Inaugural-Dissertation zur Erlangung der Doktorwürde der Tierärztlichen Fakultät der
Ludwig-Maximilians-Universität München

**Shuni-Virus: Charakterisierung der neurologischen Erkrankung im Tiermodell und
Entwicklung serologischer Tests**

von Franziska Sick

aus Rothenburg o.d.T.

München 2022

Aus dem Veterinärwissenschaftlichen Department der Tierärztlichen Fakultät

der Ludwig-Maximilians-Universität München

Lehrstuhl für Virologie

Arbeit angefertigt unter der Leitung von Univ.- Prof. Dr. Dr. h. c. Gerd Sutter

Angefertigt im Institut für Virusdiagnostik des Friedrich-Loeffler-Instituts,

Bundesforschungsinstitut für Tiergesundheit, Insel Riems

Mentor: Prof. Dr. Martin Beer

Gedruckt mit Genehmigung der Tierärztlichen Fakultät der Ludwig-Maximilians-Universität München

Dekan: Univ.-Prof. Dr. Reinhard K. Straubinger, Ph.D.

Berichterstatter: Univ.- Prof. Dr. Dr. h. c. Gerd Sutter

Korreferent/en: Prof. Dr. Andrea Fischer

Tag der Promotion: 12. Februar 2022

Meiner Familie

Die vorliegende Arbeit wurde gemäß § 6 Abs. 2 der Promotionsordnung für die Tierärztliche Fakultät der Ludwig-Maximilians-Universität München in kumulativer Form verfasst.

Folgende wissenschaftliche Arbeiten sind in dieser Dissertationsschrift enthalten:

<u>Franziska Sick</u>, Martin Beer, Helge Kampen and Kerstin Wernike „**Culicoides Biting Midges—Underestimated Vectors for Arboviruses of Public Health and Veterinary Importance**" erschienen in Viruses 2019, online verfügbar under doi:10.3390/v11040376

(Dissertation Seite 13)

<u>Franziska Sick</u>, Angele Breithaupt, Natalia Golender, Velizar Bumbarov, Martin Beer, Kerstin Wernike „ **Shuni virus-induced meningoencephalitis after experimental infection of cattle**" erschienen in Transboundary and Emerging Diseases 2020, online verfügbar unter doi:10.1111/tbed.13823

(Dissertation Seite 41)

Kerstin Wernike, Andrea Aebischer, <u>Franziska Sick</u>, Kevin P Szillat, Martin Beer „**Differentiation of Antibodies against Selected Simbu Serogroup Viruses by a Glycoprotein Gc-Based Triplex ELISA**" erschienen in Veterinary Sciences 2021, online verfügbar unter doi:10.3390/vetsci8010012

(Dissertation Seite 75)

Angele Breithaupt, <u>Franziska Sick</u>, Natalia Golender, Martin Beer, Kerstin Wernike „**Characterization of a small animal model to study Shuni virus infection**" eingereicht bei Veterinary Pathology 2021

(Dissertation Seite 53)

Weitere Arbeiten, die nicht in der Dissertationsschrift enthalten sind:

Anna Michelitsch, Christine Fast, <u>Franziska Sick</u>, Birke Andrea Tews, Karin Stiasny, Malena Bestehorn-Willmann, Gerhard Dobler, Martin Beer, Kerstin Wernike **"Long-term presence of tick-borne encephalitis virus in experimentally infected bank voles (Myodes glareolus)"** erschienen in Ticks and Tick-borne Diseases 2021, online verfügbar unter doi: 10.1016/j.ttbdis.2021.101693

Donata Hoffmann, Björn Corleis, Susanne Rauch, Nicole Roth, Janine Mühe, Nico Joel Halwe, Lorenz Ulrich, Charlie Fricke, Jacob Schön, Anna Kraft, Angele Breithaupt, Kerstin Wernike, Anna Michelitsch, <u>Franziska Sick</u>, Claudia Wylezich, Stefan O. Müller, Thomas C. Mettenleiter, Benjamin Petsch, Anca Dorhoi, Martin Beer **"CVnCoV protects human ACE2 transgenic mice from ancestral B BavPat1 and emerging B.1.351 SARS-CoV-2"** erschienen in nature communications 2021, online verfügbar unter doi: 10.1038/s41467-021-24339-7

Inhalt

1 Einleitung

Shuni-Virus (SHUV) ist ein Insekten-übertragenes Orthobunyavirus der Simbu-Serogruppe, Familie *Peribunyaviridae*, und eng verwandt mit anderen Viren veterinärmedizinischer Bedeutung wie dem Schmallenberg-Virus (SBV) und dem Akabane-Virus (AKAV) (1, 2). Die meisten Viren der Simbu-Serogruppe infizieren Wiederkäuer und können bei akuter Infektion milde Symptome wie Fieber, Durchfall oder einen Rückgang der Milchleistung für einige Tage verursachen. Wird jedoch ein trächtiger Wiederkäuer während einer sensiblen Phase der Trächtigkeit infiziert, können Aborte, Totgeburten oder schwere kongenitale Missbildungen auftreten, die unter dem Begriff "Arthrogryphose-Hydranencephalie-Syndrom" (AHS) zusammengefasst werden (3-5). SHUV wurde erstmals in den 1960er Jahren in Nigeria aus einem gesunden Schlachtrind isoliert (6). Seitdem wurde es in mehreren afrikanischen Ländern in verschiedenen Säugetieren (Haus- und Wildwiederkäuer, Pferde, Wildtiere) und blutsaugenden Insekten nachgewiesen (7-9). Neben den teratogenen Effekten bei diaplazentarer Infektion von Wiederkäuerfeten sind bei Pferden nach akuter Infektion adulter Tiere schwere neurologische Erkrankungen beschrieben (3, 9). Auch ein zoonotisches Potential wird angenommen, da in einer neuen Studie SHUV RNA in Liquorproben von Menschen mit neurologischer Erkrankung nachgewiesen wurde (10).

SHUV wird, wie die meisten Vertreter der Simbu-Serogruppe, von blutsaugenden Gnitzen der Gattung *Culicoides* übertragen, welche weltweit verbreitet sind (11, 12). Wenn kompetente Vektoren und empfängliche Wirte gleichzeitig in einem Gebiet vorhanden sind, wie es bei Gnitzen und Wiederkäuern in vielen Gebieten weltweit der Fall ist, können sich bei Neueintrag von Viren in naive Populationen leicht neue Infektionszyklen etablieren (13). SHUV hat daher das Potential, sich schnell in bisher unbetroffenen Gebieten auszubreiten. Ende 2014 schaffte SHUV den Sprung aus Gebieten südlich der Sahara bis in den Nahen Osten nach Israel (3). Neben teratogenen Symptomen wurden im Rahmen dieses Ausbruches erstmals SHUV-induzierte schwere neurologische Erkrankungen bei adulten Rindern beschrieben. Histopathologische Untersuchungen der erkrankten Rinder ergaben eine nicht-suppurative Meningoenzephalitis (14). Das Virus wurde in den folgenden Jahren mehrfach detektiert, sodass man davon ausgehen kann, dass SHUV einen endemischen Status in Israel etabliert hat (15). Studien zur Pathologie der neurologischen Erkrankungen fehlen weitgehend, werden

aber auch vor dem Hintergrund des zoonotischen Potentials dringend benötigt. Zur serologischen Überwachung und Diagnostik von simbuviralen Erkrankungen stehen diagnostische Tests zur Verfügung. Wegen Kreuzreaktivitäten von nah verwandten Viren innerhalb der Simbu-Serogruppe ist die serologische Differenzierung einzelner Vertreter dieser Gruppe allerdings oftmals ein Problem (16-18). Serologische Tests, die schnell, einfach und mit großen Probenzahlen durchgeführt werden können, wie beispielsweise ELISAs, waren für eine spezifische Detektion von SHUV-Antikörpern noch nicht vorhanden.

Die vorliegende Arbeit umfasst einen Übersichtsartikel über die Bedeutung von Gnitzen, den Überträgern von SHUV, als Vektoren verschiedener Viren mit Relevanz für die öffentliche Gesundheit und die Tiergesundheit (19). Zudem werden die neu etablierten Versuchstier-Infektions-Modelle Rind und IFNAR (-/-) Maus vorgestellt, die für die *in vivo* Charakterisierung der Neuropathologie zweier aktuell zirkulierender SHUV Isolate verwendet wurden (A. Breithaupt, Characterization of a small animal model to study Shuni virus infection"), (20). Ein weiterer Artikel befasst sich mit der Etablierung eines ELISAs zur differenzierten Detektion von gegen SHUV und andere Viren der Simbu-Serogruppe gerichtete Antikörper (21).

2 Literaturübersicht

Um einen Überblick über die Bedeutung von Gnitzen als Vektoren von Arboviren mit Relevanz für die öffentliche Gesundheit und tiermedizinischer Bedeutung zu gewinnen, wurde ein Übersichtsartikel erarbeitet, der als Hintergrundinformation für diese Arbeit verwendet wird. Die Kapitel "Orthobunyaviren: Taxonomie und molekulare Charakteristika", "Shuni-Virus: Wirtsspektrum und Krankheitsbilder", "Shuni-Virus: Epidemiologie und Potential als ‚emerging disease'", "Simbuviren: Tiermodelle" und "Simbuviren: Serologische Diagnostik" wurden ergänzt, um die Literaturübersicht für diese Arbeit zu vervollständigen.

Die Literaturverzeichnisse der Originalpublikationen der Literaturübersicht und im Ergebnisteil sind im Zitierstil des jeweiligen Journals angegeben und sind nicht im Literaturverzeichnis am Ende dieser Dissertationsschrift enthalten. Die Nummerierung von Tabellen und Abbildungen bezieht sich auf die publizierte beziehungsweise eingereichte Form des jeweiligen Manuskripts.

2.1 Orthobunyaviren: Taxonomie und molekulare Charakteristika

Die vom *international commitee on taxonomy of viruses* (ICTV) erst kürzlich (2019) neu überarbeitete Nomenklatur von Viren mit linearem, segmentiertem, einzelsträngigem RNA Genom ordnet das Shuni-Virus (SHUV) in die neu etablierte Familie der *Peribunyaviridae*, Ordnung Bunyavirales ein (2, 22). Das Genus *Orthobunyavirus* beinhaltet mehr als 170 Viren, die, basierend auf serologischen Tests und Sequenzanalysen, in 18 Serogruppen eingeordnet werden (1, 23). Die Simbu-Serogruppe ist nicht nur eine der größten, sondern auch eine der bedeutendsten Serogruppen, wenn es um die Tiergesundheit geht, und umfasst Viren wie das tierpathogene Akabane-Virus (AKAV), das Schmallenberg-Virus (SBV) und SHUV, aber auch das humanpathogene Oropouche-Virus (OROV), das in Südamerika vorkommt (1, 24, 25).

Orthobunyaviren sind behüllte, sphärische Viren mit einem Durchmesser von 80-120 nm. Für das mit SHUV nahe verwandte SBV wurde eine Partikelgröße von etwa 100 nm ermittelt (26). Die Virushülle besteht aus einer Lipiddoppelschicht, die dem Golgi-Apparat der Wirtszelle entstammt. In der Oberfläche verankert befinden sich Spikes, die aus den integralen, transmembranösen Glykoproteinen Gn und Gc bestehen, die über Disulfidbrücken miteinander zu Heterodimeren verbunden sind (27, 28). Innerhalb dieser Hülle befinden sich drei Genomsegmente, die anhand ihrer Größe benannt sind: Das S-Segment (small – klein) hat bei SHUV eine Länge von 851 Nukleotiden (nt), das M-Segment (medium – mittel) von 4351 nt und das L-Segment (large – groß) von 6910 nt. Die offenen Leserahmen (ORF) der jeweiligen Segmente werden von nicht-translatierten Regionen (NTR) flankiert, deren Sequenzen an den 3' und 5' Enden hoch konserviert und komplementär sind. Durch Basenpaarung der komplementären Enden ermöglicht sich die Ausbildung einer stabilen Panhandel-Struktur der einzelnen RNA-Segmente (29) (Abbildung 1).

Die drei Segmente kodieren lediglich für vier Strukturproteine und zwei Nichtstrukturproteine: Das S-Segment kodiert für das Nukleokapsidprotein N und in einem alternativen, überlappenden Leserahmen für das Nichtstrukturprotein NSs (30). Das M-Segment kodiert für die Oberflächen Glykoproteine Gn und Gc und das Nichtstrukturprotein NSm (Reihenfolge Gn-NSm-Gc), die von einem einzelnen ORF als Vorläufer Polyprotein translatiert und anschließend durch zelluläre Proteasen in einzelne, ausgereifte Proteine

gespalten werden (31). Das L-Segment kodiert für das L-Protein, das als RNA-abhängige RNA-Polymerase (RdRp) fungiert (32).

Das N-Protein bildet funktionelle Replikations- und Transkriptionseinheiten mit den drei RNA-Segmenten und je einem L-Protein als Ribonukleoprotein-Komplexe (30). Das NSs-Protein ist als Interferonantagonist ein wichtiger Virulenzfaktor im Säuger-Wirt (27). Die doppelsträngigen RNA-Bereiche der komplementären Genomsegmentenden, die eine Panhandle Struktur ausbilden, werden in Säugerzellen von RIG-I (retinoic acid-inducible gene I) erkannt, wodurch das angeborene Immunsystem aktiviert wird: proinflammatorische Zytokine wie Interferon (IFN) Typ 1 werden produziert und lösen in den Zellen eine Kaskade von Reaktionen aus, die die Zelle in einen antiviralen Status versetzt (33). Das NSs-Protein kann diese angeborene Immunantwort effektiv unterdrücken. Für SBV konnte gezeigt werden, dass Virusmutanten, die kein NSs exprimieren, eine IFN-Induktion in infizierten Zellen auslösen und, im Vergleich zu Wildtypviren, ein Wachstumsdefizit auf IFN-kompetenten Zelllinien aufweisen. Auf IFN-defizienten Zelllinien dagegen replizieren Viren mit und ohne NSs gleich effizient (34, 35). Für SHUV konnte dieses Funktion von NSs ebenfalls bestätigt werden (36).

Zudem kann das NSs einen direkten Einfluss auf die wirtseigene Proteinbiosynthese nehmen, indem es auf die DNA-abhängige RNA-Polymerase II (RNAP II) einwirkt: Für SBV wurde gezeigt, dass die C-terminale Untereinheit RPB1 der RNAP II abgebaut wird. Auf diesem Weg wird die Transkription gehemmt, neue Proteinbiosynthese verhindert und somit die antivirale Antwort der Wirtszelle unterdrückt (35). In Transkriptomanalysen wurde für SBV gezeigt, dass NSs Einfluss auf die Expression von antiviralen und IFN-stimulierten Genen nimmt (37). Für das Viruswachstum in Zellkultur ist das NSs-Protein nicht essentiell, NSs-Deletionsmutanten zeigen im Vergleich zum Wildtyp-Virus aber ein Wachstumsdefizit auf IFN-kompetenten Zelllinien (34, 35, 38).

In IFN-kompetenten Tieren sind NSs-Deletionsmutanten attenuiert: Bei intrazerebral infizierten NIH-Swiss Mäuse zeigte eine SBV NSs-Deletionsmutante einen attenuierten Phänotyp mit einer deutlichen Verzögerung der Sterblichkeit der Tiere (34). In erwachsenen, IFN-kompetenten Rindern konnte eine SBV NSs-Deletionsmutante keine Virämie und keine klinische Erkrankung auslösen (38).

Die Gylkoproteine Gn und Gc sind verantwortlich für Virusattatchment, Viruseintritt in die Wirtszelle und für die Induktion der Immunantwort des Wirts. Der N-terminale Teil des Gc Glykoproteins (sog. „Gc Head-Domäne") ist als hauptimmunogene Domäne verantwortlich für die Ausbildung neutralisierender Antikörper (28, 39-41).

Bei Orthobunyaviren wird die Funktion des NSm-Proteins im Säugerwirt mit Virus-Assembly und Morphogenese assoziiert (42).
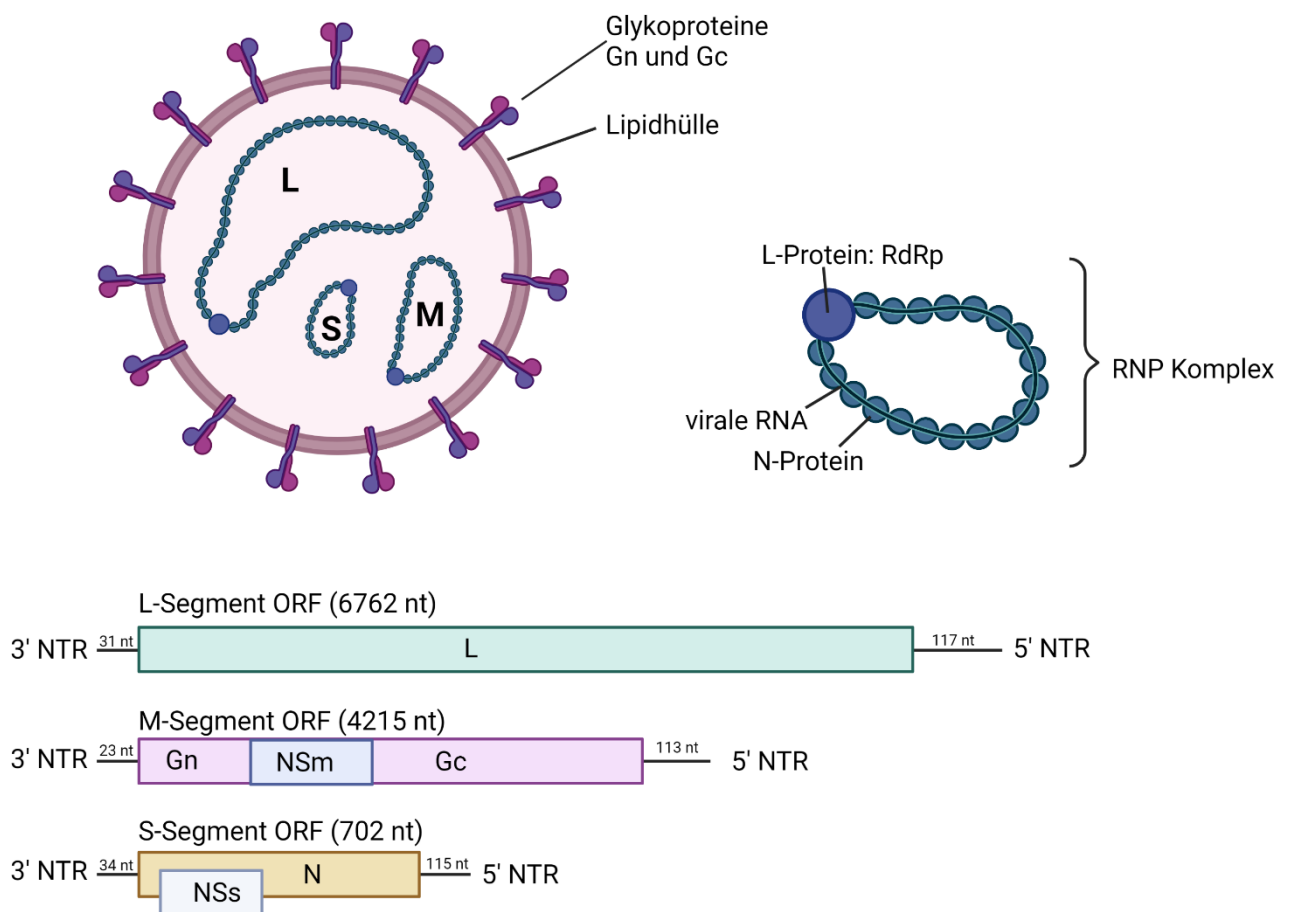

Abbildung 1: Schematische Darstellung eines SHUV-Virions und des segmentierten Genoms (erstellt mit BioRender.com)

2.2 Shuni-Virus: Wirtsspektrum und Krankheitsbilder

Das Wirtsspektrum umfasst bei SHUV hauptsächlich Wiederkäuer wie Rinder, Schafe und Ziegen (3, 14, 43), aber auch Pferde (9, 44), Menschen (10, 45, 46) und verschiedene andere Wirbeltiere einschließlich Reptilien und Vögel (8). SHUV RNA wurde mittels Polymerasekettenreaktion (PCR) in wildlebenden Wiederkäuern nachgewiesen (Rappenantilope, Büffel, Giraffe, Springbock). Zudem gelang der RNA-Nachweis in Proben von Nashörnern, Warzenschwein, Krokodil, Alpaka und Fasan. Spezifische neutralisierende Antikörper gegen SHUV wurden in Büffeln und Nashörnern nachgewiesen (8). Generell kann SHUV, wie die meisten Viren der Simbu-Serogruppe, zwei verschiedene Krankheitsbilder auslösen: Akute Infektionen verlaufen oft asymptomatisch oder die Tiere zeigen für wenige Tage unspezifische Symptome wie Fieber, Diarrhö oder einen Rückgang der Milchleistung. Werden allerdings naive Tiere während einer bestimmten Phase der Trächtigkeit infiziert, kann es zu schweren kongenitalen Missbildungen des muskuloskelettalen Apparates und des zentralen Nervensystems, die als Arthrogryphose-Hydranenzephalie-Syndrom (AHS) bezeichnet werden, Aborten, Frühgeburten, Mummifikationen oder Totgeburten kommen (3-5). Für SHUV wurde eine schnelle Überwindung der Plazentaschranke in Inokulationsversuchen an trächtigen Schafen experimentell nachgewiesen. SHUV RNA konnte bereits 7 Tage nach der Inokulation in der Plazenta und im Fetus detektiert werden (47). Neben unspezifischen Symptomen bei akuter Infektion kann SHUV zudem neurologische Erkrankungen auslösen. Pferde können bei akuten SHUV-Infektionen in progressiv fortschreitenden Krankheitsverläufen Symptome wie Ataxie, Depression, Festliegen mit Rudern der Vorderbeine, Muskelspasmen und Tremor, Lähmungen oder Hyperästhesie zeigen. Krankheitsverläufe mit schwerer neurologischer Symptomatik enden oft in der Euthanasie der Tiere, bei milderen Krankheitsverläufen mit Fieber oder nur leichter neurologischer Symptomatik können sich die Tiere zum Teil wieder vollständig erholen (9, 44). Auch bei zwei jungen Rindern in Israel wurde SHUV mit schwerer neurologischer Erkrankung in Verbindung gebracht. Nach Lähmung des Kopfes und des Halses verstarb ein 5 Monate altes Kalb. Eine 16 Monate alte Färse zeigte zu Beginn der Erkrankung Symptome wie Kreiseln, Ataxie und Schluckbeschwerden; im fortschreitenden Verlauf der Erkrankung war das Tier festliegend und wurde infolgedessen euthanasiert (14). SHUV konnte in beiden Fällen mittels Reverse-

Transkriptase-Polymerase-Kettenreaktion (RT-PCR) nachgewiesen und im zweiten Fall auch in Zellkultur isoliert werden. In der histopathologischen Untersuchung wurde in beiden Fällen eine (Meningo-)Enzephalitis diagnostiziert (14). Bei einer Vielzahl an Wildtieren wurde SHUV RNA mittels PCR in Proben des zentralen Nervensystems, Blut oder Milz nachgewiesen. Unter den SHUV positiven Wildtieren waren verschiedene Wiederkäuer, Rhinozeros, Warzenschwein, Alpaka, Giraffe, aber auch Fasan und Krokodil. Sie zeigten neurologische Symptome oder verstarben plötzlich. SHUV könnte auch hier der Grund für die neurologische Erkrankung gewesen sein (10). Obwohl die meisten Viren der Simbu-Serogruppe ausschließlich tierpathogen sind, gibt es doch einige Vertreter mit zoonotischem Potential. Viren der Spezies *oropouche orthobunyavirus* wie das OROV (48), Madre de Dios Virus (49) und Iquitos-Virus (50) spielen in Zentral- und Südamerika eine wichtige Rolle und verursachen fiebrige Erkrankungen, oft auch in Verbindung mit Meningitis oder Enzephalitis (51). Oya-Virus, ein Virus der Spezies *Cat Que orthobunyavirus*, wurde bei Menschen nachgewiesen und mit fiebriger Erkrankung in Verbindung gebracht (52). Für SHUV gibt es zudem klare Hinweise auf ein zoonotisches Potential. In den 1960er Jahren gelang z.B. die Virusisolation bei einem Kind mit fiebriger Erkrankung (45). Spezifische Antikörper wurden bei Großtierärzten in Südafrika nachgewiesen, jedoch wurden in dieser Studie keine Information über etwaige klinische Erkrankungen der Antikörper-positiven Menschen gegeben (46). Kürzlich konnten in Südafrika erstmals klinische Erkrankungen beim Menschen mit dem direkten Nachweis von SHUV in Verbindung gebracht werden: bei Menschen mit neurologischer Erkrankung gelang der Nachweis von SHUV RNA in Liquorproben per PCR. Neben neurologischen Symptomen zeigten die SHUV-positiven Patienten häufig Symptome wie Erbrechen, Durchfall, Krampfanfälle und Fieber (10). Obwohl momentan keine bewiesenen Fälle vertikaler SHUV-Transmission beim Menschen vorliegen, gibt es Hinweise auf eine Empfänglichkeit der humanen Plazenta für eine SHUV-Infektion. Humane plazentare Explantate erwiesen sich in Infektionsversuchen als empfänglich und eine Virusvermehrung wurde in den Trophoblasten nachgewiesen (47). Zudem gelang die Detektion von SHUV RNA bei einem 13 Tage alten Neugeborenen. Eine vertikale Infektion ist hier naheliegend, da das Neugeborene das Krankenhaus seit der Geburt nicht verlassen hatte und eine Ansteckung über blutsaugende Insekten somit unwahrscheinlich ist.

2.3 Shuni-Virus: Epidemiologie und Potential als "emerging disease"

Viren der Simbu-Serogruppe wie das SHUV zählen zu den sogenannte Arthropod-borne Viren (Arboviren) und persistieren in der Natur, indem sie abwechselnd Insekten-Vektoren und Vertebraten-Wirte (hauptsächlich Wiederkäuer) infizieren. Die Insekten-Vektoren von SHUV sind blutsaugende Gnitzen der Gattung *Culicoides* (11). Wird ein virämisches Tier von einem empfänglichen Vektor gebissen, kann sich dieser mit dem Virus infizieren und dieses bei der darauffolgenden Blutmahlzeit an den nächsten empfänglichen Wirt weitergeben. Das Virusvorkommen richtet sich nach der Verbreitung und der Menge der kompetenten Vektoren, sowie deren saisonaler Aktivität. In gemäßigten Zonen beginnt der Anstieg der Menge der Gnitzen im späten Frühling, um in Spätsommer oder Frühherbst seinen Höhepunkt zu erreichen. Bei kälteren Temperaturen und den ersten Frösten fallen die Zahlen an aktiven Vektoren drastisch ab (12, 53). In feuchten, warmen Sommern ist die Virusübertragungsrate folglich am höchsten. SHUV wurde bei Pferden in Südafrika, wo die Monate Oktober bis April durch wärmere Temperaturen und erhöhte Niederschlagsmengen gekennzeichnet sind, vorwiegend am Ende der Regenperiode nachgewiesen, mit einem Peak im April (44). In der aktiven Zeit saugen adulte weibliche Gnitzen mehrmals Blut, das sie für die Entwicklung der Eier benötigen (12). Transovarielle Übertragung von Viren ist für andere Orthobunyaviren beschrieben (54), für SHUV ist allerdings noch unbekannt, ob eine vertikale Transmission in der Gnitzenpopulation stattfindet. In endemischen Regionen entwickeln Viren der Simbu-Serogruppe Muster periodischer Viruszirkulation mit Jahren hoher Viruslasten, gefolgt von Jahren mit nur sporadischen Virusnachweisen, was vermutlich am Immunstatus der empfänglichen Wirtspopulation und der Menge an Vektoren liegt (55-58).

Gnitzen der Gattung *Culicoides* sind weltweit verbreitet (12) und auch Viren der Simbu-Serogruppe treten weltweit auf (19). SHUV kommt in Afrika und dem Nahen Osten vor (3, 6, 9). SHUV gilt als endemisch auf dem afrikanischen Kontinent und hat nach dem ersten Nachweis im Jahr 2014 in Israel schnell einen endemischen Status erreicht (3, 14, 15). Dass SHUV plötzlich den Sprung von südlich der Sahara bis in den Nahen Osten in ein bislang unbetroffenes Gebiet geschafft hat, zeigt das große Potential der Viren der Simbu-Serogruppe, als "emerging disease" zu agieren. Wenn vektorkompetente Insekten weit verbreitet sind und in hoher Zahl vorkommen, wie es bei Gnitzen der Fall ist, und zudem

empfängliche Wirte vorhanden sind, kann es bei Neueintrag eines Krankheitserregers in eine naive Population leicht zur Etablierung eines stabilen Infektionszyklus zwischen Vektoren und empfänglichen Wirten kommen (13). Ähnliches spielte sich auch 2011 mit dem Auftreten von SBV in Europa ab. Das Virus wurde erstmals im Spätsommer 2011 in Deutschland nahe der holländischen Grenze nachgewiesen (24, 59) und breitete sich in kurzer Zeit in der naiven Wiederkäuerpopulation in ganz Deutschland und in weiten Teilen Europas aus. SBV hat schnell einen endemischen Status in ganz Europa etabliert, kehrt mit einem periodischen Auftreten wieder (60) und stellt ein anhaltendes Problem in der Tiergesundheit dar. Die genaue Herkunft des Virus, das bis 2011 völlig unbekannt war, ist allerdings weiterhin ungeklärt.

Bei Viren der Simbu-Serogruppe bietet die Segmentierung des viralen Genoms die Möglichkeit zur Reassortierung, eines Austausches von Genomsegmenten zweier parentaler Viren, was zur Entstehung neuer Viren mit potentiell veränderter Pathogenität führen könnte (61). Der Austausch von M-Segmenten könnte zu Veränderungen im Wirts-Tropismus führen, da die M-Segment kodierten Glykoproteine den Viruseintritt in die Zelle vermitteln. Derartige Viren könnten ein großes Potential bergen, neue "emerging diseases" zu werden. Bei Orthobunyaviren kam es in der Vergangenheit bereits durch Reassortierung zur Entstehung neuer Viren. Ein Beispiel ist Ngari Virus, eine Bunyamwera Reassortante, die zu großen Ausbrüchen von hämorrhagischem Fieber in Afrika führte (62).

2.4 Zusammenfassung und Auswertung der verfügbaren Daten zu Gnitzen als Vektoren von Arboviren mit Relevanz für die öffentliche Gesundheit und tiermedizinischer Bedeutung – Übersichtsartikel

„Culicoides Biting Midges—Underestimated Vectors for Arboviruses of Public Health and Veterinary Importance"

Franziska Sick [1], Martin Beer [1], Helge Kampen [2] and Kerstin Wernike [1]

[1] Institute of Diagnostic Virology, Friedrich-Loeffer-Institut, Südufer 10, 17493 Greifswald-Insel Riems, Germany

[2] Institute of Infectology, Friedrich-Loeffer-Institut, Südufer 10, 17493 Greifswald-Insel Riems, Germany

viruses

MDPI

Review

Culicoides Biting Midges—Underestimated Vectors for Arboviruses of Public Health and Veterinary Importance

Franziska Sick [1], Martin Beer [1], Helge Kampen [2] and Kerstin Wernike [1,*

[1]   Institute of Diagnostic Virology, Friedrich-Loeffler-Institut, Suedufer 10, 17493 Greifswald-Insel Riems, Germany; Franziska.Sick@fli.de (F.S.); Martin.Beer@fli.de (M.B.)
[2]   Institute of Infectology, Friedrich-Loeffler-Institut, Suedufer 10, 17493 Greifswald-Insel Riems, Germany; Helge.Kampen@fli.de
*   Correspondence: Kerstin.Wernike@fli.de; Tel.: +49-38351-71212

check for updates

Abstract: *Culicoides* biting midges, small hematophagous dipterans, are the demonstrated or putative vectors of multiple arboviruses of veterinary and public health importance. Despite its relevance in disease spread, the ceratopogonid genus *Culicoides* is still a largely neglected group of species, predominantly because the major human-affecting arboviruses are considered to be transmitted by mosquitoes. However, when a pathogen is detected in a certain vector species, a thorough search for further vectors often remains undone and, therefore, the relevant vector species may remain unknown. Furthermore, for many hematophagous arthropods, true vector competence is often merely suspected and not experimentally proven. Therefore, we aim to illuminate the general impact of *Culicoides* biting midges and to summarize the knowledge about biting midge-borne disease agents using the order *Bunyavirales*, the largest and most diverse group of RNA viruses, as an example. When considering only viruses evidentially transmitted by *Culicoides* midges, the Simbu serogroup (genus *Orthobunyavirus*) is presumably the most important group within the virus order. Its members are of great veterinary importance, as a variety of simbuviruses, e.g., the species *Akabane orthobunyavirus* or *Schmallenberg orthobunyavirus*, induces severe congenital infections in pregnant animals. The major zoonotic representative of this serogroup occurs in South and Central America and causes the so-called Oropouche fever, an acute febrile illness in humans.

Keywords: *Culicoides*; biting midges; transmission; insect vector; *Bunyavirales*; orthobunyavirus; Simbu serogroup; Schmallenberg virus; Akabane virus

1. Introduction

During the last decade, the incidence of emerging viral diseases has increased considerably in various regions worldwide. Diseases such as Zika fever or West Nile fever have been among numerous neglected viral diseases, until they have expanded to new regions and caused large outbreaks, thereby attracting strong media attention [1,2]. Among the emerging or re-emerging pathogenic viruses are a large number of so-called arthropod-borne (arbo) viruses, i.e., viruses that are transmitted between their vertebrate hosts by insects and other arthropods [3].

For the establishment and maintenance of "virus-vector-host" transmission cycles of arboviruses in a given area, competent vectors and susceptible hosts need to encounter under favorable environmental conditions [4]. Hence, as reasons for the increasing number of reported arbovirus-induced disease outbreaks, factors such as climate change, increasing urbanization, and globalization with increasing trade, livestock movement, and increasing traveling activities are discussed [5].

Medically, one of the most highly important arthropod vectors is unquestionably mosquitoes. Nonetheless, further, sometimes neglected arthropods such as ticks or biting midges can also act as vectors of emerging disease agents [6]. However, when a pathogen has been detected in a certain vector species, a thorough search for further putative vectors often remains undone. Furthermore, the mere demonstration of a pathogen in a blood-feeding arthropod is often mistaken as evidence for vector competence of that arthropod species without further experimental studies. Therefore, we want to summarize here knowledge about ceratopogonid-transmitted disease agents and their impact on human and animal health focusing on *Culicoides* biting midges and the new viral order *Bunyavirales*, which contains the largest and most diverse families of RNA viruses [7]. In this review, we illuminate the general impact of *Culicoides* biting midges, an often-neglected insect vector, but primarily, we present evidence of their role in the transmission of selected orthobunyaviruses of public health or veterinary importance.

2. How to Differentiate Mechanical from Biological Vectors?

Vector competence refers to the physiological ability of arthropods to acquire and maintain microbial agents from a host, and later transmit them to the next susceptible host [8]. The mere virus detection in a midge does not necessarily indicate vector competence. A midge feeding on a viremic host can carry virus from the blood meal in its gut without being infected itself. However, it can subsequently serve as a so-called "mechanical vector" for virus transmission. To understand a disease and its epidemiology entirely, it is essential to distinguish biological (competent) from pure mechanical vectors.

One option to examine the vector competence is the separate analysis of the head and the salivary glands and the rest of the insect body. Virus detection in the salivary glands is a sign for a biological infection followed by virus dissemination, resulting in a potential virus transmission via saliva. Hence, virus detection in the salivary glands of an insect hints at true vector competence. On the other hand, detection of virus in the completely homogenized body of the insect may indicate just the ingestion of a viremic blood meal, which not necessarily leads to an infection of the insect.

To evaluate vector competence in more detail, infection studies under laboratory conditions either with biting midges caught in the field or with laboratory-adapted colonies are a suitable method. Insects can be artificially infected by intrathoracic inoculation of virus directly into the hemolymph bypassing the intestinal ("gut") barrier, or orally by feeding viremic blood. Subsequently, after an extrinsic incubation period, samples are collected from surviving individuals. The insects themselves could be examined as a whole or with head plus salivary glands and body separated as described above. Furthermore, saliva could be collected from individual insects and tested for infectious virus or viral genome. From the results, infection, dissemination and transmission rates are calculable [9].

3. *Culicoides* Biting Midges: Classification, Morphological Characteristics, and Distribution

Culicoides is a genus of biting midges in the order Diptera, family Ceratopogonidae. Currently, the genus contains 1368 species divided into numerous subgenera [10].

Culicoides biting midges are among the most abundant hematophagous insects worldwide, occurring from temperate areas to the tropics. Representing one of the smallest hematophagous flies, they measure only 1–3 mm [11]. Their mouthparts form a proboscis well adapted for cutting skin and sucking blood. Wings are well developed, and biting midges are commonly identified to complex or species level based on the wing maculation [12]. However, this method of identification is very time consuming and depends on the professional experience of the examiner. Alternative methods use genome amplification by polymerase chain reaction (PCR) with subsequent sequencing and phylogenetic comparison of DNA marker regions [13,14]. In addition, real-time PCR assays have been established for a few *Culicoides* species [15,16], a DNA microarray was developed to identify *Culicoides* species of the obsoletus group [17], and matrix-assisted laser desorption/ionization time-of flight mass spectrometry (MALDI-TOF-MS) has been used to identify *Culicoides* species based on peptide and protein signatures [18].

The life cycle of *Culicoides*, which passes through the egg stage, four larval stages, a pupa, and the imago, requires a certain amount of free water or moisture, and some species occur in both fresh water and estuarine environments. Breeding sites range from pools, streams, tree holes to saturated soil, animal dung and rotting vegetation [19]. Females depend on blood for the maturation of the eggs; they feed, depending on the species, on mammals and/or birds. Males do not feed on blood, and can survive on nectar alone. The development of *Culicoides* species takes a few weeks—or even months, when overwintering in a larval stage—and this process depends on the ambient temperatures, resulting in a seasonal activity pattern in temperate regions. In mild climatic zones, the insect numbers start to increase in late spring and early summer and usually peak in late summer or early autumn [11,20]. With the onset of low temperatures and the first frosts, the number of active insects drops dramatically.

In general, adult biting midges are short-lived and only a few individuals survive longer than 10 to 20 days. During this time, females may feed on hosts multiple times [11].

4. Public Health and Veterinary Impact of *Culicoides* Biting Midges

Biting midge bites can cause painful lesions; in some cases, the saliva even induces acute allergic reactions such as the "common summer eczema (insect hypersensitivity)" in horses [21]. However, their veterinary or public health importance predominantly results from their role in the transmission of pathogens, especially viruses, but also protozoans and filarial parasites [22] such as avian hamosporidians [23,24] and *Tetrapetalonema* spp. [25–27]. More than 50 viruses have been isolated from *Culicoides* spp. worldwide [28,29]. Most of the viruses belong to the families *Reoviridae* (e.g., African horse sickness virus (AHSV), bluetongue virus (BTV), or epizootic hemorrhagic disease virus (EHDV)), *Rhabdoviridae* (e.g., bovine ephemeral fever virus (BEFV)) and *Peribunyaviridae* (e.g., Akabane virus (AKAV), Schmallenberg virus (SBV), or Oropouche virus (OROV)). However, some viruses isolated from *Culicoides* are only incidental findings, such as Rift Valley fever virus (RVFV), which is mainly transmitted by mosquitoes [28,30]. Furthermore, vector competence studies are still missing for a wide range of viruses suspected to be transmitted by *Culicoides*.

Surveillance on biting midges and biting midge-borne diseases is carried out in various countries of several continents. As an example, the distribution and potential spread of BTV is monitored by the collection of biting midges in virtually every affected European country, also to define e.g., a so-called "seasonally vector free" period. To sample the adult *Culicoides* population, light-suction traps placed at sentinel sites across outbreak areas are the most commonly used standardized trap system [31]. Furthermore, detailed studies on local dispersal of *Culicoides* are available from Australia, comparing various types of light traps with different outcomes in biting midge catches [32] and analyzing distribution patterns related to weather and climate [33].

In search for potential vector species, an accurate estimate of species actually biting the vertebrate host is vital. In the context of BTV vector search in Europe, it was demonstrated in a comparative study of light-suction traps and drop catches that the biting midges caught in light-suction traps do not provide an accurate reflection of the biting *Culicoides* population [31], which has to be considered when assessing data collected in such surveillance studies.

Because of the needs of their agents' vectors, *Culicoides*-borne diseases are strongly linked to climate and weather. In temperate regions, the seasonal pattern of virus transmission coincides with warm, moist summer and autumn months. In tropical and subtropical regions, high vertebrate infection rates have been reported during wet summer and declining transmission rates in periods of lower rainfall [34]. Although their flight range does not exceed a few hundred meters, biting midges can be dispersed passively over great distances by wind [35–38], further contributing to their impact on disease epidemiology.

The establishment of laboratory colonies poses great difficulties, especially because field-collected specimens fail to mate under laboratory conditions. So far, very few *Culicoides* species have been successfully reared under laboratory conditions [29,39,40] and, consequently, are available for studies on their vector competence. Most laboratory colonies represent *C. nubeculosus* and *C. sonorensis*, rendering these two species

important models for studying arbovirus transmission by *Culicoides* biting midges [41]. Due to difficulties in acquiring biting midges from the field for laboratory studies caused by the characteristics of the insects, the major part of data available on experimental infections pertain to *C. nubeculosus* and *C. sonorensis*.

5. The Genus Orthobunyavirus

Only recently, the International Committee on Taxonomy of Viruses (ICTV) made some fundamental changes in the taxonomy of the order *Bunyavirales*, which had been established in 2017 to accommodate viruses with linear, segmented, single-stranded RNA genome [42]. In early 2019, the order was taxonomically revised by creating several new families, subfamilies, genera, and species. As of February 2019, the order *Bunyavirales* consists of 46 genera assigned to 12 distinct families [7]. One of the genera belonging to the newly established family *Peribunyaviridae* is the genus *Orthobunyavirus*, which contains more than 170 unanimous insect-transmitted viruses in 18 serogroups, of which the Simbu serogroup is not only one of the largest, but also the most important in terms of veterinary public health. For human health, the California encephalitis serogroup is very likely the most relevant one as it contains, e.g., La Crosse virus (LACV).

In general, orthobunyaviruses are spherical, about 100 nm in diameter and relatively simple in their composition as the tri-partite RNA genome encodes only four structural and two non-structural proteins. The small (S) genomic segment encodes for the nucleocapsid protein N and the non-structural protein NSs, while the medium (M) segment encodes for the glycoproteins Gn and Gc, which form spikes on the surface of the virus particle, and the non-structural protein NSm. The large (L) segment encodes for the RNA dependent RNA polymerase [43].

The glycoproteins Gn and Gc are integral transmembrane proteins which are important for viral attachment, membrane fusion and the induction of the host's immune response [44–46]. The N-protein is essential for viral RNA transcription and replication, and it forms ribonucleoprotein complexes with the three viral RNA segments. These complexes are associated with the L-protein [47,48]. The NSs-protein is a major virulence factor in vertebrate hosts as it acts as an interferon antagonist and is responsible for the shut-off of protein synthesis in mammalian hosts cells [48]. In vertebrate hosts, the NSm-protein is associated with virus assembly and morphogenesis [49]. However, the function of both non-structural proteins, NSs and NSm, in the insect vectors is largely unknown.

