Virus Genes (2011) 43:177–191
DOI 10.1007/s11262-011-0620-x

Phylogenetic analysis of *Puumala virus* subtype Bavaria, characterization and diagnostic use of its recombinant nucleocapsid protein

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Received: 16 January 2011 / Accepted: 5 May 2011 / Published online: 20 May 2011

Abstract *Puumala virus* (PUUV) is the predominant hantavirus species in Germany causing large numbers of mild to moderate cases of haemorrhagic fever with renal syndrome (HFRS). During an outbreak in South-East Germany in 2004 a novel PUUV subtype designated Bavaria was identified as the causative agent of HFRS in humans [1]. Here we present a molecular characterization of this PUUV strain by investigating novel partial and almost entire nucleocapsid (N) protein-encoding small (S-) segment sequences and partial medium (M-) segment sequences from bank voles (*Myodes glareolus*) trapped in Lower Bavaria during 2004 and 2005. Phylogenetic analyses confirmed their classification as subtype Bavaria, which is further subdivided into four geographical clusters. The entire N protein, harbouring an amino-terminal hexahistidine tag, of the Bavarian strain was produced in yeast *Saccharomyces cerevisiae* and showed a slightly different reactivity with N-specific monoclonal antibodies, compared to the yeast-expressed N protein of the PUUV strain Vranica/Hällnäs. Endpoint titration of human sera from different parts of Germany and from Finland revealed only very slight differences in the diagnostic value of the different recombinant proteins. Based on the novel N antigen indirect and monoclonal antibody capture IgG-ELISAs were established. By using serum panels from Germany and Finland their validation demonstrated a high sensitivity and specificity. In summary, our investigations demonstrated the Bavarian PUUV strain to be genetically

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Electronic supplementary material The online version of this article (doi:10.1007/s11262-011-0620-x) contains supplementary material, which is available to authorized users.

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divergent from other PUUV strains and the potential of its N protein for diagnostic applications.

Keywords Hantavirus · *Puumala virus* · Phylogeny · Recombinant nucleocapsid protein · ELISA · Diagnostics

Introduction

Hantaviruses (family *Bunyaviridae*) are enveloped viruses with a segmented RNA genome of negative polarity [2]. The spherical particles of about 80–120 nm in diameter contain the three genome segments, small (S), medium (M) and large (L), which are complexed with the viral nucleocapsid (N) protein and associated at their ends with the RNA-dependent RNA polymerase [3]. The S-segment (1.7–2.1 kilo bases, kb) encodes the N protein. In addition, for *Puumala virus* (PUUV) and related hantaviruses a second open reading frame (ORF) was observed encoding a putative non-structural protein that was suggested to play a role in pathogenicity in humans or alternatively in the adaptation of the virus to the rodent host [4–6]. The M-segment (3.6–3.7 kb) encodes for a glycoprotein precursor that is co-translationally cleaved into the glycoproteins (Gn) G1 and Gc (G2). The L-segment (6.5–6.6 kb) encodes the RNA-dependent RNA polymerase [3].

Hantaviruses are carried and transmitted to humans by persistently infected rodents which shed the virus by urine, faeces and saliva. The major route of transmission to humans is by inhalation of virus-contaminated aerosols. Further, rodent biting is a rare transmission route to humans [7]. Human-to-human transmission has been described exclusively for the South-American *Andes virus* (ANDV; [8, 9]). In addition to rodent-associated hantaviruses there is an increasing number of newly detected hantaviruses in different insectivores [10–15]). However, it is unknown if these viruses can be transmitted to humans or cause disease.

In Europe, the bank vole (*Myodes glareolus*) associated PUUV is the geographically most widely distributed human pathogenic hantavirus. In addition, PUUV-like viruses were detected in *M. rufocanus* and *M. regulus* in Asia [16–18]. PUUV infections in humans may induce nephropathia epidemica (NE) which is characterized as a mild to moderate form of haemorrhagic fever with renal syndrome (HFRS) with a case fatality rate of less than 1% [19]. Reflecting the distribution of the reservoir host in almost all parts of Europe, human PUUV infections have been observed in different countries of northern, eastern, central, western and southern Europe [20–23]. In Germany, most NE cases are due to autochthonous PUUV infections. This is reflected in results of large-scale seroepidemiological studies as well as in the number of NE cases recorded since the introduction of the German Federal Protection against Infection Act in 2001 [24, 25]. The increased number of cases observed in 2005 (447 cases), in 2007 (1,688 cases) and in 2010 (2,016 cases) were caused by PUUV outbreaks mainly affecting Baden-Wuerttemberg, Bavaria, North Rhine Westphalia and Lower Saxony (Robert Koch-Institut: SurvStat, http://www3.rki.de/SurvStat, 16th March 2011; [26–28]). During an outbreak of human PUUV infections in Lower Bavaria in 2004, studies on rodents as well as on patients revealed a novel PUUV subtype designated Bavaria (Bayerischer Wald, Bawa, PUUV-Bawa; [1, 29]).

Human hantavirus infections are mainly detected by serological assays. Indirect immunofluorescence assays based on hantavirus-infected Vero cells, ELISA, immunoblot and immunochromatographical rapid tests have been developed. Mostly N protein derivatives of different hantavirus species generated by heterologous expression in *Escherichia coli*, yeast *Saccharomyces cerevisiae* and baculovirus-mediated insect cell systems are used as diagnostic antigens [30–32]. Although the N proteins of hantaviruses, in particular of closely related species, are cross-reactive, the use of homologous antigens of local virus strains was recommended for a sensitive detection of specific antibodies, especially during the acute phase [33–38].

Here we report on the phylogenetic characterization of the almost entire N protein-encoding sequence and non-coding regions (NCR) of the S-segment and partial M-segment sequences of PUUV-Bawa. The N protein of PUUV-Bawa was synthesized in yeast, immunologically characterized and tested for its diagnostic application in indirect and capture immunoglobulin G (IgG)-ELISA formats.

Materials and methods

Rodent trapping and necropsy

Rodents were collected in May 2005 at five locations in the administrative districts of Straubing-Bogen (military training grounds Bogen), Regen (military training grounds Regen; Hangenleiten—county Kirchberg) and Freyung-Grafenau (military training grounds Freyung; Raimundsreuth and Bierhütte—county Hohenau) in South-East Germany (Fig. 1; Table 1). Trapping, necropsy and analysis of rodents during the outbreak there in 2004 has been published before [1]. Blood and lung samples were taken from 19 animals (11 *M. glareolus;* four yellow-necked field mice *Apodemus flavicollis*; two long-tailed field mice *A. sylvaticus*; one common vole *Microtus arvalis*; one water vole *Arvicola amphibius*; see Table 1).

