TABLE 6. Nucleotide and amino acid divergence among clusters of analyzed partial S segments of TULV and closely related hantaviruses[a]

	Germany I (13)	Germany II (13)	Germany III (3)	Germany IV (1)	Russia I (5)	Russia II (2)	Russia III (2)	Poland (2)	Slovakia I (3)	Slovakia II (4)	Slovakia III/ Cz.Rep. (9)	Austria I (5)	Austria II (4)	Croatia (1)	Serbia (1)	Yakeshi (3)	Fusong (2)	VLA (3)	Prospect Hill (2)	Isla Vista (4)
Germany I		12.3±1.6	18.0±2.0	19.8±2.0	17.3±1.9	17.6±1.9	18.2±1.9	19.2±2.1	18.8±2.0	18.7±2.0	18.9±2.0	18.0±2.0	18.5±2.0	19.0±2.0	17.0±1.9	26.9±2.4	27.5±2.5	27.0±2.4	22.7±2.2	26.2±2.3
Germany II	0.2±0.1		18.5±2.1	20.8±2.2	15.4±1.7	17.2±1.9	19.2±2.0	19.3±2.1	17.5±2.1	20.6±2.2	20.5±2.2	19.0±2.2	20.8±2.2	18.0±2.1	17.4±2.1	27.2±2.5	27.7±2.5	30.5±2.6	23.5±2.3	25.7±2.3
Germany III	1.2±1.2	1.2±1.2		20.1±2.2	18.4±2.1	17.7±2.1	16.9±2.1	10.0±1.8	17.9±2.2	18.7±2.3	17.8±2.2	17.2±2.2	18.7±2.3	18.4±2.3	19.9±2.3	26.8±2.5	26.3±2.6	25.4±2.5	22.1±2.4	23.0±2.5
Germany IV	4.6±2.2	4.6±2.2	3.4±2.0		20.2±2.2	19.5±2.1	19.2±2.1	17.7±2.2	17.7±2.2	15.8±2.1	14.6±2.1	14.1±2.1	14.7±2.1	17.7±2.3	18.0±2.2	24.8±2.5	26.9±2.5	25.6±2.4	19.9±2.3	25.2±2.5
Russia I	0.5±0.4	0.5±0.3	1.6±1.2	5.0±2.2		15.6±1.9	18.9±2.1	19.1±2.2	17.7±2.0	21.3±2.2	21.5±2.2	20.4±2.2	20.6±2.2	21.1±2.2	18.9±2.1	28.0±2.5	28.6±2.4	28.0±2.5	24.1±2.3	24.9±2.4
Russia II	2.4±1.3	2.4±1.3	2.8±1.7	4.0±2.1	2.7±1.3		18.2±2.0	18.8±2.3	17.5±2.0	19.4±2.2	19.7±2.2	20.7±2.4	19.0±2.1	19.0±2.2	19.0±2.2	28.8±2.6	27.8±2.6	27.6±2.4	23.1±2.3	22.3±2.3
Russia III	1.2±1.1	1.2±1.1	2.3±1.5	5.7±2.5	1.6±1.2	3.4±1.7		18.4±2.2	20.1±2.2	19.0±2.0	19.1±2.0	19.3±2.1	19.4±2.1	22.4±2.1	19.9±2.2	27.4±2.5	26.7±2.5	24.1±2.4	26.9±2.4	21.9±2.3
Poland	2.4±1.5	2.4±1.5	1.1±1.1	2.3±1.6	2.7±1.6	1.7±1.3	3.4±1.9		18.2±2.3	18.4±2.2	16.0±2.1	16.7±2.1	18.4±2.2	20.3±2.3	17.3±2.1	25.1±2.4	28.2±2.6	25.2±2.5	21.4±2.3	22.0±2.5
Slovakia I	0.1±0.1	0.1±0.1	1.1±1.2	4.5±2.2	0.5±0.3	2.3±1.3	1.1±1.1	2.3±1.5		17.3±2.3	17.0±2.3	17.3±2.2	17.7±2.3	19.4±2.3	11.5±1.9	26.3±2.5	24.4±2.5	26.7±2.5	22.6±2.5	23.0±2.4
Slovakia II	3.5±1.9	3.5±1.9	2.3±1.5	2.3±1.6	3.9±1.9	4.0±2.0	4.5±2.2	2.3±1.5	3.4±1.9		4.4±1.2	9.2±1.7	2.3±0.8	9.8±1.8	18.8±2.2	24.8±2.5	26.9±2.5	24.4±2.4	18.4±2.2	22.8±2.4
Slovakia III/ Cz.Rep.	3.7±1.9	3.7±1.9	2.4±1.5	2.4±1.6	4.0±1.9	4.1±2.0	4.7±2.2	2.4±1.5	3.6±1.9	0.2±0.2		7.2±1.5	3.6±1.0	10.0±1.8	18.2±2.2	25.2±2.5	28.1±2.6	24.8±2.4	18.0±2.2	22.5±2.3
Austria I	3.5±1.9	3.5±1.9	2.3±1.5	2.3±1.6	3.9±1.9	4.0±2.0	4.5±2.2	2.3±1.5	3.4±1.9	0	0.2±0.2		8.5±1.7	10.5±1.9	15.4±2.1	24.4±2.6	28.0±2.6	24.4±2.5	19.0±2.3	25.2±2.6
Austria II	3.5±1.9	3.5±1.9	2.3±1.5	2.3±1.6	3.9±1.9	4.0±2.0	4.5±2.2	2.3±1.5	3.4±1.9	0	0.2±0.2	0		9.4±1.7	18.0±2.2	25.1±2.6	28.8±2.6	24.8±2.4	16.9±2.1	23.3±2.4
Croatia	3.5±1.9	3.5±1.9	2.3±1.5	2.3±1.6	3.9±1.9	4.0±2.0	4.5±2.2	2.3±1.5	3.4±1.9	0	0.2±0.2	0	0		18.8±2.3	26.1±2.6	30.3±2.7	27.4±2.6	18.8±2.2	23.4±2.5
Serbia	0.1±0.1	0.1±0.1	1.1±1.2	4.5±2.2	0.5±0.3	2.3±1.3	1.1±1.1	2.3±1.5	0	3.4±1.9	3.6±1.9	3.6±1.9	3.6±1.9	3.4±1.9		24.9±2.4	23.7±2.5	26.3±2.4	20.3±2.3	24.3±2.6
Yakeshi	14.1±3.8	14.1±3.8	15.2±3.9	16.3±4.1	14.5±3.8	16.3±4.0	14.0±3.8	16.3±4.1	14.0±3.8	15.2±3.9	15.2±3.9	15.2±3.9	15.2±3.9	15.2±3.9	14.0±3.8		21.1±2.3	20.9±2.2	23.6±2.4	25.4±2.4
Fusong	14.3±3.8	14.3±3.8	15.3±3.9	16.5±4.1	14.7±3.8	16.5±4.0	14.2±3.8	16.5±4.1	14.2±3.8	15.3±4.0	15.3±4.0	15.3±4.0	15.3±4.0	15.3±4.0	14.2±3.8	2.1±1.3		12.6±2.0	23.5±2.6	26.8±2.5
VLA	13.7±3.8	13.7±3.8	14.8±3.9	15.9±4.1	14.1±3.8	15.9±4.0	13.6±3.8	15.9±4.1	13.6±3.8	14.8±3.9	14.8±3.9	14.8±3.9	14.8±3.9	14.8±3.9	13.6±3.8	1.5±1.1	0.6±0.6		22.2±2.4	24.7±2.5
Prospect Hill	11.5±3.2	11.5±3.2	10.2±3.1	11.4±3.4	11.8±3.2	13.1±3.5	11.4±3.3	11.4±3.4	11.4±3.3	10.2±3.2	10.2±3.2	10.2±3.2	10.2±3.2	10.2±3.2	11.4±3.3	7.8±2.8	8.0±2.9	7.4±2.8		21.7±2.3
Isla Vista	7.5±2.6	7.5±2.6	6.5±2.8	9.1±3.0	7.8±2.7	9.1±2.8	7.4±2.7	9.1±2.9	7.4±2.7	8.2±2.8	8.2±2.8	8.2±2.8	8.2±2.8	8.2±2.8	7.4±2.7	10.0±3.2	10.2±3.2	9.7±3.2	8.0±2.7	

[a] Nucleotide divergence, above diagonal; amino acid divergence, below the diagonal; analyzed partial S segments of TULV, boxed. The number of singular sequences for each cluster is shown in parentheses. The number of base differences and standard error estimates (500 bootstrap replicates) per site from averaging all sequence pairs among groups are hidden. All results are based on the pairwise analysis of 76 sequences, with 266-nt positions corresponding to 88 aa in the final data set.