5.1. The Simbu Serogroup

When considering only viruses evidentially transmitted by *Culicoides* midges, the Simbu serogroup is presumably the most important group within the genus *Orthobunyavirus*. Furthermore, its members are of great veterinary importance, as a variety of simbuviruses induces severe congenital infections in pregnant animals. The serogroup currently consists of 32 viruses grouped into 19 virus species (Table 1). Simbuviruses are distributed worldwide (Table 1, Figure 1), and they persist in nature by alternately infecting mammalian hosts and *Culicoides* vectors. In endemic regions, Simbu serogroup viruses establish a pattern of cyclic circulation, with seasons of high virus appearance followed by periods of only sporadic detection [50–55], which is presumably related to the overall immunity in the host population and the abundance of *Culicoides* vectors.

Important representatives of the Simbu serogroup are AKAV and SBV, which predominantly infect ruminants. Infections of adults are either asymptomatic or mild, associated with fever, diarrhea, and decrease in milk yield for a few days. Infections of naïve dams during a critical phase of gestation, however, may be followed by abortion, premature birth, mummification, stillbirth or congenital deformations referred to as arthrogryposis-hydranencephaly syndrome [34,56]. Further ruminant-infecting Simbu serogroup viruses that might induce similar clinical signs as AKAV or SBV include Shuni virus (SHUV), Aino virus (AINOV) and the eponymous Simbu virus (SIMV) [57] (Table 1). The only zoonotic virus of this serogroup is OROV (and reassortants such as Iquitos virus and Madre de Dios virus), which is present in South America (Figure 1).
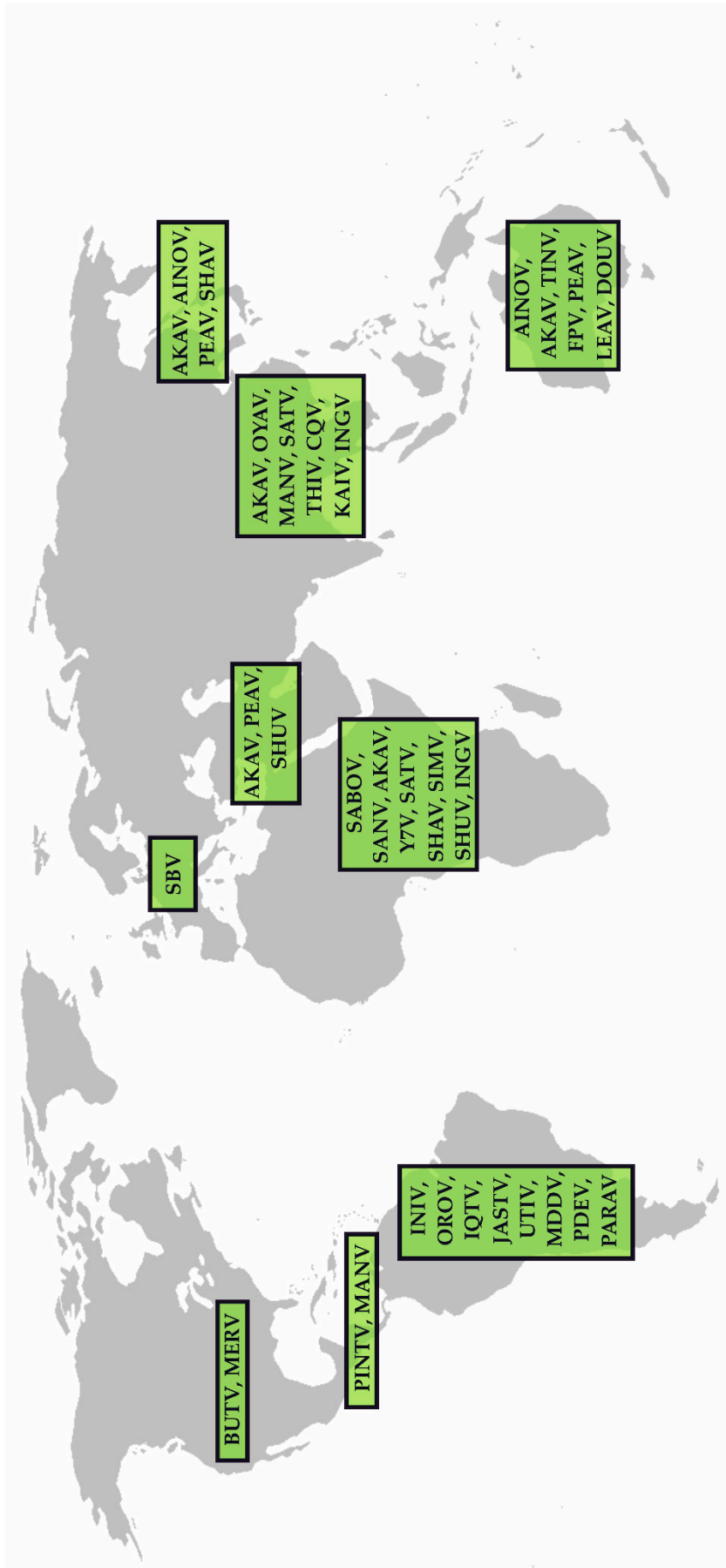
Figure 1. Distribution of Simbu serogroup viruses. The meanings of the abbreviations (virus names) are listed in Table 1.

Table 1. Classification and distribution of Simbu serogroup viruses and vector species responsible for their transmission.

Virus Species	Virus (Abbreviation)	First Isolation			Insect Vector		Distribution		Reference (1st Description)
		Year	Country	Organism	Putative Vectors	Demonstrated Vectors	Continent	Animal Hosts (Virus Detection)	
Akabane orthobunyavirus	Akabane virus (AKAV)	1959	Japan	mosquitoes	biting midges, mosquitoes	*C. brevitarsis, C. variipennis* [1]	Asia, Africa, Australia	ruminants, swine	[58]
Aino orthobunyavirus	Tinaroo virus (TINV)	1978	Australia	*Culicoïdes* midges	biting midges (*C. brevitarsis*)		Australia	ruminants	[59]
	Yaba-7 virus (Y7V)	1963	Nigeria	mosquitoes	mosquitoes		Africa		[60]
	Aino virus (AINOV)	1964	Japan	mosquitoes	mosquitoes, biting midges (*C. brevitarsis*)		Asia, Australia	ruminants	[61]
Buttonwillow orthobunyavirus	Buttonwillow virus (BUTV)	1962	USA	Cottontail rabbit	midges (*C. variipennis*)		North America	leporids	[62]
Cat Que orthobunyavirus	Cát Qué virus (CQV)	2004	Vietnam	mosquitoes	mosquitoes (*Culex* spp., *Anopheles* spp., *Mansonia* spp.)		Asia	swine, birds	[63]
	Oya virus (OYAV)	1999	Malaysia	swine	mosquitoes		Asia	swine, humans	[64]
Faceys paddock orthobunyavirus	Facey's paddock virus (FPV)	1974	Australia	mosquitoes	mosquitoes (*Culex annulirostris*), midges		Australia		[65]
Ingwavuma orthobunyavirus	Ingwavuma virus (INGV)	1959	South Africa	spectacled weaver	mosquitoes (*Culex* spp., *Mansonia* spp.), biting midges		Africa, Asia	swine, dogs, birds	[66]
Jatobal orthobunyavirus	Jatobal virus (JASTV)	1985	Brazil	coati	mosquitoes, biting midges		South America	coati	[67]
Leanyer orthobunyavirus	Leanyer virus (LEAV)	1974	Australia	mosquitoes	mosquitoes (*Anopheles meraukensis*)		Australia	cattle, wallabies, dogs	[68]
Manzanilla orthobunyavirus	Manzanilla virus (MANV)	1954	Trinidad	Howler monkey	mosquitoes (*Culex tritaeniorhynchus*), biting midges		Central America, Asia		[69]
	Inini virus (INIV)	1973	French Guaiana	Aracari bird	biting midges		South America	birds	[70]
Mermet orthobunyavirus	Mermet virus (MERV)	1964	USA	purple martin	mosquitoes (*Culex* spp.)		North America	birds	[71]
Oropouche orthobunyavirus	Iquitos virus (IQTV)	1999	Peru	human	midges		South America	humans	[72]
	Madre de Dios virus (MDDV)	2007	Peru	human	mosquitoes, biting midges		South America	humans, monkeys	[73]
	Oropouche virus (OROV)	1955	Trinidad	human	mosquitoes (*Aedes* spp., *Coquillettidia venezuelensis, Culex quinquefasciatus*), biting midges	*C. paraensis*	Central/South America	sloths, non-human primates, rodents, birds, humans	[74]
	Perdões virus (PDEV)	2012	Brazil	black-tufted marmoset	biting midges		South America	non-human primates	[75]
	Pintupo virus (PINTV)		Panama	sloth	biting midges		Central America	sloths	[76]
Peaton orthobunyavirus	Peaton virus (PEAV)	1976	Australia	*Culicoïdes* spp.	biting midges (*C. brevitarsis, C. imicola, C. jacobsoni*)		Australia, Asia	ruminants, horses	[77]
Sabo orthobunyavirus	Sabo virus (SABOV)	1966	Nigeria	goat	biting midges		Africa	goat	[60]
Sango orthobunyavirus	Sango virus (SANV)	1965	Nigeria	cattle	mosquitoes, biting midges		Africa	cattle	[60]
Schmallenberg orthobunyavirus	Douglas virus (DOUV)	1978	Australia	cattle	biting midges (*C. brevitarsis*)		Australia/Oceania	ruminants	[59]
	Sathuperi virus (SATV)	1957	India	mosquitoes	mosquitoes (*Culex vishnui*), biting midges (*C. oxystoma*)		Asia, Africa	ruminants	[78]
	Schmallenberg virus (SBV)	2011	Germany	cattle	biting midges	biting midges (*C. obsoletus, C. scoticus, C. chiopterus, C. imicola, C. sonorensis* [1])	Europe	ruminants	[79]
Shuni orthobunyavirus	Shamonda virus (SHAV)	1965	Nigeria	cattle	biting midges		Africa, Asia	ruminants	[60]
	Kaikalur virus (KAIV)	1971	India	mosquitoes	mosquitoes		Asia	ruminants	[80]
	Shuni virus (SHUV)	1966	Nigeria	cattle	mosquitoes (*Culex theileri*), biting midges	biting midges (*C. nubeculosus* [1], *C. sonorensis* [1])	Africa, Asia	ruminants, horse, (humans?)	[60]
Simbu orthobunyavirus	Para virus (PARAV)	1955	Argentina	mosquitoes	mosquitoes		South America		[81]
	Simbu virus (SIMV)	1955	South Africa	mosquitoes	mosquitoes (*Aedes* spp.), biting midges		Africa		[82]
Thimiri orthobunyavirus	Thimiri virus (THIV)	1963	India	Indian Pond Heron	biting midges (*C. histrio*)		Asia	birds	[83]
Utinga orthobunyavirus	Utinga virus (UTIV)	1965	Brazil	brown-throated sloth	?		South America	sloths	[84]

[1] laboratory-adapted species, not naturally found in areas where the respective virus circulates.

5.1.1. AKAV

AKAV was first described in the 1950s in Japan and is now endemic in large parts of Asia, the Middle East, Australia and Africa [34,57,85,86]. The virus may induce abnormal courses of pregnancy and fetal malformation in ruminants as described for both AKAV and SBV [34]. In addition, some strains might occasionally cause encephalitis in newborn calves. The Iriki strain, which is present in Japan and Korea, has been in rare cases associated with encephalitis in adult cattle [87–89].

Although AKAV was initially isolated from mosquitoes, they do not seem to play an important epidemiological role in virus transmission [90]. In contrast, depending on geographical regions, various *Culicoides* species are considered the main vectors and responsible for virus spread.

For Australia, the assumed main vector is *C. brevitarsis* [91,92], since it was demonstrated that AKAV replicates in *C. brevitarsis* to high virus titers and reaches the salivary glands after 10 days of incubation [93]. Furthermore, the virus has been isolated from *C. wadai* in Australia [94]. In Japan, *C. oxystoma* is considered the major vector [51,95], although AKAV was also isolated from the mosquito species *Aedes vexans* and *Culex tritaeniorhynchus* [58], which are most likely no competent vectors. In the Middle East, *C. imicola* is probably the main vector responsible for virus circulation. In Israel, AKAV was repeatedly detected in this vector species by RT-PCR, and in Oman, the isolation of AKAV was possible from this culicoid species [96,97]. Also, throughout Africa, *C. imicola* seems to be an important vector of AKAV. The virus was isolated multiple times from *C. imicola* in Zimbabwe [98], and in South Africa AKAV was detected in a pool of mixed *Culicoides* spp. mainly consisting of *C. imicola* [99]. Another vector could be *C. milnei*, as AKAV was isolated from this species in Zimbabwe [98]. In Kenia, AKAV was isolated from *Anopheles funestus* mosquitoes [100], but true vector competence has not been demonstrated yet.

To investigate the vector competence of midges for AKAV, some experimental infection studies were carried out with different *Culicoides* species. It could be demonstrated that AKAV replicates in *C. variipennis* after oral infection for at least 9 days, while in *C. nubeculosus*, AKAV replicated only after intrathoracic inoculation [101]. The extensive replication of AKAV within *C. variipennis* suggests that *Culicoides* spp. can act as fully competent vectors. However, although *C. variipennis* serving as a suitable surrogate model, this laboratory-adapted species is not the natural vector of the virus, as it is native to North America while AKAV is endemic in Asia, Africa, and Australia.

5.1.2. SBV

SBV emerged in late 2011 in the German/Dutch border region [79] and is now endemic in most European countries [50,102]. It predominantly infects ruminants, causing a mild, transient disease in adult animals, but it may induce severe fetal malformation when naïve dams are infected during a critical period of pregnancy [103]. Based on its close relationship to AKAV, it was assumed that *Culicoides* act as vectors also for SBV and, indeed, SBV genome was subsequently detected in field-collected *Culicoides* midges of various species repeatedly throughout Europe [104–108]. In contrast, mosquitoes do not seem to play a major role, if any, in virus transmission [109–112].

In temperate European countries, biting midges of the *C. obsoletus* complex are considered the main vectors, while in Mediterranean countries *C. imicola* and *C. punctatus* seem to significantly contribute to virus transmission. Viral genome was also detected in *C. dewulfi, C. pulicaris, C. newsteadi, C. lupicaris,* and *C. nubeculosus* by (real-time) RT-PCR [104–108,112–114]. To underpin the findings in field-collected biting midges with experimental data, several vector competence studies were carried out. The Nearctic species *C. sonorensis* was infected orally and intrathoracically. The detection of viral genome after an extrinsic incubation period in the saliva and the isolation of infectious virus from the head proved dissemination of SBV within the insect organism [115] and demonstrated that *C. sonorensis* is a suitable laboratory model for SBV. Furthermore, from these infected individuals, a range of Cq-values from decapitated heads (including salivary glands) was available to compare with those produced from heads of field-collected *Culicoides*. The values provided for field-collected *C. obsoletus, C. scoticus* and *C. chiopterus* from the Netherlands were very similar to those obtained from

the laboratory infections, indicating that these species act as true vectors [115]. In contrast, multiple infection studies with laboratory colonies of *C. nubeculosus* showed only low infection rates, although viral genome was detected in field-collected *C. nubeculosus* [112,115].

Unfortunately, for the *C. obsoletus* complex, which consists of the species *C. obsoletus*, *C. scoticus*, *C. chiopterus* and *C. montanus* and is considered to contain the major vectors of SBV in temperate European countries, laboratory colonies are not available. However, experimental laboratory infection studies were carried out with field-collected individuals of the *C. obsoletus* complex and with *C. imicola*, which, according to field data, is considered the main vector in the Mediterranean. Both, i.e., midges of the *C. obsoletus* complex and *C. imicola*, showed high infection rates and virus dissemination [116], confirming the assumptions of their vector competence, and field-collected *C. scoticus* were able to replicate SBV to a potentially transmissible level [112].

5.1.3. SHUV

SHUV was firstly isolated in Nigeria from healthy cattle [60] as well as from *Culicoides* biting midges and mosquitoes in Africa [117]. In 2014, SHUV was for the first time detected outside of Africa, and was isolated from malformed lambs in Israel [118]. In addition to typical Simbu virus-related congenital defects in cattle and sheep, SHUV may occasionally induce severe neurological symptoms in horses or cattle [119,120]. A general zoonotic potential cannot be ruled out, since SHUV was isolated from a febrile child in Nigeria [121], and specific antibodies were found in large animal veterinarians in South Africa [122].

In South Africa, SHUV was recovered twice from pools of *Culex theileri* [117]. However, the mosquito taxa *Culex pipiens* biotype *pipiens* and *Aedes aegypti* showed only very low susceptibility to SHUV following intrathoracic inoculation. Oral infection was not possible under experimental conditions [9]. In contrast, *C. nubeculosus* and *C. sonorensis* could be orally infected, with the virus disseminating well in both species [9], indicating that *Culicoides* biting midges are more likely the natural vectors than mosquitoes.

5.1.4. OROV

First discovered in a febrile forest worker in 1955 in Trinidad [74], OROV is now endemic in many South and Central American countries [123,124]. Oropouche fever is an acute febrile illness that affects humans. Common symptoms include fever, headache, muscle pain and skin rash, and many infections develop into meningitis or encephalitis [123]. With this zoonotic potential, OROV protrudes from the other viruses in the Simbu serogroup. In South and Central America, OROV occurs in a sylvatic cycle between its insect vectors, some wild bird species and mammals such as rodents, sloths and non-human primates as amplifying hosts (Table 1). In the urban cycle, humans are most likely the only vertebrate hosts, as domestic animals such as chickens, dogs or cats could be excluded as amplifying hosts [124,125].

The main vector in the sylvatic cycle is still unclear; however, OROV was isolated from two sylvatic mosquitoes in the forest: *Aedes serratus*, collected in the Brazilian Amazon region, and *Coquillettidia venezuelensis* in Trinidad [125,126].

In the urban cycle, the biting midge species *C. paraensis* and the mosquito *Culex quinquefasciatus* are believed to be the main vectors. Although the isolation rate from *C. paraensis* during epidemics is low, its involvement as a vector is suggested based on transmission studies, where *C. paraensis* transmitted OROV to hamsters five or more days after feeding on blood of viremic patients [125].

Likewise, *Culex quinquefasciatus* proved to be an ineffective vector of OROV in laboratory transmission experiments [127], but virus is frequently detected in this species during outbreaks [128]. The link between the two transmission cycles, i.e., the sylvatic and the urban cycle, are probably humans entering the forests, where they become infected, and returning to urbanized areas [125].

5.1.5. Further Members of the Simbu Serogroup

There are several additional members of veterinary importance within the Simbu serogroup (Table 1), which might induce the typical congenital malformation when naïve pregnant dams are infected. Unfortunately, vector competence studies are missing for most of them. However, in general, certain biting midge species are suspected to be able to transmit several members of the serogroup. As an example, field-collected *C. brevitarsis*, the major vector for AKAV in Australia, were tested positive for further Australian simbuviruses such as Douglas virus (DOUV) or Peaton virus (PEAV) [59,77]. In the Mediterranean and the Middle East, where *C. imicola* is the main vector of SBV or AKAV, also PEAV was found in this species [129], confirming the concept of certain *Culicoides* species transmitting several simbuviruses present in the respective region.

5.2. Further Orthobunyaviruses of Public Health Importance

In the California encephalitis serogroup of the genus *Orthobunyavirus*, several zoonotic agents distributed in North America, Europe, Africa, or Asia can be found. However, for none of them *Culicoides* spp. have been demonstrated to be vector-competent. Since they are transmitted by mosquitoes between their vertebrate hosts, which range from small mammals to ungulates and humans, they are among the viruses that are referred to as "mobovirus" (= mosquito-borne viruses) [130]. Some of the most relevant representatives of zoonotic orthobunyaviruses include LACV, Jamestown Canyon virus (JCV), Keystone virus (KEYV) and Ťahyňa virus (TAHV).

LACV, the most pathogenic agent within the California encephalitis serogroup, is the causative agent of the so-called "rural encephalitis" in the Appalachian and midwestern regions of North America and the leading cause of pediatric arboviral encephalitis. Clinical symptoms of an LACV-infection include headache, fever, myalgia, and encephalitis, while in rare cases fatalities may occur (reviewed in [131]). Within the natural infection cycle between small mammals and mosquito vectors, humans are considered as dead-end hosts, meaning that the virus is not transmitted from humans to blood-feeding insects and that, consequently, the transmission chain ends in human beings [132]. As LACV was isolated multiple times from *Aedes triseriatus* [133–135] this mosquito species, which is native to North America, is considered the main insect vector. Furthermore, LACV has been isolated from the invasive Asian tiger mosquito *Aedes albopictus* [136,137] and the Asian bush mosquito *Aedes japonicus* [138,139]. In experimental infection studies, both proved to be competent vectors of LACV [140,141]. To date, this virus has not been found in midges, suggesting that these do not play an important role in transmission.

Similar to LACV, JCV has a wide geographical distribution throughout North America and causes a mild febrile illness or central nervous system infections inducing meningitis or meningoencephalitis in humans [142]. However, in contrast to LACV, which is associated with clinical disease in children, JCV induces symptoms also in adults. White-tailed deer and other ungulates are considered the primary vertebrate hosts [143]. The first virus isolation succeeded from a pool of *Aedes abserratus* [144]. Since then, JCV has been found in varying but great numbers of different species of Culicidae (*Aedes* spp., *Anopheles* spp., *Culiseta* spp., *Psorophora* spp.) and Tabanidae (*Chrysops* spp., *Hybomitra* spp.) [145]. In the US state of Connecticut, the predominant vector species seems to be *Aedes canadensis*, while in northeastern New York and in Michigan, JCV is predominantly transmitted by *Aedes provocans* [146,147]. *Aedes intrudens*, *Aedes abserratus* and *Aedes punctor* seem to be important vectors in Massachusetts [148], *Aedes stimulans* in northern Indiana [149], and *Aedes vexans* in North Dakota [150].

KEYV is distributed on the east coast of North America from Florida, where the virus was firstly discovered in 1964 from *Aedes atlanticus* in Keystone [151], up to Maryland in the north and Texas in the west. Vertebrate hosts seem to be mammals such as squirrels, raccoons, and white-tailed deer [152–154], while the main insect vectors are mosquitoes such as *Aedes atlanticus* [155], various *Aedes* spp. (e.g., *vexans*, *tiseriatus*, *taeniorhynchus*, *infirmatus*, *canadensis*), and some *Culex* spp. [156]. In humans, antibodies against KEYV have been repeatedly detected [157], and the virus itself was isolated from individuals showing clinical symptoms such as fever and rash [158].

TAHV is endemic in Asia and throughout Africa and represents the first mobovirus pathogenic to humans found in Europe [130]. It may induce a febrile illness referred to as Valtice fever, which occurs mainly in children and is characterized by influenza-like symptoms. In rare cases, it may cause meningitis or atypical pneumonia [159]. Vertebrate hosts other than humans are small mammals such as rabbits or hedgehogs [160,161]. Arthropod vectors are most likely mosquitoes of the genera *Aedes*, *Culex*, and *Anopheles*; however, experimental vector competence studies are again missing. Originally, TAHV was isolated from *Aedes vexans* and *Aedes caspius* mosquitoes in the villages Ťahyňa and Križany, Czechoslovakia [162]. Following its initial discovery, TAHV has been isolated from several mosquito species such as *Aedes vexans*, *Aedes cantans*, *Aedes caspius*, *Aedes sticticus*, *Aedes diantaeus*, *Aedes hexodontus*, *Culex pipien*s and *Anopheles hyrcanus* throughout Europe [163–169].

6. Responses to *Culicoides*-borne Arbovirus Incursions

Since treatment options are not available for *Culicoides*-borne arbovirus infections, other possibilities are discussed to prevent clinical disease in vertebrate hosts and virus spread into unaffected regions. Trading and movement restrictions are put in place for livestock and their products (e.g., semen, embryos) to reduce the risk of virus introductions into new areas [22,103]. For *Culicoides*-borne diseases with teratogenic effects such as AKAV, SBV, or SHUV, livestock-management measures could be effective. The mating period of livestock could be adjusted to avoid that naïve females are in the critical phase of pregnancy during the season of the highest biting midge activity. Another possibility is to ensure that dams acquire immunity before they conceive for the first time, which could be achieved by exposing the youngstock to potentially infected vectors. However, this concept requires a high number of infected insect organisms every year and a very high transmission rate of the virus from the vector to the vertebrate host to ensure that every young animal is bitten and infected. Hence, a much more reliable method to acquire immunity is by vaccination [50]. For some of the livestock-affecting simbuviruses including AKAV, AINOV, and SBV, vaccines are commercially available [170–173], and European experiences during the bluetongue outbreak from 2006 to 2009 demonstrated that vaccination campaigns against a *Culicoides*-borne disease can play a major role in reducing virus circulation or even in eradicating the disease from a given region [174,175]. For the zoonotic OROV, however, there is no vaccine available yet [176].

From the entomological point of view, the application of insecticides or repellents could be taken into consideration to prevent vectors from biting susceptible animals. In addition, these could be housed in insect-proof (e.g., screened) stable buildings. However, most of these measures seem unhandy, expensive, impracticable and have either none or only limited effect [177,178]. For humans with transient exposure, the use of repellents is a suitable measure to protect themselves from insect bites; *N*,*N*-diethyl-m-toluamide (DEET) is considered the gold standard repellent [177,179].

In addition, measures controlling vector development should be considered. These may include environmental interventions to remove larval breeding sites and the control of adult midges by residual insecticide spraying of surfaces where adult biting midges rest within animal stables. However, due to the broad range of habitats and breeding sites used by biting midges, insecticide treatment and removal of breeding sites are of limited success in biting midge control [177].

In conclusion, *Culicoides*-borne diseases are difficult to control by vector management alone, while vaccinating livestock may represents an effective tool for disease prevention or even for eradicating a disease from a region.

7. Concluding Remarks

For veterinary medicine and veterinary public health, *Culicoides* biting midges are of great importance as they transmit a multitude of viruses of great impact. In contrast, they only seem to play a minor role for human public health. Due to their potential to contribute to unexpected disease emergence they should be thoroughly surveyed. Multiple detections of novel viruses or introductions

of known pathogens into previously unaffected regions during the past decade demonstrate in an impressive fashion that arbovirus outbreaks are hard to predict in space and time [180].

From the genus *Orthobunyavirus*, the emergence of SBV in Europe or the incursion of SHUV into the Middle East are prominent examples [79,118]. To be prepared for putative future outbreaks of arboviral diseases, it is critical to carry out profound research and surveillance on biting midges and their role as vectors.

Author Contributions: Conceptualization, M.B. and K.W.; methodology, F.S. and K.W.; writing—original draft preparation, F.S. and K.W.; writing–review and editing, H.K. and M.B; visualization, F.S. and K.W.; all authors read and approved the final version of the manuscript.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

References

1. Baud, D.; Gubler, D.J.; Schaub, B.; Lanteri, M.C.; Musso, D. An update on Zika virus infection. *Lancet* **2017**, *390*, 2099–2109. [CrossRef]

2. Petersen, L.R.; Brault, A.C.; Nasci, R.S. West Nile virus: Review of the literature. *JAMA* **2013**, *310*, 308–315. [CrossRef]

3. Young, P.R. Arboviruses: A family on the move. *Adv. Exp. Med. Biol.* **2018**, *1062*, 1–10. [PubMed]

4. Althouse, B.M.; Hanley, K.A. The tortoise or the hare? Impacts of within-host dynamics on transmission success of arthropod-borne viruses. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **2015**, *370*, 20140299. [CrossRef] [PubMed]

5. Medlock, J.M.; Leach, S.A. Effect of climate change on vector-borne disease risk in the UK. *Lancet Infect. Dis.* **2015**, *15*, 721–730. [CrossRef]

6. Hubálek, Z.; Rudolf, I.; Nowotny, N. Arboviruses pathogenic for domestic and wild animals. *Adv. Virus Res.* **2014**, *89*, 201–275.

7. ICTV. Virus Taxonomy: The Classification and Nomenclature of Viruses. Available online: https://talk.ictvonline.org/ictv-reports/ictv_online_report/ (accessed on 25 March 2019).

8. Beerntsen, B.T.; James, A.A.; Christensen, B.M. Genetics of mosquito vector competence. *Microbiol. Mol. Biol. Rev.* **2000**, *64*, 115–137. [CrossRef] [PubMed]

9. Möhlmann, T.W.R.; Oymans, J.; Wichgers Schreur, P.J.; Koenraadt, C.J.M.; Kortekaas, J.; Vogels, C.B.F. Vector competence of biting midges and mosquitoes for Shuni virus. *PLoS Negl. Trop. Dis.* **2018**, *12*, e0006993. [CrossRef]

10. Borkent, A. Numbers of Extant and Fossil Species of Ceratopogonidae. 6 July 2016. Available online: https://www.inhs.illinois.edu/files/4014/6785/5847/WorldCatalogtaxa.pdf (accessed on 25 March 2019).

11. Mellor, P.S.; Boorman, J.; Baylis, M. *Culicoides* biting midges: Their role as arbovirus vectors. *Annu. Rev. Entomol.* **2000**, *45*, 307–340. [CrossRef]

12. Mathieu, B.; Cêtre-Sossah, C.; Garros, C.; Chavernac, D.; Balenghien, T.; Carpenter, S.; Setier-Rio, M.L.; Vignes-Lebbe, R.; Ung, V.; Candolfi, E.; et al. Development and validation of IIKC: An interactive identification key for *Culicoides* (Diptera: Ceratopogonidae) females from the western Palaearctic region. *Parasit. Vectors* **2012**, *5*, 137. [CrossRef]

13. Pages, N.; Sarto, I.M.V. Differentiation of *Culicoides obsoletus* and *Culicoides scoticus* (Diptera: Ceratopogonidae) based on mitochondrial cytochrome oxidase subunit I. *J. Med. Entomol.* **2005**, *42*, 1026–1034. [CrossRef]

14. Dallas, J.F.; Cruickshank, R.H.; Linton, Y.M.; Nolan, D.V.; Patakakis, M.; Braverman, Y.; Capela, R.; Capela, M.; Pena, I.; Meiswinkel, R.; et al. Phylogenetic status and matrilineal structure of the biting midge, *Culicoides imicola*, in Portugal, Rhodes and Israel. *Med. Vet. Entomol.* **2003**, *17*, 379–387. [CrossRef]

15. Cêtre-Sossah, C.; Mathieu, B.; Setier-Rio, M.L.; Grillet, C.; Baldet, T.; Delécolle, J.C.; Albina, E. Development and evaluation of a real-time quantitative PCR assay for *Culicoides imicola*, one of the main vectors of bluetongue (BT) and African horse sickness (AHS) in Africa and Europe. *Res. Vet. Sci.* **2008**, *85*, 372–382. [CrossRef]

16. Wenk, C.E.; Kaufmann, C.; Schaffner, F.; Mathis, A. Molecular characterization of Swiss Ceratopogonidae (Diptera) and evaluation of real-time PCR assays for the identification of *Culicoides* biting midges. *Vet. Parasitol.* **2012**, *184*, 258–266. [CrossRef]

17. Deblauwe, I.; de Witte, J.C.; de Deken, G.; de Deken, R.; Madder, M.; van Erk, S.; Hoza, F.A.; Lathouwers, D.; Geysen, D. A new tool for the molecular identification of *Culicoides* species of the Obsoletus group: The glass slide microarray approach. *Med. Vet. Entomol.* **2012**, *26*, 83–91. [CrossRef]

18. Uhlmann, K.R.; Gibb, S.; Kalkhof, S.; Arroyo-Abad, U.; Schulz, C.; Hoffmann, B.; Stubbins, F.; Carpenter, S.; Beer, M.; von Bergen, M.; et al. Species determination of *Culicoides* biting midges via peptide profiling using matrix-assisted laser desorption ionization mass spectrometry. *Parasit. Vectors* **2014**, *7*, 392. [CrossRef]

19. Wirth, W.W.; Hubert, A.A. The *Culicoides* of Southeast Asia (Diptera: Ceratopogonidae). *Am. Entomol. Inst. (USA)* **1989**, *44*, 514.

20. Kline, D.L.; Axtell, R.C. Salt-marsh *Culicoides* (Diptera—Ceratopogonidae)—Species, seasonal abundance and comparisons of trapping methods. *Mosq. News* **1976**, *36*, 1–10.

21. Van der Rijt, R.; van den Boom, R.; Jongema, Y.; van Oldruitenborgh-Oosterbaan, M.M. *Culicoides* species attracted to horses with and without insect hypersensitivity. *Vet. J.* **2008**, *178*, 91–97. [CrossRef]

22. Carpenter, S.; Groschup, M.H.; Garros, C.; Felippe-Bauer, M.L.; Purse, B.V. *Culicoides* biting midges, arboviruses and public health in Europe. *Antiviral Res.* **2013**, *100*, 102–113. [CrossRef]

23. Veiga, J.; Martinez-de la Puente, J.; Vaclav, R.; Figuerola, J.; Valera, F. *Culicoides paolae* and *C. circumscriptus* as potential vectors of avian haemosporidians in an arid ecosystem. *Parasit. Vectors* **2018**, *11*, 524. [CrossRef]

24. Chagas, C.R.F.; Bukauskaite, D.; Ilgunas, M.; Iezhova, T.; Valkiunas, G. A new blood parasite of leaf warblers: Molecular characterization, phylogenetic relationships, description and identification of vectors. *Parasit. Vectors* **2018**, *11*, 538. [CrossRef]

25. Yates, J.A.; Lowrie, R.C., Jr.; Eberhard, M. Development of *Tetrapetalonema llewellyni* to the infective stage in *Culicoides hollensis*. *J. Parasitol.* **1982**, *68*, 293–296. [CrossRef]

26. Lowrie, R.C., Jr.; Eberhard, M.L.; Orihel, T.C. Development of *Tetrapetalonema marmosetae* to the infective stage in *Culicoides hollensis* and *C. furens*. *J. Parasitol.* **1978**, *64*, 1003–1007. [CrossRef]

27. Linley, J.R. Biting midges (Diptera: Ceratopogonidae) as vectors of nonviral animal pathogens. *J. Med. Entomol.* **1985**, *22*, 589–599. [CrossRef]

28. Meiswinkel, R.; Nevill, E.M.; Venter, G.J. Vectors: *Culicoides* spp. In *Infectious Diseases of Livestock with Special Reference to Southern Africa*; Coetzer, J.A.W., Thomson, G.R., Tustin, R.C., Eds.; Oxford University Press: Cape Town, South Africa, 1994; Volume 1, pp. 68–89.

29. Carpenter, S.; Veronesi, E.; Mullens, B.; Venter, G. Vector competence of *Culicoides* for arboviruses: Three major periods of research, their influence on current studies and future directions. *Rev. Sci. Tech.* **2015**, *34*, 97–112. [CrossRef]

30. Jennings, M.; Platt, G.S.; Bowen, E.T. The susceptibility of *Culicoides variipennis* Coq. (Diptera: Ceratopogonidae) to laboratory infection with Rift Valley fever virus. *Trans. R. Soc. Trop. Med. Hyg.* **1982**, *76*, 587–589. [CrossRef]

31. Carpenter, S.; Szmaragd, C.; Barber, J.; Labuschagne, K.; Gubbins, S.; Mellor, P. An assessment of *Culicoides* surveillance techniques in northern Europe: Have we underestimated a potential bluetongue virus vector? *J. Appl. Ecol.* **2008**, *45*, 1237–1245.

32. Murray, M.D. Local dispersal of the biting midge *Culicoides brevitarsis Kieffer* (Diptera, *Ceratopogonidae*) in Southeastern Australia. *Austr. J. Zool.* **1987**, *35*, 559–573. [CrossRef]

33. Murray, M.D.; Nix, H.A. Southern limits of distribution and abundance of the biting-midge *Culicoides brevitarsis Kieffer* (Diptera, *Ceratopogonidae*) in Southeastern Australia—An application of the Growest Model. *Austr. J. Zool.* **1987**, *35*, 575–585. [CrossRef]

34. Kirkland, P.D. Akabane virus infection. *Rev. Sci. Tech.* **2015**, *34*, 403–410. [CrossRef]

35. Sellers, R.F. Weather, host and vector—Their interplay in the spread of insect-borne animal virus diseases. *J. Hyg. (London)* **1980**, *85*, 65–102. [CrossRef]

36. Sellers, R.F.; Maarouf, A.R. Possible introduction of epizootic hemorrhagic disease of deer virus (serotype 2) and bluetongue virus (serotype 11) into British Columbia in 1987 and 1988 by infected *Culicoides* carried on the wind. *Can. J. Vet. Res.* **1991**, *55*, 367–370.

37. Sellers, R.F.; Pedgley, D.E. Possible windborne spread to western Turkey of bluetongue virus in 1977 and of Akabane virus in 1979. *J. Hyg. (London)* **1985**, *95*, 149–158. [CrossRef]

38. Braverman, Y.; Chechik, F. Air streams and the introduction of animal diseases borne on *Culicoides* (Diptera, Ceratopogonidae) into Israel. *Rev. Sci. Tech.* **1996**, *15*, 1037–1052. [CrossRef]

39. Hunt, G.J. *A Procedural Manual for the Large-Scale Rearing of the Biting Midge, Culicoides variipennis (Diptera: Ceratopogonidae)*; U.S. Department of Agriculture, National Agricultural Library (USA): Beltsville, MD, USA, 1994.

40. Carpenter, S.; Mordue, A.J.; Mordue, W. Oviposition in *Culicoides impunctatus* under laboratory conditions. *Entomol. Exp. Appl.* **2001**, *101*, 123–129. [CrossRef]

41. Jones, R.H.; Foster, N.M. Relevance of laboratory colonies of the vector in arbovirus research—*Culicoides variipennis* and bluetongue. *Am. J. Trop. Med. Hyg.* **1978**, *27*, 168–177. [CrossRef]

42. Maes, P.; Alkhovsky, S.V.; Bao, Y.; Beer, M.; Birkhead, M.; Briese, T.; Buchmeier, M.J.; Calisher, C.H.; Charrel, R.N.; Choi, I.R.; et al. Taxonomy of the family *Arenaviridae* and the order *Bunyavirales*: update 2018. *Arch. Virol.* **2018**, *163*, 2295–2310. [CrossRef]

43. Barr, J.N.; Walter, C.T. Recent advances in the molecular and cellular biology of bunyaviruses. *J. Gen. Virol.* **2011**, *92*, 2467–2484.

44. Shi, X.; Lappin, D.F.; Elliott, R.M. Mapping the Golgi targeting and retention signal of Bunyamwera virus glycoproteins. *J. Virol.* **2004**, *78*, 10793–10802. [CrossRef]

45. Shi, X.; van Mierlo, J.T.; French, A.; Elliott, R.M. Visualizing the replication cycle of Bunyamwera orthobunyavirus expressing fluorescent protein-tagged Gc glycoprotein. *J. Virol.* **2010**, *84*, 8460–8469. [CrossRef] [PubMed]

46. Shi, X.; Goli, J.; Clark, G.; Brauburger, K.; Elliott, R.M. Functional analysis of the Bunyamwera orthobunyavirus Gc glycoprotein. *J. Gen. Virol.* **2009**, *90*, 2483–2492. [CrossRef]

47. Elliott, R.M. Orthobunyaviruses: Recent genetic and structural insights. *Nat. Rev. Microbiol.* **2014**, *12*, 673–685. [CrossRef] [PubMed]

48. Elliott, R.M.; Blakqori, G. Molecular biology of orthobunyaviruses. In *Bunyaviridae. Molecular and Cellular Biology*; Plyusnin, A., Elliott, R.M., Eds.; Caister Academic Press: Norfolk, UK, 2011; pp. 1–39.

49. Shi, X.; Kohl, A.; Léonard, V.H.J.; Li, P.; McLees, A.; Elliott, R.M. Requirement of the N-terminal region of *Orthobunyavirus* nonstructural protein NSm for virus assembly and morphogenesis. *J. Virol.* **2006**, *80*, 8089. [CrossRef]

50. Wernike, K.; Beer, M. Schmallenberg virus: A novel virus of veterinary importance. *Adv. Virus Res.* **2017**, *99*, 39–60. [PubMed]

51. Kato, T.; Shirafuji, H.; Tanaka, S.; Sato, M.; Yamakawa, M.; Tsuda, T.; Yanase, T. Bovine arboviruses in *Culicoides* biting midges and sentinel cattle in southern Japan from 2003 to 2013. *Transbound. Emerg. Dis.* **2016**, *63*, e160–e172. [CrossRef]

52. Kato, T.; Yanase, T.; Suzuki, M.; Katagiri, Y.; Ikemiyagi, K.; Takayoshi, K.; Shirafuji, H.; Ohashi, S.; Yoshida, K.; Yamakawa, M.; et al. Monitoring for bovine arboviruses in the most southwestern islands in Japan between 1994 and 2014. *BMC Vet. Res.* **2016**, *12*, 125. [CrossRef]

53. Hayama, Y.; Yanase, T.; Suzuki, M.; Unten, K.; Tomochi, H.; Kakehi, M.; Shono, Y.; Yamamoto, T.; Kobayashi, S.; Murai, K.; et al. Meteorological factors affecting seroconversion of Akabane disease in sentinel calves in the subtropical Okinawa Islands of Japan. *Trop. Anim. Health Prod.* **2018**, *50*, 209–215. [CrossRef]

54. Wernike, K.; Holsteg, M.; Szillat, K.P.; Beer, M. Development of within-herd immunity and long-term persistence of antibodies against Schmallenberg virus in naturally infected cattle. *BMC Vet. Res.* **2018**, *14*, 368. [CrossRef]

55. Geoghegan, J.L.; Walker, P.J.; Duchemin, J.B.; Jeanne, I.; Holmes, E.C. Seasonal drivers of the epidemiology of arthropod-borne viruses in Australia. *PLoS Negl. Trop. Dis.* **2014**, *8*, e3325. [CrossRef]

56. Wernike, K.; Elbers, A.; Beer, M. Schmallenberg virus infection. *Rev. Sci. Tech.* **2015**, *34*, 363–373. [CrossRef]

57. Saeed, M.F.; Li, L.; Wang, H.; Weaver, S.C.; Barrett, A.D. Phylogeny of the Simbu serogroup of the genus *Bunyavirus*. *J. Gen. Virol.* **2001**, *82*, 2173–2181. [CrossRef]

58. Oya, A.; Okuno, T.; Ogata, T.; Kobayashii; Matsuyama, T. Akabane, a new arbovirus isolated in Japan. *Jpn. J. Med. Sci. Biol.* **1961**, *14*, 101–108. [CrossRef]

59. Cybinski, D.H. Douglas and Tinaroo viruses: Two Simbu group arboviruses infecting *Culicoides brevitarsis* and livestock in Australia. *Aust. J. Biol. Sci.* **1984**, *37*, 91–97. [CrossRef]

60. Causey, O.R.; Kemp, G.E.; Causey, C.E.; Lee, V.H. Isolations of Simbu-group viruses in Ibadan, Nigeria 1964-69, including the new types Sango, Shamonda, Sabo and Shuni. *Ann. Trop. Med. Parasitol.* **1972**, *66*, 357–362. [CrossRef]

61. Takahashi, K.; Oya, A.; Okazda, T.; Matsuo, R.; Kuma, M. Aino virus, a new member of Simbu group of arbovirus from mosquitoes in Japan. *Jpn. J. Med. Sci. Biol.* **1968**, *21*, 95–101. [CrossRef]

62. Reeves, W.C.; Scrivani, R.P.; Hardy, J.L.; Roberts, D.R.; Nelson, R.L. Buttonwillow virus, a new Arbovirus isolated from mammals and *Culicoides* midges in Kern County, California. *Am. J. Trop. Med. Hyg.* **1970**, *19*, 544–551. [CrossRef]

63. Bryant, J.E.; Crabtree, M.B.; Nam, V.S.; Yen, N.T.; Duc, H.M.; Miller, B.R. Isolation of arboviruses from mosquitoes collected in northern Vietnam. *Am. J. Trop. Med. Hyg.* **2005**, *73*, 470–473. [CrossRef]

64. Kono, Y.; Yusnita, Y.; Mohd Ali, A.R.; Maizan, M.; Sharifah, S.H.; Fauzia, O.; Kubo, M.; Aziz, A.J. Characterization and identification of Oya virus, a Simbu serogroup virus of the genus Bunyavirus, isolated from a pig suspected of Nipah virus infection. *Arch. Virol.* **2002**, *147*, 1623–1630. [CrossRef]

65. Doherty, R.L.; Carley, J.G.; Kay, B.H.; Filippich, C.; Marks, E.N.; Frazier, C.L. Isolation of virus strains from mosquitoes collected in Queensland, 1972–1976. *Aust. J. Exp. Biol. Med. Sci.* **1979**, *57*, 509–520. [CrossRef]

66. McIntosh, B.M.; McGillivray, G.M.; Dickinson, D.B. Ingwavuma virus: An arbovirus isolated in South Africa. *S. Afr. J. Med. Sci.* **1965**, *30*, 67–70.

