNA Direct microkit (Invitrogen). The quantity and quality of the RNA were observed at each step using a NanoDrop 1000 spectrophotometer (Peqlab, Erlangen, Germany) and an Agilent 2100 bioanalyzer (Agilent Technologies, Böblingen, Germany). Strand-specific Ion Torrent-compatible libraries were constructed using an Ion Total RNA-Seq kit (version 2; Life Technologies) following the manufacturer's instructions. After quantification using a Kapa Ion Torrent library quantification kit (Kapa Biosystems, Wilmington, MA, USA), the libraries were sequenced on an Ion Torrent S5XL system (Life Technologies) using appropriate sequencing reagents.

For data analysis, raw reads were quality trimmed, and the remaining adapters were cut using 454 sequencing system software (version 3.0; Roche; Mannheim, Germany). Trimmed reads were then mapped against 233 coding DNA sequences (CDSs) from CPXV BR and the CDSs of RatPox09 genes *gCPXV0002* (*gNMDAr*), *gCPXV0003* (*gCrmE*), *gCPXV0284* (*gD7L*), and *gCPXV0030* (*g7tGP*) using the Bowtie2 program (version 2.3.5.1) (73). The matching reads were then quantified and transformed using Salmon software (version 0.14.1) (74) running in strand-specific alignment-based mode with 100 bootstrap replicates. The transformed read counts for each gene were then compared between the CPXV BR reference strain and the CPXV knock-in mutants using R (version 3.6.0) (75) and R studio (version 1.2.1335) software.

***In vitro* characterization: replication kinetics.** Vero cells from overnight cultures were infected with CPXV chimeras or CPXV knock-in mutants at a multiplicity of infection (MOI) of either 0.01 or 3. CPXV RatPox09, wtBR and BAC-derived BR (vBR) were used as references. After infection, cells were incubated for 60 min at 37°C. Afterwards, the cells were washed three times with phosphate-buffered saline (PBS), and then fresh culture medium was added. Samples were obtained at 6 different time points (0 h, 6 h, 12 h, 24 h, 48 h, and 72 h) as biological duplicates. Virus titers from the different time points were determined by an endpoint dilution assay with two technical replicates. The virus titers were calculated as the number of $TCID_{50}$ per milliliter using the Spearman-Kärber algorithm (76, 77).

***In vivo* characterization of chimeras and mutants.** The animal protocols were evaluated by the responsible ethics committee of the State Office for Agriculture, Food Safety and Fishery in Mecklenburg-Western Pomerania, Germany, and approval was obtained (approval number LALFF M-V 7221.3-1-020/13).

The animal numbers and the design of all experiments are summarized in Table 6. Initially, we inoculated 8 mixed-sex Wistar rats (outbred; Charles River, Sulzfeld, Germany) at 6 weeks of age with the double chimera BR_sB/C, the triple chimera BR_sD/E/F, or the BR_D7L knock-in mutant virus (experimental setup 1). The dose of the inoculum was $10^{5.5}$ $TCID_{50}$ per animal and was applied intranasally. CPXV RatPox09 and wtBR were used as controls. Over a period of 21 days, the animals were clinically evaluated on a daily basis and buccal swab specimens (Bakteriette; EM-TE Vertrieb, Hamburg, Germany) were taken every second day. At day 5 postinfection (p.i.), 2 rats per group were humanely killed. Individual organ tissue samples (turbinates, trachea, lung, liver, spleen, skin) were taken. Serum was sampled from the surviving animals at day 28 p.i., when the animals were humanely killed for autopsy and individual organ tissue samples were collected: rhinarium, trachea, lung, liver, spleen, kidney, skin, and bladder.

The experimental design of the animal studies performed here exactly followed that published previously for infection experiments with CPXV BR and RatPox09 in Wistar rats (31, 35, 49). This initial animal experiment had a duration of 28 days (experimental setup 1); since there was no change in the development of disease seen at 20 dpi, the following two animal experiments were conducted for only 21 days (experimental setup 2).

In the second experimental setup, we infected 10 Wistar rats (per virus) either with the single-gene-knock-in mutants (BR_D7L, BR_CrmE, BR_NMDAr, BR_7tGP) or with the multiple-gene-knock-in mutants (BR_ATI/p4c, BR_D7L/CrmE/NMDAr/7tGP). In addition to CPXV RatPox09, wtBR and BAC-derived BR were included as controls. Moreover, we developed a quantitative clinical score point system, which allowed for an objective evaluation of the clinical status of the individual infected animals (Table 1) and analysis of the kinetics of the symptomatic period (Tables 2 and 3). Animals that reached the experimental endpoint were euthanized. Buccal swab specimens were taken every other day, and the rats were

humanly euthanized at day 21 p.i. for autopsy and sampling (rhinarium, trachea, lung, liver, spleen, kidney, skin, serum). The clinical score point system was used only for experimental setup 2.

Determination of viral loads from buccal swab and organ tissue samples. Buccal swab samples were resuspended in 2 ml cell culture medium containing antibiotics (enrofloxacin, 1% [Bayer, Leverkusen, Germany]; amphotericin-gentamicin, 0.2% [Thermo Fisher Scientific Inc., Schwerte, Germany]; lincomycin, 0.5% [WDT, Garbsen, Germany]). The organ samples were transferred into 1 ml cell culture medium supplemented with 10% fetal calf serum (FCS) and antibiotics (1% penicillin-streptomycin; Biochrom GmbH, Berlin, Germany). The reaction tubes also contained stainless steel beads (diameter, 5 mm), which allowed mechanical homogenization (TissueLyser II; Qiagen, Hilden, Germany). Viral DNA was extracted from all buccal swab and organ tissue samples by using a BioSprint 96 instrument and a NucleoMag Vet kit (Macherey-Nagel, Berlin, Germany). OPV-specific DNA was detected by quantitative PCR (31). In addition, the viral loads of the samples were determined by an endpoint dilution assay and are given as the number $TCID_{50}$ per milliliter, calculated using the Spearman-Kärber algorithm (76, 77). The serum samples were analyzed by an indirect immunofluorescence test (31).

Bioinformatics and statistical analysis. Analysis of homologous poxvirus sequences, as well as the prediction of protein domains and functions, was performed using the NCBI BLAST, PFAM (Protein Families Database; http://pfam.xfam.org), and VectorNTI (version 9.1; Invitrogen, Darmstadt, Germany) software packages and was based on the OPV sequences available at the NIAID Virus Pathogen Database and Analysis Resource (ViPR) (78) through the website http://www.viprbrc.org/ and GenBank (79). GraphPad Prism software (GraphPad Software, La Jolla, CA, USA) was used for statistical evaluation. To describe the viral shedding kinetics in the Wistar rat model, we used median parameters in temporal units to determine the time of the start of the shedding period (T_{start}), the time of peak shedding (T_{max}), and the time of the end of virus shedding (T_{end}) (80). We also ascertained the initial virus titer shed (V_{start}), the peak viral titer shed (V_{max}), and the final virus titer shed (V_{end}) and characterized the viral shedding period as that time when viral titers of >1.625 $\log_{10}$ $TCID_{50}$ ml^{-1} were detected. For clinical scoring, we determined T_{start}, T_{max}, and T_{end} (in temporal units), as well as the clinical score (CS) units at the start of the shedding period (CS_{start}), at the time of peak shedding (CS_{max}), and at the end of virus shedding (CS_{end}). The data were truncated to keep virus-positive samples as well as CS-positive animals. Survival data were screened for the number of days that animals survived an infection (S_{max}) and the clinical scores when the animals left the experiments (S_{end}). We tested the association of individual parameters between the groups and animals infected with vBR or RatPox09.