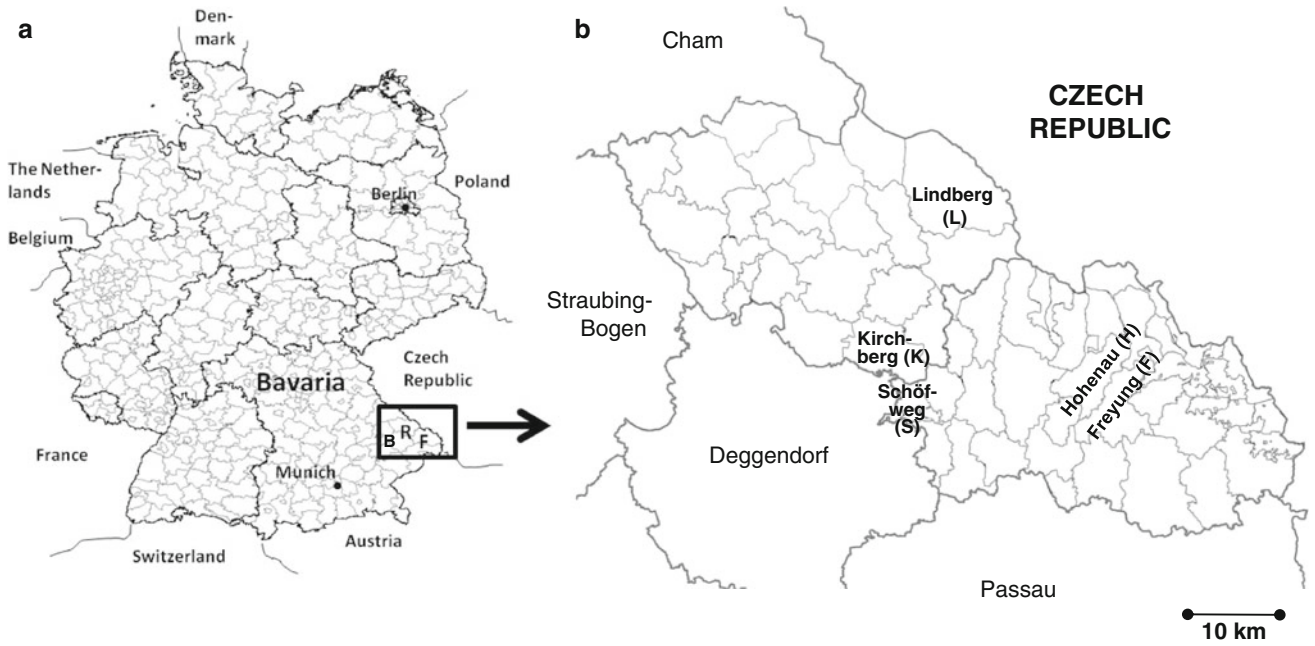

Fig. 1 **a** Geographical map of Germany with the study area in the Federal State of Bavaria in the administrative districts Straubing-Bogen (B), Regen (R) and Freyung-Grafenau (F). **b** Administrative districts of Regen and Freyung-Grafenau with counties where PUUV-reactive rodents have been found are designated (2004: Lindberg (L), Kirchberg (K), Schöfweg (S), Hohenau (H), Freyung (F); 2005: Hohenau (H), Freyung (F), see also Table 1)

Table 1 Summary of results of rodent trapping in Lower Bavaria in 2004 and 2005 and of PUUV-RT-PCR screening of bank voles

Administrative districts (Fig. 1a)—trapping site (sites with abbreviation marked in Fig. 1b)	Total number and number of PUUV-RT-PCR-positive bank voles trapped in				Number of other rodent species[b] trapped in	
	2004[a]		2005		2004[a]	2005
Regen						
Kirchberg (Hangenleithen, K)	5	2 (K-9/04, K-20/04)	1	0	0	4
Lindberg (Falkenstein, L)	6	2 (L-39/04, L-41/04)	n.t.	n.t.	3	n.t.
Regen (barrack)	n.t.	n.t.	0	0	n.t.	2
Straubing-Bogen						
Bogen	n.t.	n.t.	1	0	n.t.	0
Freyung-Grafenau						
Hohenau (Raimundsreuth, H)	13	4 (H-2/04, H-32/04, H-33/04, H-34/04)	2	2 (H-152/05, H-153/05)	5[c]	0
Hohenau (Glashütte)	1	0	n.t.	n.t.	1	n.t.
Hohenau (Bierhütte)	n.t.	n.t.	2	0	n.t.	1
Schöfweg (Langenfurth, S)	2	2 (S-17/04, S-18/04)	n.t.	n.t.	3	n.t.
Schöfweg (Mutzenwinkel)	2	0	n.t.	n.t.	1	n.t.
Schöfweg	0	0	n.t.	n.t.	1	n.t.
Freyung (military training site, F)	n.t.	n.t.	5	3 (F-151/05, F-157/05, F-159/05)	n.t.	1
Total	29	10	11	5	14	8

n.t. no trapping

[a] For details see [1]

[b] Other species are common voles, yellow-necked field mice, long-tailed field mice, water vole

[c] One yellow-necked mouse was found to be serologically PUUV-reactive

Human serum samples

For the establishment and the validation of the PUUV-Bawa antigen-based indirect and capture IgG-ELISAs and for cross-reactivity investigations, serum panels from Germany and Finland were applied. These sera have previously been analyzed by commercial reference assays and used for validation of indirect PUUV strain Vranica/Hällnäs (PUUV-Vra) IgM- and IgG-ELISAs ([32] and our unpublished data). The serum samples from Germany include a negative control panel of 149 sera from the routine diagnostics at the University Hospital in Frankfurt/Main and as positive control 79 sera from human NE cases. Out of these 79 sera, 51 samples were from PUUV-IgM- and PUUV-IgG-positive individuals with an acute infection and 28 from PUUV-IgM-negative and PUUV-IgG-positive persons at the convalescent phase. The set from Finland consists of 40 PUUV-IgM/PUUV-IgG-negative, 31 PUUV-IgG-positive and 40 PUUV-IgG/PUUV-IgM-positive sera.

Monoclonal antibodies

For antigenic comparison of the recombinant N proteins of PUUV-Bawa and PUUV-Vra monoclonal antibodies (mAbs) raised against N proteins of PUUV (mAbs 5E11, 5C5, 7A5, 2C6 [39]; 1C12, 5E1, 5A3, 2E12, 4C3 [40, 41]; A1C5 [42]), *Hantaan virus* (HTNV; E5/G6, Eco2 [43]; B5D9 [42]), *Andes virus* (ANDV; mAb 5C2/E10), *Sin Nombre virus* (SNV; mAb 5F1/F7; Immunological and Biochemical Testsystems GmbH, Reutlingen, Germany) and ANDV/SNV (4H3, 7G2 [44]) were used. The mAbs A1C5 and B5D9 were purchased from Progen Biotechnik GmbH (Heidelberg, Germany). Histidin (His)-tag specific mAbs 6E8 and 7B8 were obtained by immunization with a His-tagged yeast-expressed PUUV-Vra N protein (Zvirbliene et al. unpublished data).