67. Figueiredo, L.T.; Da Rosa, A.P. Jatobal virus antigenic characterization by ELISA and neutralization test using EIA as indicator, on tissue culture. *Mem. Inst. Oswaldo Cruz* **1988**, *83*, 161–164. [CrossRef]

68. Doherty, R.L.; Carley, J.G.; Filippich, C.; Kay, B.H.; Gorman, B.M.; Rajapaksa, N. Isolation of Sindbis (alphavirus) and Leanyer viruses from mosquitoes collected in the Northern Territory of Australia, 1974. *Aust. J. Exp. Biol. Med. Sci.* **1977**, *55*, 485–489. [CrossRef]

69. Anderson, C.R.; Spence, L.P.; Downs, W.G.; Aitken, T.H. Manzanilla virus: A new virus isolated from the blood of a howler monkey in Trinidad, W.I. *Am. J. Trop. Med. Hyg.* **1960**, *9*, 78–80. [CrossRef] [PubMed]

70. Pajot, F.X. Diseases transmitted by insects in French Guaiana. *Bol. Oficina Sanit. Panam.* **1980**, *88*, 218–227. (In Spanish) [PubMed]

71. Calisher, C.H.; Kokernot, R.H.; De Moore, J.F.; Boyd, K.R.; Hayes, J.; Chappell, W.A. Arbovirus studies in the Ohio-Mississippi Basin, 1964–1967. VI. Mermet: A Simbu-group arbovirus. *Am. J. Trop. Med. Hyg.* **1969**, *18*, 779–788. [CrossRef] [PubMed]

72. Aguilar, P.V.; Barrett, A.D.; Saeed, M.F.; Watts, D.M.; Russell, K.; Guevara, C.; Ampuero, J.S.; Suarez, L.; Cespedes, M.; Montgomery, J.M.; et al. Iquitos virus: A novel reassortant Orthobunyavirus associated with human illness in Peru. *PLoS Negl. Trop. Dis.* **2011**, *5*, e1315. [CrossRef] [PubMed]

73. Ladner, J.T.; Savji, N.; Lofts, L.; Travassos da Rosa, A.; Wiley, M.R.; Gestole, M.C.; Rosen, G.E.; Guzman, H.; Vasconcelos, P.F.; Nunes, M.R.; et al. Genomic and phylogenetic characterization of viruses included in the Manzanilla and Oropouche species complexes of the genus *Orthobunyavirus*, family *Bunyaviridae*. *J. Gen. Virol.* **2014**, *95*, 1055–1066. [CrossRef] [PubMed]

74. Anderson, C.R.; Spence, L.; Downs, W.G.; Aitken, T.H. Oropouche virus: A new human disease agent from Trinidad, West Indies. *Am. J. Trop. Med. Hyg.* **1961**, *10*, 574–578. [CrossRef]

75. Tilston-Lunel, N.L.; Hughes, J.; Acrani, G.O.; da Silva, D.E.; Azevedo, R.S.; Rodrigues, S.G.; Vasconcelos, P.F.; Nunes, M.R.; Elliott, R.M. Genetic analysis of members of the species Oropouche virus and identification of a novel M segment sequence. *J. Gen. Virol.* **2015**, *96*, 1636–1650. [CrossRef]

76. Seymour, C.; Peralta, P.H.; Montgomery, G.G. Viruses isolated from Panamanian sloths. *Am. J. Trop. Med. Hyg.* **1983**, *32*, 1435–1444. [CrossRef] [PubMed]

77. St George, T.D.; Standfast, H.A.; Cybinski, D.H.; Filippich, C.; Carley, J.G. Peaton virus: A new Simbu group arbovirus isolated from cattle and *Culicoides brevitarsis* in Australia. *Aust. J. Biol. Sci.* **1980**, *33*, 235–243. [CrossRef] [PubMed]

78. Dandawate, C.N.; Rajagopalan, P.K.; Pavri, K.M.; Work, T.H. Virus isolations from mosquitoes collected in North Arcot district, Madras state, and Chittoor district, Andhra Pradesh between November 1955 and October 1957. *Indian J. Exp. Biol.* **1969**, *57*, 1420–1426.

79. Hoffmann, B.; Scheuch, M.; Höper, D.; Jungblut, R.; Holsteg, M.; Schirrmeier, H.; Eschbaumer, M.; Goller, K.V.; Wernike, K.; Fischer, M.; et al. Novel orthobunyavirus in cattle, Europe, 2011. *Emerg. Infect. Dis.* **2012**, *18*, 469–472. [CrossRef] [PubMed]

80. Rodrigues, F.M.; Singh, P.B.; Dandawate, C.N.; Soman, R.S.; Bhatt, P.N. Kaikalur virus—A new arthropod-borne virus belonging to the Simbu group isolated in India from *Culex tritaeniorhynchus* (Giles). *Indian J. Med. Res.* **1977**, *66*, 719–725.

81. Mitchell, C.J.; Monath, T.P.; Sabattini, M.S.; Cropp, C.B.; Daffner, J.F.; Calisher, C.H.; Jakob, W.L.; Christensen, H.A. Arbovirus investigations in Argentina, 1977–1980. II. Arthropod collections and virus isolations from Argentine mosquitoes. *Am. J. Trop. Med. Hyg.* **1985**, *34*, 945–955. [CrossRef] [PubMed]

82. Weinbren, M.P.; Heymann, C.S.; Kokernot, R.H.; Paterson, H.E. Studies on arthropod-borne viruses of Tongaland. VII. Simbu virus, a hitherto unknown agent isolated from *Aedes (Banksinella) circumluteolus* Theo. *S. Afr. J. Med. Sci.* **1957**, *22*, 93–102. [PubMed]

83. Carey, D.E.; Reuben, R.; George, S.; Shope, R.E.; Myers, R.M. Kammavanpettai, Kannamangalam, Sembalam and Thimiri viruses: Four unrelated new agents isolated from birds in India. *Indian J. Med. Res.* **1971**, *59*, 1708–1711.

84. Shope, R.E.; Paes de Andrade, A.H.; Bensabath, G. The serological response of animals to virus infection in Utinga Forest, Belem, Brazil. *Atas Simp* **1967**, *243*, 225–230.

85. Oğuzoğlu, T.C.; Toplu, N.; Koc, B.T.; Dogan, F.; Epikmen, E.T.; Ipek, E.; Akkoc, A.N. First molecular detection and characterization of Akabane virus in small ruminants in Turkey. *Arch. Virol.* **2015**, *160*, 2623–2627. [CrossRef]

86. Golender, N.; Bumbarov, V.Y.; Erster, O.; Beer, M.; Khinich, Y.; Wernike, K. Development and validation of a universal S-segment-based real-time RT-PCR assay for the detection of Simbu serogroup viruses. *J. Virol. Methods* **2018**, *261*, 80–85. [CrossRef]

87. Oem, J.K.; Lee, K.H.; Kim, H.R.; Bae, Y.C.; Chung, J.Y.; Lee, O.S.; Roh, I.S. Bovine epizootic encephalomyelitis caused by Akabane virus infection in Korea. *J. Comp. Pathol.* **2012**, *147*, 101–105. [CrossRef]

88. Oem, J.K.; Yoon, H.J.; Kim, H.R.; Roh, I.S.; Lee, K.H.; Lee, O.S.; Bae, Y.C. Genetic and pathogenic characterization of Akabane viruses isolated from cattle with encephalomyelitis in Korea. *Vet. Microbiol.* **2012**, *158*, 259–266. [CrossRef]

89. Miyazato, S.; Miura, Y.; Hase, M.; Kubo, M.; Goto, Y.; Kono, Y. Encephalitis of cattle caused by Iriki isolate, a new strain belonging to Akabane virus. *Nihon Juigaku Zasshi* **1989**, *51*, 128–136. [CrossRef]

90. Charles, J.A. Akabane virus. *Vet. Clin. North Am. Food Anim. Pract.* **1994**, *10*, 525–546. [CrossRef]

91. St George, T.D.; Standfast, H.A.; Cybinski, D.H. Isolations of Akabane virus from sentinel cattle and *Culicoides brevitarsis*. *Aust. Vet. J.* **1978**, *54*, 558–561. [CrossRef]

92. Cybinski, D.H.; St George, T.D.; Paull, N.I. Antibodies to Akabane virus in Australia. *Aust. Vet. J.* **1978**, *54*, 1–3. [CrossRef]

93. St George, T.D.; Standfast, H.A. Diseases caused by Akabane and related Simbu-group viruses. In *Infectious Diseases of Livestock with Special Reference to Southern Africa*; Coetzer, J.A.W., Thomson, G.R., Tustin, R.C., Eds.; Oxford University Press: Cape Town, South Africa, 1994; Volume 1, pp. 681–687.

94. Cybinski, D.H.; Muller, M.J. Isolation of arboviruses from cattle and insects at two sentinel sites in Queensland, Australia, 1979–1985. *Austr. J. Zool.* **1990**, *38*, 25–32. [CrossRef]

95. Kurogi, H.; Akiba, K.; Inaba, Y.; Matumoto, M. Isolation of Akabane virus from the biting midge *Culicoides oxystoma* in Japan. *Vet. Microbiol.* **1987**, *15*, 243–248. [CrossRef]

96. Stram, Y.; Brenner, J.; Braverman, Y.; Banet-Noach, C.; Kuznetzova, L.; Ginni, M. Akabane virus in Israel: A new virus lineage. *Virus Res.* **2004**, *104*, 93–97. [CrossRef]

97. Al-Busaidy, S.M.; Mellor, P.S. Isolation and identification of arboviruses from the Sultanate of Oman. *Epidemiol. Infect.* **1991**, *106*, 403–413. [CrossRef]

98. Blackburn, N.K.; Searle, L.; Phelps, R.J. Viruses isolated from *Culicoides* (Diptera: Ceratopogonidae) caught at the veterinary research farm, Mazowe, Zimbabwe. *J. Entomol. Soc. South. Afr.* **1958**, *48*, 331–336.

99. Theodoridis, A.; Nevill, E.M.; Els, H.J.; Boshoff, S.T. Viruses isolated from *Culicoides* midges in South Africa during unsuccessful attempts to isolate bovine ephemeral fever virus. *Onderstepoort J. Vet. Res.* **1979**, *46*, 191–198.

100. Metselaar, D.; Robin, Y. Akabane virus isolated in Kenya. *Vet. Rec.* **1976**, *99*, 86. [CrossRef] [PubMed]

101. Jennings, M.; Mellor, P.S. *Culicoides*: Biological vectors of Akabane virus. *Vet. Microbiol.* **1989**, *21*, 125–131. [CrossRef]

102. EFSA. "Schmallenberg" Virus: Analysis of the Epidemiological Data (May 2013). EFSA Supporting Publications 2013. EN-3429. Available online: http://www.efsa.europa.eu/de/supporting/doc/429e.pdf (accessed on 15 July 2013).

103. EFSA. Schmallenberg virus: State of Art. *EFSA J.* **2014**, *12*, 54.

104. De Regge, N.; Deblauwe, I.; De Deken, R.; Vantieghem, P.; Madder, M.; Geysen, D.; Smeets, F.; Losson, B.; van den Berg, T.; Cay, A.B. Detection of Schmallenberg virus in different *Culicoides* spp. by real-time RT-PCR. *Transbound. Emerg. Dis.* **2012**, *59*, 471–475. [CrossRef]

105. Rasmussen, L.D.; Kristensen, B.; Kirkeby, C.; Rasmussen, T.B.; Belsham, G.J.; Bodker, R.; Bøtner, A. Culicoids as vectors of Schmallenberg virus. *Emerg. Infect. Dis.* **2012**, *18*, 1204–1206. [CrossRef]

106. Goffredo, M.; Monaco, F.; Capelli, G.; Quaglia, M.; Federici, V.; Catalani, M.; Montarsi, F.; Polci, A.; Pinoni, C.; Calistri, P.; et al. Schmallenberg virus in Italy: A retrospective survey in *Culicoides* stored during the bluetongue Italian surveillance program. *Prev. Vet. Med.* **2013**, *111*, 230–236. [CrossRef]

107. Elbers, A.R.; Meiswinkel, R.; van Weezep, E.; Sloet van Oldruitenborgh-Oosterbaan, M.M.; Kooi, E.A. Schmallenberg virus in *Culicoides* spp. biting midges, the Netherlands, 2011. *Emerg. Infect. Dis.* **2013**, *19*, 106–109. [CrossRef]

108. Larska, M.; Polak, M.P.; Grochowska, M.; Lechowski, L.; Zwiazek, J.S.; Zmudzinski, J.F. First report of Schmallenberg virus infection in cattle and midges in Poland. *Transbound. Emerg. Dis.* **2013**, *60*, 97–101. [CrossRef] [PubMed]

109. Wernike, K.; Jöst, H.; Becker, N.; Schmidt-Chanasit, J.; Beer, M. Lack of evidence for the presence of Schmallenberg virus in mosquitoes in Germany, 2011. *Parasit. Vectors* **2014**, *7*, 402. [CrossRef] [PubMed]

110. Manley, R.; Harrup, L.E.; Veronesi, E.; Stubbins, F.; Stoner, J.; Gubbins, S.; Wilson, A.; Batten, C.; Koenraadt, C.J.; Henstock, M.; et al. Testing of UK populations of *Culex pipiens L.* for Schmallenberg virus vector competence and their colonization. *PLoS ONE* **2015**, *10*, e0134453. [CrossRef] [PubMed]

111. Scholte, E.J.; Mars, M.H.; Braks, M.; Den Hartog, W.; Ibanez-Justicia, A.; Koopmans, M.; Koenraadt, C.J.; De Vries, A.; Reusken, C. No evidence for the persistence of Schmallenberg virus in overwintering mosquitoes. *Med. Vet. Entomol.* **2014**, *28*, 110–115. [CrossRef] [PubMed]

112. Balenghien, T.; Pagès, N.; Goffredo, M.; Carpenter, S.; Augot, D.; Jacquier, E.; Talavera, S.; Monaco, F.; Depaquit, J.; Grillet, C.; et al. The emergence of Schmallenberg virus across *Culicoides* communities and ecosystems in Europe. *Prev. Vet. Med.* **2014**, *116*, 360–369. [CrossRef] [PubMed]

113. Segard, A.; Gardes, L.; Jacquier, E.; Grillet, C.; Mathieu, B.; Rakotoarivony, I.; Setier-Rio, M.L.; Chavernac, D.; Cetre-Sossah, C.; Balenghien, T.; et al. Schmallenberg virus in *Culicoides Latreille* (Diptera: Ceratopogonidae) populations in France during 2011–2012 outbreak. *Transbound. Emerg. Dis.* **2018**, *65*, e94–e103. [CrossRef] [PubMed]

114. Elbers, A.R.; Meiswinkel, R.; van Weezep, E.; Kooi, E.A.; van der Poel, W.H. Schmallenberg virus in *Culicoides* biting midges in the Netherlands in 2012. *Transbound. Emerg. Dis.* **2015**, *62*, 339–342. [CrossRef] [PubMed]

115. Veronesi, E.; Henstock, M.; Gubbins, S.; Batten, C.; Manley, R.; Barber, J.; Hoffmann, B.; Beer, M.; Attoui, H.; Mertens, P.P.; et al. Implicating *Culicoides* biting midges as vectors of Schmallenberg virus using semi-quantitative RT-PCR. *PLoS ONE* **2013**, *8*, e57747. [CrossRef]

116. Pagès, N.; Talavera, S.; Verdún, M.; Pujol, N.; Valle, M.; Bensaid, A.; Pujols, J. Schmallenberg virus detection in *Culicoides* biting midges in Spain: First laboratory evidence for highly efficient infection of *Culicoides* of the Obsoletus complex and *Culicoides imicola*. *Transbound. Emerg. Dis.* **2018**, *65*, e1–e6. [CrossRef]

117. McIntosh, B.M.; Jupp, P.G.; De Sousa, J. Further isolations of the arboviruses from mosquitoes collected in Tongaland, South Africa, 1960–1968. *J. Med. Entomol.* **1972**, *9*, 155–159. [CrossRef]

118. Golender, N.; Brenner, J.; Valdman, M.; Khinich, Y.; Bumbarov, V.; Panshin, A.; Edery, N.; Pismanik, S.; Behar, A. Malformations caused by Shuni virus in ruminants, Israel, 2014–2015. *Emerg. Infect. Dis.* **2015**, *21*, 2267–2268. [CrossRef]

119. Van Eeden, C.; Williams, J.H.; Gerdes, T.G.; van Wilpe, E.; Viljoen, A.; Swanepoel, R.; Venter, M. Shuni virus as cause of neurologic disease in horses. *Emerg. Infect. Dis.* **2012**, *18*, 318–321. [CrossRef]

120. Golender, N.; Bumbarov, V.; Assis, I.; Beer, M.; Khinich, Y.; Koren, O.; Edery, N.; Eldar, A.; Wernike, K. Shuni virus in Israel: Neurological disease and fatalities in cattle. *Transbound. Emerg. Dis.* **2019**, in press. [CrossRef] [PubMed]

121. Moore, D.L.; Causey, O.R.; Carey, D.E.; Reddy, S.; Cooke, A.R.; Akinkugbe, F.M.; David-West, T.S.; Kemp, G.E. Arthropod-borne viral infections of man in Nigeria, 1964–1970. *Ann. Trop. Med. Parasitol.* **1975**, *69*, 49–64. [CrossRef]

122. Van Eeden, C.; Swanepoel, R.; Venter, M. Antibodies against West Nile and Shuni viruses in veterinarians, South Africa. *Emerg. Infect. Dis.* **2014**, *20*, 1409–1411. [CrossRef] [PubMed]

123. Romero-Alvarez, D.; Escobar, L.E. Oropouche fever, an emergent disease from the Americas. *Microbes Infect.* **2018**, *20*, 135–146. [CrossRef] [PubMed]

124. Travassos da Rosa, J.F.; de Souza, W.M.; Pinheiro, F.P.; Figueiredo, M.L.; Cardoso, J.F.; Acrani, G.O.; Nunes, M.R.T. Oropouche virus: Clinical, epidemiological, and molecular aspects of a neglected Orthobunyavirus. *Am. J. Trop. Med. Hyg.* **2017**, *96*, 1019–1030. [PubMed]

125. Pinheiro, F.P.; Travassos da Rosa, A.P.; Travassos da Rosa, J.F.; Ishak, R.; Freitas, R.B.; Gomes, M.L.; LeDuc, J.W.; Oliva, O.F. Oropouche virus. I. A review of clinical, epidemiological, and ecological findings. *Am. J. Trop. Med. Hyg.* **1981**, *30*, 149–160. [CrossRef] [PubMed]

126. Pinheiro, F.; Travassos da Rosa, A.; Vasconcelos, P.F. Oropouche fever. In *Textbook of Pediatric Infectious Diseases*; Feigin, R.D., Ed.; Saunders: Philadelphia, PA, USA, 2004; pp. 2418–2423.

127. Hoch, A.L.; Pinheiro, F.P.; Roberts, D.R.; Gomes, M.L. Laboratory transmission of Oropouche virus by *Culex quinquefasciatus* Say. *Bull. Pan. Am. Health Organ.* **1987**, *21*, 55–61.

128. Cardoso, B.F.; Serra, O.P.; Heinen, L.B.; Zuchi, N.; Souza, V.C.; Naveca, F.G.; Santos, M.A.; Slhessarenko, R.D. Detection of Oropouche virus segment S in patients and in *Culex quinquefasciatus* in the state of Mato Grosso, Brazil. *Mem. Inst. Oswaldo Cruz* **2015**, *110*, 745–754. [CrossRef]

129. Behar, A.; Leibovich, B.B.; Edery, N.; Yanase, T.; Brenner, J. First genomic detection of Peaton virus in a calf with hydranencephaly in Israel. *Vet. Med. Sci.* **2018**, *5*, 87–92. [CrossRef]

130. Hubalek, Z. Mosquito-borne viruses in Europe. *Parasitol. Res.* **2008**, *103*, 29–43. [CrossRef]

131. Harding, S.; Greig, J.; Mascarenhas, M.; Young, I.; Waddell, L.A. La Crosse virus: A scoping review of the global evidence. *Epidemiol. Infect.* **2018**, 1–13. [CrossRef]

132. Anonymous. La Cross Encephalitis. Available online: https://www.cdc.gov/lac/index.html (accessed on 12 March 2019).

133. Kappus, K.D.; Calisher, C.H.; Baron, R.C.; Davenport, J.; Francy, D.B.; Williams, R.M. La Crosse virus infection and disease in western North Carolina. *Am. J. Trop. Med. Hyg.* **1982**, *31*, 556–560. [CrossRef]

134. Szumlas, D.E.; Apperson, C.S.; Powell, E.E.; Hartig, P.; Francy, D.B.; Karabotsos, N. Relative abundance and species composition of mosquito populations (Diptera: Culicidae) in a La Crosse virus-endemic area in western North Carolina. *J. Med. Entomol.* **1996**, *33*, 598–607. [CrossRef]

135. Miller, B.R.; DeFoliart, G.R.; Yuill, T.M. Vertical transmission of La Crosse virus (California encephalitis group): Transovarial and filial infection rates in *Aedes triseriatus* (Diptera: Culicidae). *J. Med. Entomol.* **1977**, *14*, 437–440. [CrossRef]

136. Gerhardt, R.R.; Gottfried, K.L.; Apperson, C.S.; Davis, B.S.; Erwin, P.C.; Smith, A.B.; Panella, N.A.; Powell, E.E.; Nasci, R.S. First isolation of La Crosse virus from naturally infected *Aedes albopictus*. *Emerg. Infect. Dis.* **2001**, *7*, 807–811. [CrossRef]

137. Lambert, A.J.; Blair, C.D.; D'Anton, M.; Ewing, W.; Harborth, M.; Seiferth, R.; Xiang, J.; Lanciotti, R.S. La Crosse virus in *Aedes albopictus* mosquitoes, Texas, USA, 2009. *Emerg. Infect. Dis.* **2010**, *16*, 856–858. [CrossRef]

138. Westby, K.M.; Fritzen, C.; Paulsen, D.; Poindexter, S.; Moncayo, A.C. La Crosse Encephalitis virus infection in field-collected *Aedes albopictus*, *Aedes japonicus*, and *Aedes triseriatus* in Tennessee. *J. Am. Mosq. Control Assoc.* **2015**, *31*, 233–241. [CrossRef]

139. Harris, M.C.; Dotseth, E.J.; Jackson, B.T.; Zink, S.D.; Marek, P.E.; Kramer, L.D.; Paulson, S.L.; Hawley, D.M. La Crosse virus in *Aedes japonicus japonicus* mosquitoes in the Appalachian region, United States. *Emerg. Infect. Dis.* **2015**, *21*, 646–649. [CrossRef]

140. Grimstad, P.R.; Kobayashi, J.F.; Zhang, M.B.; Craig, G.B., Jr. Recently introduced *Aedes albopictus* in the United States: potential vector of La Crosse virus (*Bunyaviridae*: California serogroup). *J. Am. Mosq. Control Assoc.* **1989**, *5*, 422–427. [PubMed]

141. Sardelis, M.R.; Turell, M.J.; Andre, R.G. Laboratory transmission of La Crosse virus by *Ochlerotatus j. japonicus* (Diptera: *Culicidae*). *J. Med. Entomol.* **2002**, *39*, 635–639. [CrossRef] [PubMed]

142. Grimstad, P.R. Jamestown Canyon virus. In *Encyclopedia of Arthropod-Transmitted Infections of Man and Domestic Animals*; Service, M.W., Ashford, R.W., Eds.; CABI Publishing: New York, NY, USA, 2001; pp. 235–239.

143. Grimstad, P.R. California group virus disease. In *The Arboviruses: Epidemiology and Ecology*; Monath, T.P., Ed.; CRC Press: Boca Raton, FL, USA, 1988; pp. 99–136.

144. Whitman, L.; Wallis, R.C.; Leventhal, E.A. Isolation of a California-group arbovirus from *Aedes abserratus* (Felt and Young) in Simsbury, Connecticut. *Am. J. Trop. Med. Hyg.* **1968**, *17*, 449–450. [CrossRef] [PubMed]

145. Andreadis, T.G.; Anderson, J.F.; Armstrong, P.M.; Main, A.J. Isolations of Jamestown Canyon virus (*Bunyaviridae*: Orthobunyavirus) from field-collected mosquitoes (Diptera: *Culicidae*) in Connecticut, USA: A ten-year analysis, 1997–2006. *Vector Borne Zoonotic Dis.* **2008**, *8*, 175–188. [CrossRef]

146. Boromisa, R.D.; Grayson, M.A. Incrimination of *Aedes provocans* as a vector of Jamestown Canyon virus in an enzootic focus of northeastern New York. *J. Am. Mosq. Control Assoc.* **1990**, *6*, 504–509.

147. Heard, P.B.; Zhang, M.B.; Grimstad, P.R. Isolation of Jamestown Canyon virus (California serogroup) from *Aedes* mosquitoes in an enzootic focus in Michigan. *J. Am. Mosq. Control Assoc.* **1990**, *6*, 461–468. [PubMed]

148. Walker, E.D.; Grayson, M.A.; Edman, J.D. Isolation of Jamestown Canyon and snowshoe hare viruses (California serogroup) from *Aedes* mosquitoes in western Massachusetts. *J. Am. Mosq. Control Assoc.* **1993**, *9*, 131–134. [PubMed]

149. Boromisa, R.D.; Grimstad, P.R. Virus-vector-host relationships of *Aedes stimulans* and Jamestown Canyon virus in a northern Indiana enzootic focus. *Am. J. Trop. Med. Hyg.* **1986**, *35*, 1285–1295. [CrossRef]

150. Anderson, J.F.; Main, A.J.; Armstrong, P.M.; Andreadis, T.G.; Ferrandino, F.J. Arboviruses in North Dakota, 2003–2006. *Am. J. Trop. Med. Hyg.* **2015**, *92*, 377–393. [CrossRef]

151. Bond, J.O.; Hammon, W.M.; Lewis, A.L.; Sather, G.E.; Taylor, D.J. California group arboviruses in Florida and report of a new strain, Keystone virus. *Public Health Rep.* **1966**, *81*, 607–613. [CrossRef]

152. LeDuc, J.W.; Burger, J.F.; Eldridge, B.F.; Russell, P.K. Ecology of Keystone virus, a transovarially maintained arbovirus. *Ann. N. Y. Acad. Sci.* **1975**, *266*, 144–151. [CrossRef]

153. Issel, C.J.; Hoff, G.L.; Trainer, D.O. Serologic evidence of infection of white-tailed deer in Texas with three California group arboviruses, (Jamestown Canyon, San Angelo, and Keystone). *J. Wildl. Dis.* **1973**, *9*, 245–248. [CrossRef]

154. Watts, D.M.; LeDuc, J.W.; Bailey, C.L.; Dalrymple, J.M.; Gargan, T.P., 2nd. Serologic evidence of Jamestown Canyon and Keystone virus infection in vertebrates of the DelMarVa Peninsula. *Am. J. Trop. Med. Hyg.* **1982**, *31*, 1245–1251. [CrossRef]

155. Watts, D.M.; Bailey, C.L.; Roberts, N.T.; RF, T.A.; Dalrymple, J.M.; Clark, G.C. Maintenance and transmission of Keystone virus by *Aedes atlanticus* (Diptera: *Culicidae*) and the gray squirrel in the Pocomoke Cypress Swamp, Maryland. *J. Med. Entomol.* **1988**, *25*, 493–500. [CrossRef]

156. Sudia, W.D.; Newhouse, V.F.; Calisher, C.H.; Chamberlain, R.W. California group arbo-viruses: Isolations from mosquitoes in North America. *Mosq. News* **1971**, *31*, 576–600.

157. Parkin, W.E.; Hammon, W.M.; Sather, G.E. Review of current epidemiological literature on viruses of the California arbovirus group. *Am. J. Trop. Med. Hyg.* **1972**, *21*, 964–978. [CrossRef]

158. Lednicky, J.A.; White, S.K.; Stephenson, C.J.; Cherabuddi, K.; Loeb, J.C.; Moussatche, N.; Lednicky, A.; Morris, J.G., Jr. Keystone virus isolated from a Florida teenager with rash and subjective fever: Another endemic arbovirus in the Southeastern United States? *Clin. Infect. Dis.* **2019**, *68*, 143–145. [CrossRef]

159. Bardos, V.; Medek, M.; Kania, V.; Hubalek, Z. Isolation of Tahyna virus from the blood of sick children. *Acta Virol.* **1975**, *19*, 447.

160. Rodl, P.; Bardos, V.; Ryba, J. Experimental transmission of Tahyna virus (California group) to wild rabbits (*Oryctolagus cuniculus*) by mosquitoes. *Folia Parasitol. (Praha)* **1979**, *26*, 61–64.

161. Aspöck, H.; Kunz, C. Felduntersuchungen über die Bedeutung des Igels (*Erinaceus europaeus roumanicus* Barrett-Hamilton) im Zyklus des Tahyna-virus. *Zentralbl Bakteriol.* **1970**, *213*, 304–310.

162. Bardos, V.; Danielova, V. The Tahyna virus—A virus isolated from mosquitoes in Czechoslovakia. *J. Hyg. Epidemiol. Microbiol. Immunol.* **1959**, *3*, 264–276.

163. Danielová, V.; Hájková, Z.; Kolman, J.M.; Málková, D.; Minár, J.; Smetana, A. Results of virological examination of mosquitoes in Southern Moravia in 1962–1964. *Cesk. Epidemiol. Mikrobiol. Imunol.* **1966**, *15*, 178–184. [PubMed]

164. Kolman, J.M.; Malkova, D.; Nemec, A.; Smetana, A.; Hajkova, Z.; Minar, J. The isolation of the Tahyna virus from the mosquito *Aedes vexans* in Southern Moravia. *J. Hyg. Epidemiol. Microbiol. Immunol.* **1964**, *8*, 380–386.

165. Hannoun, C.; Panther, R.; Corniou, B. Isolation of Tahyna virus in the South of France. *Acta Virol.* **1966**, *10*, 362–364.

166. Balducci, M.; Verani, P.; Lopes, M.C.; Sacca, G.; Gregorig, B. Isolation of Tahyña virus from *Aedes* mosquitoes in Northern Italy (Gorizia Province). *Acta Virol.* **1968**, *12*, 457–459.

167. Traavik, T.; Mehl, R.; Wiger, R. California encephalitis group viruses isolated from mosquitoes collected in Southern and Arctic Norway. *Acta Pathol. Microbiol. Scand. B* **1978**, *86B*, 335–341. [CrossRef]

168. Arcan, P.; Topciu, V.; Rošiu, N.; Csaky, N. Isolation of Ťahyňa virus from *Culex pipiens* mosquitoes in Romania. *Acta Virol.* **1974**, *18*, 175.

169. Hubalek, Z.; Sebesta, O.; Pesko, J.; Betasova, L.; Blazejova, H.; Venclikova, K.; Rudolf, I. Isolation of Tahyna virus (California encephalitis group) from *Anopheles hyrcanus* (Diptera, *Culicidae*), a mosquito species new to, and expanding in, Central Europe. *J. Med. Entomol.* **2014**, *51*, 1264–1267. [CrossRef]

170. Anonymous. VMD authorises SBV vaccine for use in the UK. *Vet. Rec.* **2013**, *172*, 543.

171. Anonymous. Schmallenberg virus vaccine. *Vet. Rec.* **2015**, *177*, 321.

172. EMA. Zulvac SBV. 2015. Available online: https://www.ema.europa.eu/medicines/veterinary/EPAR/zulvac-sbv (accessed on 25 March 2019).

173. Kim, Y.H.; Kweon, C.H.; Tark, D.S.; Lim, S.I.; Yang, D.K.; Hyun, B.H.; Song, J.Y.; Hur, W.; Park, S.C. Development of inactivated trivalent vaccine for the teratogenic Aino, Akabane and Chuzan viruses. *Biologicals* **2011**, *39*, 152–157. [CrossRef]

174. Patta, C.; Giovannini, A.; Rolesu, S.; Nannini, D.; Savini, G.; Calistri, P.; Santucci, U.; Caporale, V. Bluetongue vaccination in Europe: The Italian experience. *Vet. Ital.* **2004**, *40*, 601–610.

175. Zientara, S.; MacLachlan, N.J.; Calistri, P.; Sanchez-Vizcaino, J.M.; Savini, G. Bluetongue vaccination in Europe. *Expert Rev. Vaccines* **2010**, *9*, 989–991. [CrossRef] [PubMed]

176. Sakkas, H.; Bozidis, P.; Franks, A.; Papadopoulou, C. Oropouche fever: A review. *Viruses* **2018**, *10*, 175. [CrossRef] [PubMed]

177. Carpenter, S.; Mellor, P.S.; Torr, S.J. Control techniques for *Culicoides* biting midges and their application in the U.K. and northwestern Palaearctic. *Med. Vet. Entomol.* **2008**, *22*, 175–187. [CrossRef]

178. Wernike, K.; Conraths, F.; Zanella, G.; Granzow, H.; Gache, K.; Schirrmeier, H.; Valas, S.; Staubach, C.; Marianneau, P.; Kraatz, F.; et al. Schmallenberg virus—Two years of experiences. *Prev. Vet. Med.* **2014**, *116*, 423–434. [CrossRef]

179. Corbel, V.; Stankiewicz, M.; Pennetier, C.; Fournier, D.; Stojan, J.; Girard, E.; Dimitrov, M.; Molgo, J.; Hougard, J.M.; Lapied, B. Evidence for inhibition of cholinesterases in insect and mammalian nervous systems by the insect repellent DEET. *BMC Biol.* **2009**, *7*, 47. [CrossRef]

180. Mayer, S.V.; Tesh, R.B.; Vasilakis, N. The emergence of arthropod-borne viral diseases: A global prospective on dengue, chikungunya and zika fevers. *Acta Trop.* **2017**, *166*, 155–163. [CrossRef] [PubMed]

2.5 Simbuviren: Tiermodelle

Für die Untersuchung von Pathogenese, Virulenz, Virus-Wirt-Interaktionen, Transmission und der Immunreaktion bei Virusinfektionen sind Tiermodelle essentiell. Geeignete Virus-Challenge-Modelle sind auch für die Testung von Vakzinekandidaten und Therapeutika unerlässlich. Für einige Viren der Simbu-Serogruppe sind natürliche Wirte wie Wiederkäuer bereits als Großtier-Infektionsmodelle etabliert. SBV-Infektionen konnten kurz nach der Erstbeschreibung des Erregers experimentell in den natürlichen Wirten Rind, Schaf und Ziege nachgestellt werden (24, 63-65), auch für AKAV stehen verschiedene Großtiermodelle zur Verfügung (66, 67). Zahlreiche Studien zur Testung von Vakzinekandidaten wurden anhand geeigneter Großtiermodelle durchgeführt (38, 68-70). Die Durchführung von Tierversuchen an Großtieren ist allerdings sehr kostenintensiv, die Tierzahlen sind limitiert und Versuche sind auf wenige Forschungseinrichtungen beschränkt, die über Großtierställe mit entsprechender biologischer Schutzstufe verfügen. Kleintiermodelle dagegen sind kosteneffizienter, benötigen weniger Platz, was höhere Tierzahlen pro Versuchsgruppe ermöglicht, sind leichter in der Handhabung, die Tiere haben eine höhere Reproduktionsrate und oftmals sind ingezüchtete Linien genetisch identischer Tiere vorhanden. Hühnerembryonen erwiesen sich in der Forschung an Viren der Simbu-Serogruppe bereits als geeignetes Modell und wurden eingesetzt, um Pathogenität und Teratogenität von SBV und AKAV zu untersuchen (71-74). Zudem wurden mehrere Kleintiermodelle etabliert. C3H/He Mäuse sind nach intrazerebraler und BALB/cAJcl Mäuse nach intrazerebraler und intraperitonealer Infektion empfänglich für AKAV und wurden vor allen für Pathogenesestudien verwendet (74, 75). Für OROV wurden Modelle mit neonatalen BALB/c Mäusen und Goldhamstern etabliert, die mit einer subkutanen Inokulationsmethode infiziert wurden (76, 77) und bereits für umfangreiche Pathogenesestudien eingesetzt wurden (78). 18 Tage alte NIH-Swiss Mäuse und neugeborene BALB/c Mäuse sind nach intrakranialer Inokulation hochempfänglich für SBV (34, 79). Diese Infektionsroute ähnelt allerdings nicht dem natürlichen Infektionsweg. Sehr junge Mäuse eignen sich zum einen aufgrund des bei der Geburt nicht vollständig entwickelten Immunsystems und zum anderen wegen zeitlich bedingten Entwicklungsunterschieden bei den Zeitpunkten der Impfungen und der Challenge-Infektion nicht für Vakzinetestungen. Adulte, immunkompetente Mäuse sind oft unempfänglich für verschiedenste

Virusinfektionen (80). Interferon α/β Rezeptor knock-out (IFNAR-/-) Mäuse erwiesen sich als eine geeignete Alternative: Ihnen fehlen die zellulären Interferon α/β Rezeptoren IFNAR1, die in Wildtyp-Mäusen beim Vorhandensein von Typ1 IFN (IFNα/β) einen antiviralen Status der Zelle induzieren und vor Virusinfektion schützen. In IFNAR (-/-) Mäusen wird kein antiviraler Status der Zellen induziert, wodurch sie empfänglicher für Virusinfektionen sind (81). Sie eignen sich trotz ihres beeinträchtigten angeborenen Immunsystems für Vakzinetestungen, da sie durch Impfung vor tödlichen verlaufenden Virusinfektionen geschützt werden können (81). IFNAR (-/-) Mäuse erwiesen sich bereits als geeignetes Kleintiermodell für eine Auswahl an Viren der Simbu-Serogruppe: Sie sind empfänglich für experimentelle SBV-Infektionen (82). Auch für Sabo-, Simbu- und Sathuperi-Virus, für die andere *in vivo* Studien noch weitgehend fehlen, sind IFNAR (-/-) Mäuse ein geeignetes Modell (83). Bei histologischen Untersuchungen zeigte sich ein ähnliches Bild wie bei Feten nach transplazentarer SBV-Infektion, was IFNAR (-/-) Mäuse zu einem guten Kleintiermodell für Pathogenesestudien macht (83). Für SBV wurden bereits verschiedene Vakzinekandidaten erfolgreich im IFNAR (-/-) Maus-Modell getestet (38, 68, 84-86).

Für SHUV waren noch keine Groß- oder Kleintiermodelle etabliert, um die Neuropathogenität zu untersuchen. Dass SHUV mit humanen neurologischen Erkrankungen im Zusammenhang zu stehen scheint, betont die Relevanz von gut charakterisierten Tiermodellen, um die Pathogenese der Krankheit im Tier, aber auch im Menschen zu verstehen. Auch wenn Tiere oftmals nicht optimale Modelle für die humane Erkrankung sind, tragen die Erkenntnisse, die aus diesen Modellen gewonnen werden, maßgeblich zum Verständnis der Pathogenese bei. Das Wissen über humane Erkrankungen kommt zum Großteil aus Studien über Krankheitsprozesse in verschiedenen Tierspezies (87).

In der vorliegenden Arbeit werden sowohl ein Großtiermodell im Rind als auch ein Kleintiermodell in der IFNAR (-/-) Maus für SHUV-Infektionen vorgestellt.

2.6 Simbuviren: Serologische Diagnostik

Für die Überwachung des Krankheitsgeschehens und für gezielte Diagnostik am Einzeltier sind verlässliche diagnostische Tests essentiell. Neben direktem Virusnachweis durch PCR oder Virusanzucht und Sequenzierung basiert die Diagnostik von simbuviralen Erkrankungen auf serologischen Untersuchungsmethoden. Direkte Erregernachweise wie PCR oder Virusanzucht sind im frühen Krankheitsstadium das Mittel der Wahl, haben aber durch die sehr kurze Virämie von nur wenigen Tagen einen Nachteil gegenüber Untersuchungsmethoden der humoralen Immunantwort. Antikörper, die nach natürlicher Infektion oder durch Impfung gebildet wurden, sind ein bis drei Wochen nach Infektion im Serum detektierbar und über mehrere Jahre im Tier nachweisbar (56, 88) und schützen vor Reinfektionen (38, 65, 89-91). Nach vertikaler Infektion immunkompetenter Feten sind vor der ersten Kolostrumaufnahme Antikörper im Blut nachweisbar (92, 93).

Häufig genutzte serologische Testverfahren sind Mikroneutralisationstests (NT), indirekte Immunfluoreszenztests (IIFT) und Enzyme-Linked Immunosorbent Assays (ELISAs). Da Viren der Simbu-Serogruppe aufgrund ihrer antigenetischen Verwandtschaft in einer Serogruppe zusammengefasst sind (1), treten beträchtliche serologische Kreuzreaktivitäten zwischen den einzelnen Virusspezies auf (94). Das trifft besonders auf Tests zu, die auf dem N-Protein basieren, wie kommerzielle N-Protein ELISAs (16-18), da das S-Segment, welches für das N-Protein kodiert, das konservierteste Genomsegment bei Viren der Simbu-Serogruppe ist (32). Weil das N-Protein schnell starke Antikörperantworten auslösen kann und Anti-N-Antikörper in hoher Zahl im Tier vorhanden sind (70), wird es dennoch häufig für die serologische Diagnostik genutzt. Antikörper gegen das N-Protein haben keine virusneutralisierende Aktivität (95). Im Gegensatz dazu ist das M-Segment, das für die beiden Glykoproteine Gn und Gc kodiert, das Genomsegment mit der höchsten genetischen Variabilität (94). Antikörper, die gegen das Glykoprotein Gc gerichtet sind, weisen eine virusneutralisierende Aktivität auf (28, 86). Im Vergleich zu Tests basierend auf dem hochkonservierten N-Protein sind Tests basierend auf den variablen Glykoproteinen spezifischer für die jeweilige Virus-Spezies und weniger Kreuzreaktivitäten treten auf. Mikroneutralisationstests, die neutralisierende Antikörper gegen Glykoproteine nachweisen, sind spezifischer als N-Protein basierte Tests (17).

Eine Virusspezies-spezifische serologische Diagnostik ist von besonderer Bedeutung zur Differenzierung simbuviraler Erkrankungen, beispielsweise bei sich überschneidenden Verbreitungsgebieten verschiedener Virusspezies oder zum Nachweis eines Neueintrags einer neuen Virusspezies. Wegen der hohen Ähnlichkeit der klinischen Erkrankung und der sehr kurzen Dauer der virämischen Phase (24, 66) eignen sich klinische Untersuchung und RT-PCR nur bedingt zur differenzierten Überwachung des Vorkommens verschiedener nahe verwandter Viren. Für SHUV waren neben PCR-Tests (47, 96) und Virusisolation mit anschließender Sequenzanalyse (14, 44) keine spezifischen diagnostischen Tests etabliert.