TULV RNA in the brain should be proven in a comparative study of different tissue samples from a larger number of voles.

Phylogenetic analyses of the novel S and M segment sequences indicated that these *Microtus*-borne sequences belong to TULV and are clearly separated from sequences originating from other *Microtus*-borne viruses such as PHV, ISLAV, Yakeshi virus, Khabarovsk virus, VLA virus, Vladivostok virus, and Fusong virus.

The high nucleotide divergence level of 12 to 20% among the three novel TULV clades from northeast, northwest, and southeast Germany, the already known clade from central east Germany (32), and clades from other European countries, as well as the Omsk region in the Asian part of Russia, is remarkable. This high level of sequence divergence was even observed among sequences from trapping sites that are only about 200 km or less apart from each other, i.e., in the districts SPN (clade Germany III) (32) and PM (clade Germany II). In contrast to the high level of nucleotide sequence divergence, the level of amino acid sequence divergence was much lower, e.g., only about 1% between clades Germany II and III. This is consistent with strong purifying selection. Taken together, these results showed the presence of at least four clades of TULV sequences in Germany, suggesting a quickly evolving virus species with a strong genetic substructure.

The detection of a large number of novel sequences forming clusters in the phylogenetic tree makes an analysis of the intercluster differences possible. The observed levels of intercluster differences of 5.4 and 7.2% for the novel S segment sequences and 3.5 and 4.9% for the novel M segment sequences of clades Germany I and Germany II, respectively, are in a similar range as those observed for TULV clades Russia I, II, and III. A previous study revealed a range of diversity of 1.5 to 4.9% for the S segment and 0.2 to 1.2% for the M segment between TULV strains circulating within a location of 20 km (43). It is interesting to note that the intercluster differences for 22 partial S segment PUUV sequences derived from bank voles from the city of Cologne, Germany, and for 10 partial S segment PUUV sequences from Lower Bavaria, southeast

TABLE 7. Nucleotide and amino acid divergence among clusters of analyzed partial M segments of TULV and related hantaviruses[a]

	Germany I (5)	Germany II (4)	Poland (2)	Czech R. (2)	Serbia	Yakeshi	Fusong	Khabarovsk (2)	Prospect Hill
Germany I		15.1±1.2	18.1±1.4	17.2±1.4	20.1±1.5	27.9±1.9	26.1±1.6	27.0±1.8	25.6±1.6
Germany II	1.1±0.7		17.4±1.4	18.8±1.4	17.8±1.5	27.6±1.8	23.9±1.6	25.5±1.7	24.7±1.7
Poland	1.5±0.8	2.0±0.9		17.8±1.5	18.1±1.5	26.1±1.8	23.9±1.7	26.3±1.8	23.5±1.6
Czech Republic	1.0±0.6	1.5±0.8	0.5±0.5		13.7±1.3	26.3±1.8	25.5±1.7	23.8±1.7	21.9±1.7
Serbia	1.5±0.7	2.0±0.9	1.0±0.6	0.5±0.5		26.5±1.8	24.4±1.7	26.9±1.8	21.8±1.6
Yakeshi	18.1±2.7	18.1±2.7	17.6±2.7	17.6±2.7	18.1±2.7		24.6±1.7	9.9±1.2	27.7±1.7
Fusong	14.2±2.3	14.2±2.3	15.2±2.3	14.7±2.3	15.2±2.4	10.3±2.2		22.8±1.6	27.7±1.8
Khabarovsk	17.4±2.7	17.4±2.7	16.9±2.6	16.9±2.6	17.4±2.7	1.7±0.9	9.6±2.1		26.6±1.8
Prospect Hill	15.6±2.5	15.7±2.5	14.7±2.5	14.7±2.5	14.2±2.4	19.6±2.9	18.1±2.7	18.9±2.8	

[a] Nucleotide divergence, above diagonal; amino acid divergence, below the diagonal; analyzed partial M segments of TULV, boxed. The number of singular sequences for each cluster is shown in parentheses. The number of base differences and standard error estimates (500 bootstrap replicates) per site from averaging all sequence pairs among groups are indicated. All results are based on the pairwise analysis of 76 sequences, with 614-nt positions and 206 aa in the final data set.

TABLE 8. Cytochrome *b* sequences of *Microtus arvalis* and *M. agrestis* of different genetic lineages[a]

Lineage/outgroup	Strain	GenBank accession no.
M. arvalis Central lineage	Denmark	AY220776
	Germany Rastatt	AY708494
	Germany Dresden	AY708491
	Germany Jena	AY708479
	Germany Heilsbronn	AY708476
	Netherlands	AY220778
	Germany Regensburg	AY708495
M. arvalis Eastern lineage	Hungary	AY220769
	Slovakia	AY220767
	Austria	AY708460
	Czech Republic	AY708471
	Poland	AY220773
	Russia	AY220771
M. arvalis Italian lineage	Switzerland1	AY708512
	Switzerland2	AY708513
	Italy	AY220766
M. arvalis Western lineage	Spain1	AY220788
	Spain2	AY708502
	Belgium	AY708508
	France1	AY220787
	France2	AY708511
M. agrestis Western lineage	France	AY167188
	Mag Sen16/05 Sennickerode Germany	DQ480084
	Netherlands	AY167183
	Germany	AY167210
	Norway	AY167202
	Denmark	AY167179
	Finland	AY167198
	Russia	AY167153
	Czech Republic	AY167151
	Lithuania	AY167176
	Sweden	AY167211
	Poland	AY167185
	Belarus	AY167155
M. agrestis Southern lineage	Switzerland2	AY167161
	Switzerland1	AY167160
	Spain2	AY167163
	Spain1	AY167162
	Portugal	AY167186
M. glareolus outgroup	*M. glareolus* Omsk	AF367079
	M. glareolus Slovenia	AJ867953

[a] The different genetic lineages are included in the phylogenetic analysis shown in Fig. 2.

Germany, were only 1.2% and 3.1%, respectively (11, 12). Compared to these studies, the intercluster sequence differences of TULV clusters Germany I and II were doubled.

In contrast to previous investigations (45), no indication of a quasispecies population in the investigated *Microtus* animals has been obtained. The novel nucleotide sequence from Bavaria (Germany IV) clustered with nucleotide sequences from Austria, Slovakia, Czech Republic, and Croatia, and the previously described sequences from cluster Germany III grouped with sequences from Poland. Although the novel sequences represented by clusters Germany I and Germany II did not cluster with sequences from other parts of Europe in the phylogenetic tree, a multiple amino acid sequence alignment reflected some similarities in the amino acid sequences between the novel strains (clades Germany I and II) and those from clades Germany III and Poland (Fig. 5).