RT-PCR and sequencing

Nucleic acids were extracted from *M. glareolus* lung and heart homogenates using the RNeasy kit (Qiagen, Hilden, Germany, see [1]). To obtain the NCRs as well as the entire N-encoding regions, several primer pairs (Table 2) were used for RT-PCR [see 1]. Partial M-segment sequences (nucleotides, nt 2369–3031, encoding a partial G2 of PUUV strain CG1820, accession number M29979) were amplified with primers C1 and C2 (Table 2) and the Superscript™ III RT/Platinum Taq Mix (Invitrogen, Karlsruhe, Germany) in a final volume of 50 µl according the manufacturer's instructions. Following reverse transcription at 50°C for 30 min and denaturation at 94°C for 2 min, DNA was amplified in 55 cycles for 30 s at 94°C, 30 s at 50°C, 1 min at 68°C with a final extension for 5 min at 68°C. Morphological species determination of the PUUV-RT-PCR-positive *M. glareolus* was confirmed by PCR using mitochondrial *cytochrome b* (*cyt b*) gene specific primers as described before [1]. Direct sequencing was performed using segment and *cyt b* gene specific primers.

Cloning of the entire nucleocapsid protein-encoding sequence for expression in yeast

RNA extracted from homogenized lung tissue of the *M. glareolus* sample Bawa H-34/04 (Tables 1, 3; [1]) was amplified using primers PUUVexprF and PUUVexprR (Table 2) and the above described RT-PCR reagents with addition of 1 µM $MgSO_4$. Cycling conditions were modified to 50 cycles for 25 s at 94°C, 50 s at 54°C, 75 s at 68°C. RT-PCR products of expected size were cloned into vector pCR2.1-TOPO® (TopoTA cloning kit, Invitrogen) as described by the manufacturer. The insert with a correct sequence encoding amino acids (aa) 2–433 of the PUUV-Bawa N protein was isolated as a XbaI fragment and subsequently inserted into XbaI-linearized pFX7-His6 plasmid [46]. The generation of the pFX7 expression plasmid encoding a His-tagged entire N protein of PUUV-Vra has been described previously [47].

Phylogenetic analysis

Nt sequences were aligned at the codon level using the ClustalW algorithm [48] implemented in BioEdit 7.0 [49] and revised manually. Phylogenetic relationships were inferred analogous to Braaker and Heckel [50] by neighbour-joining algorithms (NJ; [51]) implemented in Mega 3.1 [52] and by Bayesian algorithms (BI) implemented in MrBayes 3.1.2 [53]. The optimal mutation model was selected based on the Bayesian Information Criterion (BIC; [54]) in jModeltest 0.1.1 [55]: the TIM2ef model was best for the S-segment, the SYM + G model for the M-segment and the Tim1ef + I + G model for *cyt b* sequences. The NJ analyses were replicated 10,000 times using the Tamura-3-parameter model for the S-segment and *cyt b* data and the Maximum Composite Likelihood model [56] for the M-segment. The Bayesian analyses for the S- and M-segment were run three times for 2 million generations with every 10th generation sampled, using one cold and three heated chains. Bayesian *cyt b* data analyses were run for 10 million generations with every 100th generation samples with the web-based cluster implemented in the Cipres Portal [57]. The first 25% of the samples were discarded as burn-in and convergence was determined by examining the log likelihood values, the split frequencies and by using the web-based program AWTY [58]. Net average divergence based on the S- and M-segment was computed with Mega 3.1, using the mentioned nt

Table 2 Primers used for RT-PCRs and sequencing of amplification products

Amplicon	Primer name	Sequence	Direction of primer	Position[a] (nt)	Reference
S-segment	PUUV342	5′-TATGGTAATGTCCTTGATGT	Forward	334–353	[1]
	PUUV390	5′-ACHCCCATNACWGGRCTYAT	Forward	382–400	[1]
	cPUUV721	5′-GGNCARACAGCGAYTGGT	Reverse	688–707	[1]
	PUUV1104	5′-ATGAGRAAYACHATHATGGC	Forward	1066–1085	[1] (complementary reverse to cPUUV1122)
	cPUUV1122	5′-GCCATDATDGTTTYCTCAT	Reverse	1066–1085	[1]
	PUUVFpuni	5′-TAGTAGACTCCTTGAARAGCTRCTACGA	Forward	1–28	This paper
	cPUUV1758	5′-GAYAGCTCAGYTTCACAT	Reverse	1590–1607	This paper
	PUUVexprF	5′-AatctagaGACTTGACAGACATTCAGG	Forward	49–68	This paper
	PUUVexprR	5′-aTCTAGATTATCATATTTTTTAAGGGCTCCTGATT	Reverse	1316–1348	This paper
M-segment	C1	5′-CCCCCTGATTGTCCTGGTGTAG	Forward	2369–2390	[45]
	C2	5′-CCAACTCCTGAACCCCATGC	Reverse	3011–3030	[45]

[a] Numbering corresponds to nucleotide sequence of PUUV strain CG18-20 S-segment (Genbank M32750) and M-segment (Genbank M29979)

substitution models. GenBank accession numbers of the sequences included in this study are shown in the corresponding phylogenetic trees.

Yeast synthesis and purification of the PUUV N antigens

The production in yeast cells and purification of N proteins of PUUV-Vra and PUUV-Bawa were done as described before [47]. Briefly, yeast *S. cerevisiae* cells of the wild-type strain FH4C were transformed with the corresponding pFX7-His6-derived yeast expression plasmids. After cultivation of the transformed yeast cells in YEPD growth medium (yeast extract 1%, peptone 2%, glucose 2%) supplemented with formaldehyde for 24 h at 28°C, N protein synthesis was induced by adding YEPG medium (yeast extract 1%, peptone 2%, galactose 6%) and incubation for additional 20 h. Protein purification was carried out by nickel-chelate affinity chromatography (Qiagen) with elution under denaturing conditions (8 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris, pH 4.5; see [47]). For use in mAb-capture ELISA, the PUUV-Bawa N antigen was dialyzed against carbonate buffer as previously described [47].

Indirect PUUV-IgG-ELISA

The cross-reactivity of human sera with yeast-expressed N antigens of PUUV-Bawa and PUUV-Vra was investigated by endpoint titration in twofold steps starting at a dilution of 1:400 and using recently validated indirect IgG-ELISA protocols [32]. Definition of lower and upper cut-off values and determination of sensitivity and specificity for the PUUV-Bawa antigen-based ELISA follow published criteria [32].