3 Zielsetzung

Etablierung von Infektionsmodellen für SHUV

SHUV stellt aufgrund von fetalen Verlusten bei Wiederkäuern und akuter neurologischer Erkrankung bei Pferden und Rindern ein Problem für die Tiergesundheit dar (3, 9, 14). Des Weiteren wurde das zoonotische Potential von SHUV beim Auftreten von Fällen neurologischer Erkrankung bei Menschen deutlich (10). Um die Pathogenese von SHUV Infektionen *in vivo* zu charakterisieren, sind geeignete Infektionsmodelle essentiell. In dieser Arbeit werden sowohl ein Großtiermodell (Rind) als auch ein Kleintiermodell (IFNAR -/- Maus) vorgestellt, die eine *in vivo* Charakterisierung von SHUV-Infektionen ermöglichen.

Charakterisierung der Neuropathogenität von SHUV

2014 trat neben dem bekannten klinischen Bild der kongenitalen Missbildungen nach diaplazentaren Infektionen eine neue klinische Manifestation bei Rindern in Israel auf. SHUV wurde, ähnlich wie bei Pferden, mit akuter neurologische Erkrankung in Verbindung gebracht (3, 9, 14). Der kausale Zusammenhang zwischen SHUV-Infektionen und neurologischer Erkrankung im Rind sollte experimentell bestätigt und der Verlauf der Erkrankung klinisch, virologisch, serologisch und pathohistologisch untersucht werden. Zwei verschiedene SHUV-Isolate aus den beiden möglichen Krankheitsbildern, kongenitale Missbildung und neurologische Erkrankung, wurden in einem neu etablierten Rinder- und einem Kleintiermodell *in vivo* charakterisiert.

Entwicklung serologischer Tests

Um SHUV-Infektionen verlässlich diagnostizieren zu können, sind neben direkten Virusnachweisen (PCR, Virusanzucht) auch spezifische serologische Tests von sehr großer Bedeutung. Anhand von Referenzseren eines SHUV-Infektionsversuchs an Rindern sollten ein Mikroneutralisationstest und ein indirekter Immunfluoreszenztest entwickelt werden. Zudem wurde ein Triplex-ELISA für SHUV und die beiden nahe verwandten Viren SBV und AKAV entwickelt.

4 Ergebnisse

„Shuni virus-induced meningoencephalitis after experimental infection of cattle"

Franziska Sick [1], Angele Breithaupt [2], Natalia Golender [3], Velizar Bumbarov [3] Martin Beer [1], Kerstin Wernike [1]

[1] Institute of Diagnostic Virology, Friedrich-Loeffler-Institut, Greifswald-Insel Riems, Germany

[2] Department of Experimental Animal Facilities and Biorisk Management, Friedrich-Loeffler-Institut, Greifswald-Insel Riems, Germany

[3] Department of Virology, Kimron Veterinary Institute, Bet Dagan, Israel

ORIGINAL ARTICLE

Transboundary and Emerging Diseases **WILEY**

Shuni virus-induced meningoencephalitis after experimental infection of cattle

Franziska Sick[1] | **Angele Breithaupt[2]** | **Natalia Golender[3]** | **Velizar Bumbarov[3]** | **Martin Beer[1]** (iD) | **Kerstin Wernike[1]** (iD)

[1]Institute of Diagnostic Virology, Friedrich-Loeffler-Institut, Greifswald-Insel Riems, Germany

[2]Department of Experimental Animal Facilities and Biorisk Management, Friedrich-Loeffler-Institut, Greifswald-Insel Riems, Germany

[3]Department of Virology, Kimron Veterinary Institute, Bet Dagan, Israel

Correspondence
Kerstin Wernike, Institute of Diagnostic Virology, Friedrich-Loeffler-Institut, 17493 Greifswald - Insel Riems, Germany.
Email: kerstin.wernike@fli.de

Funding information
German Federal Ministry of Food and Agriculture (BMEL) through the Federal Office for Agriculture and Food, Grant/Award Number: 281B101816; Marie Skłodowska-Curie Actions (MSCA) Innovative Training Networks (ITN) H2020-MSCA-ITN-2016, Grant/Award Number: 721367

Abstract

Shuni virus (SHUV), an insect-transmitted orthobunyavirus of the Simbu serogroup within the family *Peribunyaviridae*, may induce severe congenital malformations when naïve ruminants are infected during gestation. Only recently, another clinical presentation in cattle, namely neurological disease after postnatal infection, was reported. To characterize the course of the disease under experimental conditions and to confirm a causal relationship between the virus and the neurological disorders observed in the field, six calves each were experimentally inoculated (subcutaneously) with two different SHUV strains from both clinical presentations, that is encephalitis and congenital malformation, respectively. Subsequently, the animals were monitored clinically, virologically and serologically for three weeks. All animals inoculated with the 'encephalitis strain' SHUV 2162/16 developed viremia for three to four consecutive days, seroconverted, and five out of six animals showed elevated body temperature for up to three days. No further clinical signs such as neurological symptoms were observed in any of these animals. However, four out of six animals developed a non-suppurative meningoencephalitis, characterized by perivascular cuffing and glial nodule formation. Moreover, SHUV genome could be visualized in brain tissues of the infected animals by in situ hybridization. In contrast to the 'encephalitis SHUV strain', in animals subcutaneously inoculated with the strain isolated from a malformed newborn (SHUV 2504/3/14), which expressed a truncated non-structural protein NSs, a major virulence factor, no viremia or seroconversion, was observed, demonstrating an expected severe replication defect of this strain in vivo. The lack of viremia further indicates that virus variants evolving in malformed foetuses may represent attenuated artefacts as has been described for closely related viruses. As the neuropathogenicity of SHUV could be demonstrated under experimental conditions, this virus should be included in differential diagnosis for encephalitis in ruminants, and cattle represent a suitable animal model to study the pathogenesis of SHUV.

KEYWORDS

cattle, encephalitis, pathogenesis, Peribunyaviridae, Shuni virus, Simbu serogroup

1 | INTRODUCTION

The insect-transmitted Shuni virus (SHUV) is a member of the Simbu serogroup within the family *Peribunyaviridae*, genus *Orthobunyavirus* (Plyusnin & Elliott, 2011). Besides SHUV, several viruses of veterinary importance such as Akabane virus (AKAV) or Schmallenberg virus (SBV) are assigned to the Simbu serogroup. SHUV was firstly isolated in Nigeria in 1960 from apparently healthy cattle (Causey et al., 1972), and, thereafter, it was also detected in further African countries. In 2014, the virus was reported for the first time outside of the African continent, namely in the Middle East, where it was isolated from malformed lambs in Israel (Golender et al., 2015). In the following years, SHUV was repeatedly detected in aborted and/or malformed calves or lambs in this country (Golender et al., 2018); hence, the virus established an endemic status in Israel. As SHUV is transmitted by biting midges (genus *Culicoides*) (Möhlmann et al., 2018), which occur worldwide, a further spread of the virus into hitherto unaffected regions seems very likely.

In general, Simbu serogroup virus infections can cause two different types of clinical manifestation in ruminants. Acute infections of animals of all age groups are either asymptomatic or mild, associated with unspecific signs like fever, diarrhoea or loss in milk yield for a few days (Kirkland, 2015; Wernike et al., 2015). However, when naïve dams are infected during a critical phase of gestation, severe congenital defects referred to as arthrogryposis-hydranencephaly syndrome (AHS), abortion, premature birth, mummification or stillbirth can occur (Beer & Wernike, 2019; Kirkland, 2015; Wernike et al., 2015).

For SHUV and AKAV, another clinical presentation is described after natural infection. In rare cases, some strains of AKAV may induce encephalitis in newborn calves and the Iriki strain has been occasionally associated with encephalitis in young and adult cattle (Miyazato et al., 1989). Only recently, severe neurological signs have been described in young cattle naturally infected with SHUV (Golender et al., 2019). However, for SHUV the pathogenesis of this clinical presentation is largely unknown. In addition to the disease induced in ruminants, SHUV has also been associated with neurological diseases in horses in South Africa (van Eeden et al., 2012). Apart from animals, SHUV was isolated from a febrile child in Nigeria (Moore et al., 1975) and specific antibodies were found in veterinarians in South Africa. Hence, a zoonotic potential cannot be excluded; however, no clear histories of disease compatible with SHUV infections could be determined from antibody-positive veterinarians (van Eeden et al., 2014).

Similar to other orthobunyaviruses, SHUV is spherical, about 100 nm in diameter and comprises a tri-partite single-stranded negative-sense RNA genome that encodes for four structural and two non-structural proteins (Walter & Barr, 2011). The small (S) genomic segment encodes for the nucleocapsid protein N and in an overlapping reading frame for the non-structural protein NSs. The medium (M) segment encodes for the glycoproteins Gn and Gc, and the non-structural protein NSm, and the large (L) segment encodes for the RNA dependent RNA polymerase (Walter & Barr, 2011). The

NSs protein acts as an interferon antagonist and induces a shut-off of protein synthesis in mammalian cells; hence, it represents a major virulence factor in vertebrate hosts (Elliott & Blakqori, 2011). For the insect vector, the function of both non-structural proteins remains largely unknown.

From ovine and bovine samples collected during the recent SHUV outbreak in Israel, infectious viruses could be successfully isolated (Golender et al., 2015, 2019). Sequence analysis revealed that the amino acid (aa) sequences of the viral N protein are highly conserved. Israeli SHUVs show an overall identity of 99.3 to 100%, however, when compared to the more ancient African sequences, aa identity decreases to about 90.5% (Golender et al., 2016). Nevertheless, single point mutations in the nucleotide sequences found in Israeli isolates from foetal brains led to changes in aa sequence or resulted in stop codons. A SHUV isolate with mutations leading to a truncated NSs protein showed a loss of function of the NSs protein in vitro (Golender et al., 2016). Whether this mutation also effects the function in vivo remains unknown.

In the present study, cattle were experimentally inoculated with two recent Israeli SHUV isolates and clinically, virologically, serologically and pathologically investigated to characterize the course of the disease. The first isolate represented the virus displaying a truncated, non-functional NSs protein, and the second isolate was obtained from acutely infected cattle showing neurological symptoms including compulsive circling, ataxia, swallowing difficulties and dysstasia.

2 | MATERIALS AND METHODS

2.1 | Viruses

Viruses from both previously described clinical pictures, namely encephalitis and congenital malformation, were selected. The first virus strain 'SHUV 2162/16' was isolated on Vero cells from the brain homogenate of a heifer showing neurological symptoms such as compulsive circling, ataxia, swallowing difficulties and later on dysstasia and recumbency (Golender et al., 2019). The second virus strain 'SHUV 2504/3/14' was obtained from the brain of a malformed, aborted sheep foetus using suckling mice and Vero cells (Golender et al., 2015). The latter isolate expresses a truncated NSs protein due to a stop codon in the NSs open reading frame (Golender et al., 2016). Both isolates were passaged once each on *Culicoides sonorensis* cells (KC, L1062, collection of cell lines in veterinary medicine (CCLV), Friedrich-Loeffler-Institut, Greifswald-Insel Riems, Germany) and baby hamster kidney cells (BHK 21, L0164 CCLV).

2.2 | Animals and experimental design

Fourteen cattle between 7 and 11 months of age (13 females, one castrated male) were obtained from a commercial supplier and rehoused to an insect-proof high containment stable. The animals

were kept in two infection groups of six cattle each, and the two remaining negative-control animals were kept separately. Before the experimental inoculation, sera of all animals were tested by a commercially available SBV antibody ELISA (ID Screen Schmallenberg virus competition multispecies; IDvet, Grabels, France) that also detects antibodies against several other Simbu serogroup viruses (Oluwayelu et al., 2018; Wernike et al., 2017) and by a microneutralization test against SHUV isolate 2162/16. All sera tested negative.

Six animals each were subcutaneously injected with 1 ml containing 10^6 TCID$_{50}$/mL of SHUV strain 2162/16 (cattle numbers C1 to C6) or strain 2504/3/14 (cattle C7 to C12), respectively. The two control animals (C13 and C14) were subcutaneously injected with 1 ml of phosphate-buffered saline (PBS). Throughout the study, the animals were monitored daily by veterinarians using a clinical scoring system (depending on the severity of clinical signs, up to 3 points were awarded for changes in activity, posture, gait and feeding behaviour). Rectal body temperatures were measured daily.

Whole blood and serum samples were collected daily for 10 days, on day 14 and on day 21. After three weeks, all animals were killed and tissue samples were collected (duodenum, jejunum, ileum (including Peyer's patches), caecum, rectum, spleen, tonsil, mesenteric, tracheobronchial, mandibular and retropharyngeal lymph nodes, spinal cord, cerebrum and cerebellum, liver, kidney, heart, ovary, muscle).

The experimental protocol was evaluated by a state ethics commission and approved by the competent authority (permission number LALLF M-VTSD/7221.3–2-010/18).

2.3 | RNA extraction and real-time RT-PCR

Tissue samples were homogenized in 1 ml of Modified Eagle Medium (MEM) using a TissueLyzer (Qiagen, Hilden, Germany). Total RNA from the tissue homogenates, serum and whole blood samples was extracted using the King Fisher 96 Flex purification system (Thermo Scientific, Braunschweig, Germany) in combination with the NucleoMag Vet kit (Macherey-Nagel, Düren, Germany) according to the manufacturers' instructions. The extracts were subsequently tested for SHUV genome using an S-segment-based generic Simbu serogroup real-time RT-PCR (Golender et al., 2018). To control for efficient RNA extraction and amplification, thereby avoiding false-negative results, an internal control based on the beta-actin gene was additionally tested (Toussaint et al., 2007).

2.4 | Serology

The sera collected at weekly intervals were heat inactivated at 56°C for 30 min and analysed by a standard microneutralization test against the virus isolate used for inoculation, sera obtained from the control animals were tested against both isolates. Each serum was tested in quadruplicate. The serum samples were first diluted in MEM in a 1/5 ratio and titrated in two-fold dilutions. Subsequently, 50 µl of the diluted sera were incubated with 50 µl

of MEM containing approximately 100 TCID$_{50}$ of SHUV, which was confirmed by performing back-titrations, in microtiter plates for 2 hr at 37°C. Thereafter, 100µl of a BHK21 cell suspension was added to the virus-serum mixture and incubated at 37°C for 3 to 4 days. Evaluation was done by assessing the cytopathic effect. The antibody titres were calculated as ND$_{50}$ (neutralization dose 50%) according to Behrens and Kaerber (Behrens & Kärber, 1934).

In addition, the sera were tested in an indirect immunofluorescence assay against the virus isolate used for inoculation. Sera of the control animals were tested against both SHUV strains. As antigen matrix, Vero cells (VERO 76, L0228 CCLV) were cultivated in 96-well microtiter plates. Odd-numbered columns were either infected with SHUV strain 2162/16 or 2504/3/14, while even numbered columns were left uninfected to control for unspecific reactions of the sera. After a 24-hr incubation period at 37°C, cells were heat-fixed at 80°C for 2 hr.

The cells were incubated with the sera pre-diluted in PBS (serial dilution of 1/50 to 1/400 in steps of 50). Binding of serum antibodies to the SHUV-infected and uninfected cells was visualized by adding a fluorescein-labelled secondary anti-bovine antibody (Anti-Bovine IgG (whole molecule)-FITC produced in rabbit, Sigma life sciences, St. Louis, USA). The assay was evaluated using a fluorescence microscope (Eclipse Ti-U, Nikon, Melville, USA). A comparison of infected and uninfected cells facilitated to exclude unspecific reactions.

Moreover, the sera were tested by a commercially available SBV antibody ELISA (ID Screen Schmallenberg virus competition multispecies; IDvet, Grabels, France) that also detects antibodies against several other Simbu serogroup viruses (Oluwayelu et al., 2018; Wernike et al., 2017).

2.5 | Autopsy, histopathology and in situ hybridization

Full autopsies were performed on all animals according to a standard protocol. The brain and cervical spinal cord were collected and fixed in 10% neutral-buffered formalin. Coronal sections of the following brain regions were processed: cortex (frontal and parietal lobe), the periventricular area of the lateral ventricle (region of caudate nucleus), hippocampus region, cerebral aqueduct with periaqueductal grey and white matter, cerebellum and cervical spinal cord. Tissues were embedded in paraffin, cut at three µm and stained with haematoxylin and eosin for light microscopic analyses. Meningeal infiltrates and perivascular immune cell cuffing in the grey and white matter were evaluated using a semi-quantitative scoring system, considering the most severely affected area: no lesion; up to three cell layers = mild; 4–9 cell layers = moderate; ≥10 layers = severe. The presence of glial nodules was recorded for the grey and white matter: no nodules, few (one focus per 20× field), multiple (two to three foci), abundant (≥ four foci).

Consecutive slides of areas with the most abundant inflammation were processed for immunohistochemistry. T-cell, B-cell and microglia/macrophage markers were applied, apoptosis was detected

SICK ET AL.

by active-caspase-3 labelling and potential astrogliosis was tested by using glial fibrillary acidic protein (GFAP) immunohistochemistry, according to standardized procedures (see Table 1). Non-specific antibody binding was blocked with undiluted goat normal serum for 30 min at room temperature. Immunolabeling was visualized by 3-amino-9-ethylcarbazole substrate (AEC, Dako, Agilent, Santa Clara, CA, USA) producing a red-brown signal, and sections were counter-stained with Mayer's haematoxylin.

On additional consecutive sections, conventional staining protocols were applied, including Luxol Fast Blue Cresyl Violet for detection of myelin sheaths and Nissl substance, von Kossa stain to show mineralization and Prussian blue reaction for demonstration of ferric iron, indicating hemosiderin.

RNA in situ hybridization for the detection of SHUV RNA in brain tissue sections was performed with the RNAScope® 2–5 HD Reagent Kit-Red (ACD, Advanced Cell Diagnostics, Newark, CA) according to the manufacturer's instructions. For hybridization, RNAScope® probes were custom-designed for the nucleocapsid protein. The specificity of the probes was verified using a positive control probe peptidylprolyl isomerase B (cyclophilin B, ppib) and a negative control probe dihydrodipicolinate reductase (DapB). Histopathology and RNAScope® interpretation were performed by a board-certified pathologist (DiplECVP).

3 | RESULTS

3.1 | Clinical manifestation

After inoculation, all animals were monitored daily for clinical signs. All cattle inoculated with SHUV strain 2162/16 except for C3 showed elevated rectal body temperatures (>39.5°C), reaching values as high as 40.4°C (C1), for one to three days starting at the earliest at day four post-infection (dpi). The course of fever varied markedly between the individual animals of this group (Figure 1). In contrast, the body temperature of the animals inoculated with SHUV

strain 2504/3/14 and of the uninfected control cattle remained in a normal temperature range (<39.5°C) throughout the study (Figure 1). Further clinical signs such as neurological changes were never observed in any animal.

3.2 | Detection of viral genome

Viral genome was detected by real-time RT-PCR in the blood of all animals inoculated with SHUV strain 2162/16 for three to four consecutive days starting two or three days after infection (Figure 2). No remarkable differences between whole blood and serum samples were observed. The whole blood and serum samples of animals inoculated with strain 2504/3/14 and of the control animals tested negative for all animals at all time points.

From the diverse panel of tissue samples collected during autopsy, only lymphatic and nervous tissue samples of the animals inoculated with strain 2162/16 tested positive by real-time RT-PCR. In samples collected from cattle C1, SHUV RNA was found in the spleen (quantification cycle (Cq) value: 38.7), the spinal cord (Cq 35.7) and in the cerebellum (Cq 34.8). In animal C2, SHUV RNA was detected only in the cerebrum (Cq 39.4), while in C3 viral RNA could be detected only in the spleen (Cq 33.5). In cattle C4, viral RNA was found in the spinal cord (Cq 35.1), cerebellum (Cq 32.8) and cerebrum (Cq 33.8). In cattle C6, viral RNA was found in spleen (Cq 35.5), spinal cord (Cq 38.5) and cerebellum (Cq 34.3). No viral genome could be detected in any other tissue sample of the aforementioned cattle. Animal C5, as well as all cattle inoculated with SHUV strain 2504/3/14 and the uninfected control animals tested negative by real-time RT-PCR in every tissue sample.

3.3 | Antibody detection

On the day of inoculation, the sera of all animals reacted negative in the neutralization test. All animals inoculated with SHUV strain

**TABLE 1** Immunohistochemical markers and applications. HIER: heat-induced epitope retrieval, RT: room temperature

Marker	Antibody	Pre-treatment	Secondary reagents
CD79a	Mouse anti-CD79A (LifeSpan BioSciences, Seattle, WA, #LS-B8330), 1/50, overnight	HIER, 10mM Tris/ 1mM EDTA buffer pH 9.0, 20 min	Dako EnVision+ System- HRP Labelled Polymer Anti-mouse, 30 min (Dako, Agilent, Santa Clara, CA, USA)
CD3	Rabbit anti-CD3 polyclonal (Dako, #A045229–2), 1/100, overnight	HIER, 10mM Tris/ 1mM EDTA buffer pH 9.0, 20 min	Dako EnVision+ System- HRP Labelled Polymer Anti-rabbit, 30 min
Iba–1	Rabbit anti Iba1 (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan, # 019–19741), 1/800, overnight	HIER, Citrate buffer pH 6.0, for 20 min	Dako EnVision+ System- HRP Labelled Polymer Anti-rabbit, 30 min
GFAP	Rabbit anti GFAP (abcam, Cambridge, UK, #ab16997), 1/200, overnight	HIER, Citrate buffer pH 6.0, for 20 min	Dako EnVision+ System- HRP Labelled Polymer Anti-rabbit, 30 min
active caspase 3	Rabbit anti-active caspase 3 (Promega, Madison, WI, USA, #G7481), 1/200, overnight	No pre-treatment	Anti-Rabbit IgG Biotinylated, 1/200, 30 min. RT; and ABC Kit Vectastain Elite PK 6100, 30 min (Vector Laboratories, Burlingame, CA, USA)

FIGURE 1 Rectal body temperature of cattle inoculated with either Shuni virus strain 2162/16 (red) or 2504/3/14 (blue). Uninfected control animals are shown in green [Colour figure can be viewed at wileyonlinelibrary.com]
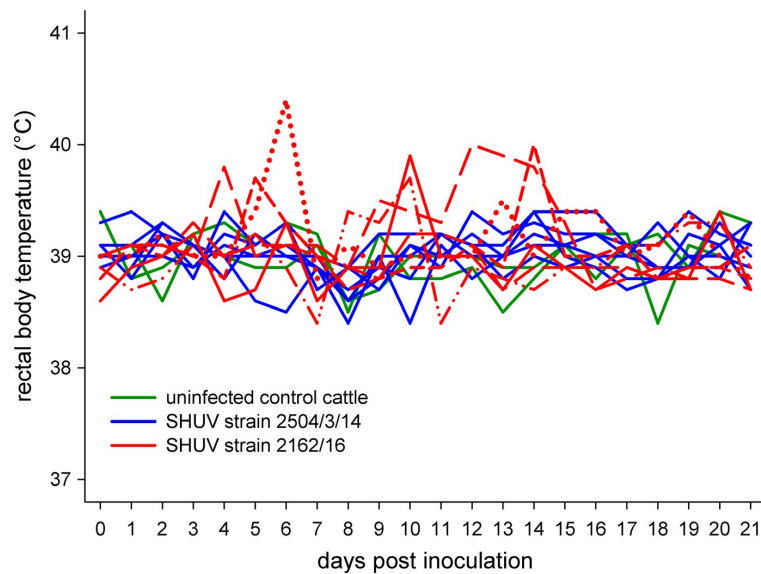
FIGURE 2 Real-time RT-PCR results for whole blood (shown in black) and serum samples (shown in red). Samples taken from the same animal are depicted by the identical line type. No Shuni virus RNA was detected in C7 - C12, which were inoculated with strain 2504/3/14, or in the uninfected control animals at any time [Colour figure can be viewed at wileyonlinelibrary.com]
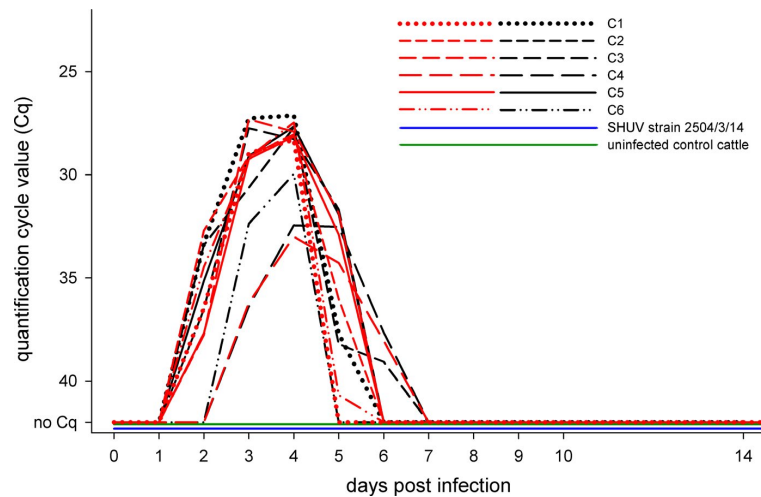
2162/16 seroconverted; neutralizing antibodies could be detected from day seven onwards, reaching titres as high as 1/320 at 14 or 21 dpi (Table 2). In the group that was inoculated with SHUV strain 2504/3/14, neutralizing antibodies were only detectable in the serum of cattle C7, albeit at very low titres of 1/17 (14 dpi) and 1/24 (21 dpi), respectively (Table 2).

The results of the neutralization assay were confirmed by an indirect immunofluorescence assay. A fluorescence signal was visible for all cattle infected with SHUV strain 2162/16 from day 14 onwards, reaching the highest titres on day 21 (1/350). No fluorescence could be seen in samples of the cattle inoculated with SHUV strain 2504/3/14 or the control animals. The commercially available SBV antibody ELISA scored positive in three cattle inoculated with

strain 2162/16 (C1, C3 and C6) at 14 and/or 21 dpi. No antibodies were detected in cattle inoculated with SHUV strain 2504/3/14 or the control animals (Table2).

3.4 | Histopathology and in situ hybridization

Histopathology revealed a mild (C2) to moderate (C1, C4, C6) non-suppurative meningoencephalitis in the animals, in which SHUV RNA was detected by real-time RT-PCR in samples of the central nervous system. The grey and white matter was equally affected by perivascular cuffing and glial nodule formation. The most consistent, moderate inflammation and glial reaction were recorded in

TABLE 2 Results of the serological tests

Animal ID	0 dpi			7 dpi			14 dpi			21 dpi		
	NT (ND_{50})	IIFT	ELISA	NT (ND_{50})	IIFT	ELISA	NT (ND_{50})	IIFT	ELISA	NT (ND_{50})	IIFT	ELISA
C1	<1/5	<1/50	87	**1/24**	<1/50	48	**1/57**	**1/100**	**36**	**1/57**	**1/200**	**40**
C2	<1/5	<1/50	104	**1/28**	<1/50	62	**1/191**	**1/100**	51	**1/270**	**1/200**	50
C3	<1/5	<1/50	92	<1/5	<1/50	66	**1/95**	**1/150**	**34**	**1/320**	**1/250**	42
C4	<1/5	<1/50	103	<1/5	<1/50	91	**1/57**	**1/50**	73	**1/80**	**1/150**	67
C5	<1/5	<1/50	100	**1/12**	<1/50	79	**1/160**	**1/100**	41	**1/135**	**1/300**	55
C6	<1/5	<1/50	103	**1/14**	<1/50	75	**1/328**	**1/300**	41	**1/226**	**1/350**	**40**
C7	<1/5	<1/50	96	<1/5	<1/50	86	**1/17**	<1/50	63	**1/24**	<1/50	67
C8	<1/5	<1/50	100	<1/5	<1/50	96	<1/5	<1/50	70	<1/5	<1/50	73
C9	<1/5	<1/50	95	<1/5	<1/50	89	<1/5	<1/50	90	<1/5	<1/50	91
C10	<1/5	<1/50	103	<1/5	<1/50	94	<1/5	<1/50	94	<1/5	<1/50	90
C11	<1/5	<1/50	99	<1/5	<1/50	93	<1/5	<1/50	92	<1/5	<1/50	99
C12	<1/5	<1/50	92	<1/5	<1/50	88	<1/5	<1/50	78	<1/5	<1/50	83
C13	<1/5	<1/50	99	<1/5	<1/50	83	<1/5	<1/50	80	<1/5	<1/50	85
C14	<1/5	<1/50	96	<1/5	<1/50	88	<1/5	<1/50	89	<1/5	<1/50	92

Note: Titres measured in a microneutralization test are indicated as ND_{50}. Results of the indirect immunofluorescence test are given as the highest dilution in which a fluorescent signal was visible. ELISA (ID Screen SBV competition multispecies, IDvet) results are indicated as the sample optical density (OD) relative to the negative control OD, values above 40% were considered negative. Positive results are shown in bold letters.

the periductal matter of the cerebral aqueduct (3 out of 4 animals). The spinal cord was mildly affected in all infected animals. The hippocampus and cerebellum showed mild to moderate lesions in 3 out of 4 animals. In individual cases, mild perivascular infiltrates or glial reaction was detected in the frontal or parietal lobe or in periventricular area of the lateral ventricle.

Consistently, perivascular infiltrates as well as glial nodules were composed of CD3-positive T-cells and Iba-1-labelled microglia/macrophages in comparable amounts. T-cells were also present in increased numbers, scattered within the grey and white matter. CD79a-positive B cells were only rarely detected. Representative lesions are shown in Figure 3. Apoptosis was not a significant feature with only scattered labelled cells (supplementary Figure S1). Further, astrogliosis was not identified; the number and distribution of GFAP-positive astrocytes did not differ between inoculated animals and the control (supplementary Figure S1). Neither haemorrhage, nor mineralization, nor demyelination, or loss of Nissl substance was found.

Shuni virus RNA was detected by in situ RNA hybridization, within the lesions in the periductal white matter of the cerebral aqueduct, phenotypically consistent with neuronal cell processes (Figure 4).

4 | DISCUSSION

The spread of SHUV from the African continent to Israel (Golender et al., 2015) or the unexpected emergence of SBV in Central Europe in 2011 (Hoffmann et al., 2012) proved the ability of Simbu serogroup viruses to cause large-scale epidemics after incursion into previously not affected areas (EFSA, 2012; Golender et al., 2018).

When naïve, pregnant ruminants are infected, SHUV is known for its ability to cause severe congenital malformations, abortion, premature birth, mummification or stillbirth (Golender et al., 2015). However, during its spread through the Middle East, another clinical presentation became evident, when neurological signs and fatalities were described in young and adult cattle (Golender et al., 2019). In addition to ruminants, SHUV has been associated with neurological disease in other animals such as horses (van Eeden et al., 2012) and was detected in several other domestic and wildlife species (Steyn et al., 2020), demonstrating the broad host range of SHUV.

In our study, virus isolates obtained from representative cases of both clinical presentations from recent outbreaks in Israel, that is a malformed ovine foetus and a heifer showing neurological symptoms, were injected into cattle to compare the outcome under standardized experimental conditions. When comparing the two virus strains used in this study on a molecular level, few but crucial differences have been found. In the S segment, the sequences differ by only three nucleotides, which does not lead to changes of amino acids within the N protein (Golender et al., 2019). However, for the non-structural protein NSs, which is coded in an overlapping alternative reading frame (Walter & Barr, 2011), the varying nucleotide at position 50 in the NSs open reading frame ORF results in a stop codon in strain SHUV 2504/3/14 instead of a tryptophan in strain 2162/16 (Golender et al., 2016, 2019). In vitro, this mutation led to a loss of the protein function (Golender et al., 2016), that is to prevent the activation of the host's innate immune response of releasing interferons (Elliott & Blakqori, 2011). In this study, it was demonstrated by a lack of viremia in all inoculated animals that the NSs function of strain 2504/3/14 is also impaired in vivo. The inability of NSs deletion mutant viruses to induce viremia in interferon-competent mammalians was already demonstrated for
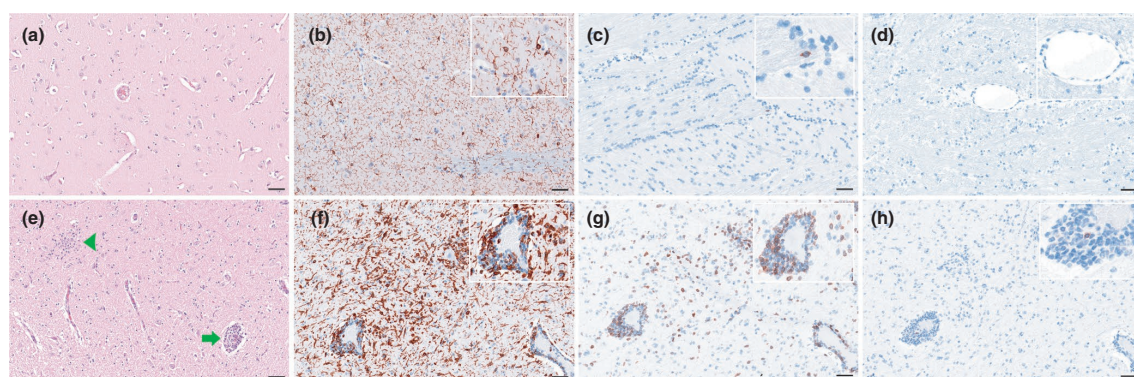
FIGURE 3 Histopathology and immunohistochemistry of brain sections of control (a-d) and Shuni virus-infected animals (e-h). Haematoxylin and eosin (a, e) or immunohistochemistry with AEC (red-brown) chromogen and Mayer's haematoxylin counterstain (b-d, f-h). All scale bars 50 μm, inlays showing details. (a) Unaffected brain, control animal. (b) Iba-1 labelled microglia/macrophages, note the equal distribution of slender cells, control animal. (c) CD3-positive, single cell, control animal. (d) Absence of CD79a-labelled B cells, control animal. (e) Non-suppurative, moderate encephalitis with mononuclear, perivascular cuffing (arrow, 4 layers) and focal glial nodule formation (arrow head), infected animal C6, (f) Iba-1-positive microglial cells/macrophages, note the increased number (hyperplasia) and size (hypertrophy) of labelled cells, infected animal C4. (g) CD3-positive cells in high number, perivascularly and scattered within the neuropil, infected animal C4. (h) CD79a labelled, single B-cell, infected animal C4 [Colour figure can be viewed at wileyonlinelibrary.com]
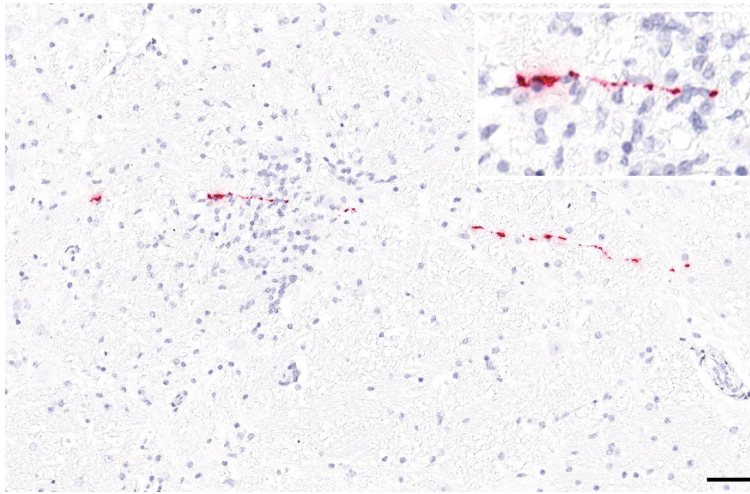
FIGURE 4 RNA in situ hybridization of Shuni virus (SHUV)-infected animal C4. Chromogenic labelling (fast red) with probes to SHUV N-coding region are visible intralesional, in neuronal cell body processes (arrow and inlay), within a focal inflammatory reaction, Mayer's haematoxylin counterstain. Scale bar = 50 μm [Colour figure can be viewed at wileyonlinelibrary.com]

other simbuviruses, such as SBV (Kraatz et al., 2015). However, for SBV, the NSs deletion mutant was generated by reverse genetics (Kraatz et al., 2015), while the SHUV mutant used in this study evolved naturally. This mutant virus occurred in a malformed foetus and not in an acutely infected adult animal, further supporting the theory that genetically highly variable Simbu serogroup viruses evolving in foetuses represent dead-end artefacts that are not fit for the usual transmission cycle. Nevertheless, the inoculation of strain 2504/4/14 led to the development of neutralizing antibodies in one of six animals, albeit at low level, which has been likewise described for an NSs-deleted SBV mutant (Kraatz et al., 2015). In the case of SBV, this was used for the generation of live attenuated candidate vaccines, since immunization with the NSs deletion mutant conferred protection from virulent virus challenge. Whether this holds also true for the naturally evolved SHUV strain needs to be further evaluated in future vaccination-challenge experiments.

In contrast to the NSs-deficient virus, SHUV strain 2162/16, which was isolated from an acutely infected animal, successfully infected calves leading to a short-lived viremia and seroconversion in all six inoculated animals. This, for vector-transmitted agents surprisingly short viremia, was also demonstrated for other simbuviruses such as SBV (Hoffmann et al., 2012) and AKAV (Lee et al., 2016) and seems to be long enough for the arthropod vector to acquire the virus and for the establishment of a successful transmission cycle between the vector and the mammalian hosts.

Even though experimentally infected animals did not show clinical signs characteristic for a neurological disease, viral genome was detected in the central nervous system of four out of six animals by real-time RT-PCR. Furthermore, the histopathological investigation revealed a non-suppurative meningoencephalitis in all of these animals. Perivascular cuffs and glial nodules as common characteristic features of viral encephalitis were observed in both, naturally infected (Golender et al., 2019) and in experimentally inoculated cattle. In addition, SHUV RNA was visualized in this study within the lesions by in situ hybridization. Hence, SHUV could be determined as the causative agent of the encephalitis.

In general, the clinical picture observed in SHUV infected cattle mirrors that of the related AKAV, where some strains may induce encephalitis in postnatally infected cattle, while others are associated with abnormal deliveries (Yanase et al., 2018). However, AKAV strains found in association with encephalitis were also isolated from asymptomatic cattle (Yanase et al., 2018), and SHUV isolates likewise did not reveal any specific genomic changes that could be associated with neuropathogenicity (Golender et al., 2019).

As acute SHUV infections often seem to be asymptomatic or associated with only mild, unspecific clinical signs, the spatial distribution of the virus might be underestimated. As demonstrated in this study, even major histological findings like encephalitis might not result in symptoms. To identify the actual distribution of SHUV, serological screenings might be considered, especially since the detection of the virus itself is time restricted by the short viremia of only a few days. Both assays presented in this study, that is the neutralization test and the indirect immunofluorescence test, proved suitable for the detection of SHUV antibodies. Alternatively, biting midges (genus *Culicoides*) could be monitored for the presence of viral genome as has been demonstrated for various Simbu serogroup viruses (Behar et al., 2020; De Regge et al., 2012; Stram et al., 2004).

In summary, it was demonstrated that cattle, one of the major target species of SHUV, represent a suitable animal model to study the pathogenesis of the virus. Moreover, the neuropathogenicity of SHUV could be demonstrated under experimental conditions. Thus, SHUV should be included in the list of differential diagnosis of encephalitis in ruminants and an outbreak of SHUV in Europe would most likely result in more infected species and a broader spectrum of clinical signs, including neuropathology.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ETHICAL APPROVAL

The experimental protocol was evaluated by a state ethics commission and approved by the competent authority (permission number LALLF M-VTSD/7221.3–2-010/18).

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Martin Beer https://orcid.org/0000-0002-0598-5254
Kerstin Wernike https://orcid.org/0000-0001-8071-0827

REFERENCES

Beer, M., & Wernike, K. (2019). Akabane virus and Schmallenberg virus. *Reference Module in Life Sciences*, https://doi.org/10.1016/b978-0-12-809633-8.20939-4

Behar, A., Rot, A., Lavon, Y., Izhaki, O., Gur, N., & Brenner, J. (2020). Seasonal and spatial variation in Culicoides community structure and their potential role in transmitting Simbu serogroup viruses in Israel. *Transboundary and Emerging Diseases*, 67(3), 1222–1230. https://doi.org/10.1111/tbed.13457

Behrens, B., & Kärber, G. (1934). Wie sind Reihenversuche für biologische Auswertungen am zweckmäßigsten anzuordnen? *Naunyn-Schmiedebergs Archiv Für Experimentelle Pathologie Und Pharmakologie*, 177(2), 379–388. https://doi.org/10.1007/BF02023142

Causey, O. R., Kemp, G. E., Causey, C. E., & Lee, V. H. (1972). Isolations of Simbu-group viruses in Ibadan, Nigeria 1964–69, including the new types Sango, Shamonda, Sabo and Shuni. *Annals of Tropical Medicine and Parasitology*, 66(3), 357–362. https://doi.org/10.1080/00034983.1972.11686835

De Regge, N., Deblauwe, I., De Deken, R., Vantieghem, P., Madder, M., Geysen, D., & Cay, A. B. (2012). Detection of Schmallenberg virus in different Culicoides spp. by real-time RT-PCR. *Transboundary and Emerging Diseases*, 59(6), 471–475. https://doi.org/10.1111/tbed.12000

EFSA (2012). *"Schmallenberg" virus: analysis of the epidemiological data* (November 2012). EFSA Supporting Publications 2012. EN-360. http://www.efsa.europa.eu/en/supporting/doc/360e.pdf; accessed 15/07/2013.

Elliott, R. M., & Blakqori, G. (2011). Molecular biology of orthobunyaviruses. In A. Plyusnin, & R. M. Elliott (Eds.), *Bunyaviridae: Molecular and Cellular Biology*. Caister Academic Press.

Golender, N., Brenner, J., Valdman, M., Khinich, Y., Bumbarov, V., Panshin, A., Edery, N., Pismanik, S., & Behar, A. (2015). Malformations Caused by Shuni Virus in Ruminants, Israel, 2014–2015. *Emerging Infectious Diseases*, 21(12), 2267–2268. https://doi.org/10.3201/eid2112.150804

Golender, N., Bumbarov, V., Assis, I., Beer, M., Khinich, Y., Koren, O., Edery, N., Eldar, A., & Wernike, K. (2019). Shuni virus in Israel: Neurological disease and fatalities in cattle. *Transboundary and Emerging Diseases*, 66(3), 1126–1131. https://doi.org/10.1111/tbed.13167

Golender, N., Bumbarov, V. Y., Erster, O., Beer, M., Khinich, Y., & Wernike, K. (2018). Development and validation of a universal S-segment-based real-time RT-PCR assay for the detection of Simbu serogroup viruses. *Journal of Virological Methods*, 261, 80–85. https://doi.org/10.1016/j.jviromet.2018.08.008

Golender, N., Wernike, K., Bumbarov, V., Aebischer, A., Panshin, A., Jenckel, M., Khinich, Y., & Beer, M. (2016). Characterization of Shuni viruses detected in Israel. *Virus Genes*, 52(6), 806–813. https://doi.org/10.1007/s11262-016-1381-3

Hoffmann, B., Scheuch, M., Höper, D., Jungblut, R., Holsteg, M., Schirrmeier, H., Eschbaumer, M., Goller, K. V., Wernike, K., Fischer, M., Breithaupt, A., Mettenleiter, T. C., & Beer, M. (2012). Novel orthobunyavirus in Cattle, Europe, 2011. *Emerging Infectious Diseases*, 18(3), 469–472. https://doi.org/10.3201/eid1803.111905

Kirkland, P. D. (2015). Akabane virus infection. *Revue Scientifique Et Technique*, 34(2), 403–410. https://doi.org/10.20506/rst.34.2.2366

Kraatz, F., Wernike, K., Hechinger, S., König, P., Granzow, H., Reimann, I., & Beer, M. (2015). Deletion mutants of Schmallenberg virus are avirulent and protect from virus challenge. *Journal of Virology*, 89(3), 1825–1837. https://doi.org/10.1128/JVI.02729-14

Lee, H., Jeong, H., Park, S., Yang, M.-S., Kim, J., Bae, J., Kwon, Y., Kim, M.-S., Oem, J.-K., Lee, M.-H., Lim, C.-W., & Kim, B. (2016). Experimental infection of cows with newly isolated Akabane virus strain (AKAV-7) causing encephalomyelitis. *Veterinary Research*, 47(1), 62. https://doi.org/10.1186/s13567-016-0349-6

Miyazato, S., Miura, Y., Hase, M., Kubo, M., Goto, Y., & Kono, Y. (1989). Encephalitis of cattle caused by Iriki isolate, a new strain belonging to Akabane virus. *Nihon Juigaku Zasshi*, 51(1), 128–136. https://doi.org/10.1292/jvms1939.51.128

Möhlmann, T. W. R., Oymans, J., Wichgers Schreur, P. J., Koenraadt, C. J. M., Kortekaas, J., & Vogels, C. B. F. (2018). Vector competence of biting midges and mosquitoes for Shuni virus. *PLoS Neglected Tropical Diseases*, 12(12), e0006993. https://doi.org/10.1371/journal.pntd.0006993

Moore, D. L., Causey, O. R., Carey, D. E., Reddy, S., Cooke, A. R., Akinkugbe, F. M., David-West, T. S., & Kemp, G. E. (1975). Arthropod-borne viral infections of man in Nigeria, 1964–1970. *Annals of Tropical Medicine and Parasitology*, 69(1), 49–64. https://doi.org/10.1080/00034983.1975.11686983

Oluwayelu, D., Wernike, K., Adebiyi, A., Cadmus, S., & Beer, M. (2018). Neutralizing antibodies against Simbu serogroup viruses in cattle and sheep, Nigeria, 2012–2014. *BMC Veterinary Research*, 14(1), 277. https://doi.org/10.1186/s12917-018-1605-y

Plyusnin, A., & Elliott, R. M. (2011). *Bunyaviridae : Molecular and cellular biology*. Caister Academic Press.