Phylogenetic reconstructions of rodent host relationships based on the mitochondrial cytochrome *b* gene showed a clear differentiation between *M. arvalis* and *M. agrestis*. This is in accord with the current taxonomy of *Microtus* (16). In both species, separation of populations during glacial cycles has led to intraspecific genetic divergence into several evolutionary lineages, which nowadays occupy large regions in Central Europe which do not overlap (19). These evolutionary lineages of *M. arvalis* and *M. agrestis* can be identified by phylogenetic analysis of the cytochrome *b* gene. The new sequences of *M. arvalis* presented here cluster clearly within the Central lineage, an old evolutionary lineage which currently inhabits most of Central Europe (Germany, Denmark, The Netherlands, and Switzerland) (19). Variation in cytochrome *b* was not high enough for a further geographical resolution among rodents at the regional level. All new sequences are distinct from the Eastern lineage of *M. arvalis*. The western border of this lineage is in Poland and the Czech Republic (19). All new cytochrome *b* sequences of *M. agrestis* clustered with the Western lineage of this species. The Western lineage in *M. agrestis* has a very large distribution range, encompassing most of Western, Central, and Eastern Europe and Scandinavia (25, 26). The absence of a substructure within the Western lineage is consistent with detailed analyses of cytochrome *b* in *M. agrestis* (25). Dedicated analyses of more variable genetic markers are needed to resolve the finer genetic structures that exist among local populations within evolutionary lineages of voles (8, 13, 19, 60). However, movements of individual *Microtus* animals are unlikely to exceed a few kilometers (60). The clustering of TULV strains according to locality within the clades Germany I and II is in accord with relatively low levels of migration among regional *Microtus* populations.

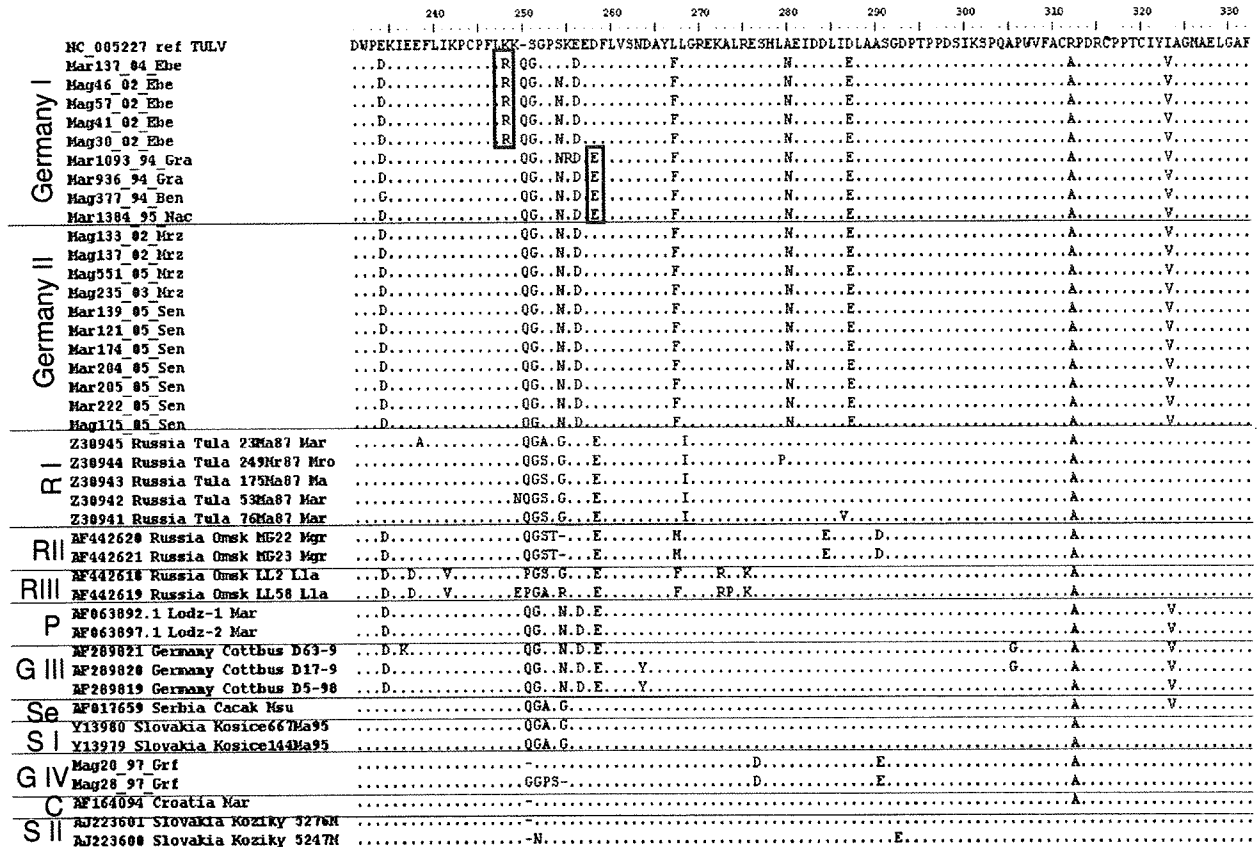

FIG. 5. Hypervariable region of the predicted nucleocapsid proteins of TULV (aa 231 to 332). TULV strain NC_005227 (71) was used as the leader strain for an amino acid alignment. Sequences were aligned in clusters according to the S segment tree in Fig. 3B, and identical amino acid residues in German subclusters are boxed. Amino acid sequences of TULV strains from clusters Austria I and II, Slovakia II, and Slovakia III/Czech Republic are identical to NC_005227 and not included in the alignment. Abbreviations: R I, Russia I; RII, Russia II; RIII, Russia III; P, Poland; G III, Germany III; Se, Serbia; S I, Slovakia I; G IV, Germany IV; C, Croatia; S II, Slovakia II.

The detection of naturally TULV-infected *M. agrestis* contrasts with a previous study, where attempts to infect this rodent species with TULV failed (33). The failure in this experimental setting might have been due to the use of a cell culture-adapted TULV (33), as previously observed for PUUV (39). In addition, the TULV strain Moravia used for the experimental infections contains a stop codon in the putative NSs ORF (71), which may reduce its potential activity as an interferon antagonist (28, 29). Interestingly, in our field study, TULV infections in *M. arvalis* were found at a similar frequency in male and female animals, whereas *M. agrestis* TULV-positive males were significantly more frequent than *M. agrestis* TULV-positive females. This might indicate gender-dependent limitations for the transmission of TULV by spillover infections from *M. arvalis* or by horizontal transmission between *M. agrestis* individuals mediated by gender-specific differences in the TULV-specific immune response, as previously demonstrated for PUUV patients (34). Further studies are required to prove whether differences in the immune response are responsible for the clearance or the establishment of a persistent infection in spillover-infected *M. agrestis*. Additionally, species-, age-, and gender-specific differences in behavior, such as aggression, territoriality, or social status, may contribute to the detected differences in the prevalences. Males tend to be more mobile and aggressive than females in many *Microtus* species (see reference 60 and references therein), which may increase the risk of infection for males overall. However, comparative analyses of space use and social behavior of *M. arvalis* and *M. agrestis* living in sympatry are needed to clarify whether these factors could cause gender-specific differences in infection rates or prevalences between species. In line with previous studies for other hantaviruses (for a review, see reference 39a), our investigations demonstrated a positive association of the age and seropositivity for *M. agrestis*.

Paleozoological and molecular investigations suggest a last common ancestor of *M. arvalis* and *M. agrestis* more than 0.5 million years ago (16, 27). Following the coevolution hypothesis, this ancient separation of the *Microtus* species should have resulted in very distinct genetic lineages of hantaviruses associated with *M. arvalis* and *M. agrestis*. Indeed, the coexistence of two different virus lineages has been described for Dobrava-Belgrade virus in natural foci with sympatric populations of *Apodemus agrarius* and *Apodemus flavicollis* in Slovenia and Slovakia (5, 63). Similar observations were made for different

TABLE 9. TULV S segment sequences included in the phylogenetic analysis[a]