PUUV-Bawa antigen-based IgG capture ELISA

For the capture ELISA Maxi-Sorp plates (Nunc) were coated with 100 µl/well capture mAb A1C5 (1:1,000 in carbonate buffer, pH 9.8) for 30 min at 37°C. After washing three times with PBS/0.1% Tween 20, blocking was performed using 200 µl/well 3% BSA in PBS/0.05% Tween 20 for 30 min at room temperature (RT). Additional washing was followed by adding 100 µl/well recombinant PUUV-Bawa N antigen (2 µg/ml in 1% BSA/0.05% Tween 20 in PBS) and incubation at 37°C for 30 min. Plates were washed and the human sera were added (diluted 1:400 in 1% BSA/0.05% Tween 20 in PBS). After incubation at 37°C for 30 min the plates were washed, the HRP-labelled goat anti-human IgG conjugate (1:2,000, Sigma-Aldrich, Deisenhofen, Germany) was incubated at 37°C for 1 h. The final steps of this ELISA as well as the definition of lower and upper cut-off values and

Table 3 Summary of results of RT-PCR investigations targeting the S- and M-segment of PUUV in lung tissue samples from bank voles from Lower Bavaria

Sample number	Counties (trapping site)	Results of RT-PCR amplification using primer pairs					Accession number		
		PUUVFpuni–cPUUV721	PUUV342–cPUUV1122	PUUV1104–cPUUV1758	PUUVexprF–PUUVexprR	C1–C2	mt *cyt b*	Partial S-segment	Partial M-segment
Bawa H-2/04	Hohenau (Raimundsreuth), (H)	− (He)	+[a]	+	−	+	DQ090757[a]	AY954725[c]	DQ518218
Bawa K-9/04	Kirchberg (Hangenleiten), (K)	+	+[a]	+	+	+	DQ090756[a]	AY954722[c]	DQ518219
Bawa K-20/04	Kirchberg (Hangenleiten), (K)	− (He)	+[a]	+	−	−	DQ090758[a]	AY954724[c]	−
Bawa H-33/04	Hohenau (Raimundsreuth), (H)	+	+[a]	+	−	+	DQ090760[a]	DQ016430[c]	DQ518223
Bawa H-34/04	Hohenau (Raimundsreuth), (H)	+	+[a]	+	+	+	DQ090755[a]	AY954723[c]	DQ518235
Bawa L-39/04	Lindberg (Falkenstein), (L)	− (He)	+[a]	+	+	+	DQ090753[a]	DQ016431[c]	DQ518236
Bawa L-41/04	Lindberg (Falkenstein), (L)	+	+[a]	+	+	+	DQ090759[a]	DQ016432[c]	DQ518237
Bawa F-151/05	Freyung (military barracks), (F)	+	+	+	−	+	EU439963	EU439968	DQ518220
Bawa H-152/05	Hohenau (Raimundsreuth), (H)	− (He)	+	+	+	+	EU439964	EU439969	DQ518233
Bawa H-153/05	Hohenau (Raimundsreuth), (H)	n.d.	(+)[b]	n.d.	n.d.	n.d.	EU439965	EU439970	n.d.
Bawa F-157/05	Freyung (military training site), (F)	− (He)	+	+	+	+	EU439966	EU439971	DQ518232
Bawa F-159/05	Freyung (military training site), (F)	+	+	+	+	+	EU439967	EU439972	DQ518234

For comparisons of nt sequences the previously determined S-segment sequences of S-17/04, S-18/04 and H-32/04 [1] were applied (Genbank DQ091192, DQ091190, DQ091191)

n.d. not determined, − no RT-PCR amplification product, *He* amplification also tried with heart tissue of the respective PUUV-positive *Myodes glareolus*

[a] Already described in [1]

[b] Novel sequence obtained by nested RT-PCR targeting nt 390 to nt 721 (Primer pair PUUV390 – PUUVcPUUV721, for details of the protocol see [1])

[c] Updated sequences of [1]

the determination of sensitivity and specificity were done the same way as for the indirect ELISA.

SDS-PAGE and western blot analysis

Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analyses using an enhanced chemiluminescence (ECL) reagent (Amersham, Braunschweig, Germany) were performed as previously described [32, 37]. Briefly, 2.5 μg of PUUV-Vra or PUUV-Bawa N proteins, each in a volume of 250 μl, were separated in a 12.5% SDS-PAGE gel with a 0.75 mm comb in a Mini-Protean 3 system (BioRad, Munich, Germany). Thereafter, the proteins were semidry blotted onto a Polyvinylidenfluoride (PVDF) membrane (Millipore, Bedford, MA). After blocking, 3 mm-wide strips of the membrane were incubated for 90 min at RT with 500 μl mAb diluted 1:500 in blocking buffer. For detecting the immune reaction, the strips were incubated for 90 min at RT in 500 μl HRP-labelled goat anti-mouse IgG (H + L) (BioRad) diluted 1:500 in blocking buffer.

Results and discussion

PUUV strain Bavaria is persistently present in *M. glareolus* in Lower Bavaria

In this study we describe the hantavirus screening of 19 rodents trapped in Lower Bavaria in 2005. Lung samples from 5 of 11 (45%) bank voles were found to be positive in at least one of the PUUV-specific RT-PCRs. All eight lung samples from other rodent species were negative (Table 1). This finding is in line with the prevalence observed in two independent studies in the same region during 2004. In a combined serological and RT-PCR study 10 of 29 (34.5%) of bank voles were found to be PUUV-positive [1]. A similar prevalence of 21% (8/38) was observed in a RT-PCR screening of bank voles from this particular area in November 2004 [29]. Interestingly, the current study demonstrated a continuing presence of PUUV-infected bank voles at least in the trapping area Hohenau-Raimundsreuth (Fig. 1; Table 1). In this area PUUV-infected bank voles were detected both in 2004 and 2005 suggesting an overwinter survival of at least some of the infected animals or, alternatively, a long-term viability of infectious virus particles in the environment. The latter might be supported by a relatively long-term stability of hantaviruses outside of their host [59, 60]. A further longitudinal study in South-East Germany during 2008–2010 has recently shown that the PUUV strain Bavaria is still present in the study area (see [61]; Essbauer et al. unpublished data).

Molecular and phylogenetic analysis of partial S- and M-segment sequences of PUUV strain Bavaria illustrate the high variability of PUUV in Germany

In this study, for two *M. glareolus* trapped in Lower Bavaria in 2005 (Bawa F-151/05, 1711 nt; Bawa F-159/05, 1672 nt) the almost entire PUUV S-segment sequences were amplified. For two samples (Bawa H-152/05, 1603 nt; Bawa F-157/05, 1639 nt) we obtained the main part of the N protein-encoding region and part of the 3′-NCR. For the lung sample of *M. glareolus* Bawa F-153/05 an amplification product was exclusively obtained using a nested RT-PCR (Table 3). These novel PUUV sequences are highly similar to the subtype Bawa sequences collected from *M. glareolus* in this region in 2004 [1, 29]. For seven out of ten animals trapped in 2004 [1], the S-segment sequences were extended up to about 1,700 nt (see Tables 1, 3). Finally, for 10 out of the 11 PUUV S-segment RT-PCR-positive *M. glareolus* trapped in 2004 and 2005, partial M-segment sequences were amplified from lung tissue (Table 3).