One-way ANOVA with the Bonferroni correction ($P < 0.05$) was performed to determine whether the results were significantly different between the groups.

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DISKUSSION

V. DISKUSSION

5.1 ISOLIERUNG UND IN VITRO CHARAKTERISIERUNG EINES NEUEN CPXV-ISOLATES AUS DEM RÖTELMAUS-RESERVOIR

Kleinnager gelten als das natürliche Reservoir für CPXV in Europa. Bereits in früheren Publikationen wurde gezeigt, dass die Seroprävalenz in Rötelmäusen ebenso wie in Feld-, Erd- und Brandmäusen besonders hoch ist (20, 23, 24). Außerdem sind Rötelmäuse in den Gebieten, in denen CPXV Infektionen detektiert werden, lokal vertreten, während dies für die weiteren Arten nicht immer zutrifft (103). 2015 wurde das CPXV-Isolat FM2292 aus dem Reservoirwirt Feldmaus beschrieben und phänotypisch wie auch genotypisch charakterisiert (28). Obwohl bereits in den Jahren 2010-2014 ein groß angelegtes Reservoirscreening durchgeführt wurde, in dessen Rahmen auch die Lebern von 1594 Rötelmäusen auf OPV-DNA untersucht wurden, konnte damals kein Isolat aus Rötelmaus-originärem Material gewonnen werden (23), so dass ihre Bedeutung als Reservoirtier kritisch betrachtet werden musste. Insgesamt konnten jedoch drei Leberproben positiv auf OPV getestet werden, so dass sich eine Prävalenz von 0,19% ergab. Serologisch wurden 318 Rötelmäuse untersucht, davon waren zwei Tiere positiv (Prävalenz 0,63%) (23).

Als Bestandteil dieser Arbeit wurden von 2017-2018 erneut Kleinsäuger mittels spezifischer quantitativer PCR auf OPV-DNA untersucht (Publikation I). Dabei wurde das Beprobungsschema angepasst und die Nasenscheidewand (*Septum nasi*) getestet, da Versuche mit Feldmäusen gezeigt haben, dass in der Nasenschleimhaut im Tierexperiment deutlich häufiger Virus-DNA und infektiöses Virus nachgewiesen werden konnte als in der Leber (28). Insgesamt konnte von 509 individuellen Tieren die Nasenscheidewand untersucht werden. Bei fünf Proben wurde mittels qPCR OPV-DNA nachgewiesen. Aus der Einzelprobe einer Rötelmaus konnte initial ein CPXV-Isolat auf Vero76-Zellen angezogen werden, welches im Folgenden als CPXV GerMygEK 938/17 bezeichnet wird (Publikation I). Dieses neue CPXV-Isolat GerMygEK 938/17, das erste aus einer Rötelmaus, wurde anschließend *in vitro* und *in vivo* charakterisiert. Später war es auf Basis des neuen Beprobungsschemas (Nutzung von Nasenscheidenwänden) möglich, weitere Isolate zu generieren (Publikation in Vorbereitung).

Die phylogenetische Analyse des Gesamtgenoms zeigte, dass das CPXV-Isolat GerMygEK 938/17 eine Nukleotidsequenzidentität von 99,2% zu CPXV Ger2010MKY besitzt, einem Isolat,

welches zuvor aus einem Lisztaffen eines Tierparks gewonnen wurde (10). Somit ist CPXV GerMygEK 938/17 phylogenetisch in die Klade „CPXV-like 3“ einzuordnen (25), welche damit auch als eigenständige Klade innerhalb der polyphyletischen CPXV-Spezies bestätigen werden kann. Ebenso lässt dies die Vermutung zu, dass Rötelmäuse als „Eintragsquelle“ bei der Infektion in der Lisztaffen-Haltung beteiligt waren. Da CPXV eine hohe Stabilität in der Umwelt besitzt, muss ein indirekter Eintrag von CPXV Ger2010MKY ebenfalls in Betracht gezogen werden. 23 wildlebende Nagetiere, darunter Rötelmäuse, wurden nach der Infektion gefangen und mittels qPCR auf OPV-DNA untersucht. Kein Tier war OPV-DNA positiv. Da sich aber sowohl in dieser Studie (Publikation I), als auch in früheren Analysen (23) gezeigt hat, dass die Prävalenz von OPV-DNA in Rötelmäusen sehr gering ist, war die Anzahl an getesteten Nagetieren zu niedrig, um eine valide Aussage treffen zu können. Zudem wurde mit der Leber zunächst ein ungünstiges Probenmaterial zur Detektion gewählt (28).

Da die Klade „CPXV-like 3“ phylogenetisch nah zur Spezies *ECTV* clustert (Publikation I Abb. 2), wurde CPXV GerMygEK 938/17 vergleichend zu ECTV und RatPox09, einem Vertreter der Klade „VARV-like“, auf der CAM untersucht. Während ECTV weiße Pockenläsionen auf der CAM ausbildete, zeigten beide CPXV-like 3 Isolate typische hämorrhagische Pocken (Publikation I Abb. 3). Viren aus den Kladen CPXV-like 1 und CPXV-like 2 wurden ebenfalls auf der CAM untersucht. Alle bildeten hämorrhagische Pockenläsionen aus (Abb. 7; Daten nicht publiziert).

Die, bis auf eine Ausnahme, bisher lediglich und ausschließlich in Labormäuse-Kolonien nachgewiesene OPV-Spezies *ECTV* umfasst ein deutlich kürzeres Genom und eine reduzierte Anzahl von Genen verglichen zu CPXV GerMygEK 938/17. Dabei fehlen vor allem wirtsspezifische Gene aus den terminalen Genom-Regionen, die weniger konserviert sind, verglichen zum Virus-Core. Es scheint daher wahrscheinlich, dass ECTV aus einem CPXV-ähnlichen Vorgängervirus entstanden ist und sich an Labormäuse adaptiert hat. Dies würde auch erklären, warum ECTV trotz phylogenetischer Nähe zu CPXV GerMygEK 938/17 nicht in der Lage ist Rötelmäuse zu infizieren (20).