Hantavirus lineage	Strain	GenBank accession no.
TULV Russia I	Tula 76Ma/87	Z30941
	Tula 53Ma/87	Z30942
	Tula 175Ma/87	Z30943
	Tula 249Mr/87	Z30944
	Tula 23Ma/87	Z30945
TULV Russia II	Omsk MG22	AF442620
	Omsk MG23	AF442621
TULV Russia III	Omsk LL2	AF442618
	Omsk LL58	AF442619
TULV Poland	Poland Lodz-1	AF063892
	Poland Lodz-2	AF063897
TULV Germany III	Germany Cottbus D5-98	AF289819
	Germany Cottbus D17-98	AF289820
	Germany Cottbus D63-98	AF289821
TULV Serbia	Cacak	AF017659
TULV Slovakia I	Slovakia Kosice 144/Ma/95	Y13979
	Slovakia Kosice 667/Ma/95	Y13980
TULV Croatia	Velika Gorica	AF164094
TULV Austria I	Austria O24	U95302
	Austria O52	U95303
	Austria O20	U95304
	Austria O64	U95309
	Austria O8	U95313
TULV Austria II	Austria K11	U95305
	Austria K58	U95311
	Austria K26	U95310
	Austria K64	U95312
TULV Slovakia II	Slovakia Malacky Ma32/94	Z48235
	Slovakia Malacky Ma370/94	Z68191
TULV Slovakia III and Czech Republic	Moravia5302v95	Z69991
	Moravia 5294Ma94	Z48741
	Moravia 5302Ma94	Z49915
	Moravia 5286Ma94	Z48573
	Moravia 5293Ma94	Z48574
	Slovakia Koziky 5247Ma/94	AJ223600
	Slovakia Koziky 5276Ma/94	AJ223601
PHV	Prospect Hill	M34011
	Prospect Hill PH-1	Z49098
ISLAV	Isla Vista Mca MC-SB-1	U31534
	Isla Vista MC-SB-47	U19302
	Isla Vista PC-SB-77	U31535
	Isla Vista PC-SB-46	U31530
Yakeshi virus	Yakeshi-Mm-182	EU072484
	Yakeshi-Mm-59	EU072483
	Yakeshi-Mm-31	EU072482
Khabarovsk virus	Khabarovsk	KHU35255
VLA virus	VLA/Nesterikha/Mf500/2005	AM930974
	VLA/Barguzin/Mo483/2005	AM930973
Vladivostok virus	Vladivostok	AB011630
Fusong virus	Fusong-Mf-731	EU072481
	Fusong-Mf-682	EU072480
PUUV	PUUV CRF366 Omsk	AF367071
	PUUV Balkan-1	AJ314600

[a] The phylogenetic analysis is shown in Fig. 3.

TABLE 10. TULV M segment sequences included in the phylogenetic analysis[a]

Virus lineage	Strain	GenBank accession no.
TULV-Poland1	Poland1	AF063891
TULV-Poland2	Poland2	AF063896
TULV-Serbia	Serbia	AF017658
TULV-Czech Republic1	Czech Republic1	Z66538
TULV-Czech Republic2	Czech Republic2	Z69993
PHV	Prospect Hill	X55129
Yakeshi virus	Yakeshi	EU072489
Khabarovsk virus	Khabarovsk	AJ011648
	Khabarovsk	U35254
Fusong virus	Fusong	EU072488
PUUV	PUUV Omsk	AF367061
	PUUV Vranica-Hällnäs	U14136

[a] The phylogenetic analysis is shown in Fig. 4.

New World hantaviruses (for a review, see reference 46). However, there is increasing evidence that besides a general coevolution of the reservoir host and the associated hantavirus species, host switch events might have been played an important role in hantavirus evolution. Such a host switch event has been postulated for the Arvicolinae-associated Khabarovsk virus (72). We found a clustering of TULV sequences depending on the geographical origin but not on the sympatrically occurring *M. arvalis* and *M. agrestis* hosts. In addition, our data may indicate an ongoing process of establishing *M. agrestis* as a novel reservoir host for TULV. These data thus indicate that spillover infections are less rare than believed so far. Moreover, our finding of TULV infections in *M. agrestis* at different time points without any presence of *M. arvalis* might even suggest an already established isolated replication and transmission cycle of TULV in *M. agrestis*. Taken together, these findings and the recent detection of novel hantaviruses in shrews and moles in different parts of the world suggest a richer evolutionary history and more complex transmission dynamics of hantaviruses (2, 20).

The potential for spillover infections between host species depends for instance on the frequency of cooccurrence determined by the specific ecological preferences. *M. arvalis* and *M. agrestis* both have very large distribution ranges in Europe, with regions of overlap extending from Spain into Russia (40). In many regions, *M. arvalis* prefers somewhat drier and more open habitats than *M. agrestis*, but cooccurrence is relatively common (18, 40, 41). Our trapping results confirmed the sympatric occurrence of *M. arvalis* and *M. agrestis* in exactly the same habitats at different places in northeast and northwest Germany. Information on the frequency and nature of interspecific interactions in the field is lacking for these species and other small rodents, but territoriality and the establishment of kin associations suggest aggressive interactions, with biting and scratching as the most likely route of transmission between species (7, 60).

In conclusion, this paper demonstrates that TULV is a promiscuous virus able to infect different *Microtus* species, including *M. arvalis*, *M. agrestis*, *M. rossiaemeridionalis*, *M. gregalis*, *M. subterraneus*, and other related species such as *Lagurus lagurus*. Moreover, initial evidence at one trapping site in Brandenburg suggests that TULV not only causes multiple spillover infections of *M. agrestis* but also seems to establish an isolated replication and transmission cycle in this putative novel reservoir host. Although we cannot rule out coevolutionary mechanisms, the observations described here may be interpreted against the background of the following two alternative evolution mechanisms for hantaviruses. (i) After initial multiple spillover infections of a hantavirus, e.g., TULV, from the established host to a sympatrically occurring potential novel host

in different geographical regions, host adaptation of the virus in the novel host at different geographical localizations may result in a convergent evolution. This would then lead to a change from a geographical clustering to a host-specific clustering of the hantavirus sequences. Therefore, the finally observed host-specific clustering of hantavirus sequences might be, under certain circumstances, misinterpreted as a coevolution mechanism. In line with this assumption, recent studies have postulated that similarities between the phylogenies of hantaviruses and their hosts also seem to result from preferential host switching and local host-specific adaptation (49). (ii) The geographical clustering of hantavirus, e.g., TULV, sequences might have been caused by an isolation-by-distance mechanism. If this hypothesis is true, one would postulate that the viruses are less adapted to their rodent host, e.g., representatives of the genus *Microtus*, allowing frequent spillover or host switch events in overlapping rodent populations. This might be supported by a strong similarity of host receptor molecules that may have evolved slowly since the separation of the different species in a rodent genus, e.g., *Microtus*.

Future investigations on different sympatrically occurring *Microtus* species should address the frequency of spillover and host switch events for TULV and may thus allow for the definition of the host range of TULV and its viral and host determinants. Similar investigations of other hantaviruses and their putative rodent or insectivore hosts should greatly improve our current knowledge on the molecular evolution and host adaptation of hantaviruses.

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Short Report: Chikungunya Virus Isolated from a Returnee to Japan from Sri Lanka: Isolation of Two Sub-Strains with Different Characteristics

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Abstract. A large-scale epidemic of chikungunya (CHIK) fever occurred in several Indian Ocean islands in 2004 and spread to India and Sri Lanka. In December 2006, a returnee to Japan from Sri Lanka developed an acute febrile illness. The patient was confirmed to have CHIK fever after reverse transcription-polymerase chain reaction, and specific IgM and IgG detection. CHIK virus was isolated from the serum specimen collected at the acute stage. The isolated virus developed two different sizes of plaques. Two sub-strains with different genetic and biological characteristics were obtained by plaque purification from one isolate. The entire genome was sequenced and phylogenetic analysis of the E1 genome showed that the sub-strains were of the Central/East African genotype, and were closely related to recent isolates in India. This is the first report of CHIK virus genome sequences isolated from a patient infected in Sri Lanka.