Phylogenetic analyses of 65 partial S-segment sequences (1,302 nt) demonstrated strong evolutionary differentiation of PUUV from the outgroup sequences (Fig. 2a). Furthermore, the analyses revealed a strong geographical clustering within PUUV into a Central European, Northern European I, Northern and Eastern European, Northern European II and Asian clade. The PUUV sequences from Bavaria (Southern Germany) were clearly separated from other German sequences (Western Germany I, Western Germany II) within the Central European cluster. Further, analyses of 32 M-segment sequences (621 nt) confirmed a clear separation of the PUUV from the outgroup sequences (Fig. 3a). As for the S-Segment, a well supported geographical grouping into a Southern German, Western German-Belgian, Swedish and Finish-Russian cluster was revealed, and the sequences from Bavaria (Southern Germany) were distinct from other sequences from Germany (Western Germany).

The clear separation of the Bavarian clade from PUUV sequences from the western parts of Germany is reflected in nt divergences between German clades of 12.3 and 23.3% based on the S-segment and of 19.7% in the M-segment (Table 4a, b). Based on corresponding aa, the Bavarian cluster differed 0.6 and 1% from the Western Germany clusters for the S-segment and 1.5% for the M-segment. The other Central European clusters have a nt divergence in the partial S-segment between 20.5% (Belgium) and 30.3% (Austria) to the Bavarian strain, resulting in an aa divergence up to 1.8% (Table 4a). The majority of aa exchanges was found in the region between aa 247 and 265 of the N protein. The divergence of the Bavarian cluster to the other PUUV clusters in the M-segment ranged from 24.6 to 24.7% at the nt and 2–4.2% at the aa level (Table 4b).

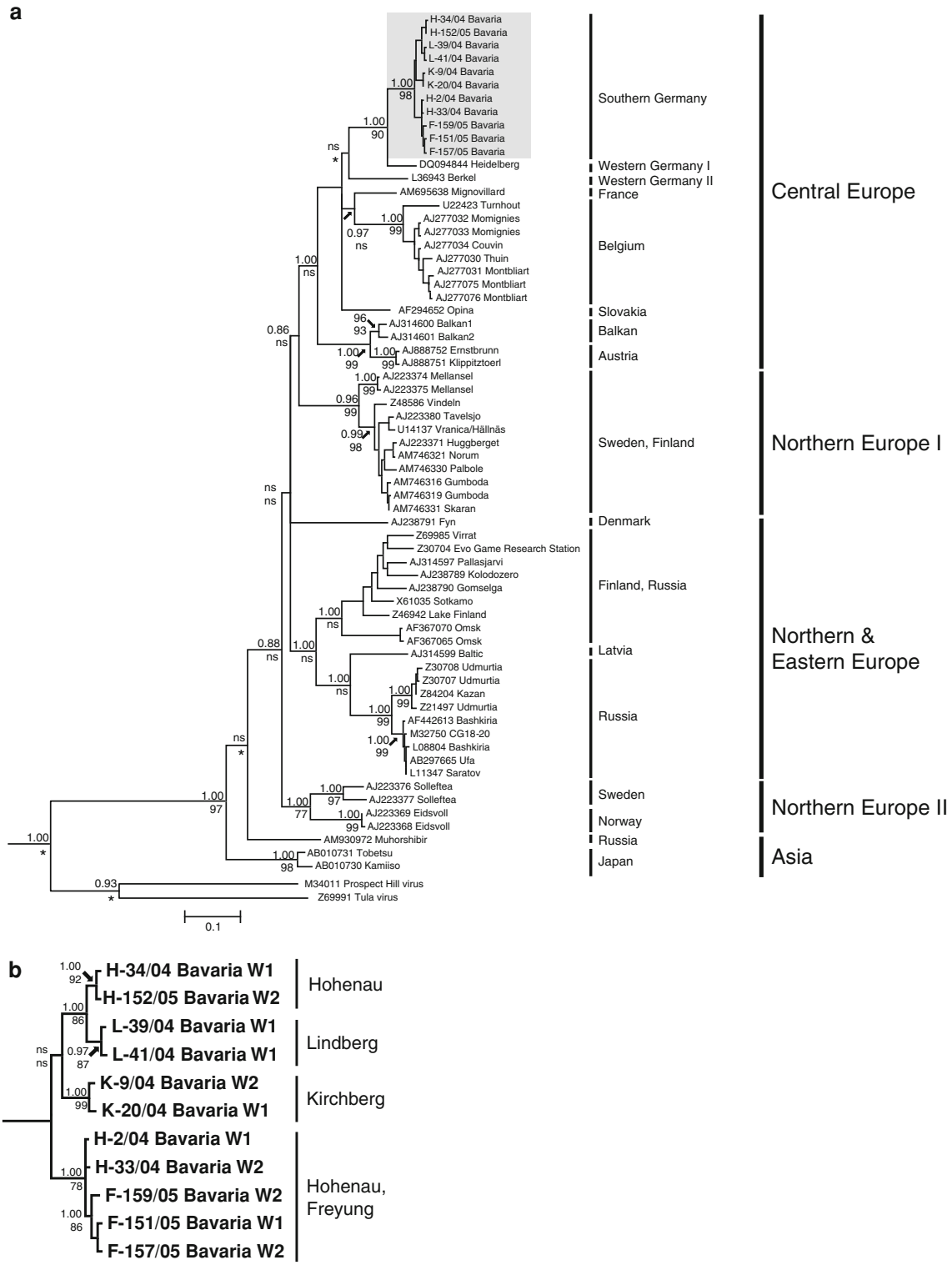

Fig. 2 Bayesian reconstruction of phylogenetic relationships based on 1,302 nt S-segment sequences. Support values are displayed only for main nodes that connect major evolutionary lineages. Posterior probabilities are indicated above the major branches and percentage of bootstrap support for neighbour-joining (NJ) algorithms below the branches. *A different topology based on NJ algorithms, ns refers to posterior probabilities <0.70 and percentages of bootstrap support <70%. **a** Phylogenetic relationships of PUUV sequences with closely related viruses used as outgroup to root the tree. The new Bavarian cluster is *highlighted in grey*. **b** The Bavarian cluster displayed with the four trapping sites Hohenau, Lindberg, Kirchberg and Freyung. The abbreviation W1 indicates the mitochondrial affiliation to the Western European 1 cluster, W2 the association to the Western European 2 cluster (see Supplementary figure)