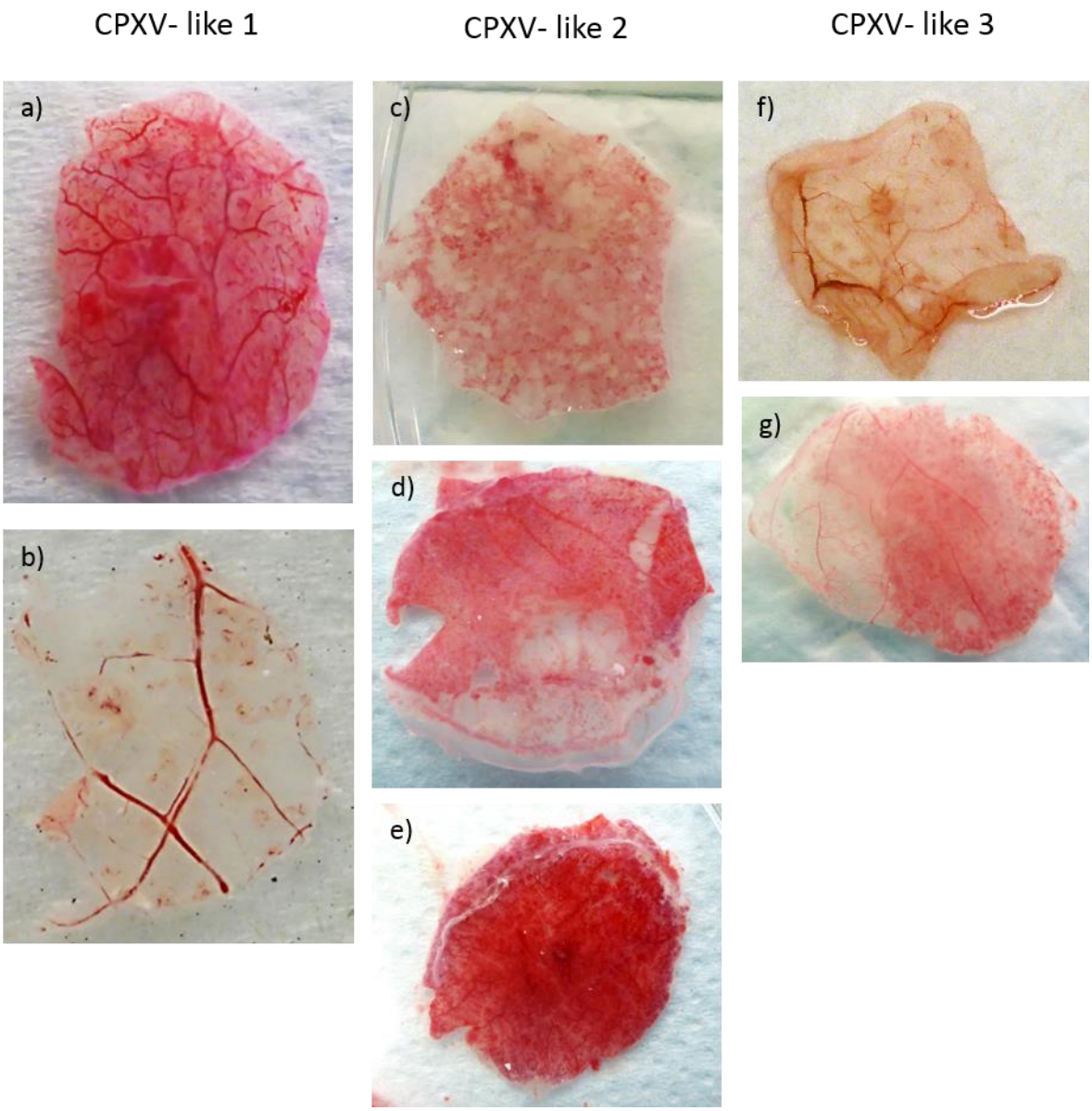

Abbildung 7 Pockenläsionen auf der Chorioallantoismembran (CAM) nach Infektion mit verschiedenen CPXV-Isolaten. Unabhängig davon, ob die verschiedenen CPXV-Isolate der Klade CPXV-like 1, CPXV-like 2 oder CPXV-like 3 angehören, bildeten sich hämorrhagische Pockenläsionen. Die Isolate CPXV Ger/2017/Vole (a) (27) und CPXV GerMiaUH 2091/18 (b) (Publikation in Vorbereitung) wurden beide aus Material von Feldmäusen gewonnen, ebenso, wie die CPXV-Isolate FM2292 (c) (28) und CPXV Ger/2007/Vole (d) (25) der Klade CPXV-like 2. In diese Klade ordnet sich auch Brighton Red (e) ein. CPXV Ger2010MKY (f) (10) und CPXV GerMygEK 934/17 (g) (Publikation in Vorbereitung) gehören der Klade CPXV-like 3 an.

Gerade auf dieser Grundlage wäre es interessant, Gene zu identifizieren, die für die Infektion von Rötelmäusen wichtig sind. Dazu bietet sich die in Publikation IV vorgestellte BAC-Technologie an, um entweder Deletionsmutanten von CPXV GerMygEK 938/17 zu bilden und dabei einzelne Gene, die im ECTV fehlen, zu entfernen oder aber Gene aus dem Isolat CPXV GerMygEK 938/17 artifiziell in das Genom von ECTV einzufügen.

Dank Rötelmaus- und Feldmaus-spezifischer Zelllinien (133) war es möglich, CPXV GerMygEK 938/17 vergleichend mit dem, aus der Feldmaus gewonnenen, CPXV-Isolat FM2292 und dem Laborstamm BR zu charakterisieren. Als Referenzzelllinie wurden Vero76-Zellen genutzt. Dabei zeigte sich, dass unabhängig von genutztem Isolat oder Zelllinie Virusreplikation stattgefunden hat. Alle Isolate zeigten auf der Referenzzelllinie Vero76 die höchsten Virus-Titer, während das neue Isolat CPXV GerMygEK 938/17 auf beiden Mäuse-Zelllinien etwa 10fache niedrigere Virustiter erreichte (Publikation I). Da es sich bei beiden Mäuse-Zelllinien um Zellen aus der Niere handelt und bereits in diversen Tierversuchen gezeigt wurde, dass sich Nierengewebe (wie auch schon für die Leber beschrieben) nicht gut für den OPV-DNA-Nachweis eignet (28), muss in Frage gestellt werden, ob diese Zelllinien für die Charakterisierung von CPXV-Isolaten geeignet sind. Von Vorteil wären hier Zelllinien aus dem oberen Respirationstrakt, in dem Replikation nachgewiesen werden kann (28). Spekulativ bleibt die Frage, ob mit einer solchen Zelllinie auch eine Aussage bezüglich der Virulenz neuer CPXV-Isolate möglich wäre.