Steyn, J., Motlou, P., van Eeden, C., Pretorius, M., Stivaktas, V. I., Williams, J., Snyman, L. P., Buss, P. E., Beechler, B., Jolles, A., Perez-Martin, E., Myburgh, J. G., Steyl, J., & Venter, M. (2020). Shuni Virus in Wildlife and Nonequine Domestic Animals, South Africa. *Emerging Infectious Diseases*, 26(7), 1521–1525. https://doi.org/10.3201/eid2607.190770

Stram, Y., Brenner, J., Braverman, Y., Banet-Noach, C., Kuznetzova, L., & Ginni, M. (2004). Akabane virus in Israel: A new virus lineage. *Virus Research*, 104(1), 93–97. https://doi.org/10.1016/j.virusres.2004.03.004

Toussaint, J. F., Sailleau, C., Breard, E., Zientara, S., & De Clercq, K. (2007). Bluetongue virus detection by two real-time RT-qPCRs targeting two different genomic segments. *Journal of Virological Methods*, 140(1–2), 115–123. https://doi.org/10.1016/j.jviromet.2006.11.007

van Eeden, C., Swanepoel, R., & Venter, M. (2014). Antibodies against West Nile and Shuni Viruses in Veterinarians, South Africa. *Emerging Infectious Diseases*, 20(8), 1409–1411. https://doi.org/10.3201/eid2008.131724

van Eeden, C., Williams, J. H., Gerdes, T. G., van Wilpe, E., Viljoen, A., Swanepoel, R., & Venter, M. (2012). Shuni virus as cause of neurologic disease in horses. *Emerging Infectious Diseases*, *18*(2), 318–321. https://doi.org/10.3201/eid1802.111403

Walter, C. T., & Barr, J. N. (2011). Recent advances in the molecular and cellular biology of bunyaviruses. *Journal of General Virology*, *92*, 2467–2484. https://doi.org/10.1099/vir.0.035105-0

Wernike, K., Beer, M., & Hoffmann, B. (2017). Schmallenberg Virus Infection Diagnosis: Results of a German Proficiency Trial. *Transboundary and Emerging Diseases*, *64*(5), 1405–1410. https://doi.org/10.1111/tbed.12517

Wernike, K., Elbers, A., & Beer, M. (2015). Schmallenberg virus infection. *Revue Scientifique Et Technique*, *34*(2), 363–373. https://doi.org/10.20506/rst.34.2.2363

Yanase, T., Kato, T., Hayama, Y., Akiyama, M., Itoh, N., Horiuchi, S., Hirashima, Y., Shirafuji, H., Yamakawa, M., Tanaka, S., & Tsutsui, T. (2018). Transition of Akabane virus genogroups and its association with changes in the nature of disease in Japan. *Transboundary and Emerging Diseases*, *65*(2), e434–e443. https://doi.org/10.1111/tbed.12778

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Sick F, Breithaupt A, Golender N, Bumbarov V, Beer M, Wernike K. Shuni virus-induced meningoencephalitis after experimental infection of cattle. *Transbound. Emerg. Dis.* 2021;68:1531–1540. https://doi.org/10.1111/tbed.13823

„Characterization of a small animal model to study Shuni virus infection"

Angele Breithaupt [1], Franziska Sick [2], Natalia Golender [3], Martin Beer [2], Kerstin Wernike [2]

[1] Department of Experimental Animal Facilities and Biorisk Management, Friedrich-Loeffler-Institut, Greifswald-Insel Riems, Germany

[2] Institute of Diagnostic Virology, Friedrich-Loeffler-Institut, Greifswald-Insel Riems, Germany

[3] Department of Virology, Kimron Veterinary Institute, Bet Dagan, Israel

Keywords:

Shuni virus, bunyavirus, arbovirus, meningoencephalomyelitis, animal model, zoonotic virus

Abstract

Shuni virus (SHUV), an orthobunyavirus of the Simbu serogroup, was initially isolated in Nigeria in the 1960th, further detected in other African countries and in the Middle East, and is now endemic in Israel. Transmitted by blood-sucking insects, SHUV infection is associated with neurological disease in cattle and horses but also with abortion, stillbirth, or the birth of malformed offspring in ruminants. Surveillance studies also indicated a zoonotic potential. Our study aimed to test the susceptibility of the well-characterized interferon-α/β receptor knock-out mouse model (IFNAR −/−), to identify target cells and to describe the neuropathology. To this end, IFNAR −/− mice were subcutaneously infected with two different SHUV strains, including a strain isolated from a calf showing neurological signs. The second strain represented a natural deletion mutant exhibiting a loss of the function of the non-structural protein NSs, which is counteracting the host's interferon response. Here we show that IFNAR-/- mice are susceptible to both SHUV strains and can develop fatal disease. Histopathology confirmed comparable lesions in mice as described for cattle. A mainly T-cell mediated, non-suppurative meningoencephalomyelitis with increased numbers of activated microglia/macrophages was found. RNA Scope™-based SHUV detection identified neurons and glial cells, as well as macrophages of the spleen and intestinal mucosa-associated lymphoid tissue as target cells. Thus, our mouse model is particularly beneficial for in vivo studies and the evaluation of virulence determinants in the pathogenesis of SHUV infection in animals.

Shuni virus (SHUV) is an insect-transmitted orthobunyavirus of the Simbu serogroup within the family *Peribunyaviridae*.[9] SHUV was initially isolated in Nigeria in the 1960th and subsequently also detected in further African countries.[1] The spread of SHUV from the African continent to the Middle East was initially reported in Israel in 2014 and, since then, the virus established an endemic status in this country.[2,4] SHUV has been associated with neurological disease in cattle and horses, and was also detected in other domestic and wildlife species.[11,15,3] In addition, an infection of naïve ruminants during gestation may lead to abortion, stillbirth or the birth of malformed lambs and calves.[2,3] A zoonotic potential was suspected since SHUV antibodies have been found in veterinarians in South Africa and became even more likely after SHUV RNA detection in cerebrospinal fluid specimens from human neurologic disease cases.[14,8]

During the recent disease outbreaks in Israel, SHUV was isolated from both clinical cases, namely encephalitis in cattle (strain 2162/16) and abnormal course of gestation (strain 2504/3/14).[5,3,16] The SHUV strain 2504/3/14 originated from a malformed, aborted sheep fetus and harbored genome mutations with particular effect on the S-segment encoded non-structural protein NSs expression, leading to a loss of the NSs protein function.[5] *In vitro* data suggested that this NSs-truncated SHUV 2504/3/14 did not prevent the activation of the host's innate immune interferon (IFN) response anymore.[5]

We recently demonstrated that experimental infection of cattle with the "encephalitis strain" SHUV 2162/16 led to short-term viremia and seroconversion. Five out of six animals showed elevated body temperature, but neurological signs were not recorded within 21 days. However, as reported for naturally infected animals,[3] we found a non-suppurative meningoencephalitis, and SHUV genome was identified intralesionally by *in situ* hybridization.[10] In clear contrast, the NSs-truncated "malformation strain" 2504/3/14 did not lead to viremia. Seroconversion was found at very low titers in only one out of six animals, indicating that this natural mutant represents a dead-end artefact, which is not fit for the usual transmission cycle.[10]

To date, no information is available on the neuropathology of SHUV infection in humans. This highlights the importance of using well-characterized animal models to reveal clues to the potential pathogenesis of this disease in humans but also in domestic animals. Studies using large animals are complicated by the high costs and the limited availability of adequate high

containment housing for laboratory animals. However, these obstacles could be mitigated by small animal models as soon as suitable species are identified. As research on orthobunyaviruses has significantly benefitted from the introduction of the interferon-α/β receptor knock-out (IFNAR −/−) mice,[18,12] we investigated whether this mouse model is also applicable to study SHUV infections.

Materials and Methods

Virus

Two virus strains were used. The "encephalitis strain" SHUV 2162/16 was isolated on Vero cells from the brain homogenate of a heifer showing neurological symptoms.[3] The "malformation strain" SHUV 2504/3/14 expresses a truncated NSs protein due to a stop codon in the NSs open reading frame and was obtained from the brain of a malformed, aborted sheep fetus. Isolation was performed using suckling mice and Vero cells (for details see [2,3]). Both isolates were passaged once each on *Culicoides sonorensis* cells (KC, L1062, collection of cell lines in veterinary medicine (CCLV), Friedrich-Loeffler-Institut, Greifswald-Insel Riems, Germany) and baby hamster kidney cells (BHK 21, L0164 CCLV).

Animal experiment

The experimental protocol was evaluated by a state ethics commission and approved by the competent authority (permission number LALLF M-VTSD/7221.3–2-010/18).

Twenty-six IFNAR−/− mice on a C57BL/6 genetic background were obtained from the specific-pathogen-free breeding unit of the FLI. The age of the mice on the day of inoculation was 35 to 38 weeks. Twenty-four IFNAR−/− mice were allocated to 4 groups with 6 animals per group, mice of both sexes were distributed evenly among the groups. Animals were inoculated subcutaneously into the scruff of the neck with 10^4 (low dose group) or 10^5 (high dose group) 50% tissue culture infectious doses (TCID$_{50}$) of either SHUV/2504/3/14 or SHUV/2162/16. The two remaining mice were injected with phosphate-buffered saline (PBS) and kept as controls (environmental control). During the entire study, all animals were offered water and rodent pellets ad libitum, and were checked for clinical signs and body weight daily by veterinarians. A clinical scoring system was applied comprising body weight, behavior, the appearance of neurological signs and eyes/respiration. Up to three points were awarded for each category. A clinical score higher than 10 points, a weight loss of more than 25 % of the weight on the

starting day and predefined clinical signs such as paralysis of the limbs or dyspnea were defined as endpoint criteria. Animals reaching the endpoint criteria were euthanized immediately and subjected to autopsy. All remaining mice were euthanized after 21 days.

Whole blood samples were collected on days 3 and 7 post inoculation (dpi). At autopsy, serum, full blood (EDTA) and tissue samples were collected, including heart, lung, liver, kidney, urinary bladder, spleen, jejunum, colon, cervical spinal cord, brain (half brain, sagittal section), skeletal muscle and gonads.

Serology

The sera collected at autopsy were analyzed for SHUV-specific antibodies by a previously described multispecies ELISA.[17]

Real-Time RT-PCR

Tissue samples were homogenized in 1 ml of Modified Eagle Medium using a TissueLyzer instrument (Qiagen, Hilden, Germany). Total RNA from 100 µl of the tissue homogenates, 20 µl of the sera or 20 µl of the whole blood samples were extracted using the King Fisher 96 Flex purification system (ThermoFisher Scientific, Braunschweig, Germany) in combination with the NucleoMag Vet kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. The extracts were subsequently tested for SHUV genome using an S-segment based generic Simbu serogroup real time RT-PCR.[4] To control for efficient RNA extraction and amplification, thereby avoiding false negative results, an internal control based on the beta-actin gene was additionally tested.[13]

Pathology

Tissue samples were collected and fixed in 10% neutral-buffered formalin and trimmed for paraffin embedding. Samples were cut at 2-3-µm-thick sections and stained with hematoxylin and eosin (HE) for light microscopical examination. On consecutive brain and spinal cord sections, conventional staining protocols were applied, including Luxol Fast Blue Cresyl Violet for detection of myelin sheaths and Nissl substance, von Kossa stain to show mineralization, and Prussian blue reaction for demonstration of ferric iron, indicating hemosiderin. Consecutive brain and spinal cord slides were processed for immunohistochemistry. Briefly, sections were mounted on adhesive glass slides, dewaxed in xylene, followed by rehydration in descending graded alcohols. Endogenous peroxidase was quenched with 3 % hydrogen

peroxide in distilled water for 10 minutes at room temperature. Antigen heat retrieval was performed in a steamer for 20 minutes followed by a cooling period. Nonspecific antibody binding was blocked by pure goat normal serum for 30 minutes at room temperature. T-cell and microglia/macrophage markers were applied for all animals. B-cell and astrocyte markers were used for uninfected animals and those with meningoencephalomyelitis (details given in Table 1). Immunolabeling was visualized by 3-amino-9-ethylcarbazole substrate (AEC, Dako, Agilent, Santa Clara, CA, USA) producing a red-brown signal and sections were counter-stained with Mayer's hematoxylin.

Lesions were scored based on ordinal grades applied on the whole tissue: 0 = no lesion; 1 = minimal or rare (area affected ≤5% or ≤3 foci); 2 = mild or multifocal (6-40% or >3 foci); 3 = moderate or coalescing (41-80%); 4 = severe or diffuse (>80%).

RNA *in situ* hybridization for the detection of SHUV RNA in brain tissue sections was performed on selected brain and spinal cord sections based on PCR results with the RNAScope™ 2-5 HD Reagent Kit-Red (ACD, Advanced Cell Diagnostics, Newark, CA) according to the manufacturer's instructions. For hybridization, RNAScope™ probes were custom-designed for the nucleocapsid protein. The specificity of the probes was verified using a positive control probe peptidylprolyl isomerase B (cyclophilin B, ppib) and a negative control probe dihydrodipicolinate reductase (DapB). Evaluation and interpretation were performed by a board-certified pathologist (DiplECVP) following postexamination masking approach.[7]

Results

Clinical findings

After inoculation, all animals were monitored daily for body weight loss and clinical signs (Figures 1-4). SHUV 2504/3/14 infection led to body weight loss in 2 out of 6 (2/6) mice after low dose infection, one animal (mouse no. 2-3) was euthanized at 6 dpi due to slowed down movement and body weight loss (about 10% within one day). No clinical signs apart from marginal weight loss were reported in the remaining animals of this group. High dose infection yielded in body weight loss in 1/6 mice and a further animal (no. 5-1) was euthanized at 9 dpi because of slowed down movement and body weight loss.

Low dose infection with SHUV 2162/16 was associated with body weight loss of about 5 to max. 10% in 3/6 mice (Figure 1). Two of these animals (no. 8-1 and 8-2) were found dead at

10 dpi. High dose infection led to weight loss in 3/6 mice, no further clinical signs were recorded.

The clinical score did not exceed 3 points in any animal at any time point (Figure 2).

Serology and viral genome detection

By ELISA, SHUV-specific antibodies could be detected in mice 5-1 and 8-2, and in all inoculated animals surviving until the end of the study (Figure 4). The uninfected control animals remained seronegative. SHUV genome was detected by real-time RT-PCR in serum, full blood and tissue samples of all groups except the uninfected control mice (Figure 3). All SHUV inoculated animals tested positive in at least one sample during the study. SHUV RNA was found in sera collected at 3 dpi in all inoculated mice with exception of animal 8-2 (Figure 1), and quantification cycle (Cq) values ranged from about 28 to 31. No difference was seen between both virus strains and dose levels. Viral RNA was also found at 7 dpi in all but three animals. In specimens collected at 21 dpi, viral RNA was most consistently found in the spleen (Cq values 34 to 38) and jejunum (Cq values 32 to 37) but also in the heart, skeletal muscle, spinal cord and brain. However, only spinal cord and brain samples yielded higher viral loads compared to full blood samples (Figure 3).

In animals euthanized prematurely, viral RNA was found in a broad spectrum of tissues, however, only brain (Cq values 16 to 19) and spinal cord samples (Cq values 16 to 22) markedly exceed the viral load of the serum or blood (Figure 3).

Histopathology

Histopathologic evaluation was performed on sections of the heart, lung, liver, kidney, urinary bladder, spleen, jejunum, colon, cervical spinal cord, brain, skeletal muscle and gonads. Uninfected control animals did not show any lesions. In the spleen of control mice, we found minimal numbers of tingible body macrophages (TBM) and minimal extramedullary hematopoiesis (EMH), interpreted to be within normal limits.

After SHUV 2504/3/14 low dose infection, 5/6 animals survived until 21 dpi. Most strikingly, in animal no. 2-2, we found oligofocal glial nodules and moderate perivascular lymphocytic cuffs in the cerebrum and cerebellum, in particular affecting the brain stem, the periductal gray matter and cerebellar white substance (arbor vitae). In the spleen, all animals showed a minimal to mild increased number and size of follicular germinal centers and one animal

exhibited a slightly increased number of TBM and EMH. Animal no. 2-3, which was euthanized 6 dpi, showed diffuse, moderate mainly neutrophilic and fewer lymphocytic perivascular cuffs and meningeal infiltrates as well as coalescing areas of neuronal and glial cell degeneration and necrosis, predominantly in the cerebral brain stem and spinal cord. The cerebellum was only focally affected. There was a moderately increased number and size of splenic germinal centers, mildly increased numbers of TBM and a moderate EMH. The intestinal mucosa-associated lymphoid tissue (MALT) of the jejunum showed focal, minimal lymphoid necrosis with few neutrophilic infiltrates and in the large intestinal MALT, the number of TBM was slightly increased. No lesion was diagnosed in any other tissue.

We identified comparable changes after SHUV 2504/3/14 high dose infection. Again, 5/6 animals survived until 21 dpi. Findings were limited to the spleen, with three animals having a minimal to mild increased number and size of follicular germinal centers, and one animal with a slightly increased number of TBM. Another mouse presented with mild EMH. Animal no. 5-1, euthanized 9 dpi, exhibited coalescing areas of mild perivascular mainly lymphocytic and fewer neutrophilic cuffs, glial nodules as well as meningeal infiltrates. The spinal cord was diffusely, the cerebellum multifocally affected. There was multifocal neuronal and glial cell degeneration and necrosis mainly in the cerebral brain stem and spinal cord. In the spleen, germinal centers were moderately increased in size and number, TBM were mildly increased in number and there was a moderate EMH. Intestinal MALT was not present, even after several consecutive sections evaluated and no lesion was diagnosed in any other tissue.

After SHUV 2162/16 low dose infection, 4/6 animals survived until 21 dpi. Two of these mice showed a slightly increased number and size of splenic germinal centers. No lesions were found in other tissues including the brain and spinal cord. However, both animals that were found dead 10 dpi exhibited diffuse, moderate mainly neutrophilic and fewer lymphocytic perivascular cuffs, glial nodules and meningeal infiltrates as well as coalescing areas of neuronal and glial cell degeneration and necrosis in the cerebrum and spinal cord. The cerebellum was multifocally to coalescent affected. There was a slightly increased number and size of splenic germinal centers in one animal and a moderately increased number of TBM and mild EMH in both prematurely euthanized mice. Additionally, we found mildly increased numbers of TBM in the jejunal MALT in one mouse. No lesion was diagnosed in any other tissue.

All animals survived until 21 dpi after high dose infection with SHUV 2162/16. Animal no. 10-1 exhibited a focal, minimal perivascular lymphocytic cuff in the cerebellum and spinal cord. The majority of mice presented with a slightly increased size and number of germinal centers (4/6) as well as up to mildly increased number of TBM (5/6) and mild EMH (2/6). No further changes were recorded.

Neither strain nor dose-related differences were found based on *in situ* hybridization, immunohistochemistry and special stains. Shuni virus RNA was abundantly detected by RNA Scope™-based *in situ* hybridization in all animals euthanized or found dead at 6, 9 or 10 dpi (Figures 5-8) but not in animals affected by meningoencephalomyelitis and killed at 21 dpi. Labelled cells were phenotypically consistent with neurons and glial cells and found in the cervical spinal cord (Figures 5-7), cerebrum and cerebellum. Remarkably, viral RNA was also detected in single cells of the MALT (animal no. 2-3) and spleen (no. 8-1), that were phenotypically consistent with macrophages (Figure 8). Viral RNA detection was associated with neuronal satellitosis, degeneration and necrosis of neurons and glial cells as well as increased numbers and activation of microglia/macrophages and astrocytes (Figure 9a-d).

Early and late stage lesions were further evaluated based on immunohistochemistry (Figures 10-13). The lymphocytic perivascular and meningeal infiltrates as well as glial nodules were composed of mainly CD-3 positive T cells and hypertrophic Iba-1 labelled microglia/macrophages in comparable amounts. T-cells were also present scattered within the gray and white matter. The detection of scattered CD79a positive B-cells was restricted to animal no. 2-2, killed 21 dpi. To identify subtle infiltrates and microglial reactions that might have been missed by HE-based examination, we tested all animals without apparent lesions in the central nervous system for CD3 and Iba-1 positive cells and applied all special stains, but no additional findings were identified. GFAP immunohistochemistry did not reveal obvious differences in the shape of astrocytes, still showing slender appearance. However, 2 out of 3 animals euthanized or found dead at 9 or 10 dpi (no. 5-1, 8-2) and all investigated animals affected by meningoencephalomyelitis and killed at 21 dpi (no. 2-2, 10-1) had an unusually high number of GFAP-labelled cells.

Using Luxol Fast Blue Cresyl Violet, von Kossa stain and Prussian blue reaction neither hemorrhage, nor mineralization, nor demyelination, or loss of Nissl substance were found in affected brain and spinal cord sections (details not shown).

Discussion

IFNAR −/− mice are used as a small animal model for several orthobunyaviruses, as the neuropathology in these animals appeared to be similar to that found in the natural hosts.[18,12] Therefore, we have explored the potential of this animal system for studies of SHUV infection as well. Two different virus dose levels were selected and interestingly, they did not result in clear differences in the parameters of body weight, clinical findings, viral genome load, serology and pathology in the established mouse model.

As reported for experimentally SHUV-infected cattle,[10] the overall clinical picture was inconspicuous in most inoculated mice, however, some IFNAR -/- mice developed fatal disease. Most important, the pathology of the central nervous system lesions resemble those found in cattle after natural and experimental infection.[10,3] A non-suppurative meningoencephalitis was characterized by perivascular cuffs, comprised of mainly CD3-positive T-cells and Iba-1 labelled microglia/macrophages. Rarely, CD79a positive B-cells were found. Neither hemorrhage, nor mineralization, nor demyelination, or loss of Nissl substance were found.

RNA Scope™-based SHUV detection in infected IFNAR −/− mouse tissues demonstrated abundant viral RNA in neurons and cells phenotypically consistent with glial cells, at 6, 9 and 10 dpi. By contrast, in cattle the viral RNA detection was restricted to focal neuronal cell processes. This difference between both species is most likely related to the fact, that cattle were evaluated only at 21 dpi in a previous experimental infection study.[10] Unfortunately, data for earlier time points after infection are not yet available for cattle. In turn, most of the mice were likewise investigated as late as 21 dpi in the present study and either no or only marginal levels of viral RNA could be detected in those animals. It remains open whether mice that did not show meningoencephalomyelitis or viral genome in the central nervous system at 21 dpi were able to completely prevent neuroinvasion or effectively cleared the virus. Localized foci of inflammation and viral genome may have been missed and thus underestimated the neuroinvasive capacity of SHUV in the small animal model. Hence, the investigation of the acute infection phase should be addressed in future studies in both, the natural ruminant host and the IFNAR -/- mouse model.

Interestingly, *in situ* hybridization identified viral RNA in macrophages of the spleen and intestinal mucosa-associated lymphoid tissue of mice. This feature is probably shared with

SHUV-infected cattle, indicated by positive PCR results in the spleen and intestine.[10] This phenomenon of viral genome detection in lymphoid tissues has been previously reported for other viruses closely related to SHUV such as Schmallenberg virus (SBV).[19] After experimental SBV inoculation, viral RNA could be detected in cattle and sheep several weeks after infection in the lymphoid tissues, most often in the mesenteric lymph nodes.[19,18,20] Hence, the role of macrophages in the early and late stage infection with Simbu serogroup viruses should be addressed in upcoming studies.

As expected, both viruses, the "encephalitis" SHUV 2162/16 and the "malformation" strain SHUV 2504/3/14 did not differ markedly in any parameter tested in our mouse model lacking a functional IFN-α/β receptor. Comparable results have already been achieved using other NSs-deficient mutant orthobunyaviruses including SBV.[6] Therefore, the effect of NSs on the pathogenesis in the natural host cannot be evaluated using the IFNAR -/- mouse model. However, these mice represent an excellent model to study the neuropathogenicity and the impact of virulence determinants beyond NSs, as the post mortem lesions mirror those seen in cattle. The present study highlights the importance of detailed histopathological evaluation and the value of techniques that enable a lesion associated pathogen detection. The latter is the prerequisite to distinguish indirect, e.g. immunopathological processes, from direct lesions triggered by infection. SHUV RNA has been detected only recently in the cerebrospinal fluid from human patients with neurologic disease.[8] However, target cells and neuropathology have not been described so far for humans. This highlights the importance of using well-characterized animal models to reveal clues to the potential pathogenesis of this disease also in humans. Further, SHUV should be considered as etiologic differential diagnoses for meningoencephalomyelitis not only for wildlife and domestic animals but also for humans in endemically affected regions.

In conclusion, we show that IFNAR-/- mice are susceptible to SHUV infection and can develop fatal disease. Histopathology confirmed comparable lesion in mice and the natural ruminant host. Thus, our mouse model is particularly beneficial for *in vivo* studies of SHUV infection and evaluation of virulence determinants in the pathogenesis. The current study provides first insights into the pathogenesis, but a deeper understanding of the virulence determinants and pathogenesis is urgently needed, especially as a further spread of the insect-transmitted pathogen into hitherto unaffected regions should be expected.

Acknowledgments

We thank Bianka Hillmann, Silvia Schuparis and Gabriele Czerwinski for their excellent technical assistance and the animal caretakers for their dedicated work.

Conflict of Interest Statement

The authors declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

This study was funded by the German Federal Ministry of Food and Agriculture (BMEL) through the Federal Office for Agriculture and Food (BLE), grant number 281B101816.

References

1. Causey OR, Causey CE, Kemp GE, Lee VH. Isolations of Simbu-Group Viruses in Ibadan, Nigeria 1964-69, Including New Types Sango, Shamonda, Sabo and Shuni. *Ann Trop Med Parasit*. 1972;66**:** 357-&.

2. Golender N, Brenner J, Valdman M, et al. Malformations Caused by Shuni Virus in Ruminants, Israel, 2014-2015. *Emerg Infect Dis*. 2015;21**:** 2267-2268.

3. Golender N, Bumbarov V, Assis I, et al. Shuni virus in Israel: Neurological disease and fatalities in cattle. *Transboundary and emerging diseases*. 2019;66**:** 1126-1131.

4. Golender N, Bumbarov VY, Erster O, Beer M, Khinich Y, Wernike K. Development and validation of a universal S-segment-based real-time RT-PCR assay for the detection of Simbu serogroup viruses. *J Virol Methods*. 2018;261**:** 80-85.

5. Golender N, Wernike K, Bumbarov V, et al. Characterization of Shuni viruses detected in Israel. *Virus genes*. 2016;52**:** 806-813.

6. Kraatz F, Wernike K, Hechinger S, et al. Deletion Mutants of Schmallenberg Virus Are Avirulent and Protect from Virus Challenge. *Journal of Virology*. 2015;89**:** 1825-1837.

7. Meyerholz DK, Beck AP. Fundamental Concepts for Semiquantitative Tissue Scoring in Translational Research. *ILAR J*. 2018;59**:** 13-17.

8. Motlou TP, Venter M. Shuni Virus in Cases of Neurologic Disease in Humans, South Africa. *Emerg Infect Dis*. 2021;27**:** 565-569.

9. Plyusnin A, Elliott RM. Molecular biology of orthobunyaviruses. In: *Bunyaviridae*. Caister Academic Press; 2011.

10. Sick F, Breithaupt A, Golender N, Bumbarov V, Beer M, Wernike K. Shuni virus-induced meningoencephalitis after experimental infection of cattle. *Transbound Emerg Dis*. 2020.

11. Steyn J, Motlou P, van Eeden C, et al. Shuni Virus in Wildlife and Nonequine Domestic Animals, South Africa. *Emerg Infect Dis*. 2020;26**:** 1521-1525.

12. Tauscher K, Wernike K, Fischer M, et al. Characterization of Simbu serogroup virus infections in type I interferon receptor knock-out mice. *Archives of Virology*. 2017;162**:** 3119-3129.

13. Toussaint JF, Sailleau C, Breard E, Zientara S, De Clercq K. Bluetongue virus detection by two real-time RT-qPCRs targeting two different genomic segments. *J Virol Methods*. 2007;140**:** 115-123.

14. van Eeden C, Swanepoel R, Venter M. Antibodies against West Nile and Shuni Viruses in Veterinarians, South Africa. *Emerg Infect Dis*. 2014;20**:** 1409-1411.

15. van Eeden C, Williams JH, Gerdes TG, et al. Shuni virus as cause of neurologic disease in horses. *Emerg Infect Dis*. 2012;18**:** 318-321.

16. Walter CT, Barr JN. Recent advances in the molecular and cellular biology of bunyaviruses. *J Gen Virol*. 2011;92**:** 2467-2484.

17. Wernike K, Aebischer A, Sick F, Szillat KP, Beer M. Differentiation of Antibodies against Selected Simbu Serogroup Viruses by a Glycoprotein Gc-Based Triplex ELISA. *Vet Sci*. 2021;8.

18. Wernike K, Eschbaumer M, Breithaupt A, Hoffmann B, Beer M. Schmallenberg virus challenge models in cattle: infectious serum or culture-grown virus? *Vet Res*. 2012;43.

19. Wernike K, Eschbaumer M, Schirrmeier H, et al. Oral exposure, reinfection and cellular immunity to Schmallenberg virus in cattle. *Veterinary microbiology*. 2013;165**:** 155-159. Research Support, Non-U.S. Gov't.

20. Wernike K, Hoffmann B, Bréard E, et al. Schmallenberg virus experimental infection of sheep. *Veterinary microbiology*. 2013;166**:** 461-466.
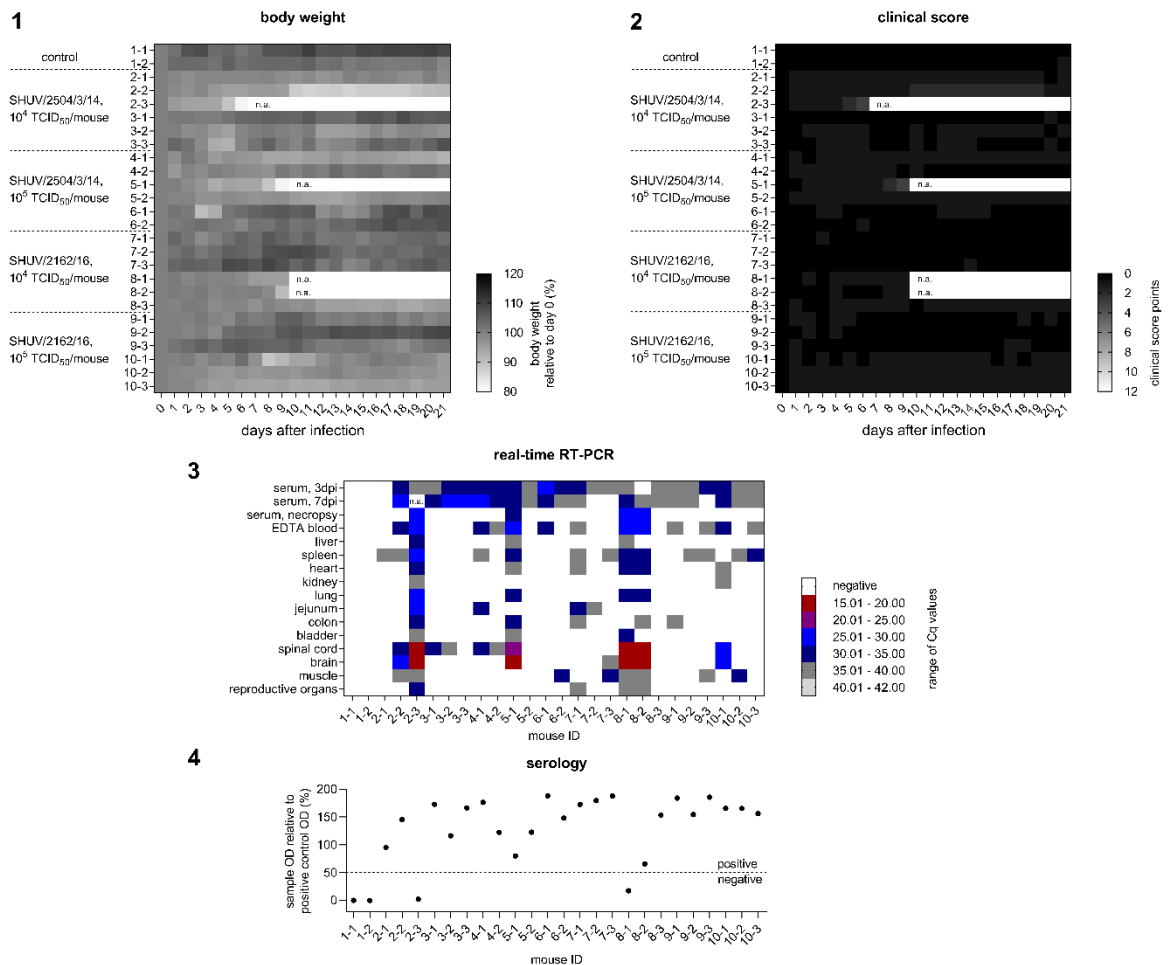
Figures 1-4. Clinical, virological and serological data of INFAR-/- mice subcutaneously infected with SHUV. **Figure 1.** Body weight loss (heat map). Independent of the SHUV strain used and infectious dose, single animals showed body weight loss. **Figure 2.** Clinical scores (shown as heat map) reflect that no clinical signs were visible except for slowed down movement and body weight loss (score never exceeded 3 points for any animal). **Figure 3.** Viral genome detection in serum, EDTA blood and tissues reveals high SHUV RNA loads in samples of clinically diseased animals (mice no. 2-3, 5-1, 8-1, 8-2), independent of the strain used for infection. **Figure 4.** A specific antibody response is detectable in all SHUV inoculated animals with exception of the prematurely lost mice 2-3 and 8-1, while both uninoculated control animals remained seronegative.
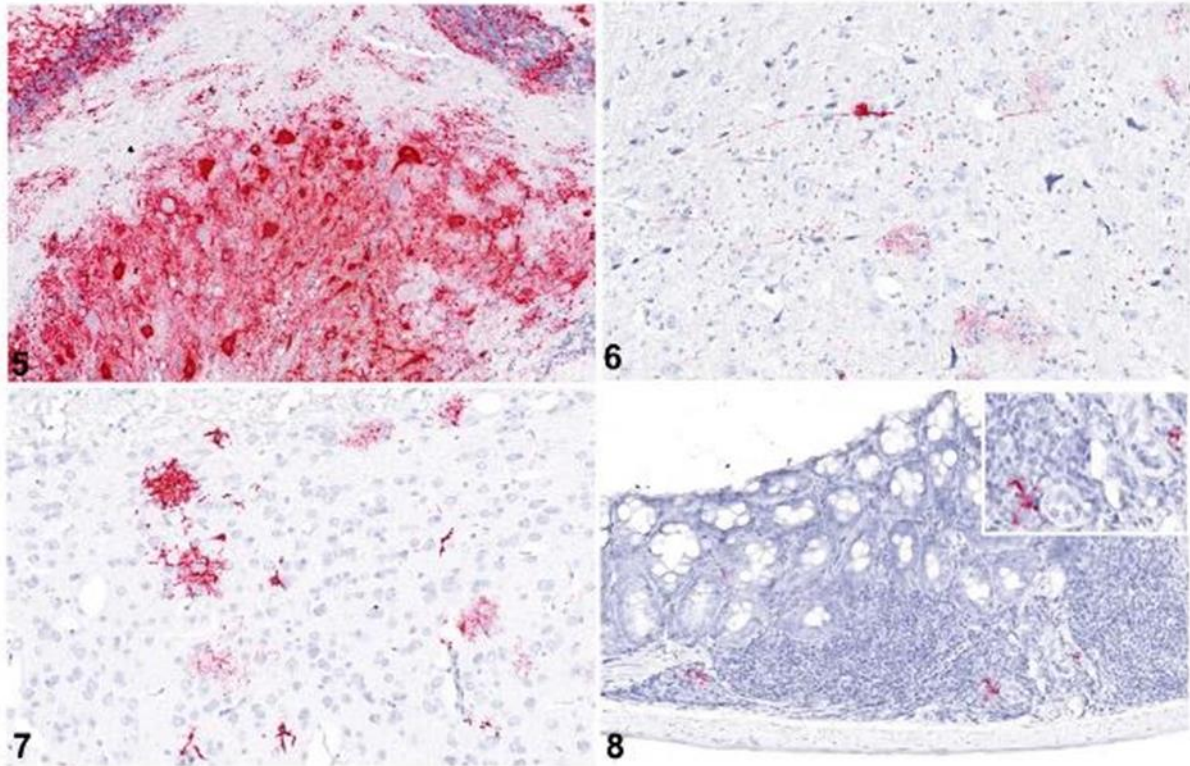
Figure 5-8. RNAScope™ based SHUV RNA detection in IFNAR-/- mouse brain and large intestinal MALT. *In situ* hybridization, RNAScope® probes designed for nucleocapsid protein gene detection, Mayer's hematoxylin counter stain. **Figure 5.** SHUV 2162/16 RNA is detected in neurons and glial cells mainly in the arbor vitae but also in all layers of the cerebellar gray substance, 10 days after infection. **Figure 6.** Viral RNA is found in neurons, neuronal processes and glial cells of the thalamic region, 9 days after SHUV 2504/3/14 infection. **Figure 7** SHUV 2162/16 RNA detection in neurons and glial cells of the larger pyramidal cell layer, 10 days after infection. **Figure 8.** In the MALT of the large intestine, 6 days after SHUV 2504/3/14 infection, viral RNA is found in macrophages of the lymphoid tissue.
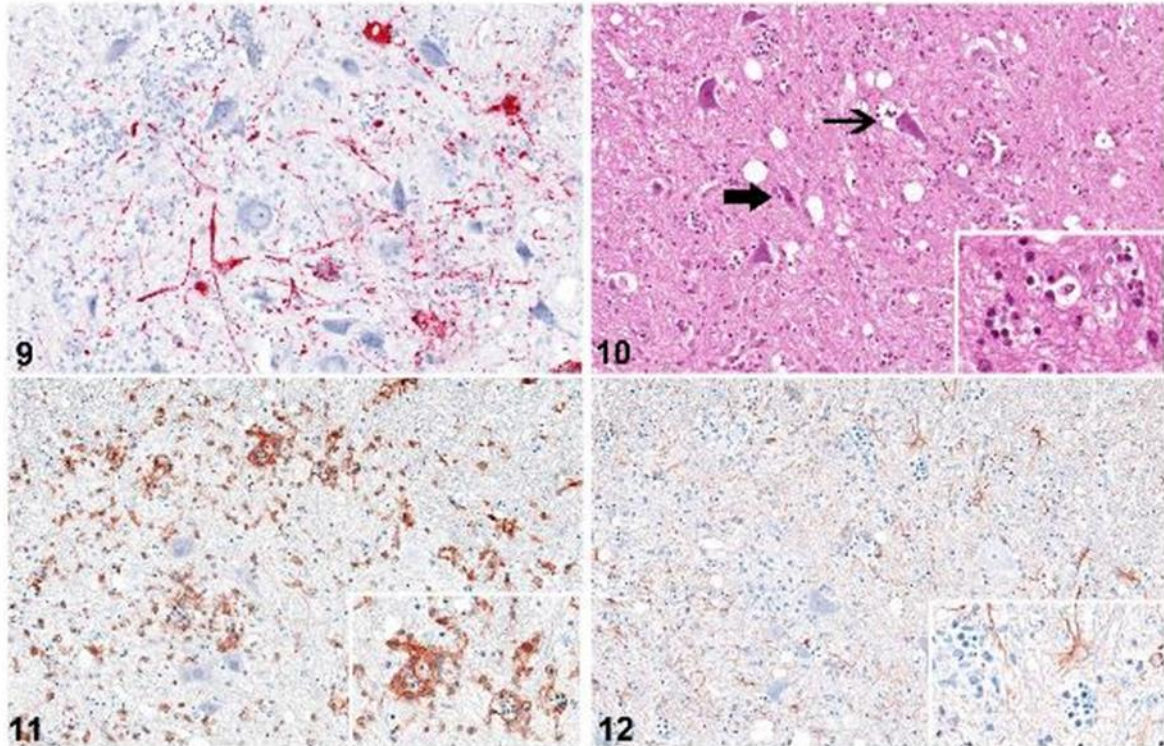
Figure 9-12. Viral RNA associated lesion profile in the cervical spinal cord of an IFNAR-/- mouse, 9 days after SHUV 2504/3/14 infection, shown in consecutive tissue sections. **Figure 9**. SHUV RNA is found in neurons and neuronal processes by *in situ* hybridization for nucleocapsid protein gene detection. **Figure 10**. Neuronal satellitosis (slender arrow), neuronal degeneration (bold arrow) and necrosis of glial cells (inlay), hematoxylin and eosin. **Figure 11**. Increased number and strong activation of microglia/macrophages, immunohistochemistry (IHC) for Iba-1. **Figure 12**. The number of astrocytes was slightly increased, but the shape was not altered. IHC for GFAP.
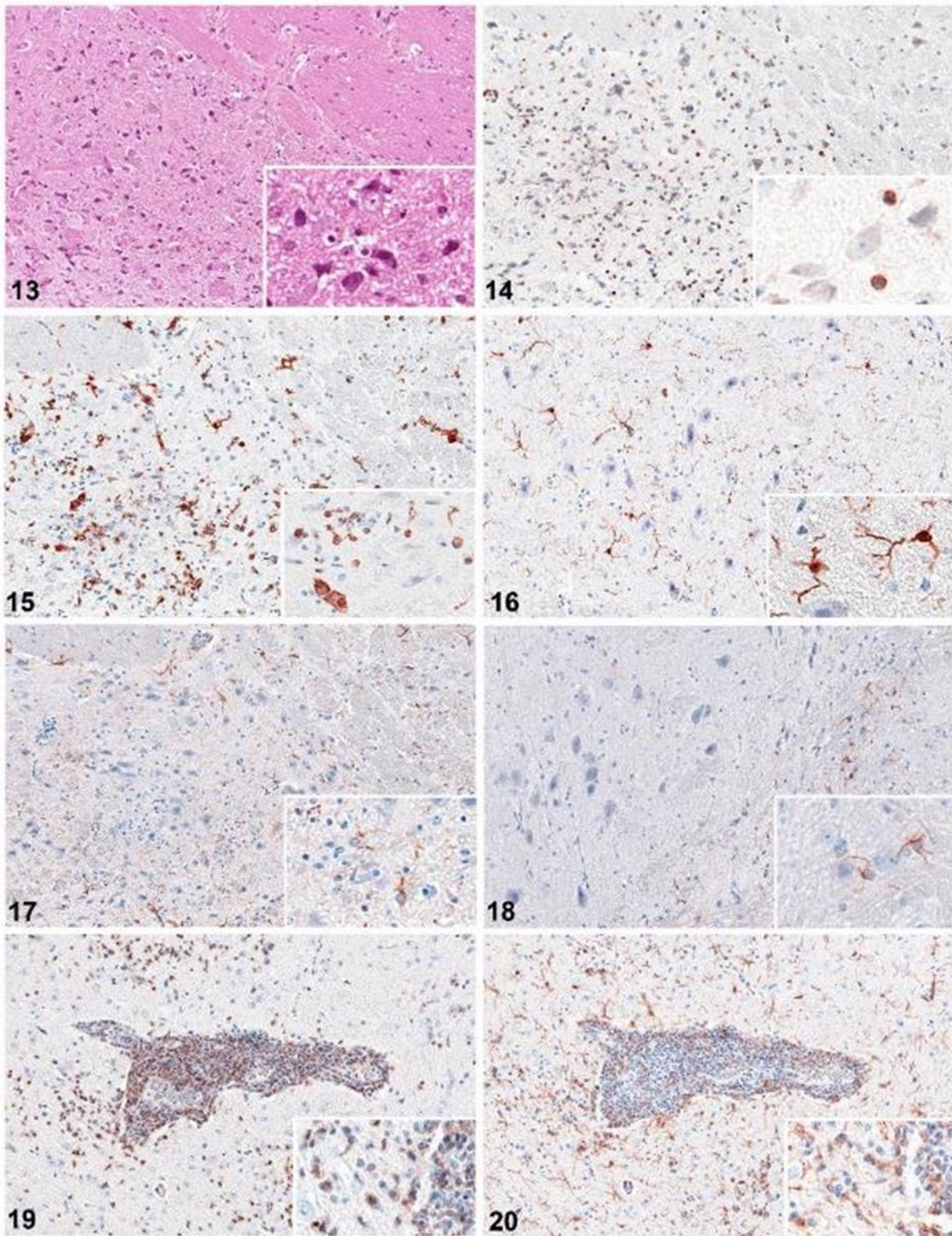
Figure 13-20. Early and late stage lesions and inflammatory response after SHUV infection.