Recent outbreaks of chikungunya (CHIK) fever occurred in coastal Kenya in 2004 and spread to several Indian Ocean islands including Reunion.[1] In Reunion Island, there were ~244,000 cases and at least 213 deaths in the elderly population between March 28, 2005 and April 16, 2006.[2,3] In India, at least 1.4 million cases were suspected during 2005–2007, after a gap of 32 years.[4,5] In Sri Lanka, there were > 40,000 suspected cases of CHIK fever between October 2006 and June 2008, after 40 years without any clinical or serologic reports.[6–10] It has been reported that CHIK virus entered Sri Lanka from India, but there do not seem to be CHIK virus reference sequences from Sri Lanka in any accessible database.[11]

CHIK fever patients have been reported in travelers returning to the United States, Europe, Australia, Hong Kong, Taiwan, and Japan.[12–16] One of the main vectors in transmission between humans is *Aedes albopictus*, which is widely distributed in parts of Europe, the United States, and East Asia. This fact raises the concern that the virus could be introduced and become established in these areas.[17] CHIK fever became epidemic for the first time in Italy, which has a temperate climate, with 205 cases occurring between July 4 and September 27, 2007, and *A. albopictus* was considered the vector.[18] In this study, we confirmed CHIK virus infection in a woman returning to Japan from Sri Lanka.

A 59-year-old Japanese woman stayed in Colombo, Sri Lanka, from November 27 to December 3, 2006. She had lassitude on December 4, 2006 and was admitted with high fever (40°C), myalgias, and arthralgias to Nagaoka Red Cross Hospital in Japan on December 5, 2006. She showed generalized erythematous rash and epistaxis. She reported a history of mosquito bites during her 1-week stay in Sri Lanka. The patient's clinical condition gradually improved, and she was discharged on December 12, 2006, although slight lassitude still persisted.

A serum specimen was collected on December 6 (Day 2) and 12 (Day 8), 2006 and November 5, 2007 (Month 11). CHIK virus-specific IgM was negative for the Day 2 serum but positive for the Day 8 serum using IgM capture ELISA (Table 1). The neutralizing antibody to CHIK virus strain S27 (GenBank accession AF369024) was undetectable in the Day 2 serum but was detected at the titer of 1:20 in the Day 8 serum (Table 2) by 50% plaque reduction neutralizing antibody assay.[19] The Day 2 serum was positive for CHIK virus RNA according to RT-PCR targeted to the *E1* gene, using forward and reverse primers (10294f; 5′-ACGCAATTGAGCGAAGCACAT-3′ and 10573r; 5′-AAATTGTCCTGGTCTTCCTG-3′) (Table 1). Direct sequence analysis of the reverse transcription-polymerase chain reaction (RT-PCR) product using an NCBI BLAST search showed that the genome sequence was 99.3% identical with CHIK virus strain IMT/6466 (DQ462746), which was isolated from cerebrospinal fluid collected from a patient with encephalitis in Reunion in 2006.[20] Real time RT-PCR analysis, using forward and reverse primers (5′-GC RCCMTCTKTAACGGACAT-3′ and 5′-GCCCCCRAAGT CKGAGGAR-3′) with a dual fluorophore-labeled probe (5′-FAM-TACCAGCCTGCACYC-MGB-3′), showed that the Day 2 serum contained 1.8×10^9 RNA copies/mL of CHIK virus RNA (Table 1). Dengue virus-specific IgG, IgM, and real-time RT-PCR were all negative (data not shown). Based on clinical symptoms and the laboratory results, the patient was diagnosed with CHIK fever.

To isolate the virus for virus characterization, the serum specimens obtained on Day 2 and Day 8 were inoculated onto Vero cell cultures (American Type Culture Collection, Rockville, MD), and a cytopathic effect (CPE) was detected 4 days after inoculation with the Day 2 serum but not with the Day 8 serum (data not shown). The virus antigen was detected by indirect immunofluorescence only in cells inoculated with the culture supernatant fluid from Day 2 serum-inoculated Vero cells (Table 1). The culture supernatant fluid was passaged on Vero cells one more time, and the collected culture supernatant fluid was designated as SL. The virus purified from the SL supernatant through the 30–60% (wt/vol) discontinuous sucrose gradient was analyzed by immuno-electron microscopy. Icosahedral-enveloped particles with a diameter of ~70 nm were labeled with anti-CHIK murine hyper-immune ascetic fluid. The morphology and immunology were compatible with that of CHIK virus, indicating that CHIK virus had been isolated (data not shown).

The isolated virus contained in the culture supernatant SL showed two types of plaques: large (5–6 mm in diameter) and

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TABLE 1

Results of laboratory diagnosis of the chikungunya fever patient serum

Serum	IgM	(P/N ratio)	IF	RT-PCR	Real-time RT-PCR (RNA copies/mL)
Day 2	–	(1.14)	+	+	1.8×10^9
Day 8	+	(3.44)	–	–	–
Month 11	–	(1.88)	ND	ND	ND

P/N ratio = positive to negative ratio (> 2.0: positive); IF = indirect immunofluorescence staining; ND = not done; – = results are negative; + = results are positive.

TABLE 2

Neutralizing antibody titer of patient serum against chikungunya virus strain S27, SL10571, and SL11131

	Neutralizing antibody titer		
Serum	S27	SL10571	SL11131
Day 2	< 10	ND	ND
Day 8	20	160	40
Month 11	5,120	10,240	10,240

S27 = S27 strain (African phenotype); ND = not done.

small (1 mm) plaques (data not shown). These two variants were plaque-purified, cloned three times, and passaged once on a Vero cell monolayer to prepare a seed virus. The large plaque variant (SL10571, AB455494) and the small plaque variant (SL11131, AB455493) were established and used in the next experiments.

Two variants, SL10571 and SL11131, were inoculated at an m.o.i. of 0.001 PFU/cell onto Vero and C6/36 cells. The time of inoculation was defined as hour 0. Culture supernatant fluids were collected every 3 hours until 51 hours after inoculation. SL10571 and SL11131 were first detected in Vero cell cultures at 6 and 9 hours after inoculation, respectively (Figure 1A, B). The virus titers were consistently higher with SL10571 than with SL11131 during the first 24 hours (Figure 1A). In Vero cells at 51 hours after inoculation, the peak titers were 1.9×10^8 and 1.2×10^9 PFU/mL for SL10571 and SL11131, respectively. In contrast, in C6/36 cell cultures, CPE was not detected within either variant. The peak titers were 1.8×10^9 and 3.8×10^9 PFU/mL for the SL10571 and SL11131, respectively. These results suggest that SL10571 and SL11131 have different characteristics against Vero cells. Host humoral immunoresponse against SL11131 was four times lower compared with SL10571 at Day 8 (Table 2). The entire genes were sequenced, showing that the SL10571 exhibited four unique amino acid substitutions: nsP1-W456R, R501L, nsP2-M703L, and 6K-T58A; the SL11131 exhibited three unique substitutions, nsP1-R171Q, E3-A53V, and E2-G55R, compared with those of the other CHIK virus strains available in the GenBank database (Table 3). The SL10571, SL11131, and other Indian-06, Italy-07, and Indian Ocean-05-06 isolates exhibited 99.7–99.9% and 99.7–99.9% for non-structural protein and 99.6–99.9% and 99.6–99.9% for structural protein at the nucleotide and amino acid levels, respectively.

A phylogenetic tree was constructed based on nucleotide sequences of the E1 region (Figure 2). Molecular virologic analysis showed that the sub-strains from Sri Lanka belong to the Central/East African cluster and that SL10571 and SL11131 formed a sub-cluster with those isolated in Seychelles 2005, Reunion 2005–2006, Mauritius 2006, India in 2006, Singapore 2006, and Italy in 2007. The two unique amino acids that were reported in all the India-06 and Italy-07 isolates responsible for genetic divergence of Indian viruses were also present in both SL10571, and SL11131: nsP1-M376, and C-S23 (Table 3).[5] Taken together, the results of the phylogenetic analysis and the amino acid sequence analysis suggest that the CHIK sub-strains in this study are related to the recent epidemic strains.

In CHIK virus isolated in September 2005 after the Reunion outbreak, there was an amino acid change from alanine to valine at Position 226 in the E1 envelope glycoprotein. This may have led to an increased rate of CHIK transmission and an increase in severe non-classic symptoms.[3,4] However, the codon specifying valine at Position 226 of E1 was absent in both CHIK SL11131 and SL10571 sub-strains (Table 3).