Um CPXV GerMygEK 938/17 mit anderen CPXV-Isolaten bekannter Virulenz und dem genetisch eng verwandten CPXV Ger2010MKY außerdem vergleichend zu charakterisieren, wurde eine Wachstumskinetik auf Vero76-Zellen durchgeführt. Dabei wurden die Zellen mit zwei verschiedenen „*multiplicities of infection*“ (MOIs) inokuliert. Getestet wurden die CPXV-like 3 Isolate, RatPox09, ein virulenter CPXV-Stamm im Modelltier Wistar Ratte, und der Laborreferenzstamm BR (Publikation III Abb. 1). Unabhängig von der eingesetzten MOI, zeigten alle vier getesteten Isolate eine übereinstimmende Replikationskinetik, so dass keine Aussage bezüg lich der Virulenz des neuen Isolates CPXV GerMygEK 938/17 getroffen werden konnte. Dies deckt sich mit den Ergebnissen der Wachstumskinetiken aus Publikation IV sowie den Daten von Hoffmann et al. (28). Somit muss geschlussfolgert werden, dass Tierversuche nach wie vor den Goldstandard darstellen, um ein neues CPXV-Isolat bezüglich seiner Virulenz zu charakterisieren.

Mit der erstmaligen Isolierung eines CPXV aus Rötelmausgewebe ist zudem der direkte Nachweis erbracht, dass Rötelmäuse an der Virusökologie von CPXV beteiligt sind.

5.2 IN VIVO CHARAKTERISIERUNG VON CPXV-ISOLATEN IN RESERVOIRTIEREN

Wie bereits im Literaturteil (siehe Kapitel 3.1 S.21) ausgeführt, existiert hinsichtlich des Reservoirwirts keine einheitliche Definition. Während das OED ein Reservoir als **eine** Population beschreibt (126), werden bei Haydon et al. sowohl mehrere Populationen, unabhängig davon ob es sich dabei um dieselbe Spezies handelt, als auch die Umgebung mit einbezogen (127). Zusätzlich wird ein Reservoir auch als eine Spezies mit subklinischem Krankheitsverlauf beschrieben (134). Einheitlich gilt, dass das Reservoir den Erreger übertragen kann, unerheblich ob direkt oder indirekt (127).

Auf Grund dieser verschiedenen Definitionen war es bisher fraglich, ob die Rötelmaus (RM) als Reservoir für CPXV angesehen werden kann. Zwar weist die Seroprävalenz innerhalb verschiedener Rötelmaus Populationen darauf hin (20, 23), dass Infektionen stattfinden, doch auch in akzidentellen Wirten, wie der Katze, kann eine Serokonversion festgestellt werden (124). Bisher gelang es nicht infektiöses Virus aus der Rötelmaus zu isolieren. Zudem fanden nur sehr wenige CPXV-Versuche in Rötelmäusen statt (20).

Für die Feldmaus (FM) als Reservoirwirt ergab sich durch die Isolation von CPXV FM2292 die besondere Situation, dass erstmalig die Kombination aus Reservoirspezies und passendem Virus vorhanden war. Dieses FM-Reservoir-CPXV-Paar konnte vergleichend mit einem akzidentellen Wirt-CPXV-Paar, bestehend aus Wistar Ratte und RatPox09, untersucht werden (28).

Vergleichend hierzu und zu umfangreichen Untersuchungen mit nicht passenden Virusisolaten in Rötelmäusen (Publikation III), wurden nun experimentelle Inokulationen mit Rötelmäusen durchgeführt. Dazu konnte CPXV GerMygEK 938/17 als kongruentes RM-Reservoir-CPXV-Paar charakterisiert und vergleichende Studien in Rötel- und Feldmäusen und dem Modelltier Wistar Ratte durchgeführt werden (Publikation II). Dabei wurden sechs Rötelmäuse und sechs Feldmäuse mit einer Infektionsdosis von $10^{5,5}$ $TCID_{50}$ (Tissue Culture Infection Dose 50%) mit CPXV GerMygEK 938/17 oder CPXV Ger2010MKY intranasal inokuliert. Um die Virulenz des neuen Isolates vergleichend zu anderen CPXV-Isolaten einschätzen zu können,

wurden zudem zehn Wistar Ratten wie oben beschrieben mit CPXV GerMygEK 938/17 inokuliert. Ein Infektionsversuch mit CPXV Ger2010MKY in Wistar Ratten wurde innerhalb dieser Arbeit nicht durchgeführt, da dazu bereits Studien existieren und Wiederholungsversuche zu vermeiden sind (10).

Unabhängig von der Tierart führte die Inokulation mit CPXV GerMygEK 938/17 zu keinen klinischen Symptomen. Anders verhielt es sich mit Rötelmäusen, die mit CPXV Ger2010MKY infiziert wurden. Zwei Tiere zeigten einen deutlichen Gewichtsverlust von mehr als 25%, so dass sie 12 Tage nach Inokulation (*days post infection*; dpi) euthanasiert werden mussten. Ein Gewichtsverlust von mehr als 25% war als Abbruchkriterium festgelegt worden. Somit konnte für Rötelmäuse -inokuliert mit CPXV Ger2010MKY- eine Mortalität von 33% verzeichnet werden. Weitere klinische Symptome konnten nicht festgestellt werden.

In allen Rötelmäusen und in vier von zehn Wistar Ratten, inokuliert mit CPXV GerMygEK 938/17, konnte im Zeitraum von 2-14 dpi Virus-DNA in buccalen Tupfern detektiert werden. Allerdings war der Nachweis von infektiösem Virus mittels Virustitration nur in den Proben dreier Rötelmäuse möglich. Da die von uns durchgeführte Virustitration ein Detektionslimit von $10^{1,625}$ $TCID_{50}ml^{-1}$ aufweist (135, 136), kann infektiöses Virus in geringeren Lasten nicht ausgeschlossen werden. Im Zusammenhang mit der sehr sensitiven qPCR (118), den daraus resultierenden hohen cq-Werten (quantification Cycle) und der somit geringen Konzentration an OPV-DNA in den Tupferproben, kann dies erklären, warum nicht für alle Tupfer der Rötelmäuse und für keinen Rattentupfer infektiöses Virus nachgewiesen werden konnte.

Bei vier mit CPXV Ger2010MKY inokulierten Rötelmäusen wurde die Ausscheidung von infektiösem Virus nachgewiesen. Interessanterweise konnte für die beiden Rötelmäuse, die aus dem Versuch genommen wurden, in den Tupfern Virus-DNA, jedoch kein infektiöses Virus, ermittelt werden. Dies kann allerdings auch auf das geringere Detektionslimit der Virustitration zurückzuführen sein. Die Tupferproben aller mit CPXV GerMygEK 938/17 und CPXV Ger2010MKY infizierten Feldmäuse waren Virus-DNA negativ.

CPXV-spezifische Antikörper konnten hingegen mittels Immunfluoreszenztest (IFT) bei allen Rötelmäusen und Wistar Ratten inokuliert mit CPXV GerMygEK 938/17 festgestellt werden. Dabei wurden Titer von 1:640 (Rötelmäuse) bis zu 1:1280 (Wistar Ratten) detektiert. CPXV Ger2010MKY infizierte Rötelmäuse waren ebenfalls Antikörper positiv.