Figure 13. Mouse no. 5-1, 9 days after SHUV 2504/3/14 infection. In the brain stem, groups of neurons are shrunken and adjacent glial cells exhibit karyopyknosis and -rhexis (inlay). Hematoxylin and eosin.

Figure 14. Consecutive section to Figure 13. The affected brain stem area contains numerous CD-3 positive T-cell infiltrates (inlay). Immunohistochemistry (IHC) for CD-3.

Figure 15. Consecutive section to Figure 13. There are increased numbers of Iba-1 labelled microglia / macrophages in the affected brain stem area. Positive cells are increased in size (activation). IHC for Iba-1.

Figure 16. Mouse no. 6-2, 21 days after SHUV 2504/3/14 infection. For comparison, this unaffected brain stem shows multifocal, slender Iba-1positive microglia / macrophages.

Figure 17 Consecutive section to Figure 13. The number of GFAP positive cells is only slightly increased, however the typical slender shape of labelled cells is comparable with unaffected brain stem areas.

Figure 18 Consecutive section to Figure 16. For comparison, this unaffected brain stem shows slender GFAP positive astrocytes, mouse no. 6-2, 21 dpi, PCR negative

Figure 19 Mouse no 2-2, 21 days after 2504/3/14 infection. In the periaqueductal gray matter, perivascular cuffs, glial nodules and disseminated immune cell infiltrates are mainly comprised of CD-3 labelled T-cell. IHC for CD3

Figure 20 Consecutive section to Figure 19. Iba-1 positive microglia / macrophages represent the second major inflammatory infiltrate. IHC for Iba-1

Table 1. Immunohistochemical markers and applications. HIER: Heat induced epitope retrieval, RT: room temperature.

Marker	Antibody	Pre-treatment	Secondary reagents
CD79a	Mouse anti-CD79A (LifeSpan BioSciences, Seattle, WA, #LS-B8330), 1:50, overnight	HIER, 10mM Tris/ 1mM EDTA buffer pH 9.0, 20 min	Dako EnVision+ System- HRP Labelled Polymer Anti-mouse, 30 min (Dako, Agilent, Santa Clara, CA, USA)
CD3	Rabbit Anti-human CD3, A0452, DAKO Agilent, Santa Clara, CA, USA, 1:100, overnight	HIER, Citrate buffer pH 6.0, for 20 min	Anti-rabbit IgG Biotinylated, 1:200, 30 min. RT; and ABC Kit Vectastain Elite PK 6100, 30 min (Vector Laboratories, Burlingame, CA, USA)
Iba-1	Rabbit anti-Iba1 (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan, # 019-19741), 1:800, overnight	HIER, Citrate buffer pH 6.0, for 20 min	Dako EnVision+ System- HRP Labelled Polymer Anti-rabbit, 30 min
GFAP	Rabbit anti-GFAP (abcam, Cambridge, UK, #ab16997) 1:200, overnight	HIER, Citrate buffer pH 6.0, for 20 min	Dako EnVision+ System- HRP Labelled Polymer Anti-rabbit, 30 min

73

75

„Differentiation of Antibodies against Selected Simbu Serogroup Viruses by a Glycoprotein Gc-Based Triplex ELISA"

Kerstin Wernike [1], Andrea Aebischer [2], Franziska Sick [1], Kevin P. Szillat [1] and Martin Beer [1]

[1] Institute of Diagnostic Virology, Friedrich-Loeffler-Institut, 17493 Greifswald-Insel Riems, Germany

[2] Department of Experimental Animal Facilities and Biorisk Management, Friedrich-Loeffler-Institut, 17493 Greifswald-Insel Riems, Germany

Article

Differentiation of Antibodies against Selected Simbu Serogroup Viruses by a Glycoprotein Gc-Based Triplex ELISA

Kerstin Wernike [1,*], Andrea Aebischer [2], Franziska Sick [1], Kevin P. Szillat [1] and Martin Beer [1,*]

1 Institute of Diagnostic Virology, Friedrich-Loeffler-Institut, 17493 Greifswald-Insel Riems, Germany; franziska.sick@fli.de (F.S.); kevin.szillat@fli.de (K.P.S.)
2 Department of Experimental Animal Facilities and Biorisk Management, Friedrich-Loeffler-Institut, 17493 Greifswald-Insel Riems, Germany; andrea.aebischer@fli.de
* Correspondence: kerstin.wernike@fli.de (K.W.); martin.beer@fli.de (M.B.)

Abstract: The Simbu serogroup of orthobunyaviruses includes several pathogens of veterinary importance, among them Schmallenberg virus (SBV), Akabane virus (AKAV) and Shuni virus (SHUV). They infect predominantly ruminants and induce severe congenital malformation. In adult animals, the intra vitam diagnostics by direct virus detection is limited to only a few days due to a short-lived viremia. For surveillance purposes the testing for specific antibodies is a superior approach. However, the serological differentiation is hampered by a considerable extent of cross-reactivity, as viruses were assigned into this serogroup based on antigenic relatedness. Here, we established a glycoprotein Gc-based triplex enzyme-linked immunosorbent assay (ELISA) for the detection and differentiation of antibodies against SBV, AKAV, and SHUV. A total of 477 negative samples of various ruminant species, 238 samples positive for SBV-antibodies, 36 positive for AKAV-antibodies and 53 SHUV antibody-positive samples were tested in comparison to neutralization tests. For the newly developed ELISA, overall diagnostic specificities of 84.56%, 94.68% and 89.39% and sensitivities of 89.08%, 69.44% and 84.91% were calculated for SBV, AKAV and SHUV, respectively, with only slight effects of serological cross-reactivity on the diagnostic specificity. Thus, this test system could be used for serological screening in suspected populations or as additional tool during outbreak investigations.

Keywords: *Peribunyaviridae*; orthobunyavirus; Schmallenberg virus; Shuni virus; Akabane virus; serology; ruminants; cattle; sheep; goat

1. Introduction

Viruses of the Simbu serogroup, which belongs to the family *Peribunyaviridae*, genus *Orthobunyavirus*, are distributed worldwide and include several pathogens of veterinary importance [1,2], e.g., members of the virus species *Akabane orthobunyavirus*, *Schmallenberg orthobunyavirus* or *Shuni orthobunyavirus* [3]. Historically, viruses were assigned into this serogroup solely based on antigenic relation determined by plaque reduction neutralization, hemagglutination inhibition, complement fixation or radial immunodiffusion tests [4,5]. More recently, the classification was additionally based on the comparative analyses of nucleic acid and protein sequences [2]. The genome of simbuviruses consists of three segments of single-stranded RNA, of which the small (S) genomic segment, that encodes for the nucleocapsid protein N and the non-structural protein NSs, is the most conserved one. The large (L) segment encodes for the RNA-dependent RNA polymerase and the medium (M) segment for the glycoproteins Gn and Gc and the non-structural protein NSm [6,7]. The orthobunyaviral glycoproteins, which form spikes on the surface of the virus particle, are integral transmembrane proteins and they are important for viral attachment, membrane fusion and the induction of the host's immune response [7–10]. The N-terminal variable part of the Gc-protein (Gc head (GcH)) is highly immunogenic and the major target of neutralizing antibodies [11,12].

Simbuviruses infect predominantly ruminants and persist in nature by alternately infecting their insect vectors (*Culicoides* biting midges) and mammalian hosts (reviewed in [1]). In enzootic regions, Simbu serogroup viruses usually establish patterns of cyclic circulation, with seasons of high virus appearance followed by periods of only sporadic detections [13–19], which is most likely related to the overall immunity in the mammalian host population and the abundance of the insect vector. Co-circulation of several simbuviruses within a given area occurs frequently [2,20–23].

Infections of adult ruminants with Akabane virus (AKAV), Schmallenberg virus (SBV) or Shuni virus (SHUV) lead to a short-lived viremia of a few days and are either asymptomatic or induce mild clinical signs such as fever, diarrhea and decreased milk yield [24–27]. However, some strains of AKAV and SHUV can also occasionally cause encephalitis [25,26,28]. Nevertheless, the most prominent clinical signs appear when naïve dams are infected during a critical phase of gestation, which may lead to abortion, premature or stillbirth or severe congenital deformation summarized under the term arthrogryposis-hydranencephaly syndrome [24,26,29,30]. Besides the main hosts, i.e., domestic and various captive and wild ruminants, AKAV infections of pigs [31], SHUV infection of horses [32] and anti-SBV antibodies in wild boar [33,34] and a few SBV-positive dogs [35,36] have been described. The epidemiological relevance, however, is not known.

In ruminants of all age groups, antibodies against simbuviruses are induced between one and three weeks after infection [25,37–40], and immunity acquired due to an earlier infection or vaccination protects from re-infection [37,41,42]. In cases of in utero infections of fetuses that are already immunocompetent (from about 90 days of gestation onwards in cattle), antibodies can be detected in the blood of the newborn before the intake of the colostrum of its mother [40,43,44]. Test systems commonly used to measure the humoral immune response include microneutralization and indirect immunofluorescence tests as well as (commercial) enzyme-linked immunosorbent assays (ELISAs). As viruses are assigned into this group based on antigenic relatedness [4], considerable serological cross-reactivity occurs between different simbuviruses [45], especially for tests that rely on the viral N-protein, such as commercial ELISAs or complement fixation tests [4,21,46,47]. The N-protein is widely used for serological diagnostics, as it elicits a strong humoral immune response [42,48,49] and anti-N antibodies are highly abundant in infected animals, however, they do not have neutralizing activity [50]. In contrast, antibodies directed against the glycoproteins, specifically Gc, efficiently neutralize the corresponding simbuvirus [11,12,50]. In addition, the M-segment, which encodes the glycoproteins, is considered to be the most variable genomic segment of orthobunyaviruses [45], presumably leading to a lower extent of cross-reactivity compared to the N-protein. Indeed, serum neutralization tests (SNTs), which detect neutralizing antibodies directed against the glycoproteins, are more specific for a given virus species [46]. However, these test systems require the handling of the respective virus, are time-consuming and labor-intensive. In contrast, ELISAs can be applied under less stringent biosafety conditions and enable high-throughput testing of clinical specimens. Hence, we established a test system for the differentiation of antibodies against the Simbu serogroup viruses SBV, AKAV, and SHUV based on the viral Gc-proteins and a triplex ELISA platform.

2. Materials and Methods

2.1. Blood Samples

A total of 477 serum or plasma samples negative for antibodies against Simbu serogroup viruses taken from cattle, sheep, goat or wildlife and zoo animals (red deer, doe deer, fallow deer, buffalo and alpaca) were included (Table 1). They were collected prior to the first detection of a simbuvirus in Germany, i.e., before 2011, or represented pre-infection sera of SBV, AKAV or SHUV cattle or sheep trials. In addition, 238 samples positive for SBV-antibodies, 36 sera displaying anti-AKAV antibodies and 53 SHUV-positive samples were included (Table 1). These specimens represented either routine diagnostic submission to the Friedrich-Loeffler-Institut, Germany, to the Kimron Veterinary Institute, Israel, or

originated from cattle and sheep experimentally infected with SBV [51,52], cattle infected with SHUV [25] or cattle vaccinated against AKAV, the Simbu serogroup member Aino virus (AINOV) and the likewise teratogenic Chuzan virus [53]. The sera of experimentally infected animals were collected between 14 and 28 days (SBV) or 7 and 21 days (SHUV) after infection. The Israeli field sera were pre-selected based on a positive result in a commercial SBV ELISA (ID Screen Schmallenberg virus Competition Multispecies, IDvet, Grabels, France) that detects antibodies against several Simbu serogroup viruses [46,47]. The German field sera were pre-selected based on the result of either the ID Screen Schmallenberg virus Competition Multispecies or ID Screen Schmallenberg virus Indirect Multispecies ELISA (both IDvet, Grabels, France).

Table 1. Numbers and serological status towards simbuviruses of samples included in this study. Wild and zoo animals comprise red deer, roe deer, fallow deer, buffalo and alpaca.

Animal Species	No. Negative	No. Positive against SBV	No. Positive against AKAV	No. Positive against SHUV
cattle	192	76	30	42
sheep	159	76	5	11
goat	78	37	1	0
wild and zoo animals	48	49	0	0
total	477	238	36	53

The status of each sample was determined by microneutralization tests against SBV, AKAV and SHUV performed as described previously [25,37,53]. When neutralizing antibodies against several simbuviruses could be detected, the sera were evaluated positive for the virus that displayed the highest titer. If the titer difference between two viruses was less than 4-fold, the serum was assessed positive for both.

2.2. Production of Recombinant Proteins

2.2.1. Cloning

The sequences of Gc head domains of SBV (amino acids (aa) 465–702), AKAV (aa 465–701) and SHUV (aa 465–704) were amplified from codon-optimized synthetic genes (Thermo Fisher Scientific, Darmstadt, Germany) based on GenBank entries CCF55030 (SBV), BAV17033.1 (AKAV) and KF153117 (SHUV), respectively, and cloned in the pMT *Drosophila* S2 expression vector (Thermo Fisher Scientific) in frame with an N-terminal BiP secretion signal. C-terminally, a double (SBV, AKAV) or a single (SHUV) Strep-Tag was added. All constructs were verified by Sanger sequencing. Primer sequences are available upon request.

2.2.2. Expression and Purification in Drosophila S2 Cells

Drosophila S2 cells (R69007) were purchased from Thermo Fisher Scientific and grown in Insect-Xpress medium (Lonza, Basel, Switzerland) at 28 °C. For protein expression, adherent cultures were transfected with the respective pMT/BiP expression plasmids and pCoBlast (Invitrogen, Karlsruhe, Germany) in a ratio of 20:1 using Effectene Transfection Reagent (#301425, Qiagen, Hilden, Germany) according to the instructions of the manufacturer. Stable polyclonal cell lines were subsequently selected by addition of 30 μg/mL Blasticidin and expanded to suspension cultures of 300 mL and grown at 28 °C, 80 rpm. After 5 days, the cultures were topped to 700 mL and protein expression was induced with a final concentration of 2.5 μM $CdCl_2$. Supernatants were harvested 7 days after induction by centrifugation and were subsequently concentrated to about 50 mL using a Vivaflow 200 device (5000 MWCO PES; Sartorius, Göttingen, Germany). Biotin was blocked by addition of BioLock (IBA Lifesciences, Göttingen, Germany) as recommended. The concentrated supernatant was purified using Streptactin-Superflow high capacity slurry (IBA Lifesciences) according to the manufacturer's protocol. All protein-containing eluates were pooled, aliquoted and stored at –80 °C until further use.

2.2.3. SDS-PAGE and Western Blot

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using a Mini-PROTEAN®Tetra System (Bio-Rad, Feldkirchen, Germany). Instant-Blue (expedeon, Heidelberg, Germany) was applied for Coomassie stainings. For Western blot analysis a TRANS-BLOT ® SD semi-dry transfer cell (Bio-Rad) was used. Stainings were performed with an Anti-StrepTag specific antibody (StrepMAB-Classic-HRP, IBA Lifesciences). Images were acquired with a ChemiDoc Imaging System (Bio-Rad).

2.3. ELISA Procedure

Medium-binding ELISA plates (Greiner Bio-One GmbH, Leipzig, Germany) were coated with 100 ng/well of either the SBV, SHUV or AKAV antigen overnight at 4 °C in a Tris buffer (0.02 mol Tris and 0.15 mol NaCl ad. 1 L H_2O, pH 7.6). The plates were subsequently washed three times using Tris-buffered saline with Tween (TBST) and blocked for 1 h at 37 °C using 5 % skimmed milk in phosphate-buffered saline (PBS). Fifty μL of the sera (pre-diluted 1/100 in TBST) were incubated on the coated wells for 1 h at room temperature followed by three washing steps using TBST. The reactivity was shown by adding 50 μL of a multi-species conjugate (SBVMILK; IDvet, Grabels, France) diluted 1/80. After an incubation period of 1 h at room temperature, the plates were washed again and 100 μL o-phenylenediamine dihydrochloride (OPD) substrate was added. Subsequent to an incubation period of 20 min at room temperature in the dark, the reaction was stopped using 50 μL 4M H_2SO_4. The ELISA readings were taken at a wavelength of 492 nm on a Tecan Spectra Mini instrument (Tecan Group Ltd., Männedorf, Switzerland). The results were expressed as the percentage of the sample adsorbance (= optical density (OD)) relative to the positive control OD (S/P*100).

2.4. Data Analyses and Cut-off Determination

To determine the cut-off values and the diagnostic sensitivities and specificities of the final ELISA protocol, the sera mentioned above were tested by the triple ELISA, and receiver operating characteristic (ROC) analyses were performed using GraphPad Prism version 8.0 for Windows (GraphPad Software, San Diego, CA, USA). To evaluate the influence of cross-reactivity with antibodies against other Simbu serogroup viruses, the ROC analyses was performed in two distinct settings. In the first set-up, only sera negative against all three simbuviruses were included as negative samples, while in the second approach negative samples additionally include sera negative against the given antigen, but positive for another Simbu serogroup virus.

### 2.5. Repeatability and Reproducibility

For evaluation of the intra-assay reproducibility, a negative cattle and a negative sheep serum as well as cattle sera positive for anti-SBV [51], anti-AKAV [53] or anti-SHUV [25] antibodies were tested in five replicates each. The inter-assay repeatability was determined with the identical samples and replicate number on five days. Mean values and standard deviations of the 25 replicates were calculated using GraphPad Prism version 8.0 for Windows (GraphPad Software, USA). In every approach, two positive controls per virus and two negative controls were included, resulting in a total of 10 replicates per control.

3. Results

3.1. Expression and Purification of Proteins

The Gc head domains of SBV (aa 465–702), AKAV (aa 465–701) and SHUV (aa 465–704) were expressed to high yields in *Drosophila* S2 cells and Streptactin-purified without further downstream processing. Integrity and purity were verified by SDS-PAGE and Western blot (Figure 1). It has been shown before, that the orthobunyavirus Gc head domain represents the major target of neutralizing antibodies produced upon virus infection [11], and the recombinant SBV, AKAV and SHUV ELISA antigens were designed and produced based

on this previously defined molecular architecture of the Gc spike protein. Their antigenicity was verified by ELISA using the antibody-positive and -negative sera as shown below.
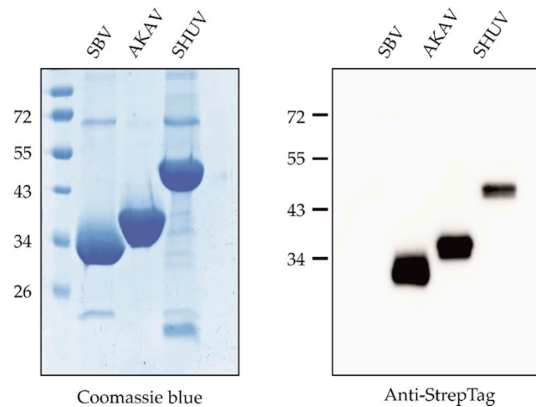
Figure 1. SDS-PAGE and Western blot analyses of recombinant proteins used for plate coating. The recombinant proteins were separated by reducing SDS-PAGE and analyzed by Coomassie staining (left panel) or Western blot (right panel) using an anti-StrepTag specific monoclonal antibody. Full-length blots are presented in the Appendix A (Figure A1).

3.2. Cut-off Determination and Diagnostic Characteristics

In order to evaluate the sensitivity and specificity of the triplex SBV-AKAV-SHUV-ELISA and to establish a threshold for positivity, the above-mentioned antibody-negative and -positive sera of multiple ruminant species were tested. ROC curve analyses indicated that the individual ELISAs have a good diagnostic accuracy with only a very limited negative effect of cross-reactive antibodies (Figure 2). The areas under the curve (AUC) that were calculated for two selected test set-ups are given in Table 2. Based on the ROC curves (Figure 2), a cut-off of <50% for negativity and ≥50 for positivity was set for cattle, sheep and wild and zoo animals and of <80% and ≥80%, respectively, for goat samples.
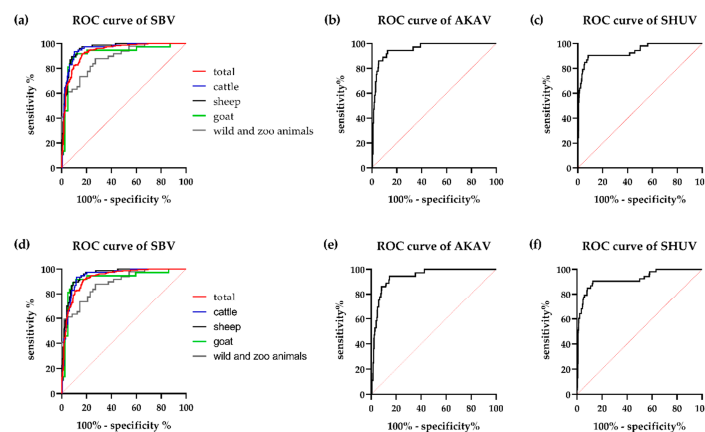
Figure 2. Receiver operating characteristic (ROC) analyses of the triplex SBV–AKAV–SHUV ELISA using 477 negative sera and 238 SBV antibody-positive sera, 36 sera displaying antibodies against AKAV and 53 SHUV seropositive samples. For the SBV assay (**a**,**d**), the ROC curves are shown separately for cattle (blue), sheep (black), goat (green) and wild and zoo animals (grey), the combined ROC curve that includes all species is depicted in red. The ROC analyses were performed using two different set-ups, for the first (**a**–**c**) only sera negative against all three simbuviruses were included as negative samples, while in the second approach (**d**–**f**) negative samples additionally include sera negative against the given antigen, but positive towards another Simbu serogroup virus. SBV, Schmallenberg virus; AKAV, Akabane virus; SHUV, Shuni virus.

Table 2. Diagnostic characteristics of the triplex SBV-AKAV-SHUV ELISA. The first row per antigen and, if applicable, animal species includes values calculated for samples positive against the given antigen and negative against all simbuviruses. In the second row, negative samples additionally include sera negative against the given antigen, but positive for another Simbu serogroup virus. AUC: area under the curve, CI: 95% confidence interval.

| Antigen	Animal Species	AUC	Sensitivity	Specificity
SBV	cattle-1	0.9519 (CI: 0.9259 to 0.9779)	92.11% (CI: 83.60% to 97.05%)	89.58% (CI: 84.37% to 93.52%)
	cattle-2	0.9418 (CI: 0.9149 to 0.9686)	92.11% (CI: 83.60% to 97.05%)	87.98% (CI: 83.10% to 91.86%)
	sheep-1	0.9566 (CI: 0.9326 to 0.9806)	93.42% (CI: 85.31% to 97.83%)	86.79% (CI: 80.52% to 91.63%)
	sheep-2	0.9532 (CI: 0.9289 to 0.9775)	93.42% (CI: 85.31% to 97.83%)	84.80% (CI: 78.52% to 89.82%)
	goat-1	0.9186 (CI: 0.8553 to 0.9818)	94.59% (CI: 81.81% to 99.34%)	76.92% (CI: 66.00% to 85.71%)
	goat-2	0.9196 (CI: 0.8571 to 0.9821)	94.59% (CI: 81.81% to 99.34%)	77.22% (CI: 66.40% to 85.90%)
	wild/zoo animals-1	0.8835 (CI: 0.8199 to 0.9471)	73.47% (CI: 58.92% to 85.05%)	79.17% (CI: 65.01% to 89.53%)
	wild/zoo animals-2	0.8835 (CI: 0.8199 to 0.9471)	73.47% (CI: 58.92% to 85.05%)	79.17% (CI: 65.01% to 89.53%)
	overall-1	0.9339 (CI: 0.9159 to 0.9520)	89.08% (CI: 84.40% to 92.74%)	85.53% (CI: 82.05% to 88.57%)
	overall-2	0.9292 (CI: 0.9110 to 0.9474)	89.08% (CI: 84.40% to 92.74%)	84.56% (CI: 81.20% to 87.53%)
AKAV	overall-1	0.9536 (CI: 0.9247 to 0.9825)	69.44% (CI: 51.89% to 83.65%)	97.06% (CI: 95.12% to 98.39%)
	overall-2	0.9386 (CI: 0.9079 to 0.9692)	69.44% (CI: 51.89% to 83.65%)	94.68% (CI: 92.80% to 96.19%)
SHUV	overall-1	0.9384 (CI: 0.8997 to 0.9771)	84.91% (CI: 72.41% to 93.25%)	93.29% (CI: 90.66% to 95.37%)
	overall-2	0.9234 (CI: 0.8792 to 0.9676)	84.91% (CI: 72.41% to 93.25%)	89.39% (CI: 86.89% to 91.55%)

Using these cut-off values, overall diagnostic specificities of 84.56%, 94.68% and 89.39% and sensitivities of 89.08%, 69.44% and 84.91% were achieved for SBV, AKAV and SHUV, respectively. Taking in account differences between set-ups 1 and 2, only a limited impact of cross-reacting antibodies could be observed (Table 2). When comparing the diagnostic characteristics of the SBV test separately for each animal species included in this study, a higher sensitivity was observed when testing domestic ruminants (cattle, goat, sheep) in comparison to wild and zoo animals (Table 2).

All sera were additionally tested by the gold-standard test SNT against all three viruses and the measured neutralizing titers were compared to the ELISA values. Overall, the proportion of false-negative ELISA results was higher for samples with low neutralizing titers (<1/80), an effect most prominent for previously AKAV-infected individuals or SBV-antibody-positive wildlife and zoo animals (Figure 3).

3.3. Repeatability and Reproducibility

The repeatability and reproducibility were assessed using five replicates each of five sera on five independent ELISA plates. The included control samples reacted as expected in every approach and with low standard deviations (SD) (Figure 4a). The SBV control resulted in an adsorbance value of 0.75 ± 0.05 against the SBV antigen, while only values of 0.05 ± 0.00 and 0.06 ± 0.00 were measured when testing this sample against the AKAV and SHUV antigens, respectively. The AKAV control sample, which originated from an animal

vaccinated against AKAV as well as AINOV and Chuzan virus [53], resulted in adsorbance values of 0.96 ± 0.05, 0.10 ± 0.00 and 0.27 ± 0.02 when tested against the AKAV, SBV and SHUV antigens, respectively. The values measured for the SHUV control samples were 0.87 ± 0.05 against the SHUV antigen and 0.06 ± 0.01 and 0.06 ± 0.00 against the SBV and AKAV proteins (Figure 4a).
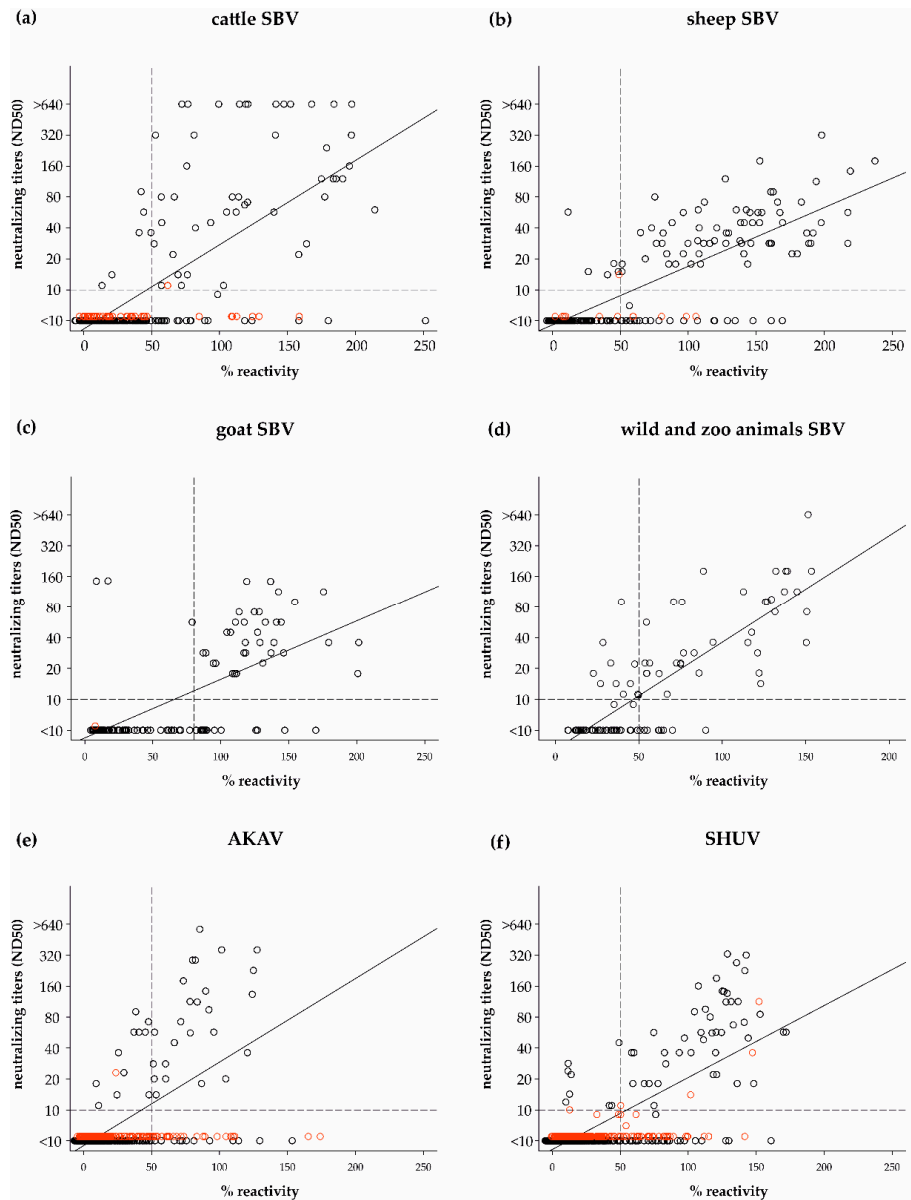
Figure 3. Correlation of the results of serum neutralization tests (SNT) against SBV (**a–d**), AKAV (**e**) and SHUV (**f**) and the ELISA based on the respective antigen. Red dots represent samples that tested positive by serum neutralization tests (SNT) against a different simbuvirus. The cut-off value for the SNT is indicated by a horizontal dashed line, while the cut-off values of the ELISA are shown by vertical dashed lines.
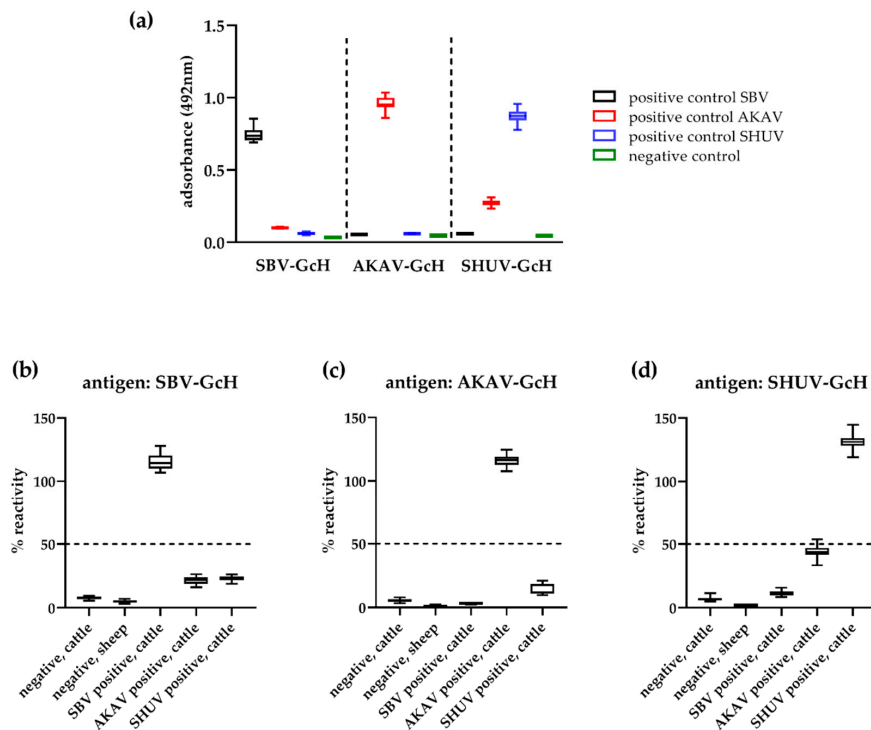
Figure 4. Reproducibility and repeatability of the SBV-AKAV-SHUV ELISA. A negative cattle and a negative sheep serum as well as sera antibody-positive towards SBV, AKAV or SHUV were tested against the SBV (**b**), AKAV (**c**) and SHUV (**d**) antigens in five replicates each in five independent approaches. The boxplots represent the results of all 25 replicates. The cut-off values are indicated by horizontal dashed lines. (**a**) Adsorbance values of the negative and positive controls that were included in every approach, the boxplots represent the results of all 10 replicates.

The results of the clinical specimens to be analyzed resulted likewise in low variations between the individual approaches. The negative cattle and sheep sera tested correctly negative in every case (Figure 4b–d). Mean OD values and standard deviations of 7.80% ± 1.18 (cattle) and 5.10% ± 1.10 (sheep) were calculated for these samples when tested against the SBV antigen, 5.58% ± 1.24 and 1.34% ± 0.53 against AKAV and 6.89% ± 1.26 and 2.02% ± 0.29 against SHUV-GcH. The SBV seropositive sample reacted correctly positive against SBV (115.12% ± 5.48) in every approach and negative against AKAV (3.14% ± 0.34) and SHUV (11.22% ± 1.93), while the SHUV antibody-positive sample reacted positive against SHUV (131.25% ± 5.73) and negative against SBV (23.22% ± 1.85) and AKAV (15.35% ± 3.73) in every case. The cattle sample that contains antibodies against AKAV reacted positive against this antigen (116.30% ± 4.19%) and negative against SBV (21.53% ± 2.76) (Figure 4b,c), however, against the SHUV antigen elevated values close to the cut-off for positivity were obtained (mean 44.10%, SD 4.39, max. 53.86%) (Figure 4d).

4. Discussion

During the last decades, numerous new members of the large and diverse family *Peribunyaviridae* were discovered and known bunyaviruses, among them several Simbu serogroup viruses, were found in previously unaffected regions or in areas with unknown infection status [7,27,30,54–56]. However, in some cases, predominantly in large-scale screenings, only antibodies against simbuviruses in general were detected using broad-reactive serological test systems, making it difficult to pinpoint a specific virus [47,57,58]. In those screenings conducted at a given time point, the direct virus detection and subsequent

identification by specific RT-PCRs or sequence analyses is hampered by the very short viremia of only a few days [25–27]. In contrast, antibodies against simbuviruses are detectable in most animals for several years after infection, if not even lifelong [17,59–62], albeit one should keep in mind that acute infections cannot be diagnosed by serological methods. For serological differentiation of antibodies against the different viruses, SNTs may be used [21,47]. However, these tests are time-consuming and require the storage and handling of the respective viruses under adequate biosecurity conditions. In contrast, discriminatory ELISA tests would be easier to perform, can be easily standardized and can potentially be performed by untrained personal. Therefore, we propose to perform (large-scale) screenings by broadly-reacting commercial ELISAs in order to detect the vast majority of bunyavirus-specific antibodies. In a second step, a virus-specific ELISA should be applied for further antibody-differentiation. Finally, questionable results would need to be verified by SNT. In order to test the applicability of this approach, SBV, AKAV and SHUV were selected in this study as model orthobunyaviruses to design an indirect triplex ELISA system allowing differentiation of antibodies raised against these viruses. SBV was chosen for this proof-of-principle study since it represents by now one of the best characterized orthobunyaviruses and, more importantly, SBV serological tests are widely used to screen for antibodies against simbuviruses [21,47], in some cases without further differentiation or confirmation by more specific tests [63,64]. AKAV and SHUV were added as they are widespread [1] and occur in the same geographical regions, including the Middle East [20] and the African continent. Furthermore, SHUV-specific ELISA systems are not yet described. For the selection of ELISA antigens, we focused on the N-terminal domain of the orthobunyavirus Gc-protein, since it shows a higher sequence variability between different viruses of a given group, while the C-terminal part is well conserved. Furthermore, the Gc ectodomain in general is highly immunogenic and represents the major target of neutralizing antibodies [11,12,50]. Hence, based on these previous findings, the Gc head domain was considered to be the most promising antigen for a discriminatory ELISA approach. It was shown before, that the complete Gc head-stalk domain represents a superior antigen in comparison to the head domain only [11]. However, we chose the isolated SBV and AKAV head domains since their antigenicity and immunogenicity have been confirmed before both, in vitro and in vivo [12,49,65]. Based on structural data [11], we assumed an equivalent performance also for SHUV, but no experimental data was available for verification. To ensure an optimal antigenicity, we therefore decided to use the complete SHUV Gc head-stalk domain. Its suitability for the design of antibody detection systems could be demonstrated in this study.

With regard to protein expression, one should take into account that a functional Gc glycoprotein requires correct post-translational modifications to ensure a proper folding [12,65], in contrast to the N-protein, which can be easily produced in a bacterial expression system. Thus, the recombinant glycoproteins have to be produced in a suitable expression host. Here, we used insect *Drosophila* S2 cells, which provide an eukaryotic environment as well as the majority of post-translational modifications found in mammalian cells [66]. In previous studies, the S2-expressed SBV Gc head domain was fully functional and showed an equal or even better performance than the same antigen expressed in mammalian HEK293T cells [11,49]. Furthermore, the S2 system can be easily up-scaled for high yield production of secreted proteins at relatively low costs. This represents a further benefit with regard to production under resource-limited settings. All the S2-expressed recombinant proteins reacted readily with sera from infected or immunized animals and can therefore be considered as fully functional. Moreover, only a limited extend of cross-reactivity was observed between the SBV-, AKAV- and SHUV antigens clearly out-performing the specificity of N-based serological assays [21,46,47]. However, it cannot be fully excluded that Gc-specific antibodies against more closely related members of this serogroup, e.g., from the identical virus species, would result in unspecific reactions. Thus, this needs to be investigated in future studies. Nonetheless, the AKAV positive control serum and the AKAV serum used to determine the reproducibly did react in the SHUV-specific system only to a limited extend and remained under the cut-off for

positivity (Figure 4), although both of these sera were obtained from animals vaccinated with a trivalent vaccine also containing AINOV, which is more closely related to SHUV [45].

In terms of sensitivity, satisfactory results were achieved for SBV and SHUV, when specimens from domestic ruminants were tested. In contrast, lower sensitivities were observed for samples collected from wild or zoo animals or in the AKAV test. However, among the samples used for validation a relatively high percentage of specimens with low titers (<1/80) as measured in the gold standard test SNT were present in the sample subsets positive for AKAV antibodies or among the wildlife samples. Hence, the lower sensitivities might be related to some extent also to the highly demanding sample panel. In addition, test sensitivity might be influenced by sampling time-points, as it was previously described that antibody titers are decreasing over time after infection in some animals [17]. In terms of age, also the group of young animals could be problematic, as maternally-derived antibodies transferred via colostrum from the dam to its newborn decay within a few months [59,60]. Thus, dependent on the age of the offspring, only very small amounts of these antibodies might still be present in the bloodstream. However, an influence of the sampling time-point on the performance of the ELISAs could not be further evaluated in this study, due to the missing information on the age and infection status of animals sampled for routine diagnostics. Nevertheless, a good overall correlation between the ELISAs and the corresponding SNTs could be observed, except for some samples with very low antibody-titers. Thus, we recommend an initial screening with highly sensitive N-based ELISAs to detect also small amounts of antibodies. Since the N-protein is produced at high levels in infected cells, N-specific antibodies occur abundantly and very early after infection [37]. In contrast, Gc-specific neutralizing antibodies can be detected reliably only from two to three weeks after infection onwards [37]. We therefore suggest using the Gc-ELISA in a second step primarily to further differentiate virus-specific antibodies.

In this study, we present a strategy to rapidly design ELISA tests allowing discrimination of antibodies against different orthobunyaviruses. Based on the conserved molecular architecture of their spike protein we suppose that similar assays can easily be adopted for additional bunyaviruses. Such tests are valuable tools to assess occurrence and distribution of specific viruses in regions where several simbuviruses (or other bunyaviruses) are supposed to circulate and, as a consequence, serological tests are needed to differentiate those pathogens.

5. Conclusions

A novel, highly specific triplex ELISA system for the differentiation of antibodies against SBV, AKAV and SHUV was developed. This assay could serve as a basis for the fast establishment of ELISAs against various further orthobunyaviruses in the future, as the antigenicity of three distinct proteins that were produced according to a previously defined general molecular architecture of the complete genus was demonstrated. Such ELISAs may be used for high-throughput serological screenings in suspected populations or as additional tools during outbreak investigations in regions in which several orthobunyaviruses circulate.

Author Contributions: Conceptualization, K.W., A.A. and M.B.; methodology, K.W., A.A. and M.B.; validation, K.W. and A.A.; formal analysis, K.W.; investigation, K.W., A.A., F.S. and K.P.S.; resources, K.W. and M.B.; writing—original draft preparation, K.W. and A.A.; writing—review and editing, all authors; visualization, K.W. and A.A. All authors have read and agreed to the published version of the manuscript.