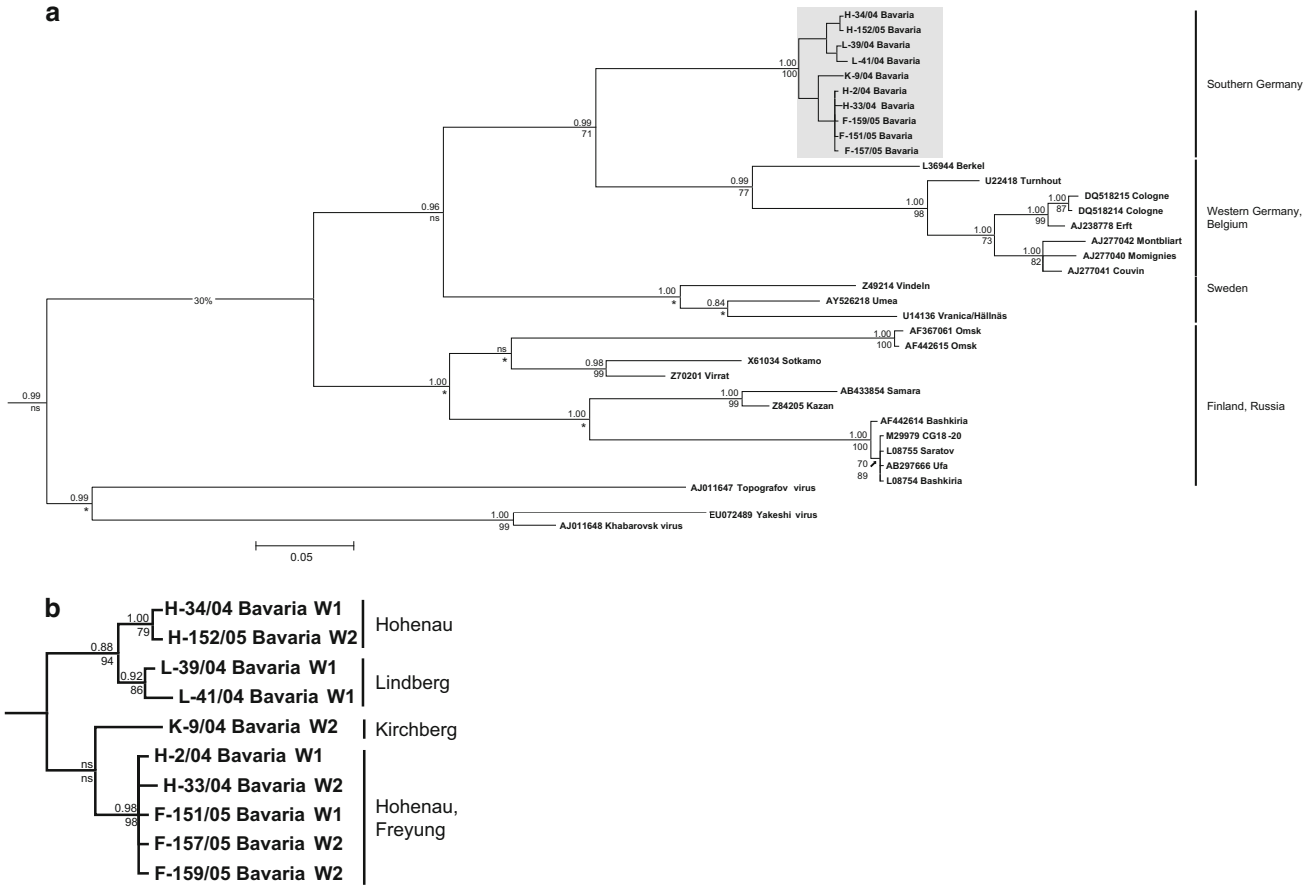

Fig. 3 Bayesian reconstruction of phylogenetic relationships based on 621 nt M-segment sequences. Only support values for main nodes that connect major evolutionary lineages are displayed. Posterior probabilities are indicated above the major branches and percentage of bootstrap support for neighbour-joining (NJ) algorithms below the branches. *A different topology based on NJ algorithms, ns refers to posterior probabilities <0.70 and percentages of bootstrap support <70%. **a** Phylogenetic relationships of PUUV sequences with closely related viruses used as outgroup to root the tree. The new Bavarian cluster is *highlighted in grey*. **b** The Bavarian cluster is displayed with the four trapping sites Hohenau, Lindberg, Kirchberg and Freyung. The abbreviation W1 indicates the mitochondrial affiliation to the Western European 1 cluster, W2 the association to the Western European 2 cluster (see Supplementary figure)

Sequence variation of PUUV at small geographical scale and corresponding mitochondrial (mt) DNA sequence analysis of *M. glareolus*

A more detailed evaluation of the Bavarian S- and M-segment sequences showed a geographical clustering associated with the regions Hohenau, Lindberg, Kirchberg and Hohenau/Freyung (Figs. 1, 2b, 3b). For the almost entire S-segment and the entire N protein-encoding sequences, the difference between the sub-clusters within the Bavarian clade (Fig. 2b) ranged from 0.4 to 3.1% at the nt level, and from 0% up to 1% on the aa level. The partial 3′-NCR sequences (377 nt) of 11 samples revealed an intra-group nt divergence from 0 up to 12.3%. In comparison, the nt divergence of the partial M-segment among the four Bavarian sub-clusters (Fig. 3b) ranged from 0.9 to 3.2%, resulting in an aa divergence of 0–0.5%.

For other PUUV lineages we have so far only very limited data concerning divergence within one trapping site at a defined time point. For 17 sequences of the PUUV originating from the city of Cologne in 2005 a divergence of up to 1.2% for the 722 nt long partial S-segment was found [26, 62]. In comparison the genetic diversity of 23 PUUV sequences from Finland was comparable to the divergence described herein, i.e. 0.2–4.9% for the S- and 0.2–4.8% for the M-segment [63]. Similarly, a divergence of 0–2.6% for the S-, and 0–0.8% for the partial M-segment were reported for Russian PUUV strains [64]. This relatively low local differentiation among virus strains is similar to patterns detected in *Tula virus* where strain similarity was associated with trapping locality but not trapping year or vole host species [65]. It is noteworthy that PUUV similarity in the present study was associated with trapping locality even at distances between trapping sites of only about 50 km (Figs. 2b, 3b). This may suggest relatively independent virus evolution between sites at the regional scale and low or no direct migration among the host populations.

Table 4 Divergence among PUUV clusters in the phylogenetic trees in Figs. 2a and 3a

(a) S-segment

	Southern Germany	Western Germany I	Western Germany II	France	Belgium	Slovakia	Balkan	Austria
Southern Germany	–	1.0	0.6	1.1	1.3	1.5	1.1	1.8
Western Germany I	12.3	–	1.7	1.7	2.4	2.0	1.7	2.4
Western Germany II	23.3	23.3	–	1.0	0.7	1.7	1.7	2.4
France	20.9	20.9	20.9	–	1.1	2.0	2.0	2.7
Belgium	20.5	20.4	21.1	18.2	–	1.3	2.4	3.1
Slovakia	21.4	26.4	26.7	20.2	24.6	–	2.7	3.4
Balkan	26.0	25.5	23.0	24.2	26.5	25.8	–	0.7
Austria	30.3	30.8	21.5	26.1	28.6	29.8	7.1	–

(b) M-segment

	Southern Germany	Western Germany, Belgium	Sweden	Finland, Russia
Southern Germany	–	1.5	2.0	4.2
Western Germany, Belgium	19.7	–	3.3	5.3
Sweden	24.7	21.5	–	2.7
Finland, Russia	24.6	22.6	19.4	–