Im Kontrast dazu, waren jedoch alle Serumproben der Feldmäuse, unabhängig vom CPXV-Isolat, Antikörper negativ.

Leider konnte nicht von allen Rötelmäusen eine Serumprobe gewonnen werden, so dass alternativ und vergleichend eine Peritoneallavage individueller Rötelmäuse durchgeführt wurde und die Proben dann mittels IFT auf Antikörper untersucht wurden. Dabei zeigte sich, dass die Sensitivität des Antikörpernachweises in der Lavage deutlich schlechter war, als in Serumproben. Von insgesamt elf Peritoneallavage-Proben zeigten sich nur sechs reaktiv bei einer Verdünnung von 1:20 und zwei wurden bis zu einer Verdünnung von 1:40 (beides Rötelmäuse inokuliert mit CPXV Ger2010MKY) als positiv gewertet. Daraus lässt sich ableiten, dass möglichst Serumproben zur Ermittlung von CPXV-Antikörperreaktionen genutzt werden sollten. Peritoneallavage-Proben können zwar alternativ, wenn kein Serum zur Verfügung steht, getestet werden. Es ist jedoch unbedingt die stark herabgesetzte Sensitivität des Antikörpernachweises in dieser Probenmatrix zu beachten.

Bei dem hier beschriebenen Tierversuch war es erstmalig möglich, die Ausscheidung von CPXV im Rötelmausmodell nachzuweisen. Zudem zeigten zwei mit CPXV Ger2010MKY infizierte Tiere Gewichtsverluste von mehr als 25%. Diesen deutlichen Unterschied in der klinischen Ausprägung zwischen den beiden genetisch zu 99,2% identischen CPXV-Isolaten gilt es in weiterführenden Studien näher zu betrachten. In Genen wie *gvCCL*, *gCrmB*, *gCPXV0022* und *gCPXV194* konnte eine genetische Variabilität gezeigt werden (Publikation II Abb. 2), auf die die Unterschiede zurückzuführen sein könnten. Zudem muss bei der Interpretation von klinischen Symptomen bei Rötel-, ebenso wie bei Feldmäusen, bedacht werden, dass es sich um Wildtiere handelt, die in ihrem natürlichen Habitat darauf angewiesen sind, Zeichen von Schwäche oder Krankheit zu maskieren.

Im Vergleich dazu zeigten mit CPXV GerMygEK 938/17 oder CPXV Ger2010MKY inokulierte Feldmäuse weder Virus-Ausscheidung noch Serokonversion. Dies steht in deutlichem Kontrast zu Infektionsversuchen mit Isolaten aus der Feldmaus (CPXV FM2292) und der Ratte (RatPox09) (28). Dieser Punkt muss dringend weiterführend betrachtet werden. Eine mögliche Erklärung für die Unterschiede der beiden CPXV-Isolate aus den Kladen CPXV-like 2 und CPXV-like 3 ist das Fehlen des Gens *gCPXV0030* in Klade CPXV-like 3. In Publikation IV konnte für dieses Gen bereits gezeigt werden, dass es einen Einfluss auf die Virulenz in Wistar Ratten hat.

In einer vorangegangenen Studie (Publikation III), in welcher Rötelmäuse experimentell mit verschiedenen Virus-Isolaten (aus der Feldmaus und aus akzidentellen Wirten) der Kladen CPXV-like 1 und CPXV-like 2 auf verschiedenen Applikationswegen inokuliert wurden, konnten zwar zum Teil Serokonversionen nachgewiesen werden, aber nie Virus-Ausscheidung durch die Rötelmäuse. Die Versuchsreihe untersuchte Parameter, die einen Einfluss auf die Virus-Wirt-Interaktion haben könnten, unter anderem: die genetische Linie, das Alter der Tiere und das Geschlecht (Publikation III Tabelle 2). Es zeigte sich, unabhängig von den oben beschriebenen Parametern, immer ein subklinischer Krankheitsverlauf ohne Virus-Ausscheidung. In Abhängigkeit von der Applikationsroute ließ sich ein geringer Unterschied bezüglich der Serokonversionsrate detektieren. So zeigten deutlich mehr Rötelmäuse, die intranasal infiziert worden sind eine Serokonversion verglichen zu Tieren, die mittels „Footpad"-Methode oder subkutan infiziert wurden.

In zwei Kontaktieren, die 24h nach der experimentellen CPXV FM2292 Inokulation zugesetzt worden sind, konnten CPXV-spezifische Antikörper nachgewiesen werden. Da die Rötelmäuse alle aus der hauseigenen Zucht am Friedrich-Loeffler-Institut stammten und diese regelmäßig auf CPXV Antikörper überprüft wird, ist nicht davon auszugehen, dass die beiden Tiere bereits vor Versuchsbeginn mit CPXV in Kontakt gekommen sein können. Es kann also von einer Virus-Ausscheidung durch inokulierte Tiere ausgegangen werden. Die negativ getesteten Nasentupfer könnten ein Indiz dafür sein, dass die Ausscheidung nicht zwangsläufig über Nasensekret erfolgen muss, obwohl dies für Feldmäuse (28) und auch für die RM-Reservoir-Virus-Paare (Publikation II) gezeigt wurde. Innerhalb dieser Studie wurden allerdings weder Kot noch Urin untersucht. Peres et al. konnte zeigen, dass bei natürlichen Infektionen mit VACV bei wilden Nagern Kot und Urin eine Rolle spielen (137). Dabei wurde allerdings VACV-DNA nachgewiesen und kein infektiöses Virus. Auf Grund der hohen Sensitivität der genutzten nested PCR, konnte eine geringe DNA-Konzentration nachgewiesen werden, welche als niedrige Viruslast in den Proben interpretiert werden kann. So ist fraglich, ob eine Ansteckung über Kot oder Urin erfolgen kann (137).

Alternativ zu den Ausscheidungen könnte das Virus theoretisch auch über kontaminiertes Heu oder Einstreu in den Käfig gelangt sein. Eine weitere Eintragsquelle, die sich nicht ausschließen lässt, wäre die Infektion mittels „Footpad"-Methode selbst. Dabei könnte infektiöses Virus als „Kontaminante" an das Tier gelangt sein. Da CPXV-Viruspartikel eine hohe Stabilität in der

Umwelt aufweisen, könnte sich ein Kontaktier mit CPXV infiziert haben, das durch das infizierte Tier in den Käfig eingeschleppt wurde. Um diese Eintragsquelle auszuschließen, sollte der Käfig vor Einsetzen der Kontaktiere gereinigt werden.