Institutional Review Board Statement: The field sera were obtained by the responsible farm veterinarian in the context of the health monitoring of the respective herd (farmed animals) or were retrieved by hunters from shot animals (wild animals), no permissions were necessary to collect these specimens. The sera from experimentally infected animals were taken according to protocols

reviewed by the responsible state ethics commission and approved by the competent authority (ref. LALLF M-VTSD/7221.3-1.1-004/12 and LALLF M-VTSD/7221.3-2-010/18).
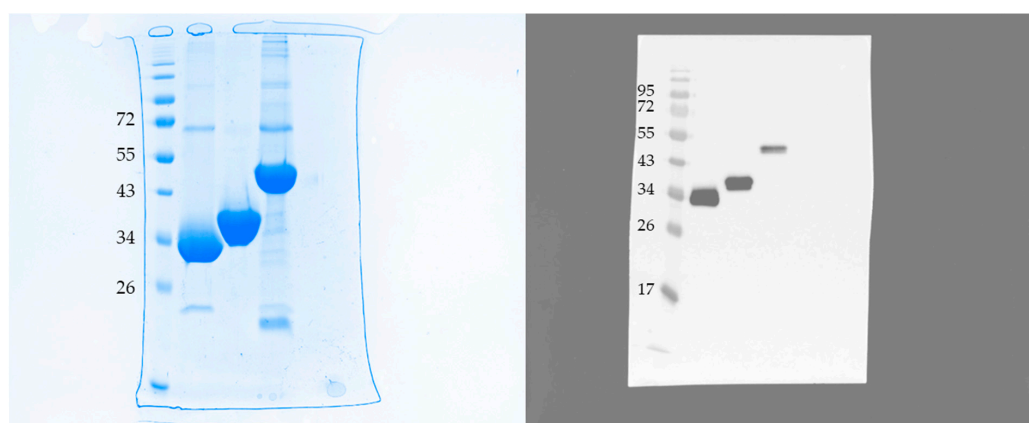
Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available in the article and Appendix A.

Appendix A

(**a**)

Lane	Relative Front	Adj. Volume (Int)	Volume (Int)
1 (SBV-GcH)	0.528926	62,255,376	63,547,920
2 (AKAV-GcH)	0.450413	47,386,160	49,039,977
3 (SHUV-GcH)	0.260331	19,823,042	20,606,450

(**b**)

Figure A1. (**a**) Full-length blots of SDS-PAGE and Western blot analyses of the recombinant proteins shown in Figure 1. (**b**) The band intensities (Western blot) of the SBV, AKAV and SHUV proteins were analyzed using the Image Lab software (Bio-Rad, Feldkirchen, Germany).

References

1. Sick, F.; Beer, M.; Kampen, H.; Wernike, K. Culicoides biting midges—Underestimated vectors for arboviruses of public health and veterinary importance. *Viruses* **2019**, *11*, 376. [CrossRef]
2. Saeed, M.F.; Li, L.; Wang, H.; Weaver, S.C.; Barrett, A.D. Phylogeny of the simbu serogroup of the genus bunyavirus. *J. Gen. Virol.* **2001**, *82*, 2173–2181. [CrossRef]
3. Maes, P.; Adkins, S.; Alkhovsky, S.V.; Avšič-Županc, T.; Ballinger, M.J.; Bente, D.A.; Beer, M.; Bergeron, E.; Blair, C.D.; Briese, T.; et al. Taxonomy of the order bunyavirales: Second update 2018. *Arch. Virol.* **2019**, *164*, 927–941. [CrossRef]
4. Kinney, R.M.; Calisher, C.H. Antigenic relationships among simbu serogroup (*Bunyaviridae*) viruses. *Am. J. Trop. Med. Hyg.* **1981**, *30*, 1307–1318. [CrossRef]
5. Causey, O.R.; Kemp, G.E.; Causey, C.E.; Lee, V.H. Isolations of simbu-group viruses in Ibadan, Nigeria 1964–69, including the new types Sango, Shamonda, Sabo and Shuni. *Ann. Trop. Med. Parasitol.* **1972**, *66*, 357–362. [CrossRef]
6. Barr, C.T.W.a.J.N. Recent advances in the molecular and cellular biology of bunyaviruses. *J. Gen. Virol.* **2011**, *92*, 2467–2484.
7. Elliott, R.M. Orthobunyaviruses: Recent genetic and structural insights. *Nat. Rev. Microbiol.* **2014**, *12*, 673–685. [CrossRef]

8.   Shi, X.; Lappin, D.F.; Elliott, R.M. Mapping the Golgi targeting and retention signal of bunyamwera virus glycoproteins. *J. Virol.* **2004**, *78*, 10793–10802. [CrossRef]

9.   Shi, X.; van Mierlo, J.T.; French, A.; Elliott, R.M. Visualizing the replication cycle of bunyamwera orthobunyavirus expressing fluorescent protein-tagged gc glycoprotein. *J. Virol.* **2010**, *84*, 8460–8469. [CrossRef]

10. Shi, X.; Goli, J.; Clark, G.; Brauburger, K.; Elliott, R.M. Functional analysis of the bunyamwera orthobunyavirus gc glycoprotein. *J. Gen. Virol.* **2009**, *90*, 2483–2492. [CrossRef]

11. Hellert, J.; Aebischer, A.; Wernike, K.; Haouz, A.; Brocchi, E.; Reiche, S.; Guardado-Calvo, P.; Beer, M.; Rey, F.A. Orthobunyavirus spike architecture and recognition by neutralizing antibodies. *Nat. Commun.* **2019**, *10*, 879. [CrossRef]

12. Roman-Sosa, G.; Brocchi, E.; Schirrmeier, H.; Wernike, K.; Schelp, C.; Beer, M. Analysis of the humoral immune response against the envelope glycoprotein gc of Schmallenberg virus reveals a domain located at the amino terminus targeted by mabs with neutralizing activity. *J. Gen. Virol.* **2016**, *97*, 571–580. [CrossRef]

13. Wernike, K.; Beer, M. Schmallenberg virus: A novel virus of veterinary importance. *Adv. Virus Res.* **2017**, *99*, 39–60.

14. Kato, T.; Shirafuji, H.; Tanaka, S.; Sato, M.; Yamakawa, M.; Tsuda, T.; Yanase, T. Bovine arboviruses in culicoides biting midges and sentinel cattle in Southern Japan from 2003 to 2013. *Transbound. Emerg. Dis.* **2016**, *63*, e160–e172. [CrossRef]

15. Kato, T.; Yanase, T.; Suzuki, M.; Katagiri, Y.; Ikemiyagi, K.; Takayoshi, K.; Shirafuji, H.; Ohashi, S.; Yoshida, K.; Yamakawa, M.; et al. Monitoring for bovine arboviruses in the most southwestern islands in Japan between 1994 and 2014. *BMC Vet. Res.* **2016**, *12*, 125. [CrossRef]

16. Hayama, Y.; Yanase, T.; Suzuki, M.; Unten, K.; Tomochi, H.; Kakehi, M.; Shono, Y.; Yamamoto, T.; Kobayashi, S.; Murai, K.; et al. Meteorological factors affecting seroconversion of akabane disease in sentinel calves in the subtropical Okinawa islands of Japan. *Trop. Anim. Health Prod.* **2018**, *50*, 209–215. [CrossRef]

17. Wernike, K.; Holsteg, M.; Szillat, K.P.; Beer, M. Development of within-herd immunity and long-term persistence of antibodies against Schmallenberg virus in naturally infected cattle. *BMC Vet. Res.* **2018**, *14*, 368. [CrossRef]

18. Geoghegan, J.L.; Walker, P.J.; Duchemin, J.B.; Jeanne, I.; Holmes, E.C. Seasonal drivers of the epidemiology of arthropod-borne viruses in Australia. *PLoS Negl. Trop. Dis.* **2014**, *8*, e3325. [CrossRef]

19. Wernike, K.; Beer, M. Re-Circulation of Schmallenberg virus, Germany, 2019. *Transbound. Emerg. Dis.* **2020**, *67*, 2290–2295. [CrossRef]

20. Golender, N.; Bumbarov, V.Y.; Erster, O.; Beer, M.; Khinich, Y.; Wernike, K. Development and validation of a universal S-segment-based real-time RT-PCR assay for the detection of simbu serogroup viruses. *J. Virol. Methods* **2018**, *261*, 80–85. [CrossRef]

21. Mathew, C.; Klevar, S.; Elbers, A.R.; van der Poel, W.H.; Kirkland, P.D.; Godfroid, J.; Mdegela, R.H.; Mwamengele, G.; Stokstad, M. Detection of serum neutralizing antibodies to Simbu sero-group viruses in cattle in Tanzania. *BMC Vet. Res.* **2015**, *11*, 208. [CrossRef] [PubMed]

22. Wang, J.; Firth, C.; Amos-Ritchie, R.; Davis, S.S.; Yin, H.; Holmes, E.C.; Blasdell, K.R.; Walker, P.J. Evolutionary history of simbu serogroup orthobunyaviruses in the Australian episystem. *Virology* **2019**, *535*, 32–44. [CrossRef] [PubMed]

23. Jun, K.; Yanaka, T.; Lee, K.K.; Lee, J.B. Seroprevalence of bovine arboviruses belonging to genus orthobunyavirus in South Korea. *J. Vet. Med. Sci.* **2018**, *80*, 1619–1623. [CrossRef] [PubMed]

24. Beer, M.; Wernike, K. Akabane virus and Schmallenberg virus. *Ref. Mod. Life Sci.* **2019**. [CrossRef]

25. Sick, F.; Breithaupt, A.; Golender, N.; Bumbarov, V.; Beer, M.; Wernike, K. Shuni virus-induced meningoencephalitis after experimental infection of cattle. *Transbound. Emerg. Dis.* **2020**. [CrossRef]

26. Kirkland, P.D. Akabane virus infection. *Rev. Sci. Tech.* **2015**, *34*, 403–410. [CrossRef]

27. Hoffmann, B.; Scheuch, M.; Höper, D.; Jungblut, R.; Holsteg, M.; Schirrmeier, H.; Eschbaumer, M.; Goller, K.V.; Wernike, K.; Fischer, M.; et al. Novel orthobunyavirus in cattle, Europe, 2011. *Emerg. Infect. Dis.* **2012**, *18*, 469–472. [CrossRef]

28. Golender, N.; Bumbarov, V.; Assis, I.; Beer, M.; Khinich, Y.; Koren, O.; Edery, N.; Eldar, A.; Wernike, K. Shuni virus in Israel: Neurological disease and fatalities in cattle. *Transbound. Emerg. Dis.* **2019**, *66*, 1126–1131. [CrossRef]

29. Wernike, K.; Elbers, A.; Beer, M. Schmallenberg virus infection. *Rev. Sci. Tech.* **2015**, *34*, 363–373. [CrossRef]

30. Golender, N.; Brenner, J.; Valdman, M.; Khinich, Y.; Bumbarov, V.; Panshin, A.; Edery, N.; Pismanik, S.; Behar, A. Malformations caused by Shuni virus in ruminants, Israel, 2014–2015. *Emerg. Infect. Dis.* **2015**, *21*, 2267–2268. [CrossRef]

31. Huang, C.C.; Huang, T.S.; Deng, M.C.; Jong, M.H.; Lin, S.Y. Natural infections of pigs with Akabane virus. *Vet. microbiol.* **2003**, *94*, 1–11. [CrossRef]

32. Van Eeden, C.; Williams, J.H.; Gerdes, T.G.; van Wilpe, E.; Viljoen, A.; Swanepoel, R.; Venter, M. Shuni virus as cause of neurologic disease in horses. *Emerg. Infect. Dis.* **2012**, *18*, 318–321. [CrossRef] [PubMed]

33. Desmecht, D.; Garigliany, M.M.; Beer, M.; Schirrmeier, H.; Paternostre, J.; Volpe, R.; Linden, A. Detection of antibodies against Schmallenberg virus in wild boars, Belgium, 2010–2012. In Proceedings of the 31th Congress of the International Union Game Biologists, Brussels, Belgium, 27–29 August 2013.

34. Mouchantat, S.; Wernike, K.; Lutz, W.; Hoffmann, B.; Ulrich, R.G.; Borner, K.; Wittstatt, U.; Beer, M. A broad spectrum screening of Schmallenberg virus antibodies in wildlife animals in Germany. *Vet. Res.* **2015**, *46*, 99. [CrossRef] [PubMed]

35. Wensman, J.J.; Blomqvist, G.; Hjort, M.; Holst, B.S. Presence of antibodies to Schmallenberg virus in a dog in Sweden. *J. Clin. Microbiol.* **2013**, *51*, 2802–2803. [CrossRef] [PubMed]

36. Sailleau, C.; Boogaerts, C.; Meyrueix, A.; Laloy, E.; Bréard, E.; Viarouge, C.; Desprat, A.; Vitour, D.; Doceul, V.; Boucher, C.; et al. Schmallenberg virus infection in dogs, France, 2012. *Emerg. Infect. Dis.* **2013**, *19*, 1896–1898. [CrossRef]

37. Wernike, K.; Eschbaumer, M.; Schirrmeier, H.; Blohm, U.; Breithaupt, A.; Hoffmann, B.; Beer, M. Oral exposure, reinfection and cellular immunity to Schmallenberg virus in cattle. *Vet. Microbiol.* **2013**, *165*, 155–159. [CrossRef]

38. Wernike, K.; Hoffmann, B.; Bréard, E.; Bøtner, A.; Ponsart, C.; Zientara, S.; Lohse, L.; Pozzi, N.; Viarouge, C.; Sarradin, P.; et al. Schmallenberg virus experimental infection of sheep. *Vet. Microbiol.* **2013**, *166*, 461–466. [CrossRef]

39. Laloy, E.; Riou, M.; Barc, C.; Belbis, G.; Bréard, E.; Breton, S.; Cordonnier, N.; Crochet, D.; Delaunay, R.; Moreau, J.; et al. Schmallenberg virus: Experimental infection in goats and bucks. *BMC Vet. Res.* **2015**, *11*, 221. [CrossRef]

40. Kurogi, H.; Inaba, Y.; Takahashi, E.; Sato, K.; Goto, Y. Experimental infection of pregnant goats with Akabane virus. *Nat. Inst. Anim. Health Quart.* **1977**, *17*, 1–9.

41. Kurogi, H.; Inaba, Y.; Takahashi, E.; Sato, K.; Goto, Y.; Satoda, K.; Omori, T.; Hatakeyama, H. Development of inactivated vaccine for Akabane disease. *Nat. Inst. Anim. Health Quart.* **1978**, *18*, 97–108.

42. Wernike, K.; Nikolin, V.M.; Hechinger, S.; Hoffmann, B.; Beer, M. Inactivated Schmallenberg virus prototype vaccines. *Vaccine* **2013**, *31*, 3558–3563. [CrossRef]

43. Wernike, K.; Holsteg, M.; Schirrmeier, H.; Hoffmann, B.; Beer, M. Natural infection of pregnant cows with Schmallenberg virus— A follow-up study. *PLoS ONE* **2014**, *9*, e98223. [CrossRef] [PubMed]

44. De Regge, N.; van den Berg, T.; Georges, L.; Cay, B. Diagnosis of Schmallenberg virus infection in malformed lambs and calves and first indications for virus clearance in the fetus. *Vet. Microbiol.* **2013**, *162*, 595–600. [CrossRef] [PubMed]

45. Goller, K.V.; Höper, D.; Schirrmeier, H.; Mettenleiter, T.C.; Beer, M. Schmallenberg virus as possible ancestor of Shamonda virus. *Emerg. Infect. Dis.* **2012**, *18*, 1644–1646. [CrossRef] [PubMed]

46. Wernike, K.; Beer, M.; Hoffmann, B. Schmallenberg virus infection diagnosis: Results of a German proficiency trial. *Transbound. Emerg. Dis.* **2017**, *64*, 1405–1410. [CrossRef] [PubMed]

47. Oluwayelu, D.; Wernike, K.; Adebiyi, A.; Cadmus, S.; Beer, M. Neutralizing antibodies against Simbu serogroup viruses in cattle and sheep, Nigeria, 2012–2014. *BMC Vet. Res.* **2018**, *14*, 277. [CrossRef]

48. Gonzalez-Scarano, F.; Shope, R.E.; Calisher, C.E.; Nathanson, N. Characterization of monoclonal antibodies against the G1 and N proteins of Lacrosse and Tahyna, two California serogroup bunyaviruses. *Virology* **1982**, *120*, 42–53. [CrossRef]

49. Wernike, K.; Aebischer, A.; Roman-Sosa, G.; Beer, M. The n-terminal domain of Schmallenberg virus envelope protein gc is highly immunogenic and can provide protection from infection. *Sci. Rep.* **2017**, *7*, 42500. [CrossRef]

50. Wernike, K.; Brocchi, E.; Cordioli, P.; Senechal, Y.; Schelp, C.; Wegelt, A.; Aebischer, A.; Roman-Sosa, G.; Reimann, I.; Beer, M. A novel panel of monoclonal antibodies against Schmallenberg virus nucleoprotein and glycoprotein Gc allows specific orthobunyavirus detection and reveals antigenic differences. *Vet. Res.* **2015**, *46*, 27. [CrossRef]

51. Wernike, K.; Mundt, A.; Link, E.K.; Aebischer, A.; Schlotthauer, F.; Sutter, G.; Fux, R.; Beer, M. N-Terminal domain of Schmallenberg virus envelope protein Gc delivered by recombinant equine herpesvirus type 1 and modified vaccinia virus Ankara: Immunogenicity and protective efficacy in cattle. *Vaccine* **2018**, *36*, 5116–5123. [CrossRef]

52. Hechinger, S.; Wernike, K.; Beer, M. Single immunization with an inactivated vaccine protects sheep from Schmallenberg virus infection. *Vet. Res.* **2014**, *45*, 79. [CrossRef] [PubMed]

53. Hechinger, S.; Wernike, K.; Beer, M. Evaluating the protective efficacy of a trivalent vaccine containing Akabane virus, Aino virus and Chuzan virus against Schmallenberg virus infection. *Vet. Res.* **2013**, *44*, 114. [CrossRef] [PubMed]

54. Yu, X.J.; Liang, M.F.; Zhang, S.Y.; Liu, Y.; Li, J.D.; Sun, Y.L.; Zhang, L.; Zhang, Q.F.; Popov, V.L.; Li, C.; et al. Fever with thrombocytopenia associated with a novel bunyavirus in China. *N. Engl. J. Med.* **2011**, *364*, 1523–1532. [CrossRef] [PubMed]

55. Marklewitz, M.; Zirkel, F.; Rwego, I.B.; Heidemann, H.; Trippner, P.; Kurth, A.; Kallies, R.; Briese, T.; Lipkin, W.I.; Drosten, C.; et al. Discovery of a unique novel clade of mosquito-associated bunyaviruses. *J. Virol.* **2013**, *87*, 12850–12865. [CrossRef]

56. Edridge, A.W.D.; Deijs, M.; Namazzi, R.; Cristella, C.; Jebbink, M.F.; Maurer, I.; Kootstra, N.A.; Buluma, L.R.; van Woensel, J.B.M.; de Jong, M.D.; et al. Novel orthobunyavirus identified in the cerebrospinal fluid of a Ugandan child with severe Encephalopathy. *Clin. Infect.* **2018**, *68*, 139–142. [CrossRef]

57. Abutarbush, S.M.; La Rocca, A.; Wernike, K.; Beer, M.; Al Zuraikat, K.; Al Sheyab, O.M.; Talafha, A.Q.; Steinbach, F. Circulation of a Simbu serogroup virus, causing Schmallenberg virus-like clinical signs in Northern Jordan. *Transbound. Emerg. Dis.* **2017**, *64*, 1095–1099. [CrossRef]

58. Hussien, M.O.; Alfaki, S.H.; Enan, K.A.; Gafar, R.A.; Elhassan, A.M.; Taha, K.M.; El Hussein, A.R.M. Prevalence of antibodies to Simbu serogroup viruses in cattle in Sudan. *Vet. Med. Int.* **2020**, *2020*, 8858742. [CrossRef]

59. Elbers, A.R.; Stockhofe, N.; van der Poel, W.H. Schmallenberg virus antibodies in adult cows and maternal antibodies in calves. *Emerg. Infect. Dis.* **2014**, *20*, 901–902. [CrossRef]

60. Elbers, A.R.; Stockhofe-Zurwieden, N.; van der Poel, W.H. Schmallenberg virus antibody persistence in adult cattle after natural infection and decay of maternal antibodies in calves. *BMC Vet. Res.* **2014**, *10*, 103. [CrossRef]

61. Wernike, K.; Holsteg, M.; Saßerath, M.; Beer, M. Schmallenberg virus antibody development and decline in a naturally infected dairy cattle herd in Germany, 2011–2014. *Vet. Microbiol.* **2015**, *181*, 294–297. [CrossRef]

62. Inaba, Y.; Matumoto, M. Chapter 43: Akabane virus. In *Virus Infections of Vertebrates, Virus Infections of Ruminants*; Dinter, S., Morien, B., Eds.; Elsevier Science Publishers, B.V.: Amsterdam, The Netherlands, 1990; Volume 3, pp. 467–480.

63. Blomström, A.L.; Stenberg, H.; Scharin, I.; Figueiredo, J.; Nhambirre, O.; Abilio, A.P.; Fafetine, J.; Berg, M. Serological screening suggests presence of Schmallenberg virus in cattle, sheep and goat in the Zambezia Province, Mozambique. *Transbound. Emerg. Dis.* **2014**, *61*, 289–292. [CrossRef] [PubMed]

64. Rasekh, M.; Sarani, A.; Hashemi, S.H. Detection of Schmallenberg virus antibody in equine population of Northern and Northeast of Iran. *Vet. World* **2018**, *11*, 30–33. [CrossRef] [PubMed]

65. Roman-Sosa, G.; Karger, A.; Kraatz, F.; Aebischer, A.; Wernike, K.; Maksimov, P.; Lillig, C.H.; Reimann, I.; Brocchi, E.; Keller, M.; et al. The amino terminal subdomain of glycoprotein gc of Schmallenberg virus: Disulfide bonding and structural determinants of neutralization. *J. Gen. Virol.* **2017**, *98*, 1259–1273. [CrossRef] [PubMed]

66. De Jongh, W.A.; Salgueiro, S.; Dyring, C. The use of Drosophila S2 cells in R&D and bioprocessing. *Pharma. Bioprocess.* **2013**, *1*, 197–213.

5   Diskussion

Etablierung von Infektionsmodellen und _in vivo_ Charakterisierung verschiedener SHUV-Isolate

Seit dem plötzlichen Auftreten von SBV Ende 2011 in Europa (24) sind Viren der Simbu-Serogruppe, die durch Gnitzen übertragen werden, weltweit verbreitet und spielen vorwiegend in der Tiergesundheit aber auch in der öffentlichen Gesundheit eine Rolle (19).

SHUV wurde erstmals in den 1960er Jahren entdeckt und ist in Afrika verbreitet (6). Durch den Sprung in den Nahen Osten nach Israel zog das seit langer Zeit weitgehend vernachlässigte SHUV vermehrt Interesse auf sich (3). SHUV-Infektionen stehen im Zusammenhang mit schweren neurologischen Erkrankungen bei Pferden, verschiedenen Wildtierspezies und Rindern (8, 9, 14). Wie die meisten Viren der Simbu-Serogruppe, kann SHUV bei Infektion naiver Tiere in einer kritischen Phase der Trächtigkeit auch Aborte, Totgeburten, Mumifikationen und kongenitale Missbildungen des muskuloskelettalen Apparats und des zentralen Nervensystems (sog. Arthrogryphose-Hydranenzephalie-Syndrom) beim Fetus auslösen (3). Die Detektion von Antikörpern in humanen Seren von Großtierärzten und von SHUV RNA in humanen Liquorproben von Patienten mit neurologischer Symptomatik in Südafrika deuten auf ein zoonotisches Potential von SHUV hin (10, 46, 97). Die Pathogenese ist bei allen Wirtsspezies allerdings noch weitgehend ungeklärt.

In den vorliegenden Studien wurden zwei neu etablierte Modelle für die _in vivo_ Charakterisierung von SHUV beschrieben: ein Großtiermodell für die Charakterisierung im natürlichen Säugerwirt Rind und ein IFNAR (-/-)-Mausmodell als eine Alternative im Kleintier. Bei den SHUV-Isolaten, die in dieser Arbeit charakterisiert wurden, handelt es sich um aktuell zirkulierende Isolate aus Israel (3, 14). Die nt Sequenzen älterer afrikanischer Isolate und des Prototyps weisen zu aktuell in Israel zirkulierenden Stämmen nur eine Identität von 90,5 bis 97,4% auf, aktuelle israelische Sequenzen untereinander stimmen dagegen zu 99,3 bis zu 100% überein (43). Für die _in vivo_ Charakterisierung von SHUV-Infektionen im natürlichen Säugerwirt wurde ein SHUV-Infektionsmodell im Rind etabliert, wobei möglichst aktuelle israelische Virusisolate zum Einsatz kamen, um eventuelle Einflüsse der Virusevolution auf die Viruseigenschaften zu verringern. Ein Isolat stammt aus einem missgebildeten Fetus, das

andere aus einem Rind mit akuter neurologischer Erkrankung. Viren beider möglicher klinischen Bilder sind somit vertreten.

Das „Enzephalitis Isolat" SHUV 2162/16 aus dem Gehirn einer akut neurologisch erkrankten Färse wurde in sechs junge Rinder inokuliert, um die Empfänglichkeit der Tiere für eine Infektion und um deren Folgen unter standardisierten experimentellen Bedingungen zu untersuchen. Die Inokulation erfolgte subkutan, was den natürlichen Infektionsweg durch Gnitzenstich widerspiegeln sollte.

Die Rinder erwiesen sich als empfänglich für SHUV: alle sechs Rinder waren virämisch und serokonvertierten. Die Immunantwort wurden mit verschiedenen neu entwickelten serologischen Methoden für die Detektion von Antikörpern untersucht, ein bis zwei Wochen nach der Inokulation zeigten die Tiere eine gute humorale Immunantwort. Die virämische Phase von nur drei bis vier aufeinanderfolgenden Tagen ist sehr kurz, vor allem für ein Vektor-übertragenes Virus. Eine derart kurze Virämie ist auch für andere Viren der Simbu-Serogruppe beschrieben: Bei SBV hält die Virämie für etwa zwei bis fünf Tage an (24, 98), bei AKAV für drei bis fünf Tage (99). Die Dauer der Virämie scheint aber lange genug zu sein, so dass sich die Vektoren am infektiösen Blut infizieren und sich Transmissionszyklen zwischen Vektor und Wirt entwickeln können.

Das Rind, aus dem das „Enzephalitis Isolat" gewonnen wurde, zeigte schwere klinische neurologische Symptome wie Kreiseln, Ataxie und Schwierigkeiten beim Schlucken. Im fortschreitenden Verlauf der Erkrankung war das Tier festliegend und wurde infolgedessen euthanasiert (14). Die experimentell infizierten Rinder dagegen zeigten keinerlei neurologische Auffälligkeiten. Fünf der sechs jungen Rinder entwickelten erhöhte Körpertemperaturen für einige Tage, die Fieberverläufe waren bei den einzelnen Tieren sehr unterschiedlich. Keine weiteren klinischen Zeichen wurden beobachtet. Trotz der fehlenden neurologischen Symptomatik konnte nach Versuchsende bei vier von sechs Rindern eine Meningoenzephalitis diagnostiziert werden. SHUV RNA konnte in Proben des zentralen Nervensystems mittels real-time RT-PCR detektiert werden und in der histopathologischen Untersuchung zeigte sich das Bild einer nichteitrigen Meningoenzephalitis. Sowohl im natürlich infizierten Rind als auch in den experimentell infizierten Rindern war diese durch perivaskuläre Zellinfiltrate und Gliaknötchen gekennzeichnet (14), die mittels immunhistochemischen Färbungen zu gleichen Teilen als Iba-1 markierten Makrophagen und

CD3-positiven T-Lymphozyten identifiziert wurden. Diese Befunde sind charakteristisch für virale Encephalitiden. Zudem konnte SHUV RNA mittels in-situ-Hybridisierung (ISH) (RNAScope™) in einem entzündeten Areal im Zellfortsatz eines Neurons detektiert werden. Ein Neurotropismus und kausaler Zusammenhang zwischen neurologischer Erkrankung und SHUV-Infektion konnte somit erstmals experimentell bewiesen werden.

Die unterschiedliche Ausprägung der klinischen Symptomatik im natürlich infizierten Rind und in experimentell infizierten Rindern könnte sich durch verschiedene Faktoren erklären lassen: Der individuelle Immunstatus und die allgemeine körperliche Konstitution, genetische Faktoren oder das Alter könnten Einfluss auf den Krankheitsverlauf nehmen. Für andere neurotrope Viren der Simbu-Serogruppe sind ebenso unterschiedliche Schweregrade des klinischen Verlaufs beschrieben. AKAV kann wie SHUV sowohl kongenitale Missbildungen bei diaplazentarer Infektion als auch akute neurologische Krankheit bei postnatal infizierten Rindern auslösen (100). Für AKAV konnten bestimmte Virusstämme identifiziert werden, die speziell mit neurologischer Erkrankung assoziiert sind (Genogruppe I). Allerdings wurden AKAV-Stämme der Genogruppe I auch aus asymptomatischen Rindern isoliert (101). Dies spricht dafür, dass, wie bei SHUV-Infektionen auch, bei neurotropen AKAV-Infektionen nicht immer eine erkennbare klinische Symptomatik auftritt. Bei SHUV konnten bisher keine Veränderungen im Virusgenom festgestellt werden, die mit neurologischer Erkrankung assoziiert sind (14).

Unter den Sequenzen der israelischen SHUV-Isolaten ist die genetische Identität sehr hoch (99,3-100%). Die wenigen auftretenden Mutationen betreffen fast ausschließlich die Aminosäuresequenz des NSs-Proteins, wobei die Aminosäuresequenz des N-Proteins unverändert bleibt (43). Diese Konservierung des N-Proteins bei gleichzeitiger Variabilität des NSs-Proteins ist auch bei SBV-Isolaten aus missgebildeten Feten bekannt (102). Das zweite SHUV Isolat, das im Rindermodell charakterisiert wurde, stammt aus dem Gehirn eines missgebildeten ovinen Fetus (3). Das „Missbildungs-Isolat" 2504/3/14 weist eine der eben beschriebenen genetischen Besonderheiten auf: Ein Stopp Codon im ORF des NSs-Proteins führt zur Expression einer verkürzten Form des Proteins, ohne die Aminosäuresequenz des N-Proteins zu verändern. Im immunkompetenten Säugerwirt ist NSs ein wichtiger Virulenzfaktor, der als IFN-Antagonist die angeborene Immunantwort der Zelle

unterdrücken und die wirtseigene Proteinbiosynthese hemmen kann (27). Statt der vollen Länge von 91 Aminosäuren (AS) ist die verkürzte Version des „Missbildungs-Isolats" nur 16 AS lang. *In vitro* Experimente zeigten, dass dieses verkürzte Protein nicht funktional ist und daher konnte die Aktivierung der angeborenen IFN-Immunantwort der Wirtszellen auch nicht inhibiert werden (43).

Im Rinder-Infektionsmodell zeigte die NSs-Deletionsmutante auch *in vivo* eine deutliche Attenuierung. Nach subkutaner Inokulation konnte bei immunkompetenten Rindern keine Virämie induziert werden und nur eines der sechs inokulierten Tiere serokonvertierte, wobei sehr niedrige Titer neutralisierender Antikörper gebildet wurden (siehe Publikation I). Für SBV ist Ähnliches beschrieben: Eine mittels reverser Genetik hergestellte SBV NSs-Deletionsmutante konnte nach Inokulation in Rindern keine Virämie auslösen, induzierte aber die Bildung von niedrigen Titern neutralisierender Antikörper (38). In diesem Fall konnte die Immunisierung mit der SBV NSs-Deletionsmutante sogar vor einer Challenge-Infektion schützen (38). Ob die SHUV NSs-Deletionsmutante ebenfalls ein Vakzinekandidat sein könnte, muss in weiteren Studien untersucht werden.

Virusmutanten, die nach diaplazentarer Infektion in missgebildeten Feten entstehen, weisen oftmals eine höhere genetische Variabilität als Isolate aus virämischen Tieren oder Insektenvektoren auf (43, 102). Für das SHUV „Missbildungs-Isolat" mit einem funktionslosen NSs-Protein konnte eine starke Attenuierung im immunkompetenten Säugerwirt gezeigt werden. Für SBV wurde gezeigt, dass solche in missgebildeten Feten vorhandene Virus-Varianten attenuiert sind und dadurch nicht mehr am natürlichen Infektionszyklus teilnehmen können und „dead-end Artefakte" darstellen (Fehler in der Virusevolution, die zu nicht mehr transmissiblen Viren führen) (38). Da auch für die SHUV-Variante eine starke Attenuierung beobachtet wurde, ist davon auszugehen, dass genetisch variable Virusvarianten, die in missgebildeten Feten entstehen, auch bei SHUV, vielleicht auch bei allen Viren der Simbu-Serogruppe, „dead-end Artefakte" sind.

Zusammenfassend lässt sich sagen, dass mit dem Rind ein Großtiermodell zur Verfügung steht, um SHUV-Infektionen in einem natürlichen Wirt zu untersuchen, was unter anderem auch die Grundlage für zukünftige Vakzine-Challenge Versuche darstellt, sobald geeignete Impfstoffkandidaten zur Verfügung stehen. Infektionen mit einem neurotropen SHUV-Isolat konnten klinisch, virologisch, serologisch und pathohistologisch im Detail charakterisiert

werden. Für ein NSs-defizientes SHUV-Isolat aus einem missgebildeten Fetus wurde die *in vivo* Attenuierung demonstriert.

Als Alternative zu kostenintensiven und in der Durchführung aufwändigen Großtierversuchen wurde in der vorliegenden Arbeit ein Kleintiermodell in der IFNAR (-/-) Maus entwickelt. IFNAR (-/-) Mäuse sind sehr gut charakterisiert und ein geeignetes Kleintiermodell für Viren der Simbu-Serogruppe, da in Studien mit SBV, Simbu-Virus, Sabo-Virus und Sathuperi-Virus gezeigt werden konnte, dass sich die Neuropathologie ähnlich wie in natürlichen Wirten darstellt (82, 83). IFNAR (-/-) Mäuse sind empfänglicher für Virusinfektionen als Wildtyp-Mäuse, da die zellulären Interferon α/β Rezeptoren IFNAR1 fehlen, die bei Virusinfektionen einen antiviralen Status der Zelle induzieren und so vor Infektionen schützten. Dieses Modell wurde daher auch auf die Empfänglichkeit für SHUV getestet und die Neuropathologie zweier SHUV-Isolate wurde charakterisiert. Ein neuropathogenes SHUV Isolat (SHUV 2162/16) und ein Isolat aus einem missgebildeten Fetus (SHUV 2504/3/14), die auch im Rindermodell charakterisiert wurden, kamen für die Studie zum Einsatz (3, 14). IFNAR (-/-) Mäuse erwiesen sich als hochempfänglich für eine SHUV-Infektion. Die klinische Erkrankung, Gewichtsverläufe, Viruslasten, Serologie, und pathologische Befunde unterschieden sich zwischen den beiden getesteten Infektionsdosen (10^4 und 10^5 TCID50/ml) und eingesetzten Virusisolaten nur kaum. Eine erfolgreiche Infektion wurde mittels PCR-Nachweis viraler RNA in Serumproben von Tag drei oder sieben bei allen inokulierten Tieren bestätigt. Zum Teil dauerte die virämische Phase sehr lange an, bei 9 Mäusen war noch an Tag 21 bei Versuchende virale RNA in EDTA-Blutproben nachweisbar. Alle Tiere (bis auf zwei vorzeitig verstorbene Mäuse) serokonvertierten.

Sowohl das „Missbildungs-Isolat" als auch das „Enzephalitis-Isolat" lösten bei den meisten Mäusen einen klinisch inapparenten Krankheitsverlauf, bei einzelnen Mäusen allerdings eine tödliche Erkrankung aus. Klinisch unauffällige Krankheitsverläufe ähneln der Infektion in experimentell mit dem „Enzephalitis Isolat" infizierten Rindern, und eine tödliche endende Erkrankung ist in einigen Fällen auch bei natürlich infizierten Rindern beschrieben (14). Die pathologischen Befunde des zentralen Nervensystems (ZNS) im IFNAR (-/-) Mausmodell ähneln den Befunden in natürlich und experimentell infizierten Rindern: Bei allen vorzeitig verstorbenen oder euthanasierten Tieren und zwei weiteren klinisch unauffälligen Mäusen wurde eine nicht-suppurative Meningoenzephalitis festgestellt. SHUV-RNA wurde per PCR in Proben des ZNS bei insgesamt 10 von 24 Mäusen nachgewiesen. Die histopathologischen

Befunde des ZNS sind wie bei infizierten Rindern gekennzeichnet durch perivaskuläre Zellinfiltrate, die mittels immunhistochemischer Färbungen als CD3-positive T-Zellen und Iba-1 markierte aktivierte Makrophagen/ Mikroglia identifiziert wurden. SHUV RNA wurde in den Mäusen mittels *in-situ* Hybridisierung in einer Vielzahl an Neuronen und Gliazellen nachgewiesen. Bei Rindern wurde mittels *In-situ* Hybridisierung SHUV RNA lediglich in einem entzündeten Areal im Zellfortsatz eines Neurons detektiert. Dieser Unterschied könnte auf die unterschiedlichen Untersuchungszeitpunkte im Infektionsgeschehen zurückzuführen sein: Rinder wurden ausschließlich bei Versuchsende nach 21 Tagen untersucht, wobei im Mausmodell auch vorzeitig verstorbene oder euthanasierte Tiere während der akuten Phase der Infektion untersucht wurden, die zu diesem Zeitpunkt vergleichsweise sehr hohe Viruslasten im ZNS aufwiesen. Neuronen konnten im IFNAR (-/-) Mausmodell auch bei SBV, Sabo-Virus, Simbu-Virus und Sathuperi-Virus mittels ISH als Zielzellen der Infektion ausgemacht werden; histologisch waren ebenfalls Meningoenzephalitiden mit perivaskulären Infiltraten von Lymphozyten und Makrophagen zu sehen (83). Verschiedene Viren der Simbu-Serogruppe infizieren somit die gleichen Zielzellen und lösen nahezu identische pathohistologischen Befunde aus, was auf einen ähnlichen Pathomechanismus aller Vertreter der Serogruppe hindeutet.

Bei vorzeitig euthanasierten oder verstorbenen Mäusen wurde SHUV RNA in einem sehr breiten Panel an Organen detektiert. Höhere Viruslasten als im Blut der noch virämischen Tiere konnten allerdings nur in Proben des ZNS nachgewiesen werden. Bei Versuchsende nach 21 Tagen wurde SHUV RNA nur in wenigen Organen detektiert, vor allem aber in Proben der Milz und des Jejunums. Die Zielzellen der SHUV-Infektion in lymphoretikulären Organen wurden mittels ISH und Immunhistochemie identifiziert: virale RNA wurde in Makrophagen der Milz und des Schleimhaut-assoziierten lymphatischen Gewebes (MALT) detektiert. Die Affinität von SHUV und anderen Viren der Simbu-Serogruppe zu Organen des lymphoretikulären Systems ist auch aus anderen Studien bekannt. In den experimentell mit SHUV infizierten Rindern konnte virale RNA neben dem ZNS bei drei von sechs Rindern auch in Proben der Milz nachgewiesen werden. Auch bei experimentell mit SBV infizierten Schafen und Rindern war in Milz und Lymphknoten nach einem längeren Zeitraum noch virale RNA nachweisbar (63, 89, 103).

Ein SHUV-Isolat, das sich aufgrund eines verkürzten und dysfunktionalen NSs-Proteins im Rindermodell als attenuiert erwiesen hat, löste im IFNAR Mausmodell einen Krankheitsverlauf inklusive Serokonversion vergleichbar zu einem Isolat mit intaktem NSs-Protein aus. Virusvarianten mit intaktem NSs-Protein haben allerdings in IFNAR (-/-) Mäusen keinen Vorteil gegenüber NSs-defizienten Virusvarianten. Der Virulenzfaktor NSs kann in IFN-kompetenten Säugerzellen eine IFN-Antwort effektiv unterdrücken (36). Bei IFNAR (-/-) Mäusen fehlen allerdings zelluläre IFNAR1 Rezeptoren, die bei Vorhandensein von IFN einen antiviralen Status der Zelle induzieren, wodurch eine gesteigerte Virulenz von Virusvarianten mit funktionalem NSs-Protein aufgehoben wird (81). Virusisolate mit intaktem und dysfunktionalem NSs-Protein zeigen in IFNAR (-/-) Mäusen also keinen Unterschied. Die Effekte des NSs-Proteins im natürlichen Wiederkäuerwirt können daher im IFNAR (-/-) Mausmodell nicht untersucht werden, es bietet aber die Möglichkeit, andere Virulenzfaktoren neben NSs zu studieren.

Das SHUV IFNAR (-/-) Mausmodell könnte in Zukunft für Vakzinetestungen als Alternative zum Rindermodell herangezogen werden. Für andere Viren der Simbu-Serogruppe wie SBV wurden bereits zahlreiche Impfstofftestungen in IFNAR (-/-) Mäusen erfolgreich durchgeführt, wobei die schützende Wirkung bei vielversprechenden Vakzinekandidaten im Anschluss im Großtiermodell bestätigt werden konnte (38, 68, 84-86). Mit dem IFNAR (-/-) Mausmodell steht damit ein Kleintiermodell zur Verfügung, das für verschiedene Pathogenese- oder Vakzinestudien genutzt werden kann.