We isolated two CHIK virus sub-strains from a patient who returned to Japan from Sri Lanka. The patient had only visited

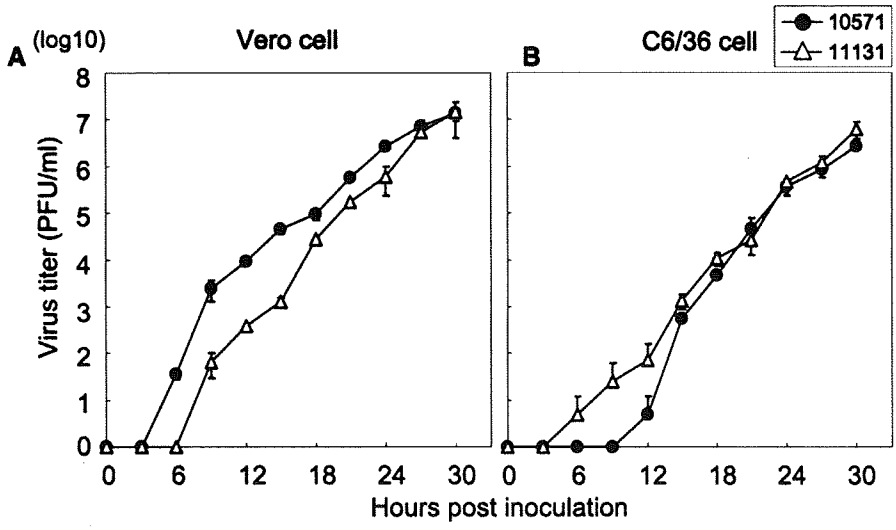

FIGURE 1. Comparison in growth kinetics between two CHIK variants: SL10571 and SL11131. Two variants, SL10571 and SL11131, were inoculated at m.o.i. of 0.001 pfu/cells on Vero (A) and C6/36 cells (B), respectively. Hour 0 was defined as the time of inoculation. Culture supernatant fluids were collected every 3 hours, and the virus titers of the samples were assessed by plaque assay on Vero cells. In Vero cell cultures, SL10571 (•) and SL11131 (Δ) were first detected at 6 and 9 hours after inoculation, respectively (A). The virus titers of SL10571 were consistently higher than those of SL11131 during the 24 hours after inoculation on Vero cells (A) compared with C6/36 cells (B).

TABLE 3
Amino acid substitutions identified in Sri Lanka isolates with respect to India 2006 (IND06AP3) strain

	Non-structural proteins												Structural proteins						
Protein	nsP1								nsP2		nsP3		C		E3		E2		6K	E1
Polypeptide position	128	171	230	314	376	456	488	501	1238	1664	1691	1709	23	27	314	380	471	806	1035
Protein position	128	171	230	314	376	456	488	501	703	331	358	376	23	27	53	55	146	58	226
EF027134 India 2006	K	R	G	M	M	W	R	R	M	A	S	T	S	V	A	G	Q	T	A
AB455494 Sri Lanka (Japan) 2006 'SL10571'*	.	.	.	.	.	R	Q	L	L	V	.	.	.	I	.	.	.	A	.
AB455493 Sri Lanka (Japan) 2006 'SL11131'*	.	Q	.	.	.	.	Q	.	.	V	.	.	.	I	V	R	.	.	.
EF210157 India 2006	.	.	.	L	.	.	.	.	.	.	.	.	.	I	.	.	.	.	.
EU244823 Italy 2007	.	.	R	.	.	.	.	.	.	.	.	I	.	I	.	.	.	.	V
AM258990 Reunion 2005	T	.	.	.	T	.	.	.	.	.	.	.	P	.	.	.	.	.	.
AM258991 Seychelles 2005	T	.	.	.	T	.	.	.	.	.	P	.	P	.	.	.	R	.	.
AM258992 Reunion 2006	T	.	.	.	T	.	.	.	.	.	.	.	P	.	.	.	.	.	V
DQ443544 Reunion (France) 2005	T	.	.	.	T	.	.	.	.	.	.	.	P	.	.	.	.	.	V
EF012359 Mauritius (UK) 2006	T	.	.	.	T	.	.	.	.	.	.	.	P	.	.	.	.	.	V
AF369024 Tanzania 1953 'S27'	T	.	.	.	T	.	Q	.	.	V	.	I	P	.	.	.	.	.	.

* Isolates from a Japanese patient returning from Sri Lanka.
S27 = strain (African phenotype); a dot indicates a match with the amino acid of the CHIK IND06AP3 strain.

Sri Lanka. A noteworthy characteristic of this CHIK case is a high level of viremia (Table 1). The vector mosquito of this virus, *Ae. albopictus*, infests most regions of Japan. Thus, the potential for the establishment of the imported CHIK virus in Japan is a cause for serious concern.[21] The viremic patient returned to Japan during winter, when the activity of *Aedes* spp. was low. This probably reduced the risk of a CHIK outbreak in Japan.

In this study, the viremia did not last long, and CHIK virus-neutralizing antibody and IgM were detected 8 days after the onset of symptoms. Furthermore, the levels of CHIK virus-neutralizing antibody were as high as 1:10,240 at 11 months, but IgM was negative (Tables 1 and 2). It is likely that a high level of viremia induced a rapid and strong host immunoresponse against the CHIK virus. These results are consistent with previous reports that CHIK IgM persisted for several weeks to 3 months and that CHIK virus infection elicited long-lasting protective immunity.[22] Physicians should be aware of the presence of CHIK fever among travelers from disease-endemic areas such as Sri Lanka and India, especially during late summer. In addition to the usual tests for dengue virus, genetic and antibody detection tests for CHIK virus should be performed for patients with fever, particularly when they have joint pain.[14] Furthermore, it should be stressed that imported cases

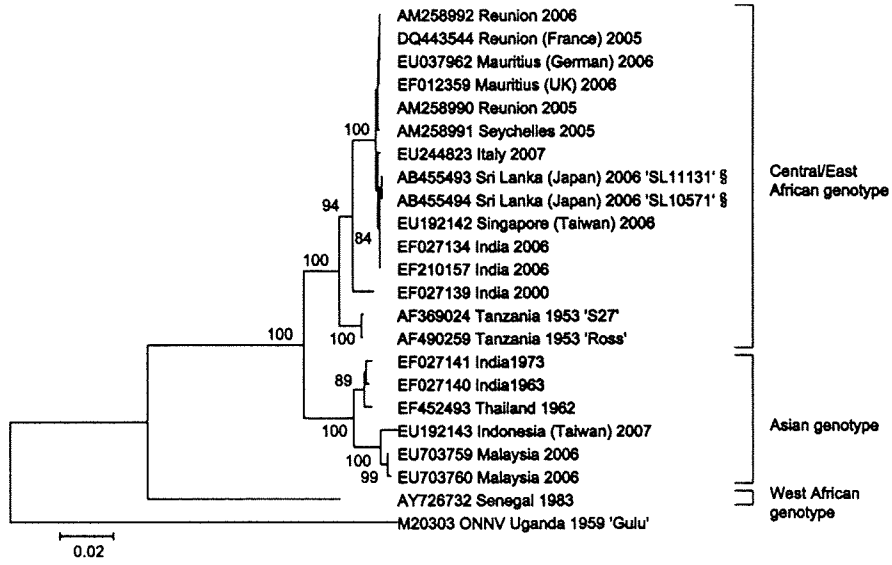

FIGURE 2. Phylogenetic analysis of the chikungunya virus isolates based on the *E1* gene. The neighbor-joining tree was constructed using nucleic acid sequences of the envelope glycoprotein *E1* gene with those of the other CHIK virus strains available in the GenBank database, including S27, Ross (GenBank accession AF490259), IND06AP3, DRDEHydlSW06 (EF210157), ITA07-RA1 (EU244823), 0611aTw (EU19214), 05-115 (AM258990), 05-209 (AM258991), 06-021 (AM258992), Wuerzburg1 (EU037962), LR2006 OPY1 (DQ443544), D570-06 (EF012359), IND73MH5 (EF027141), IND63WB1 (EF027140), AF15561 (EF452493), 0706aTw (EU192143), MY002IMR06BP (EU703759), MY003IMR06BP (EU703760), and 37997 (AY726732) with o'nyong nyong virus (M20303) as the outgroup virus. Sub-strains from the Japanese patient returning from Sri Lanka are indicated with §. Bootstrap values, which are ≥ 75%, are indicated and derived from 1,000 samplings. The scale reflects the number of nucleotide substitutions per site along the branches.

may contribute to the occurrence of a CHIK fever epidemic wherever the competent mosquito vectors are distributed.