The net average divergence based on S-segment (a) and partial M-segment (b) was calculated using the Tamura-3-parameter model. All values are given in percent. Nucleotide sequence divergence is given below and amino acid sequence divergence above the diagonal

Voles carrying PUUV strain Bawa were all confirmed as *M. glareolus* by sequence analysis of approximately 968 nt of the *cyt b* gene. Phylogenetic analysis further revealed that *M. glareolus* in Bavaria clustered together with other sequences from a large region in Western Europe, which is evolutionarily distinct from other genetic lineages in the species occurring in Italy, the Balkans, Spain or Eastern Europe (Supplementary Figure; [64, 66, 67]). However, *M. glareolus* from Bavaria belong to 2 weakly defined different subclusters (W1 and W2) of the Western European lineage. The presence of haplotypes from these two sub-clusters within single populations is not unusual [66]. Given the relatively low geographic resolution of *cyt b* within lineages [66, 68, 69], it is not surprising that this marker failed to resolve potential differentiation among voles from the Bavarian sites. Evolutionary analyses of *M. glareolus* populations have shown that genetic differentiation and thus limited dispersal may exist at regional and local scales [67; Jenkins et al. unpublished]. However, the study of population connectivity and migration at such geographical scales requires typically the analysis of highly variable genetic markers and dedicated population genetics analyses of the virus hosts (see [70–72]).

Reactivity of human sera and mAbs with different PUUV N antigens

The recombinant N protein of PUUV-Bawa was shown to be highly pure by SDS-PAGE and to have the expected molecular weight of 49.4 kDa (data not shown). The high-level expression and purification of large quantities of this antigen highlighted the value of the used yeast expression system (see [46]). A western blot analysis of the protein with the His-tag specific mAbs 6E8 and 7B8 confirmed the presence of the amino-terminal His-tag on the recombinant N protein (Fig. 4).

The antigenicity of the PUUV-Bawa N protein was characterized with mAbs raised against PUUV, HTNV, SNV and ANDV. In general, the reactivity of the N protein of PUUV-Bawa was identical to that of PUUV-Vra, PUUV strain Sotkamo (PUUV-Sot) and PUUV strain Kazan (PUUV-Kaz) for the majority of mAbs (Fig. 4; [39, 44, 73]). In contrast, the mAb 2C6 raised against the 120 amino-terminal aa of PUUV-Vra N protein and mapped to an epitope between aa 1 and 45 did not react with PUUV-Bawa N antigen as previously reported for N antigens of PUUV-Kaz and PUUV-Sot and confirmed the strain specificity of this mAb [74]. Interestingly, mAb 5A3 mapped to an epitope in the same region [73] reacted vice versa and was found to be non-reactive with PUUV-Vra N protein [73] but reactive with that of PUUV-Bawa as previously seen for PUUV-Sot and PUUV-Kaz [46]. Taken together, based on the reactivity pattern of these two N-specific mAbs, the N protein of the PUUV-Bawa subtype was antigenically closer related to those from Finnish PUUV-Sot and Russian PUUV-Kaz strains than to that of the Swedish PUUV-Vra strain.

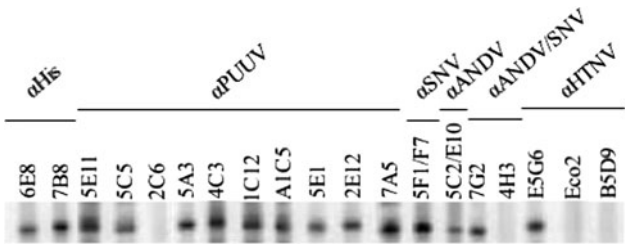

Fig. 4 Western blot reactivity of the purified recombinant PUUV-Bawa N protein (aa 2–433) of about 49 kDa with His-tag specific mAbs 6E8 and 7B8 and mAbs raised against N proteins of PUUV (mAbs 5E11, 5C5, 2C6 [39], 5A3, 4C3, 1C12, A1C5 [42], 5E1, 2E12, [40, 41], 7A5), *Sin Nombre virus* (SNV; mAb 5F1/F7), *Andes virus* (ANDV; mAb 5C2/E10), ANDV/SNV (mAb 4H3, 7G2 [44]) and *Hantaan virus* (HTNV; mAb E5/G6, Eco2 [43]; B5D9 [42]). The epitope localization of the PUUV-, HTNV and SNV/ANDV-reactive mAbs has been described in detail in [43, 78, 79]. Epitope specificity for mAbs 5C2/E10 and 5F1/F7 is given according to the information of the manufacturer (Immunological and Biochemical Testsystems GmbH, Reutlingen, Germany)

In addition, the aa sequence differences between the N proteins of these four PUUV strains in the 45 amino-terminal aa residues underline an important role of aa residue 35 in the epitope integrity for mAbs 2C6 and 5A3 ([44, 73]; see Fig. 5). Interestingly, at aa position 35 an aspartic acid (D) residue was found in the N protein of the Bavaria strain, as in the majority of the PUUV strains. In comparison, PUUV strains Vranica/Hällnas, P360 and K27 contain at the corresponding position a tyrosine (Y) residue (Fig. 5). The importance of aa residue 35 was also observed in a recent study describing the reactivity patterns of cell culture passage-derived PUUV variants with a N protein-specific mAb [75].

Establishment, validation and comparison of a PUUV-Bawa N protein-based indirect IgG and capture ELISA

Purified, yeast-expressed viral antigens have broadly been used for diagnostics of virus infections in humans and animals [76–81]. We have recently described indirect and mAb-capture IgG-ELISAs for the detection of human infections with SNV, ANDV, PUUV, DOBV and HTNV [32, 36–38]. A corresponding ELISA using the PUUV-Bawa N protein was established here following a recently published protocol for a PUUV-Vra N protein-based IgG-ELISA [32]. When validating this novel in-house ELISA with 100 negative and 76 positive control sera from