Die beiden im Rahmen dieser Arbeit durchgeführten Tierversuche stützen erstmal experimentell die Hypothese, dass es innerhalb der CPXV-Klassifikation Reservoir-Spezies-spezifische phylogenetische Kladen gibt. Die CPXV-Isolate aus den unterschiedlichen Kladen zeigten deutliche Unterschiede in ihrer Virulenz im Reservoirwirt und dem Wistar Ratten-Modell (Publikation II Tabelle 4). Innerhalb der Taxonomie des Genus *Orthopoxvirus* könnte diese Beobachtung einfach integriert werden, indem die Nomenklatur angepasst wird. Sollte sich die linnäische binominale Nomenklatur auch für Viren (derzeit diskutiert durch die ICTV) durchsetzen, wäre ein mögliches Beispiel die CPXV-like 3 Klade als *Orthopoxvirus myodetis* zu benennen. Um den Infektionsweg innerhalb einer Rötelmaus-Population und somit auch ihr Transmissionspotenzial für akzidentelle Wirte genauer zu charakterisieren, sollten Infektionsversuche mit Kontakttieren derselben Spezies und zum Beispiel der Ratte als Modell für einen akzidentellen Wirt durchgeführt werden. Ebenso muss den phylogenetischen Unterschieden der beiden Rötelmaus-Kladen weiter auf den Grund gegangen werden. Zusätzliche Untersuchungen von Kleinsäugern sowie die phylogenetische Analyse von weiteren CPXV-Isolaten könnten helfen, die Einordnung der Genome detaillierter zu erstellen und zu bewerten.

Im Rahmen dieser Arbeit war es zudem möglich, noch weitere CPXV-Isolate aus der Rötelmaus zu isolieren (Daten noch nicht publiziert). Die dafür genutzten Rötelmaus-Proben stammen aus dem Projekt „Verbesserung der Öffentlichen Gesundheit durch ein besseres Verständnis der Epidemiologie nagetierübertragener Krankheiten“. Dabei wurden vorrangig Rötelmäuse im Westen Deutschlands gefangen und die Nasenscheidewände auf OPV-DNA untersucht. Aus bisher ca. 1000 untersuchten Proben, konnten zehn neue Rötelmaus-Isolate gewonnen werden, die nun phylogenetisch klassifiziert werden. Diese Isolate bestätigten jetzt nicht nur, dass es sich bei der Rötelmaus um ein wichtiges CPXV-Reservoirtier in Deutschland handelt, sondern können auch helfen, die Klade CPXV-like 3 weiter zu charakterisieren. Allerdings ist es jetzt wichtig, auch andere Reservoirtiere, wie die Feldmaus, zu beproben und deren Population in Deutschland weiter auf OPV zu testen.

5.3 VIRULENZFAKTOREN VON CPXV IM RATTEN-MODELL

2009 kam es in Deutschland und Frankreich zu mehreren humanen Infektionen mit CPXV (5, 26, 115). Überträger waren dabei -als Haustiere gehaltene- Ratten. Bei allen Fällen konnte der identische CPXV Stamm nachgewiesen werden (5, 26, 115). Das CPXV-Isolat RatPox09, welches im Rahmen der Infektionsfälle isoliert wurde, und der Laborreferenzstamm BR zeigten phylogenetisch eine Nukleotid-Sequenzidentität von 92%. Dabei konnten vier abgegrenzte ORFs identifiziert werden, die bei RatPox09 vorhanden sind und somit verantwortlich sein könnten für die erhöhte Virulenz in Wistar Ratten verglichen zu BR, bei dem diese ORFs fehlen (29, 30).

Die vier ORFs im Einzelnen: *gCPXV0002* zeigt eine 99% Nukleotid-Sequenz-Übereinstimmung zu dem CPXV-Gen, welches für das N-methyl-D-aspartate receptor-like (NMDAr) Protein mit unbekannter Funktion kodiert (105). Da die Aminosäure-Sequenz-Identität zu, aus VACV stammenden, Golgi apparatus antiapoptotic Proteinen (GAAPs) 98% beträgt, ist zu vermuten, dass auch NMDAr mit der Inhibition der Zell-Apoptose in Zusammenhang steht (138). *gCPXV0003* zeigt eine 99% Nukleotid-Sequenz-Übereinstimmung zu dem cytokine response modifier E (CrmE) kodierenden Gen, einem bekannten Virulenzfaktor in VACV (139). Dieser gehört zur Tumor-Nekrose-Faktor (TNF) Rezeptor Superfamilie (140). *gCPXV0284* weist eine 92%ige Übereinstimmung mit dem, für das D7L Protein, kodierenden Gen auf. Die Funktion von D7L ist bisher unbekannt (141). *gCPXV0030* zeigt eine 99% Nukleotid-Sequenzidentität zu dem CPXV Gen, welches für das 7-transmembrane G protein-coupled receptor-like Protein (7tGP) kodiert (105). Zudem konnten noch zwei ORFs, *gCPXV158* (*gATI*) und *gCPXV159* (*gp4c*), mit deutlichen Unterschieden zwischen RatPox09 und BR identifiziert werden (28). Beide ORFs sind für die Eingliederung von IMVs in ATIs wichtig (102).

Um zu überprüfen, ob es sich bei den genannten Genen um Virulenzfaktoren handelt, wurden mittels „*bacterial artificial chromosome*“ (BAC) Technologie RatPox09-Gene in das BR Genom eingefügt und so potentielle „*gain of function*“ Mutanten gebildet. Anschließend wurden die gebildeten BR-Mutanten sowohl *in vitro* mittels Wachstumskinetik wie auch im Tierversuch mit Wistar Ratten näher charakterisiert (Publikation IV).

Dabei zeigte sich, wie schon in den zuvor beschriebenen Publikationen, dass die Ergebnisse der Tierversuche nicht durch *in vitro* Assays reproduziert werden konnten, die alleine auf der Beurteilung des CPE in Zellkultur beruhten. Es lässt sich daraus ableiten, dass der Einfluss von

Virulenzfaktoren auf die Replikationsfähigkeit von CPXV nur im komplexen und divers multizellulären Organismus nachvollziehbar ist. Wirtseigene Abwehrmechanismen gegen eine CPXV-Infektion und virale Strategien, diese zu umgehen oder zu bekämpfen, müssen in einem für die Infektion relevanten Tiermodell untersucht werden, um eine Inkompatibilität zwischen Virus und Wirt zu vermeiden. Mit dem CPXV-Tiermodell Wistar Ratte als „Spill-over"-Wirt stand hier ein entsprechendes Virus-Wirt-Paar zur Verfügung. Die Bedeutsamkeit entsprechender Paarungen konnte bereits in den Publikationen II und III gezeigt werden. Gleichzeitig stellt die genetische Variation der Versuchstiere die Reproduzierbarkeit von Versuchsergebnissen vor Probleme, im Vergleich zu Tiermodellen mit Inzuchtlinien. Ein Effekt, der ebenfalls in diesen Tierversuchen zu sehen war. Trotzdem ist eine solche Virus-Wirt-Paarung vorzuziehen, da die Feldsituation genauer dargestellt wird und die Ergebnisse die originäre Situation (hier CPXV Infektionen bei als Haustier gehaltenen Ratten) sehr gut abzubilden vermögen.