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特集 輸入感染症の可能性がある希少感染症

3．チクングニヤ熱

林　昌宏*

チクングニヤ熱は蚊によって媒介されるウイルス性急性熱性疾患で，近年アフリカおよびアジアの熱帯・亜熱帯地域で流行が認められる。現在，わが国におけるチクングニヤウイルスの流行はないが，流行地域からの帰国者における輸入症例はこれまでに2例が報告されている。チクングニヤウイルスは1952年にアフリカのタンザニアで初めてその存在が報告された。「チクングニヤ」とはタンザニア南東部のマコンデ族の言語で，「屈む」あるいは「ねじ曲げる」という意味であり，その病名は日常生活に困難をきたすほどの激しい関節痛を主体とするその臨床症状に由来している。現在，チクングニヤウイルスに対するワクチンはなく，特異的治療法も確立されていない。したがってチクングニヤウイルスの流行地域に渡航する場合は，蚊に吸血されにくい服装や虫除け剤の使用等により蚊の吸血を避ける必要がある。

Key Words：チクングニヤ熱／チクングニヤウイルス／アルボウイルス／ヒトスジシマ蚊／ネッタイシマ蚊

Ⅰ　チクングニヤウイルスの性状

チクングニヤウイルスはトガウイルス科アルファウイルス属に分類される節足動物媒介性ウイルスであり，オニョニョンウイルスとともにセムルキ森林熱ウイルス血清型群に分類される。直径70nmの球状ウイルスで，約12kbの1本鎖のプラス鎖RNAウイルスである（図1）[1]。チクングニヤウイルスのRNA上には5'末端側から非構造タンパク質であるNSP1，NSP2，NSP3，NSP4の4種類の非構造タンパク質がコードされており，続いてコアタンパク質（Cタンパク質），糖タンパク質3（E3），E2，6K，E1の5種類の構造タンパク質がコードされている。チクングニヤウイルスのRNAは5'末端側からの翻訳により非構造タンパク質NSP1-NSP4より構成される大きなポリタンパク質を作る。ポリタンパク質は宿主の酵素および酵素活性を有するウイルスの非構造タンパク質により切断され，各非構造タンパク質となる。非構造タンパク質は宿主細胞内でウイルスRNAの複製を行い，この過程で（－）RNAが形成される。この（－）RNAからサブゲノミックRNAが転写され，構造タンパク質より構成されるポリタンパク質が翻訳される。その後，ポリタンパク質は各ウイルス構造タンパク質となりウイルス粒子を形成する。（図2）

Ⅱ　チクングニヤウイルスの流行状況

1．チクングニヤウイルスの分布状況

チクングニヤウイルスの分布地域は，おもな媒介蚊であるネッタイシマ蚊 *Aedes. aegypti* およびヒトスジシマ蚊 *Ae. albopictus* の存在するアフリカ，南アジア，東南アジアの熱帯・亜熱帯地域である（図3）[2]。チクングニヤウイルスは1950年

Chikungunya fever

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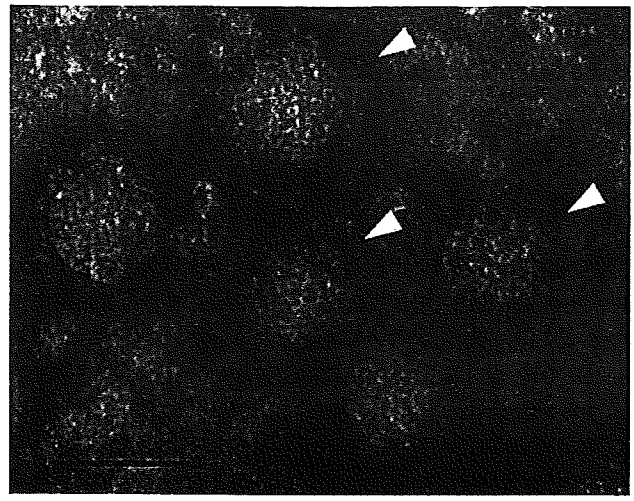

図1　チクングニヤウイルスの電子顕微鏡像
チクングニヤウイルスは直径約70nmのエンベロープを被った球状ウイルス（矢頭）である（Barは100nm）。

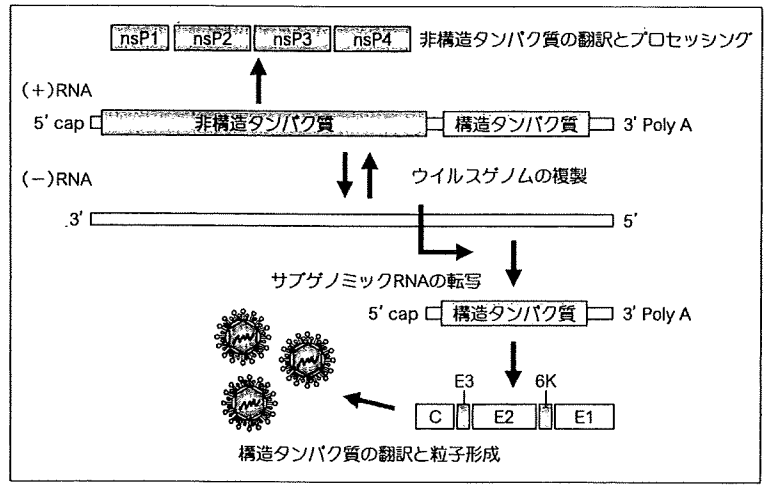

図2　チクングニヤウイルスの遺伝子構造
チクングニヤウイルスのRNA上には5'末端側から非構造タンパク質であるNSP1，NSP2，NSP3，NSP4の4種類の非構造タンパク質がコードされており，続いてコアタンパク質（Cタンパク質），糖タンパク質3（E3），E2，6K，E1の5種類の構造タンパク質がコードされている。チクングニヤウイルスのRNAは5'末端側からの翻訳により非構造タンパク質NSP1-NSP4より構成される大きなポリタンパク質を作り，各非構造タンパク質を形成する。非構造タンパク質は宿主細胞内でウイルスRNAの複製を行い，この過程で（−）RNAが形成される。この（−）RNAからサブゲノミックRNAが転写され，構造タンパク質よりなるポリタンパク質が翻訳される。ポリタンパク質は各ウイルス構造タンパク質となりウイルス粒子が形成される。