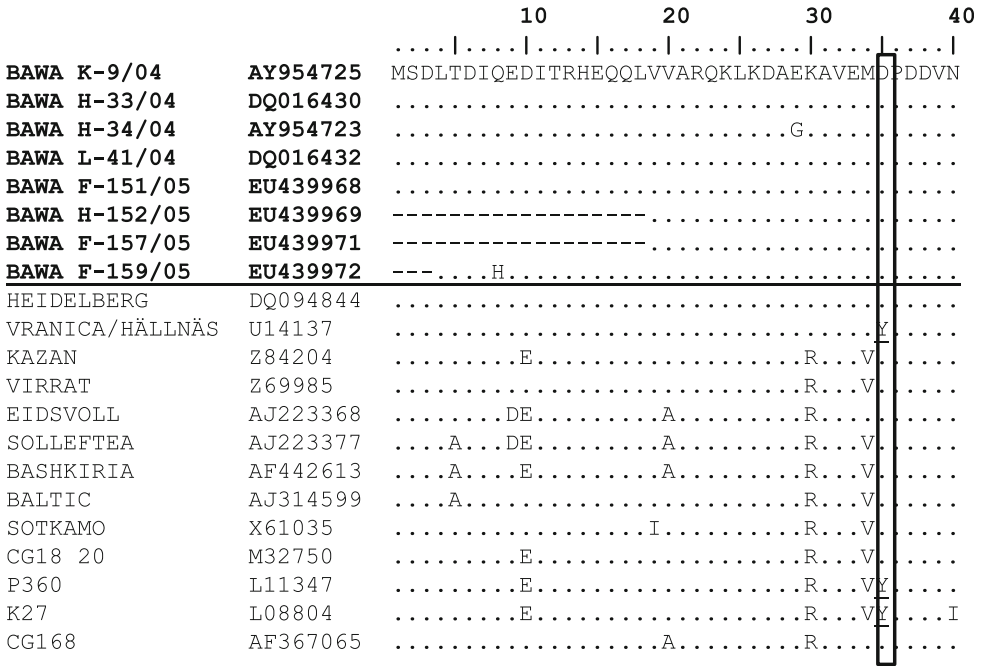

Fig. 5 Amino acid sequences spanning residues 1–40 of N proteins of German PUUV strains Bavaria (BAWA K-9/04, AY954722; BAWA H-33/04, DQ016430; BAWA H-34/04, AY954723; BAWA L-41/04, DQ016432; BAWA F-151/05, EU439968; BAWA F-152/05, EU439969; BAWA F-157/05, EU439971; BAWA F-159/05, EU439972; marked in bold) and Heidelberg (DQ094844, PUUV strain Heidelberg/hu) in comparison to corresponding PUUV N protein sequences from other European countries (U14137, strain Vranica-Hällnäs; Z84204, strain Puu/Kazan; Z69985, strain Puu/Virrat/25Cg/95; AJ223368, strain Puu/Eidsvoll/1124v; AJ223377, strain Puu/Solleftea/Cg6/95; AF442613, strain CG17/Baskiria-2001; AJ314599, strain Baltic/205Cg/00; X61035, strain Sotkamo; M32750, strain CG1820; L11347, strain P360; L08804, strain K27; AF367065, strain CG168). The amino acid residue 35, most likely responsible for distinguishing reaction with mAbs 2C6 and 5A3 is marked

Table 5 Comparison of the performance of the novel PUUV-Bawa antigen-based *in-house* assays using serum panels from Germany and Finland

Novel *in-house* assays	Total number of the tested serum samples	Origin of the serum panels	Results of the novel assays	Results of the reference assay	
				Positive	Negative
Indirect IgG-ELISA	176	Germany	Positive	75	0
			Negative	1	99
			Equivocal	0	1
Capture IgG-ELISA	228	Germany	Positive	74	0
			Negative	4	148
			Equivocal	1	1
Capture IgG-ELISA	111	Finland	Positive	62	0
			Negative	8	40
			Equivocal	1	0

Germany one sample was identified as false negative (Table 5). Therefore, the diagnostic sensitivity and specificity of the novel ELISA was 99 and 100%, respectively.

The novel IgG-ELISA was compared to the corresponding PUUV-Vra N protein-based ELISA by endpoint titration of 68 sera from different regions in Germany and 10 sera from Finland. In general, almost all sera showed a very similar reactivity with both N antigens. Thus the endpoint titer of 30 sera from Germany and 7 sera from Finland were identical to both antigens. The endpoint titer to the PUUV-Bawa antigen was twofold higher for 34 sera from Germany and two sera from Finland. For three sera from Germany the endpoint titer to PUUV-Bawa was found to be fourfold higher than that to PUUV-Vra. Only one serum originating from south Germany was detected by the PUUV-Vra IgG-ELISA at the initial dilution, but not at all with the corresponding PUUV-Bawa test (data not shown). The similar reactivity of the N proteins of PUUV-Bawa and PUUV-Vra with the majority of N protein-specific mAbs and the results of the cross-reactivity investigations with human sera demonstrated a close antigenic similarity of these N proteins. Future large-scale serological studies will have to prove if local homologous PUUV antigens are required for a highly sensitive detection of hantavirus-specific antibodies, as discussed previously [33–38, 82].

The initial validation of the herein described mAb-capture PUUV-IgG-ELISA with the panel from Germany revealed 148 true negative, 74 true positive and four false negative sera. The evaluation of the sera originating from Finland revealed 62 true positive, 40 true negative, but 8 false negative sera (Table 5).

In line with data obtained for yeast-expressed HTNV antigen [83, 84], we found a very similar sensitivity and specificity of the PUUV-Bawa antigen-based indirect ELISA (99 and 100%) and mAb-capture ELISA (95 and 100%) for the serum panel from Germany. A comparison of the results of the validation of the indirect PUUV-Vra ELISA and the PUUV-Bawa mAb-capture ELISA for all investigated sera from Finland showed a lower sensitivity for the capture ELISA (89%) than for the indirect ELISA (100%), but a higher specificity (100 and 95%, respectively). The lower sensitivity of the PUUV-Bawa capture ELISA may be a result of the heterologous antigen used in this assay with Finish serum samples. Alternatively, also a competition of the mAb and the serum samples cannot be excluded. The high sensitivity and specificity might be due to the use of a purified yeast-expressed antigen preventing problems observed for *E. coli*-expressed antigens [85].

In conclusion, these investigations suggest a continuing prevalence of PUUV subtype Bavaria in *M. glareolus* from Lower Bavaria. The encoded N protein can be expressed at high-level in yeast *S. cerevisiae* and is suitable for diagnostic purposes in an indirect and capture IgG-ELISA for human sera from Germany. Future large-scale seroepidemiological studies in different European regions should consider if this antigen might be applied for diagnostic purposes in these regions as well.

Acknowledgements We would like to thank Ausra Razanskiene and Kestutis Sasnauskas (Vilnius) and Bernd Köllner (Riems) for continous support and helpful discussions, Åke Lundkvist (Stockholm), Kumiko Yoshimatsu and Jiro Arikawa (Sapporo) for provision of N protein-specific mAbs and Olli Vapalahti (Helsinki), Regina Allwinn and Holger F. Rabenau (Frankfurt) for provision of human serum panels. We kindly acknowledge Harald Weber, Aileene Lorber, Rahime Terzioglu, Elmar Schröpfer, Ralf Hagen, Mirko Köhler, Anne Grumbach, Peter Klein and Kerstin Weiss for their support during rodent trapping, rodent necropsy and collection of human sera in South-East Germany. The excellent technical lab assistance of Jana Blumhardt, Ina Römer, Dörte Kaufmann, Sabine Fink and Astrid Thomas is gratefully acknowledged. The work was supported by Research-Project M/SAB1/5/A017 for the Bundeswehr Medical Service to RGU.

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