Entwicklung serologischer Untersuchungsmethoden

Für die Ermittlung von Prävalenzen viraler Erkrankungen innerhalb einer Population, für die Durchführung von epidemiologischen Überwachungsstudien und zur Einschätzung von spatio-temporalen Verbreitungen von Erregern, aber auch für die gezielte Diagnostik bei Ausbruchsgeschehen sind sensitive und spezifische diagnostische Testverfahren unerlässlich. Viren der Simbu-Serogruppe treten weltweit auf und die Verbreitungsgebiete einzelner Vertreter überschneiden sich häufig (19). In Israel beispielsweise kommen gleichzeitig mehrere Viren der Simbu-Serogruppe vor, die zu Ausbrüchen mit missgebildeten Jungtieren führen. Seit 1969 kommt AKAV in Israel vor (104) und führte 2002 erneut zu einem großen Ausbruch (105), seit 2014 kommt auch SHUV im Land vor (3). Sporadisch wurde auch AINOV

detektiert (105). Erst kürzlich wurde das Auftreten von Peaton-Virus beschrieben (106). Besonders in Regionen, in denen mehrere Virusspezies auftreten und um Einträge neuer Virusspezies in bereits betroffene Gebiete frühzeitig zu erkennen, sind diagnostische Tests mit der Möglichkeit, zwischen einzelnen Virusspezies zu unterscheiden, essentiell. Obwohl direkte Erregernachweise wie die PCR im frühen Krankheitsstadium das Mittel der Wahl sind, haben sie wegen des engen diagnostischen Zeitfensters verursacht durch die sehr kurze Virämie von nur wenigen Tagen einen deutlichen Nachteil. Untersuchungsmethoden der humoralen Immunantwort dagegen sind für Screeninguntersuchungen besser geeignet, da Antikörper zwar erst nach etwa 2 Wochen auftreten, dafür aber oft für mehrere Jahre nach Infektion im Tier nachweisbar sind (56, 88, 89). Für einige Vertreter der Simbu-Serogruppe existieren serologische Testsysteme wie Mikroneutralisationstest, indirekte Immun-fluoreszenztests und ELISAs (16, 18). Da die Viren der Simbu-Serogruppe aufgrund ihrer antigenetischen Verwandtschaft in einer Serogruppe zusammengefasst wurden (1), stellen Kreuzreaktivitäten eine Hauptschwierigkeit in der serologischen Diagnostik dar (94). Kommerziell erhältliche ELISAs basieren vorwiegend auf dem viralen N-Protein, das unter den Viren der Simbu-Serogruppe hoch konserviert ist (32). Dadurch kann es zu Kreuzreaktionen kommen und verschiedene Virusspezies können mit diesen Tests nicht voneinander unterschieden werden. So können N-Protein basierte kommerzielle SBV ELISAs beispielsweise auch Antikörper gegen Shamonda-Virus, Sathuperi-Virus oder Douglas-Virus detektieren (16, 18). Wie anhand der Seren, die nach der experimentellen SHUV-Infektion von Rindern gewonnen wurden, gezeigt werden konnte, gibt es auch zwischen SBV und SHUV serologische Kreuzreaktionen in N-Protein-basierten Testsystemen. In einem kommerziell erhältlichen SBV N-Protein ELISA, ergab die Testung von sechs Seren SHUV-infizierter Tiere drei positive Testergebnisse. Um vergangene SBV-Infektionen der Rinder auszuschließen, wurden diese vor Beginn des SHUV-Infektionsversuchs mit dem SBV N-Protein ELISA auf das Vorhandensein von AK gegen SBV untersucht. Die Untersuchung fiel in allen Fällen negativ aus. Aus den positiven Ergebnissen nach der SHUV-Infektion lässt sich schlussfolgern, dass eine serologische Differenzierung von SHUV und SBV mit in Europa in der Routinediagnostik häufig eingesetzten SBV N-Protein ELISAs nicht möglich ist. Ein Neueintrag von SHUV in europäische Länder kann aufgrund der ähnlichen teratogenen Symptomatik leicht mit Fällen von SBV verwechselt werden, was zu einer verzögerten Erkennung und Bekämpfung des Ausbruchs führen würde. Neben spezifischen direkten Erregernachweisen (z.B. über PCR-Systeme) sind Tests zur

serologischen Differenzierung simbuviraler Erkrankungen somit von besonderer Bedeutung. Neben einem Neutralisationstest (46) sind für SHUV bislang keine spezifischen serologischen Tests beschrieben. Im Rahmen der experimentellen Rinder-Infektionsstudie wurde ein Mikroneutralisationstest (NT) und ein indirekter Immunfluoreszenztest (IIFT) für SHUV etabliert. Der NT stellt durch den Nachweis neutralisierender Antikörper eine sensible und spezifische serologische Methode dar. Bei Orthobunyavirusinfektionen werden neutra- lisierende Antikörper gegen die hoch immunogene Ektodomäne des Gc Glykoproteins gebildet (28, 95, 107). Vor allem die N-terminale Domäne des Gc Proteins (Gc Head-Domäne) weist eine besonders hohe genetische Variabilität unter den verschiedenen Viren der Serogruppe auf (108, 109), sodass eine serologische Differenzierung einzelner Virusspezies über diese Domäne möglich ist (17). Obwohl Neutralisationstests den Goldstandard darstellen, sind sie mit einigen Nachteilen verbunden. NTs sind in der Durchführung sehr arbeits- und zeitaufwendig und die Arbeiten mit infektiösem Virus erfordern geschultes Personal und Labore entsprechender Biosicherheitsstufen. ELISAs können als alternative Testsysteme dienen, die zeitsparender und unter niedrigeren Sicherheitsstandards durchgeführt werden können. Untersuchungen mit ELISAs, die auf dem hochkonservierten N-Protein basieren, ermöglichten aber keine zuverlässige Differenzierung einzelner Viren der Simbu-Serogruppe (16, 18). Auch ein für SHUV adaptierter *epitop blocking* ELISA, der auf lysierten SHUV- infizierten Zellen basiert, konnte Antikörper gegen nahe verwandte Viren nicht sicher abgrenzen (8). Das M-Segment, das für die Glykoproteine Gn und Gc codiert, ist das Segment mit der höchsten genetischen Variabilität und verursacht wie oben beschrieben im Vergleich zum N-Protein weniger Kreuzreaktivitäten. Für die Entwicklung eines Triplex ELISAs zur Unterscheidung von gegen SHUV, SBV und AKAV gerichteten Antikörpern wurden demzufolge als Antigene die jeweiligen viralen Gc-Proteine gewählt. In vergangen Studien wurde gezeigt, dass die komplette Gc Head-Stalk-Domäne ein stärkeres immunogenes Antigen darstellt als die Head-Domäne alleine (28). Als Antigen für SBV und AKAV wurde dennoch die Gc Head- Domäne alleine gewählt, da ihre immunogenen und antigenen Eigenschaften bereits *in vitro* und *in vivo* demonstriert wurden (86, 107, 110). Für SHUV waren keine Studien verfügbar, darum wurde für SHUV die komplette Head-Stalk-Domäne als Antigen gewählt. Der vorgestellte Triplex ELISA ermöglichte eine sichere Diskriminierung zwischen anti-SBV-, anti- AKAV- und anti-SHUV-Antikörpern, wobei im Gegensatz zu N-basierten Tests nur ein sehr geringes Ausmaß an Kreuzreaktivitäten beobachtet wurde. Die Ergebnisse der ELISAs und der

entsprechenden Untersuchungen im NT zeigten eine gute Korrelation, bis auf Proben mit sehr niedrigen Antikörpertitern. Für Screeninguntersuchungen könnten zukünftig in einem ersten Schritt kommerzielle N-ELISAs verwendet werden, die eine Bandbreite an verschiedenen Viren der Simbu-Serogruppe erkennen und auch geringe Mengen an Antikörpern detektieren. Im Vergleich zu Gc-spezifischen werden N-Protein spezifische Antikörper in größerer Menge und sehr früh nach der Infektion gebildet (89). Um die positiv reagierenden Seren weiter zu differenzieren, können diese im nächsten Schritt in einem Virusspezies-spezifischen Gc-ELISA untersucht werden. Zweifelhafte Ergebnisse sollten mit einem NT bestätigt werden. Die in dieser Arbeit neu beschriebenen serologischen Testmethoden NT, IIFT und Gc-ELISA können für die Detektion von gegen SHUV gerichtete Antikörper bei Ausbruchsgeschehen und in serologischen Screeninguntersuchungen verwendet werden.

6 Schlussfolgerung und Ausblick

Nach Ausbrüchen von schweren neurologischen Erkrankungen bei Pferden und Rindern konnte SHUV als ursächlicher Erreger identifiziert werden. Auch neurologische Erkrankungen bei Menschen könnten im Zusammenhang mit SHUV-Infektionen stehen, was den dringenden Bedarf an geeigneten Infektionsmodellen für die *in vivo* Charakterisierung von SHUV unterstreicht. Die Gefahr einer weiteren sprunghaften Ausbreitung von SHUV in bisher nicht betroffene, naive Populationen betonen die Notwendigkeit von zuverlässigen diagnostischen Tests.

In dieser Arbeit konnte ein Großtier-Infektionsmodell im natürlichen Wirt Rind und ein Kleintiermodell in der IFNAR (-/-) Maus erfolgreich etabliert werden. An diesen beiden Modellen konnte die Neuropathogenität eines aktuellen SHUV-Isolates aus Israel experimentell demonstriert werden. Für ein NSs-defizientes SHUV-Isolat aus einem missgebildeten Fetus konnte die Attenuierung im natürlichen Wirt Rind gezeigt werden. Die in dieser Arbeit entwickelten Infektionsmodelle stellen eine Möglichkeit dar, die Neuropathogenese von SHUV-Infektionen besser zu verstehen. Eine hauptsächlich T-Zell mediierte nichteitrige Meningoenzephalitis wurde bei infizierten Tieren festgestellt. Neuronen und Gliazellen sowie Makrophagen der Milz wurden als Zielzellen der Infektion identifiziert. Die neu etablierten Tiermodelle können für weiterführende Pathogenesestudien verwendet werden.

Die vorgestellten serologischen Tests ermöglichen eine sensitive und spezifische Diagnostik von SHUV-Infektionen und erlauben eine Abgrenzung zu Infektionen anderer nah verwandter Viren der Simbu-Serogruppe, was insbesondere bei der Ausbruchsdiagnostik und in serologischen Screeninguntersuchungen wichtig ist.

7   Zusammenfassung

Shuni-Virus (SHUV), ein Orthobunyavirus der Simbu-Serogruppe, wurde erstmals in den 1960er Jahren in Nigeria aus einem Rind isoliert. SHUV ist in Afrika endemisch und wird durch Gnitzen der Gattung *Culicoides* übertragen. Das breite Wirtsspektrum umfasst Haus- und Wildwiederkäuer, Pferde, verschiedene Wildtiere und den Menschen. Wie bei den meisten Viren der Simbu-Serogruppe sind auch bei SHUV zwei Krankheitsbilder beschrieben: akute Infektionen von Wiederkäuern verlaufen meist asymptomatisch oder sind mit milden Symptomen wie Fieber, Diarrhö oder einem Rückgang der Milchleistung verbunden. Werden aber naive Tiere in einer kritischen Phase der Trächtigkeit infiziert, kann es zu schweren kongenitalen Missbildungen, die unter dem Begriff Arthrogryphose-Hydranencephalie-Syndrom (AHS) zusammengefasst werden, Aborten, Mumifikationen oder Totgeburten kommen. SHUV kann zudem schwere neurologische Erkrankung bei akut infizierten Pferden und Rindern verursachen und auch bei Menschen wurde SHUV mit neurologischer Erkrankung in Verbindung gebracht. 2014 wurde SHUV erstmals außerhalb des afrikanischen Kontinents in Israel in missgebildeten Lämmern nachgewiesen. Zudem wurde SHUV in diesem Ausbruchsgeschehen mit schwerer neurologischer Erkrankung bei adulten Rindern in Verbindung gebracht. Um den kausalen Zusammenhang zwischen SHUV-Infektionen und neurologischer Erkrankung bei Rindern zu bestätigen, wurden experimentelle Infektionsversuche in zwei neu etablierten SHUV-Infektionsmodellen durchgeführt. In Rindern und in IFNAR (-/-) Mäusen konnte die Neuropathogenität eines SHUV-Isolates aus einem neurologisch erkrankten Rind experimentell bestätigt werden. Beide Tierarten waren hochempfänglich für eine SHUV-Infektion. Die Tiere waren virämisch und serokonvertierten. Trotz weitgehend fehlender neurologischer Klinik wurde bei beiden Tierarten histopathologisch eine nichteitrige Meningoenzephalitis diagnostiziert und Neuronen, Gliazellen sowie Makrophagen der Milz wurden als Zielzellen der Infektion identifiziert. Für ein SHUV-Isolat, das aus einem missgebildeten Fetus isoliert wurde und das ein verkürztes, dysfunktionales NSs-Protein aufweist, konnte eine *in vivo* Attenuierung in Rindern demonstriert werden. Die NSs-Deletionsmutante konnte in Rindern keine Virämie auslösen. Das deutet darauf hin, dass genetisch variable Virusvarianten, die in missgebildeten Feten entstehen, „dead-end Artefakte" sind, die durch ihre Attenuierung nicht mehr am natürlichen

Infektionszyklus teilnehmen können. In IFNAR (-/-) Mäusen konnte die NSs-Deletionsmutante einen Krankheitsverlauf inklusive Serokonversion vergleichbar wie bei dem SHUV-Isolat mit intaktem NSs-Protein auslösen. Die Effekte des Virulenzfaktors NSs (Interferon Inhibition) können in IFNAR (-/-) Mäusen aufgrund der fehlenden IFNAR1 Rezeptoren, die bei Vorhandensein von Interferon einen antiviralen Status der Zelle induzieren, nicht untersucht werden. Es bietet aber die Möglichkeit, andere Virulenzfaktoren neben NSs zu studieren. Mit den neu etablierten Tiermodellen Rind und IFNAR (-/-) Maus stehen Modelle für weitere Pathogenesestudien und Vakzinetestungen im natürlichen Wirt und im Kleintiermodell zur Verfügung.

In den vorliegenden Studien wurden zudem neue serologische Untersuchungsmethoden vorgestellt, die eine Unterscheidung von Antikörpern gegen nahe verwandte Viren der Simbu-Serogruppe erlauben. Ein Mikroneutralisationstest ermöglicht dabei den sensitiven und spezifischen Nachweis neutralisierender anti-Shuni-Virus Antikörper. Ein Gc-Protein-basierter Triplex ELISA erlaubt zudem eine schnelle und sichere Diskriminierung zwischen anti-Schmallenberg-Virus, anti-Akabane-Virus und anti-Shuni-Virus Antikörpern. Diese serologischen Untersuchungsmethoden können für die Diagnostik in Ausbruchsgeschehen und die serologische Überwachung genutzt werden.

8 Summary

Shuni virus (SHUV) belongs to the Simbu serogroup within the genus *Orthobunyavirus* and was firstly isolated in the 1960s in Nigeria from cattle. SHUV is endemic in Africa and is transmitted by *Culicoides* biting midges. The broad host spectrum comprises domestic and wild ruminants, horses, several wild animal species and humans. Like most Simbu serogroup viruses, SHUV can cause two different types of clinical manifestation: acute infection of ruminants are either asymptomatic or mild, with clinical signs like fever, diarrhoea or loss in milk yield. When naïve dams are infected during a critical phase of gestation, severe congenital defects referred to as arthrogryphosis-hydranencephalie-syndrom (AHS), abortion, mummification or stillbirth can occur. Besides, acute SHUV infection may induce severe neurological disease in horses and cattle. Moreover, human neurological disease was associated with SHUV. In 2014, SHUV was detected for the first time outside of the African continent in malformed lambs in Israel. During this outbreak, SHUV was associated with severe neurological disease in adult cattle. To confirm a causal relationship between SHUV infection and neurological disease in cattle, experimental infection was performed using two newly established SHUV infection models. In cattle and IFNAR (-/-) mice, neuropathogenicity of a SHUV isolate from a heifer with neurological disease was demonstrated experimentally. Both species were highly susceptible for SHUV infection. The animals were viremic and seroconverted. Even though neurological clinical signs were not observed, histopathology revealed a nonsuppurative meningoencephalitis and neurons, glial cells and macrophages of the spleen were identified as target cells of the infection. For a SHUV isolate from a malformed foetus that expresses a truncated, dysfunctional NSs protein, attenuation *in vivo* was demonstrated in cattle. The NSs deletion mutant did not induce any viremia in cattle. This indicates that genetically variable virus variants evolving in malformed foetuses may represent "dead-end artefacts" that are not fit for the natural transmission cycle. In IFNAR (-/-) mice, the NSs deletion mutant caused a course of disease with seroconversion similar to the SHUV isolate with an intact NSs protein. The impact of virulence factor NSs (interferon inhibition) cannot be studied using the IFNAR (-/-) mouse model, due to the lack of IFNAR1 receptors, which induce an antiviral state of the cell in presence of interferon. However, other virulence factors besides NSs can be studies using the IFNAR (-/-) mouse model.

Summary

The newly established animal models cattle and IFNAR (-/-) mouse can be used for further pathogenesis studies and vaccine testing in the natural host and in a small animal model.

Moreover, the studies in this thesis present newly established serological diagnostic tests that enable a differentiation of antibodies directed against closely related viruses of the Simbu serogroup. A microneutralization test allows a sensitive and specific detection of neutralizing antibodies against SHUV. A Gc protein based triplex ELISA allows a safe discrimination of anti-Schmallenberg virus, anti-Akabane virus and anti-Shuni virus antibodies. These serological tests can be used for diagnostic investigation in disease outbreaks and for serological surveillance studies.

9 Literaturverzeichnis

1. **Kinney RM, Calisher CH.** Antigenic relationships among Simbu serogroup (Bunyaviridae) viruses. The American Journal of Tropical Medicine and Hygiene. 1981;30(6):1307-18.

2. **Maes P, Alkhovsky SV, Bao Y, Beer M, Birkhead M, Briese T, et al.** Taxonomy of the family Arenaviridae and the order Bunyavirales: update 2018. Archives of Virology 2018;163(8):2295-310.

3. **Golender N, Brenner J, Valdman M, Khinich Y, Bumbarov V, Panshin A, et al.** Malformations Caused by Shuni Virus in Ruminants, Israel, 2014-2015. Emerging Infectious Diseases. 2015;21(12):2267-8.

4. **Kirkland PD.** Akabane virus infection. Revue Scientifique et Technique (International Office of Epizootics). 2015;34(2):403-10.

5. **Wernike K, Elbers A, Beer M.** Schmallenberg virus infection. Revue Scientifique et Technique (International Office of Epizootics). 2015;34(2):363-73.

6. **Causey OR, Kemp GE, Causey CE, Lee VH.** Isolations of Simbu-group viruses in Ibadan, Nigeria 1964-69, including the new types Sango, Shamonda, Sabo and Shuni. Annals of Tropical Medicine and Parasitology. 1972;66(3):357-62.

7. **McIntosh BM, Jupp PG, De Sousa J.** Further isolations of the arboviruses from mosquitoes collected in Tongaland, South Africa, 1960-1968. Journal of Medical Entomology. 1972;9:155-9.

8. **Steyn J, Motlou P, van Eeden C, Pretorius M, Stivaktas VI, Williams J, et al.** Shuni Virus in Wildlife and Nonequine Domestic Animals, South Africa. Emerging Infectious Diseases. 2020;26(7):1521-5.

9. **van Eeden C, Williams JH, Gerdes TG, van Wilpe E, Viljoen A, Swanepoel R, et al.** Shuni virus as cause of neurologic disease in horses. Emerging Infectious Diseases. 2012;18(2):318-21.

10. **Motlou T, Venter M.** Shuni Virus in Cases of Neurologic Disease in Humans, South Africa. Emerging Infectious Disease Journal. 2021;27(2):565.

11. **Mohlmann TWR, Oymans J, Wichgers Schreur PJ, Koenraadt CJM, Kortekaas J, Vogels CBF.** Vector competence of biting midges and mosquitoes for Shuni virus. PLoS Neglected Tropical Diseases. 2018;12(12):e0006993.

12. **Mellor PS, Boorman J, Baylis M.** Culicoides biting midges: their role as arbovirus vectors. Annual Revue of Entomology. 2000;45:307-40.

13. **Althouse BM, Hanley KA.** The tortoise or the hare? Impacts of within-host dynamics on transmission success of arthropod-borne viruses. Philosophical transactions of the Royal Society of London Series B, Biological Sciences. 2015;370(1675):20140299.

14. **Golender N, Bumbarov V, Assis I, Beer M, Khinich Y, Koren O, et al.** Shuni virus in Israel: Neurological disease and fatalities in cattle. Transboundary and Emerging Diseases. 2019.

15. **Golender N, Bumbarov VY, Erster O, Beer M, Khinich Y, Wernike K.** Development and validation of a universal S-segment-based real-time RT-PCR assay for the detection of Simbu serogroup viruses. Journal of Virolocical Methods. 2018;261:80-5.

16. **Mathew C, Klevar S, Elbers AR, van der Poel WH, Kirkland PD, Godfroid J, et al.** Detection of serum neutralizing antibodies to Simbu sero-group viruses in cattle in Tanzania. BMC Veterinary Research. 2015;11:208.

17. **Wernike K, Beer M, Hoffmann B.** Schmallenberg Virus Infection Diagnosis: Results of a German Proficiency Trial. Transboundary and Emerging Diseases. 2017;64(5):1405-10.

18. **Oluwayelu D, Wernike K, Adebiyi A, Cadmus S, Beer M.** Neutralizing antibodies against Simbu serogroup viruses in cattle and sheep, Nigeria, 2012-2014. BMC Veterinary Research. 2018;14(1):277.

19. **Sick F, Beer M, Kampen H, Wernike K.** Culicoides Biting Midges-Underestimated Vectors for Arboviruses of Public Health and Veterinary Importance. Viruses. 2019;11(4).

20. **Sick F, Breithaupt A, Golender N, Bumbarov V, Beer M, Wernike K.** Shuni virus-induced meningoencephalitis after experimental infection of cattle. Transboundary and Emerging Diseases. 2021;68(3):1531-40.

21. **Wernike K, Aebischer A, Sick F, Szillat KP, Beer M.** Differentiation of Antibodies against Selected Simbu Serogroup Viruses by a Glycoprotein Gc-Based Triplex ELISA. Veterinary Sciences. 2021;8(1).

22. **Hughes HR, Adkins S, Alkhovskiy S, Beer M, Blair C, Calisher CH, et al.** ICTV Virus Taxonomy Profile: Peribunyaviridae. 2020;101(1):1-2.

23. **Saeed MF, Li L, Wang H, Weaver SC, Barrett AD.** Phylogeny of the Simbu serogroup of the genus Bunyavirus. The Journal of General Virology. 2001;82.

24. **Hoffmann B, Scheuch M, Höper D, Jungblut R, Holsteg M, Schirrmeier H, et al.** Novel orthobunyavirus in Cattle, Europe, 2011. Emerging Infectious Diseases. 2012;18(3):469-72.

25. **Travassos da Rosa JF, de Souza WM, Pinheiro FP, Figueiredo ML, Cardoso JF, Acrani GO, et al.** Oropouche Virus: Clinical, Epidemiological, and Molecular Aspects of a Neglected Orthobunyavirus. The American Journal of Tropical Medicine and Hygiene. 2017;96(5):1019-30.

26. **Wernike K, Conraths F, Zanella G, Granzow H, Gache K, Schirrmeier H, et al.** Schmallenberg virus-two years of experiences. Prev Vet Med. 2014;116(4):423-34.

27. **Elliott RM, Blakqori G.** Molecular biology of orthobunyaviruses. In: Plyusnin A, Elliott RM, editors. Bunyaviridae: Molecular and Cellular Biology. Norfolk, UK: Caister Academic Press; 2011.

28. **Hellert J, Aebischer A, Wernike K, Haouz A, Brocchi E, Reiche S, et al.** Orthobunyavirus spike architecture and recognition by neutralizing antibodies. Nature Communications. 2019;10(1):879.

29. **Raju R, Kolakofsky D.** The ends of La Crosse virus genome and antigenome RNAs within nucleocapsids are base paired. Journal of Virology. 1989;63(1):122-8.

30. **Elliott RM.** Orthobunyaviruses: recent genetic and structural insights. Nature reviews. Microbiology. 2014;12(10):673-85.

31. **Fazakerley JK, Ross AM.** Computer analysis suggests a role for signal sequences in processing polyproteins of enveloped RNA viruses and as a mechanism of viral fusion. Virus Genes. 1989;2(3):223-39.

32. **Barr JN, Walter CT.** Recent advances in the molecular and cellular biology of bunyaviruses. Journal of General Virology. 2011;92:2467-84.

33. **Weber M, Weber F.** RIG-I-like receptors and negative-strand RNA viruses: RLRly bird catches some worms. Cytokine & Growth Factor Reviews. 2014;25(5):621-8.

34. **Varela M, Schnettler E, Caporale M, Murgia C, Barry G, McFarlane M, et al.** Schmallenberg virus pathogenesis, tropism and interaction with the innate immune system of the host. PLoS Pathogens. 2013;9(1):e1003133-e.

35. **Barry G, Varela M, Ratinier M, Blomström A-L, Caporale M, Seehusen F, et al.** NSs protein of Schmallenberg virus counteracts the antiviral response of the cell by inhibiting its transcriptional machinery. The Journal of General Virology. 2014;95(Pt 8):1640-6.

36. **Oymans J, Wichgers Schreur PJ, van Oort S, Vloet R, Venter M, Pijlman GP, et al.** Reverse Genetics System for Shuni Virus, an Emerging Orthobunyavirus with Zoonotic Potential. Viruses. 2020;12(4):455.

37. **Blomstrom AL, Gu Q, Barry G, Wilkie G, Skelton JK, Baird M, et al.** Transcriptome analysis reveals the host response to Schmallenberg virus in bovine cells and antagonistic effects of the NSs protein. BMC Genomics. 2015;16:324.

38. **Kraatz F, Wernike K, Hechinger S, Konig P, Granzow H, Reimann I, et al.** Deletion mutants of Schmallenberg virus are avirulent and protect from virus challenge. Journal of Virology. 2015;89(3):1825-37.

39. **Shi X, Goli J, Clark G, Brauburger K, Elliott RM.** Functional analysis of the Bunyamwera orthobunyavirus Gc glycoprotein. The Journal of General Virology. 2009;90(Pt 10):2483-92.

40. **Shi X, Lappin DF, Elliott RM.** Mapping the Golgi targeting and retention signal of Bunyamwera virus glycoproteins. Journal of Virology. 2004;78(19):10793-802.

41. **Shi X, van Mierlo JT, French A, Elliott RM.** Visualizing the replication cycle of bunyamwera orthobunyavirus expressing fluorescent protein-tagged Gc glycoprotein. Journal of Virology. 2010;84(17):8460-9.

42. **Shi X, Kohl A, Léonard VHJ, Li P, McLees A, Elliott RM.** Requirement of the N-Terminal Region of Orthobunyavirus Nonstructural Protein NSm for Virus Assembly and Morphogenesis. Journal of Virology. 2006;80(16):8089.

43. **Golender N, Wernike K, Bumbarov V, Aebischer A, Panshin A, Jenckel M, et al.** Characterization of Shuni viruses detected in Israel. Virus Genes. 2016;52(6):806-13.

44. **Motlou TP, Williams J, Venter M.** Epidemiology of Shuni Virus in Horses in South Africa. Viruses. 2021;13(5).

45. **Moore DL, Causey OR, Carey DE, Reddy S, Cooke AR, Akinkugbe FM, et al.** Arthropod-borne viral infections of man in Nigeria, 1964-1970. Annals of Tropical Medicine and Parasitology. 1975;69(1):49-64.

46. **van Eeden C, Swanepoel R, Venter M.** Antibodies against West Nile and Shuni Viruses in Veterinarians, South Africa. Emerging Infectious Diseases. 2014;20(8):1409-11.

47. **Oymans J, van Keulen L, Vermeulen GM, Wichgers Schreur PJ, Kortekaas J.** Shuni Virus Replicates at the Maternal-Fetal Interface of the Ovine and Human Placenta. Pathogens (Basel, Switzerland). 2020;10(1).

48. **Anderson CR, Spence L, Downs WG, Aitken TH.** Oropouche virus: a new human disease agent from Trinidad, West Indies. The American Journal of Tropical Medicine and Hygiene. 1961;10:574-8.

49. **Ladner JT, Savji N, Lofts L, Travassos da Rosa A, Wiley MR, Gestole MC, et al.** Genomic and phylogenetic characterization of viruses included in the Manzanilla and Oropouche species complexes of the genus Orthobunyavirus, family Bunyaviridae. The Journal of General Virology. 2014;95(Pt 5):1055-66.

50. **Aguilar PV, Barrett AD, Saeed MF, Watts DM, Russell K, Guevara C, et al.** Iquitos virus: a novel reassortant Orthobunyavirus associated with human illness in Peru. PLoS Neglected Tropical Diseases. 2011;5(9):e1315.

51. **Romero-Alvarez D, Escobar LE.** Oropouche fever, an emergent disease from the Americas. Microbes and Infection. 2018;20(3):135-46.

52. **Yadav P, Shete A, Bondre V, Patil D, Kokate P, Chaudhari S, et al.** Isolation and characterization of Oya virus a member of Simbu serogroup, family Bunyaviridae, isolated from Karnataka, India. Infection, Genetics and Evolution. 2016;44:122-6.

53. **Kline DL, Axtell, R. C.** Salt marsh Culicoides (Diptera: Ceratopogonidae): Species, seasonal abundance and comparison of trapping methods. Mosquito News, 36(1), 1-10. 1976.

54. **Watts DM, Thompson WH, Yuill TM, DeFoliart GR, Hanson RP.** Overwintering of La Crosse virus in Aedes triseriatus. The American Journal of Tropical Medicine and Hygiene. 1974;23(4):694-700.

55. **Wernike K, Beer M.** Schmallenberg Virus: A Novel Virus of Veterinary Importance. Advances in Virus Research. 2017;99:39-60.

56. **Wernike K, Holsteg M, Szillat KP, Beer M.** Development of within-herd immunity and long-term persistence of antibodies against Schmallenberg virus in naturally infected cattle. BMC Veterinary Research. 2018;14(1):368.

57. **Kato T, Yanase T, Suzuki M, Katagiri Y, Ikemiyagi K, Takayoshi K, et al.** Monitoring for bovine arboviruses in the most southwestern islands in Japan between 1994 and 2014. BMC Veterinary Research. 2016;12(1):125.

58. **Hayama Y, Yanase T, Suzuki M, Unten K, Tomochi H, Kakehi M, et al.** Meteorological factors affecting seroconversion of Akabane disease in sentinel calves in the subtropical Okinawa Islands of Japan. Tropical Animal Health and Production. 2018;50(1):209-15.

59. **Beer M, Conraths FJ, van der Poel WH.** 'Schmallenberg virus' - a novel orthobunyavirus emerging in Europe. Epidemiology & Infection. 2013;141.

60. **Wernike K, Beer M.** Re-circulation of Schmallenberg virus, Germany, 2019. Transboundary and Emerging Diseases. 2020;67(6):2290-5.

61. **Briese T, Calisher CH, Higgs S.** Viruses of the family Bunyaviridae: Are all available isolates reassortants? Virology. 2013;446(1):207-16.

62. **Gerrard SR, Li L, Barrett AD, Nichol ST.** Ngari virus is a Bunyamwera virus reassortant that can be associated with large outbreaks of hemorrhagic fever in Africa. Journal of Virology. 2004;78(16):8922-6.

63. **Wernike K, Eschbaumer M, Breithaupt A, Hoffmann B, Beer M.** Schmallenberg virus challenge models in cattle: infectious serum or culture-grown virus? Veterinary Research. 2012;43(1):84.

64. **Martinelle L, Poskin A, Dal Pozzo F, De Regge N, Cay B, Saegerman C.** Experimental Infection of Sheep at 45 and 60 Days of Gestation with Schmallenberg Virus Readily Led to Placental Colonization without Causing Congenital Malformations. PloS one. 2015;10(9):e0139375.

65. **Laloy E, Riou M, Barc C, Belbis G, Bréard E, Breton S, et al.** Schmallenberg virus: experimental infection in goats and bucks. BMC Veterinary Research. 2015;11:221.

66. **Lee H, Jeong H, Park S, Yang MS, Kim J, Bae J, et al.** Experimental infection of cows with newly isolated Akabane virus strain (AKAV-7) causing encephalomyelitis. Veterinary Research. 2016;47(1):62.

67. **Jeong H, Oem JK, Yang MS, Yang D, Kim MS, Lee KH, et al.** Experimental Infection of Goats with a Newly Isolated Strain of Akabane Virus that Causes Encephalomyelitis. Journal of Comparative Pathology. 2017;157(2-3):220-9.

68. **Aebischer A, Wernike K, König P, Franzke K, Wichgers Schreur PJ, Kortekaas J, et al.** Development of a Modular Vaccine Platform for Multimeric Antigen Display Using an Orthobunyavirus Model. Vaccines. 2021;9(6).

69. **Kim YH, Kweon CH, Tark DS, Lim SI, Yang DK, Hyun BH, et al.** Development of inactivated trivalent vaccine for the teratogenic Aino, Akabane and Chuzan viruses. Biologicals. 2011;39(3):152-7.

70. **Wernike K, Nikolin VM, Hechinger S, Hoffmann B, Beer M.** Inactivated Schmallenberg virus prototype vaccines. Vaccine. 2013;31(35):3558-63.

71. **Collins Á B, Mee JF, Kirkland PD.** Pathogenicity and teratogenicity of Schmallenberg virus and Akabane virus in experimentally infected chicken embryos. Veterinary Microbiology. 2018;216:31-7.

72. **Ikeda S, Yonaiyama K.** Deformities of chick embryos in experimental Akabane virus infection. National Institute of Animal Health quarterly. 1978;18(3-4):89-96.

73. **Konno S, Koeda T, Madarame H, Ikeda S, Sasaki T, Satoh H, et al.** Myopathy and encephalopathy in chick embryos experimentally infected with Akabane virus. Veterinary Pathology. 1988;25(1):1-8.

74. **Nakajima Y, Takahashi E, Konno S.** Encephalomyelitis in mice experimentally infected with Akabane virus. National Institute of Animal Health quarterly. 1979;19(1-2):47-52.

75. **Murata Y, Uchida K, Shioda C, Uema A, Bangphoomi N, Chambers JK, et al.** Histopathological Studies on the Neuropathogenicity of the Iriki and OBE-1 Strains of Akabane Virus in BALB/cAJcl Mice. Journal of Comparative Pathology. 2015;153(2-3):140-9.

76. **Santos RI, Almeida MF, Paula FE, Rodrigues AH, Saranzo AM, Paula AE, et al.** Experimental infection of suckling mice by subcutaneous inoculation with Oropouche virus. Virus Research. 2012;170(1-2):25-33.

77. **Rodrigues AH, Santos RI, Arisi GM, Bernardes ES, Silva ML, Rossi MA, et al.** Oropouche virus experimental infection in the golden hamster (Mesocrisetus auratus). Virus Res. 2011;155(1):35-41.

78. **Santos RI, Bueno-Júnior LS, Ruggiero RN, Almeida MF, Silva ML, Paula FE, et al.** Spread of Oropouche virus into the central nervous system in mouse. Viruses. 2014;6(10):3827-36.

79. **Aksoy E, Azkur AK.** Schmallenberg virus affects T-bet, Gata3, RoRrγt, Foxp3 and Eomes in mice brain. Acta Virologica. 2019;63(3):286-91.

80. **Marín-Lopez A, Calvo-Pinilla E, Moreno S, Utrilla-Trigo S, Nogales A, Brun A, et al.** Modeling Arboviral Infection in Mice Lacking the Interferon Alpha/Beta Receptor. Viruses. 2019;11(1):35.

81. **Müller U, Steinhoff U, Reis LF, Hemmi S, Pavlovic J, Zinkernagel RM, et al.** Functional role of type I and type II interferons in antiviral defense. Science. 1994;264(5167):1918-21.

82. **Wernike K, Breithaupt A, Keller M, Hoffmann B, Beer M, Eschbaumer M.** Schmallenberg virus infection of adult type I interferon receptor knock-out mice. PloS One. 2012;7(7):e40380.

83. **Tauscher K, Wernike K, Fischer M, Wegelt A, Hoffmann B, Teifke JP, et al.** Characterization of Simbu serogroup virus infections in type I interferon receptor knock-out mice. Archives of Virology. 2017;162(10):3119-29.

84. **Boshra H, Lorenzo G, Charro D, Moreno S, Guerra GS, Sanchez I, et al.** A novel Schmallenberg virus subunit vaccine candidate protects IFNAR(-/-) mice against virulent SBV challenge. Scientific Reports. 2020;10(1):18725.

85. **Boshra HY, Charro D, Lorenzo G, Sánchez I, Lazaro B, Brun A, et al.** DNA vaccination regimes against Schmallenberg virus infection in IFNAR(-/-) mice suggest two targets for immunization. Antiviral Research. 2017;141:107-15.

86. **Wernike K, Aebischer A, Roman-Sosa G, Beer M.** The N-terminal domain of Schmallenberg virus envelope protein Gc is highly immunogenic and can provide protection from infection. Scientific Reports. 2017;7:42500.

87. **Bryant JL.** Animal Models in Virology. In: Conn PM, editor. Sourcebook of Models for Biomedical Research. Totowa, NJ: Humana Press; 2008. p. 557-63.

88. **Elbers AR, Stockhofe-Zurwieden N, van der Poel WH.** Schmallenberg virus antibody persistence in adult cattle after natural infection and decay of maternal antibodies in calves. BMC Veterinary Research. 2014;10:103.

89. **Wernike K, Eschbaumer M, Schirrmeier H, Blohm U, Breithaupt A, Hoffmann B, et al.** Oral exposure, reinfection and cellular immunity to Schmallenberg virus in cattle. Veterinary Microbiology. 2013;165(1-2):155-9.

90. **Kurogi H, Inaba Y, Takahashi E, Sato K, Goto Y, Satoda K, et al.** Development of inactivated vaccine for Akabane disease. National Institute of Animal Health quarterly. 1978;18(3-4):97-108.

91. **Endalew AD, Morozov I, Davis AS, Gaudreault NN, Wernike K, Bawa B, et al.** Virological and Serological Responses of Sheep and Cattle to Experimental Schmallenberg Virus Infection. Vector-Borne and Zoonotic Diseases (Larchmont, NY). 2018;18(12):697-703.

92. **Kurogi H, Inaba Y, Takahashi E, Sato K, Goto Y.** Experimental infection of pregnant goats with Akabane virus. National Institute of Animal Health quarterly. 1977;17(1):1-9.

93. **Wernike K, Holsteg M, Schirrmeier H, Hoffmann B, Beer M.** Natural infection of pregnant cows with Schmallenberg virus--a follow-up study. PloS One. 2014;9(5):e98223.

94. **Goller KV, Höper D, Schirrmeier H, Mettenleiter TC, Beer M.** Schmallenberg virus as possible ancestor of Shamonda virus. Emerging Infectious Diseases. 2012;18(10):1644-6.

95. **Wernike K, Brocchi E, Cordioli P, Senechal Y, Schelp C, Wegelt A, et al.** A novel panel of monoclonal antibodies against Schmallenberg virus nucleoprotein and glycoprotein Gc allows specific orthobunyavirus detection and reveals antigenic differences. Veterinary Research. 2015;46:27.

96. **Van Eeden C, Zaayman D, Venter M.** A sensitive nested real-time RT-PCR for the detection of Shuni virus. Journal of Virological Methods. 2014;195:100-5.

97. **Venter M.** Assessing the zoonotic potential of arboviruses of African origin. Current Opinion in Virology. 2018;28:74-84.

98. **Wernike K, Silaghi C, Nieder M, Pfeffer M, Beer M.** Dynamics of Schmallenberg virus infection within a cattle herd in Germany, 2011. Epidemiology & Infection. 2014;142(7):1501-4.

99. **Parsonson IM, Della-Porta AJ, Snowdon WA, O'Halloran ML.** Experimental infection of bulls with Akabane virus. Research in Veterinary Science. 1981;31(2):157-60.

100. **Miyazato S, Miura Y, Hase M, Kubo M, Goto Y, Kono Y.** Encephalitis of cattle caused by Iriki isolate, a new strain belonging to Akabane virus. Nihon juigaku zasshi The Japanese Journal of Veterinary Science. 1989;51(1):128-36.

101. **Yanase T, Kato T, Hayama Y, Akiyama M, Itoh N, Horiuchi S, et al.** Transition of Akabane virus genogroups and its association with changes in the nature of disease in Japan. Transboundary and Emerging Diseases. 2018;65(2):e434-e43.

102. **Coupeau D, Claine F, Wiggers L, Kirschvink N, Muylkens B.** S segment variability during the two first years of the spread of Schmallenberg virus. Archives of Virology. 2016;161(5):1353-8.

103. **Wernike K, Hoffmann B, Bréard E, Bøtner A, Ponsart C, Zientara S, et al.** Schmallenberg virus experimental infection of sheep. Veterinary Microbiology. 2013;166(3-4):461-6.

104. **Shimshony S.** An epizootic of Akabane disease in bovines, ovines and caprines in Israel, 1969-70: epidemiological assessment. Acta morphologica Academiae Scientiarum Hungaricae. 1980;28(1-2):197-9.

105. **Brenner J, Tsuda T, Yadin H, Chai D, Stram Y, Kato T.** Serological and clinical evidence of a teratogenic Simbu serogroup virus infection of cattle in Israel, 2001-2003. Veterinaria Italiana. 2004;40:119-23.

106. **Behar A, Leibovich BB, Edery N, Yanase T, Brenner J.** First genomic detection of Peaton virus in a calf with hydranencephaly in Israel. Veterinary Medicine and Science. 2019;5(1):87-92.

107. **Roman-Sosa G, Brocchi E, Schirrmeier H, Wernike K, Schelp C, Beer M.** Analysis of the humoral immune response against the envelope glycoprotein Gc of Schmallenberg virus reveals a domain located at the amino terminus targeted by mAbs with neutralizing activity. The Journal of General Virology. 2016;97(3):571-80.

108. **Wernike K, Reimann I, Banyard AC, Kraatz F, La Rocca SA, Hoffmann B, et al.** High genetic variability of Schmallenberg virus M-segment leads to efficient immune escape from neutralizing antibodies. PLoS Pathogens. 2021;17(1):e1009247.

109. **Fischer M, Hoffmann B, Goller K, Höper D, Wernike K, Beer M.** A mutation 'Hot Spot' in the Schmallenberg virus M segment. The Journal of General Virology. 2013;94.

110. **Roman-Sosa G, Karger A, Kraatz F, Aebischer A, Wernike K, Maksimov P, et al.** The amino terminal subdomain of glycoprotein Gc of Schmallenberg virus: disulfide bonding and structural determinants of neutralization. The Journal of General Virology. 2017;98(6):1259-73.

10 Abkürzungsverzeichnis

AHS   Arthrogryphose-Hydranencephalie-Syndrom

AKAV   Akabane-Virus

Arboviren   Arthropod-borne Viren

AS   Aminosäure

ELISA   enzyme-linked Immunosorbent Assay

ICTV   international commitee on taxonomy of viruses

IFN   Interferon

IFNAR 1   Interferon α/β Rezeptor

IFNAR (-/-)   Interferon α/β Rezeptor knockout

IIFT   indirekter Immunfluoreszenztest

ISH   in-situ-Hybridisierung

MALT   Schleimhaut-assoziiertes lymphatisches Gewebe

nt   Nukleotid

NT   Mikroneutralisationstest

NTR   nicht-translatierte Region

ORF   offener Leserahmen

OROV   Oropouche-Virus

PCR   Polymerase-Kettenreaktion

RdRp   RNA-abhängige RNA-Polymerase

RIG-I   retinoic acid-inducible gene I

RNAP II   DNA-abhängige RNA-Polymerase II

SBV   Schmallenberg-Virus

SHUV   Shuni-Virus

RT-PCR   Reverse-Transkriptase-Polymerase-Kettenreaktion

ZNS   zentrales Nervensystem

11 Danksagung

An dieser Stelle möchte ich mich bei Prof. Dr. Gerd Sutter und den Gutachtern für die Beurteilung dieser Arbeit bedanken.

Vielen Dank an meinen Mentor Prof. Dr. Martin Beer für die Bereitstellung des Themas und die Möglichkeit, meine Promotion am Institut für Virusdiagnostik zu machen. Danke für die Ratschläge und hilfreichen Besprechungen!

Mein herzlicher Dank gilt Dr. habil. Kerstin Wernike. Vielen Dank für die tolle Betreuung während allen Phasen meiner Promotion, für die Korrekturen meiner Manuskripte, die Ratschläge in allen Fragen und für die Unterstützung bei der Fertigstellung dieser Arbeit.

Allen Mitarbeitern des Labors Reimann und Wernike, vor allem Gabriela Adam und Doreen Schulz, danke ich für die tolle Einarbeitung in sämtliche Labormethoden und die schöne Zusammenarbeit. Ihr seid mir immer mit Rat und Tat zur Seite gestanden.

Ein besonderer Dank gilt auch Dr. Ilona Reimann, die mich vor allem während meiner Anfangszeit unterstützt hat.

Vielen Dank meiner Mitbewohnerin Julia für die schöne Zeit in Greifswald!

Größter Dank gilt meiner Familie und meinem Freund Tobias, auf die ich mich immer verlassen kann und die mich auf meinem Weg immer unterstützt haben.

Und nicht zu vergessen: vielen Dank an alle Tiere, ohne die diese Projekte nicht möglich gewesen wären!