Im Tierversuch zeigte sich, dass das Protein 7tGP für einen signifikanten Anstieg der Virulenz des in Ratten avirulenten CPXV-Stammes BR verantwortlich war (Publikation IV Abb. 3 und 4). Die BR_7tGP Mutante zeigte sich dabei ähnliche virulent wie das Feldisolat RatPox09. Eine ebenfalls mittels BAC-System gebildete 4-fach-Mutante BR_Quad (BR_D7L/ CrmE/ NMDAr/ 7tGP), mit allen vier vermuteten Virulenzfaktoren, einschließlich 7tGP, zeigte eine signifikant höhere Mortalität verglichen zu BR. Strukturelle Ähnlichkeiten, die auf Basis von Aminosäuresequenzen ermittelt wurden, verweisen auf die Pockenvirus-Proteine der B21R/B22R Familie. Ob 7tGP daher eine immunmodulierende Funktion, vergleichend zu B21R/B22R aufweist, muss weiter untersucht werden. Eine Funktion des B22-Proteins von MPXV und CPXV besteht darin, humane T-Zellen unempfänglich zu machen gegenüber einer Stimulation durch MHC-abhängiger und MHC-unabhängiger Antigenpräsentation (142, 143).

Interessanterweise erreichte die Virusausscheidung, ermittelt über Maultupfer und anschließender Virustitration, bei der Einzelgenmutante BR_7tGP und BR_Quad nicht das Level, welches in Ratten detektiert wurde, die mit RatPox09 infiziert worden sind (Publikation IV Abb. 5). Der Anstieg von Virulenz und Virusausscheidung war somit ein Effekt verschiedener Gene und möglicherweise deren Interaktion. Dies widerspricht interessanterweise den *in vivo* Ergebnissen anderer Viren, wie zum Beispiel dem infektiöse hämatopoetischen Nekrose Virus

der lachsartigen Fische oder dem Simiane Immundefizienz-Virus der Affenartigen, bei denen ein Anstieg der Virulenz meist zu verstärkter Virusausscheidung geführt hat (144, 145).

In diesem Teil der Arbeit konnte somit gezeigt werden, dass es sich bei CPXV 7tGP um einen relevanten Virulenzfaktor im Tiermodell Ratte handelt. Der ORF *gCPXV0030* konnte nicht nur in CPXV-Stämmen der Kladen VARV-like, wie in dem hier beschriebenen CPXV-Isolat RatPox09, gefunden werden, sondern auch in Isolaten der Klade CPXV-like 2, zu welcher zwei, aus dem Reservoirwirt Feldmaus gewonnene, CPXV-Isolate zählen: FM2292 und Ger/2007/Vole. Dies stützt die Vermutung, dass dieses Gen auch Einfluss auf die Virulenz im Reservoirwirt hat. Die detaillierte Funktionsweise der Proteine wird Gegenstand zukünftiger Studien sein und sich auch explizit der besonderen „Entkopplung“ von Virusausscheidung und Virulenz widmen.

ZUSAMMENFASSUNG

VI. ZUSAMMENFASSUNG

Das zoonotische Kuhpockenvirus (CPXV), ein Mitglied des Genus *Orthopoxvirus* innerhalb der Familie *Poxviridae*, kommt endemisch in West-Eurasien vor. Phylogenetische Studien zeigen, dass es sich um eine polyphyletische Spezies handelt, die in mehrere Kladen unterteilt werden kann. Während sich Isolate aus dem Reservoirwirt Feldmaus (*Microtus arvalis*) vor allem der Klade CPXV-like 2 zuordnen lassen, finden sich in der Klade CPXV-like 1 vornehmlich Isolate aus akzidentellen Wirten. RatPox09, isoliert aus dem akzidentellen Wirt Ratte (*Rattus norvegicus forma domestica*), kann der Klade VARV-like zugeordnet werden. Dieser für Ratten hoch-pathogene CPXV-Stamm ist für mehrere „Spill-over"-Infektionen von Heimtierratten auf den Menschen verantwortlich. Auf Grund serologischer Studien wird die Beteiligung der Rötelmaus (*Myodes glaerolus*) als CPXV-Reservoirwirt an der Virusökologie angenommen. Bisher war es jedoch nicht möglich, ein entsprechendes Isolat aus der Rötelmaus zu gewinnen.

Im Rahmen dieser Arbeit konnte das erste CPXV-Isolat aus einer Rötelmaus isoliert und so ihre Rolle als Reservoirwirt für CPXV eingehender untersucht werden. Neben der *in vitro* Charakterisierung des neuen CPXV-Isolates „GerMygEK 938/17" auf verschiedenen Zelllinien und der Chorioallantoismembran, wurden Infektionsstudien in Rötelmaus, Feldmaus und Wistar Ratte durchgeführt. Dabei konnte erstmalig Virus-Ausscheidung bei der Rötelmaus nachgewiesen werden, während bei der Feldmaus weder Virusausscheidung noch CPXV-spezifische Antikörper für diesen Rötelmaus-Stamm detektiert werden konnten.

Mittels phylogenetischer Studien wurde CPXV GerMygEK 938/17 in die Klade CPXV-like 3 mit dem bisher einzigen Vertreter CPXV Ger2010MKY, isoliert aus einem Lisztaffen (*Saguinus oedipus*), eingeordnet und diese Klade innerhalb der polyphyletischen CPXV-Spezies bestätigt. Versuche mit CPXV Ger2010MKY in den beiden wahrscheinlichen Reservoirwirts-Spezies Feld- und Rötelmaus erbrachten ähnliche Resultate wie das Rötelmaus-CPXV-Isolat. Zusätzlich mussten in diesem Versuch zwei Rötelmäuse aufgrund deutlicher Gewichtsreduktion euthanasiert werden. Im Gegensatz hierzu war eine deutliche klinische Ausprägung bei Infektionen mit dem CPXV-Isolat GerMygEK 938/17 nicht beobachtet worden. Ergänzend wurden weitere CPXV-Isolate aus den Kladen CPXV-like 1 und 2 in der Rötelmaus charakterisiert. Unabhängig vom eingesetzten CPXV-Stamm konnte ein subklinischer Verlauf mit Serkonversion gezeigt werden, aber keine klinischen Zeichen und keine Virus-

Ausscheidung. Die phylogenetische Abgrenzung der Klade CPXV-like 3 ist daher auch in Bezug auf die Empfänglichkeit von Rötelmäusen und Feldmäusen klar abbildbar.

In weitergehenden Analysen wurde untersucht, warum RatPox09-Infektionen in Ratten deutlich virulenter sind als Infektionen mit dem CPXV-Laborstamm Brighton Red (BR), der nur zu einem milden symptomatischen Verlauf führt. Vier Gene konnten dabei identifiziert werden, die im Wildtyp-Stamm BR fehlen. Darunter befindet sich auch das Gen *gCPXV0030*, welches zu 99% mit dem von einigen CPXV-Stämmen kodierten Protein 7-transmembrane G protein-coupled receptor-like Protein (7tGP) identisch ist. Mittels BR knock-in Mutanten konnte die Bedeutung dieser ausgewählten Gene in Tierversuchen mit Ratten als Modell-Tier charakterisiert werden. Dabei zeigten Ratten, die mit der BR_7tGP-Mutante inokuliert wurden, eine signifikant schwerwiegendere symptomatische Ausprägung und geringere Überlebensraten verglichen zu BR-Infektionen.

Diese Arbeit belegt, dass es innerhalb der CPXV Klassifikation Reservoir-Spezies-spezifische phylogenetische Kladen gibt. Die Funktion von Rötelmäusen als Reservoirwirt konnten auf Grund der gemessenen Virusausscheidung bestätigt werden. Voraussetzung dafür ist eine Infektion mit einem CPXV.Stamm aus der Rötelmaus-spezifischen CPXV-like 3 Klade. Zudem konnte gezeigt werden, dass das Gen *gCPXV0030* einen maßgebenden Einfluss auf die Virulenz von CPXV im Wistar-Ratten-Modell aufweist. Die Abwesenheit dieses Gens in CPXV-Isolaten der Klade CPXV-like 3 ist zudem ein deutlicher phylogenetischer Unterschied zu Isolaten der Klade CPXV-like 2.

SUMMARY

VII. SUMMARY

Zoonotic cowpox virus (CPXV) belongs to the genus *Orthopoxvirus* within the *Poxviridae* family and is endemic in western Eurasia. Phylogenetic analysis revealed that CPXV is polyphyletic and isolates cluster into different clades. Common voles (*Microtus arvalis*) are thought to act as reservoir hosts and isolates of common vole origin clustered in CPXV-like 2 clade, while most isolates from accidental hosts assembled in the CPXV-like 1 clade. RatPox09, an isolate from a diseased pet rat (*Rattus norvegicus forma domestica*) and an example for an accidental host, belongs to the VARV-like clade. It is highly pathogenic in rats and caused several spillover infections to humans. Serological studies suggest that bank voles (*Myodes glaerolus*) are also CPXV reservoirs, but up to now, no bank vole-derived isolate was available.

In this study, the first detection and isolation of a bank vole-derived isolate is reported. *In vitro* studies using this bank vole origin isolate on different cell lines and on the chorioallantoic membrane were performed. In addition, experimental inoculations of bank voles, common voles and Wistar rats as model animals for CPXV infection were implemented. We were for the first time able to detect virus shedding from excretions of bank voles, while neither shedding nor seroconversion was detectable in common voles for those isolates. The direct pathogen detection in combination to shedding of infectious virus confirmed the status of bank voles as relevant reservoir host species of CPXV.

Phylogenetic analyses classified the bank vole-derived isolate to clade CPXV-like 3 and completed this clade. It clustered close to CPXV Ger2010MKY, isolated from a cotton-top tamarin (*Saguinus oedipus*). *In vivo* studies using CPXV Ger2010MKY inoculations of bank voles affirmed viral shedding results. Furthermore, two bank voles inoculated with CPXV Ger2010MKY had to be euthanized because of prominent weight loss. Additionally, infection studies with isolates of the CPXV-like 1 and 2 clade were performed in bank voles, but independent of the used isolate only seroconversion was detectable. Therefore, the phylogenetic differentiation of the CPXV-like 3 clade in relation to susceptibility of bank voles and common voles was clearly visible.

RatPox09 shows significantly increased virulence in Wistar rats and causes a high mortality compared to the mild clinical symptoms induced by the laboratory strain Brighton Red (BR). Four genes, absent in the genome of BR and present in the genome of RatPox09, were

identified. BR-based knock-in mutants for these relevant genes were generated and in vivo characterized in Wistar rats. One mutant with insertion of the *gCPXV0030* encoding gene displayed significantly higher clinical scores and lower survival rates than the original BR strain. The gene *gCPXV0030* has a sequence identity of 99% to the CPXV gene encoding 7-transmembrane G protein-coupled receptor-like protein (7tGP).

In summary, my studies support the hypothesis of reservoir species–specific phylogenetic clades within the CPXV classification. Because of confirmed viral shedding from bank voles this species was confirmed as a possible reservoir host for CPXV. However, for productive infection, a CPXV isolate out of the CPXV-like 3 clade was necessary. Furthermore, *gCPXV0030* was identified as a novel relevant factor modifying virulence in the well-established Wistar rat model. Interestingly, exactly this gene is missing in CPXV-like 3 isolates, while it is present in isolates from the CPXV-like 2 clade.

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VIII. LITERATURVERZEICHNIS

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ANHANG

IX. ANHANG

1. ABKÜRZUNGSVERZEICHNIS

a) Liste der Spezies und Viren

BR	Brighton Red
CMPV	Kamelpockenvirus
CPXV	Kuhpockenvirus
ECTV	Mäusepocken-/Ektromelievirus
GBLV	Taterapoxvirus
MPXV	Affenpockenvirus
MVA	Modified-Vaccinia-Ankara-Virus
OPV	Orthopockenvirus
RCNV	Waschbärpockenvirus
VACV	Vaccinia Virus
VARV	Variola Virus
VPXV	Wühlmauspockenvirus

b) Liste an Genen

7tGP	7-transmembrane G protein-coupled receptor-like Protein
CrmE	cytokine response modifier E
GAAP	Golgi apparatus antiapoptotic Protein
NMDAr	N-methyl-D-aspartate receptor-like Protein
TNF	Tumor-Nekrose-Faktor

c) Sonstige Abkürzungen

AK	Antikörper
ATI	Typ A Einschlusskörperchen; A-type inclusion bodies
BAC	bacterial artificial chromosome
bp	Basenpaare
ca.	Circa
CAM	Chorioallantoismembran
CEV	cell associated enveloped virus
CEV	cell-associated enveloped virion
CPE	Cytopathischer Effekt
cq	quantification Cycle
DNA	Desoxyribonukleinsäure
dpi	Tage nach Infektion; “Days post infection”
dsDNA	Doppelstrang-DNA
EEV	Etracellular enveloped virus
ELISA	Enzyme-linked Immunosorbent Assays
FM	Feldmaus
GAGs	Glykosaminoglykane
HTS	High throughput sequencing
IEV	intracellular enveloped virus

IFT	Immunfluoreszenztest
IMV	Intracellular mature virus
ITR	Inverted terminal repeats
IV	Unreifes (immature) Virion
Kbp	Kilobasenpaare
MOI	Multiplizität der Infektion; „Multiplicity of infection”
MV	Reifes, infektiöses (mature) Virion
n. Chr.	nach Christus
ORF	Offener Leserahmen; “open reading frame”
PCR	Polymerase-Kettenreaktion; “Polymerase Chain Reaction"
RM	Rötelmaus
RNA	Ribonukleinsäure
$TCID_{50}$	Tissue Culture Infection Dose 50%
v. Chr.	vor Christus
VNT	Virusneutralisationstest
WHO	Weltgesundheitsorganisation; „World Health Organization“

2. ABBILDUNGSVERZEICHNIS

3. TABELLENVERZEICHNIS

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X. DANKSAGUNG

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