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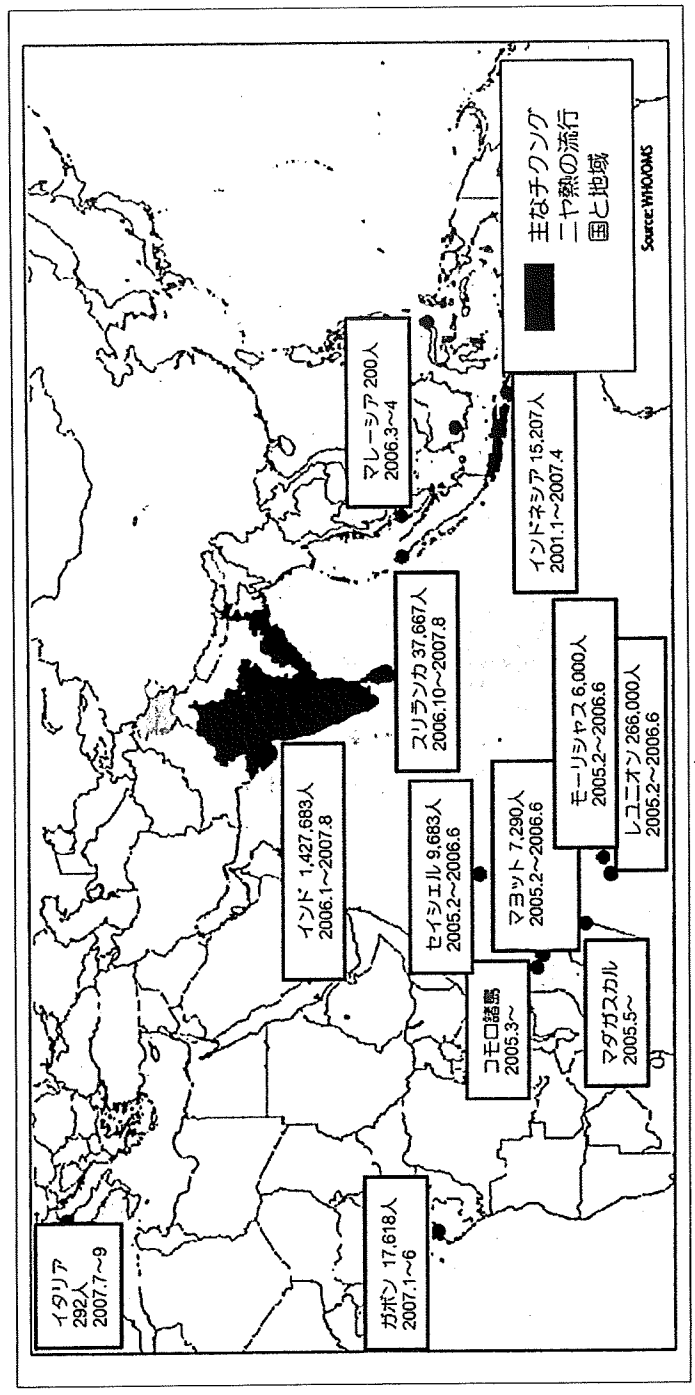

図3 2001～2007年におけるチクングニヤ熱のおもな流行状況

インド洋周辺で発生した中央・東アフリカ型のチクングニヤウイルスは，インド，スリランカ，イタリア，東南アジアに広がった。また東南アジアではインドネシア，マレーシアでアジア型の再興も認められ，2種の遺伝子型が流行している。

［世界保健機構（WHO）の発表より改変］

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代～1970年代の流行株の遺伝子解析により，中央・東アフリカ型，西アフリカ型，アジア型の3種の遺伝子型に分類されている。1952年にタンザニアで初めて報告されたチクングニヤウイルスは中央·東アフリカ型であり，その後，ウガンダ，ジンバブエ，南アフリカ，中央アフリカ，コンゴ等で報告された。また1999年～2000年にかけてはコンゴで39年ぶりに再興し，50,000人の患者が報告され，その流行はさらに2006年にカメルーンに拡大した。西アフリカ型の流行は1964年にナイジェリア，1966年，1983年にセネガルで発生している。東南アジアでは1958年に初めてタイでアジア型のウイルスが報告され，カンボジア，ベトナム，ラオス，ミャンマー，マレーシア，フィリピン，インドネシア等で報告された。2001年にはインドネシアで20年ぶりに再興し，1998年，2006年にはマレーシアでも流行したが，いずれの流行もアジア型のウイルスによるものである。

2．インド洋諸島におけるチクングニヤ熱とインド・イタリアへの流行地域拡大

現在，インド洋諸島からインドにかけて中央・東アフリカ型のチクングニヤウイルスが流行している。インド洋周辺における最初の発生は2004年にケニアで報告され，2005年にはコモロ諸島，レユニオン，セイシェル，マダガスカル等のインド洋諸島に拡大し，レユニオン島では総人口770,000のうち264,000人が感染し237人の死亡が報告された[3]。インドでは1963年～1973年にかけてアジア型の流行が主であったが，その後チクングニヤウイルスの流行は認められなかった。しかし，2005年に32年ぶりにインドで患者が報告され，流行は南部諸州に拡大した。この流行は中央・東アフリカ型によるものであり，少なくとも140万人の患者が報告された。インドでの流行は，スリランカ，イタリア，シンガポールへと拡大し，スリランカでは2006年から2008年7月までのあいだに4万人の患者が推計されている。またイタリア北部では2007年7月～9月のあいだに205人の患者が報告された[4]。イタリアでの流行の媒介蚊はわが国にも分布するヒトスジシマ蚊であり，熱帯・亜熱帯地域以外での初めての流行となった（図2）[5][6]。

3．日本および世界におけるチクングニヤ輸入症例

これまでに日本においてチクングニヤウイルスが常在していることを示す報告はなく，スリランカから帰国後の女性から2006年12月4日にチクングニヤ熱輸入症例が発生した。厚生労働省の発表によれば，患者はスリランカに1週間滞在し帰国した50代の女性である。高熱と関節痛を呈し実験室内診断の結果，チクングニヤウイルスと診断された。また同年12月11日に帰国した女性から，やはりスリランカからの輸入症例が確認された[7]。患者はスリランカに在住する30代の女性で，11月17日に発熱，関節痛などで発病した。軽快後帰国したが，関節痛が持続するため東京都内医療機関を受診した。抗体検査の結果，チクングニヤウイルスに対する特異的IgM（免疫グロブリンM）抗体陽性，中和抗体陽性であり，チクングニヤ熱と確定診断した。いずれの症例においても，デングウイルスに関するIgM抗体，IgG（免疫グロブリンG）抗体は陰性でデングウイルス感染は否定された。チクングニヤ熱の輸入症例は日本以外にも，フランス，イギリス，ドイツ，香港，台湾，オーストラリア，米国等で報告されている[8]。

Ⅲ　チクングニヤウイルスの生態と感染環

チクングニヤウイルスは自然宿主であるヒトやサルと蚊のあいだで感染環を形成・維持している（図4）。主要なチクングニヤウイルスの媒介蚊はシマカ群（*Ae. spp.*）に属する蚊である。チクングニヤウイルスのおもな媒介蚊はネッタイシマ蚊であるが，一方，近年のインド洋諸島およびイタリアでのチクングニヤウイルスの流行はヒトスジシマ蚊の媒介によるものである。チクングニヤウイルスの感染環は森林型，都市型に分けられる。森林

IgM（免疫グロブリンM）　IgG（免疫グロブリンG）

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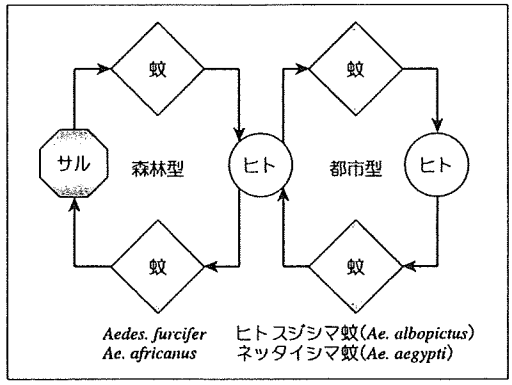

図4 セネガルにおけるチクングニヤウイルスの感染環

チクングニヤウイルスはサルと蚊のあいだで森林型の感染環を維持しており，ヒトと蚊のあいだで都市型の感染環を形成する。ヒトが感染した場合，一般的にウイルス血症は高く感染源となるため大流行を起こす。

型はアフリカに広がり，サル，リス，コウモリ等と蚊のあいだで感染環が維持されている。森林型を媒介するおもな蚊は，アフリカでは *Ae. furcifer* および *Ae. africanus* である。アジアではフィリピンにおける疫学調査により，カニクイザルのチクングニヤウイルスによる感染が報告されている[9]。都市型はヒトと蚊のあいだで感染環が成立した流行型である。都市型サイクルを媒介する主要な蚊はアフリカ，アジアいずれの流行地域でもネッタイシマ蚊およびヒトスジシマ蚊である[10]。急性期のヒトにおけるウイルス血症は非常に高いため，吸血した蚊がチクングニヤウイルスに感染する可能性はきわめて高く，輸入症例からチクングニヤウイルスの感染が拡大する危険性がある[8]。

Ⅳ チクングニヤ熱の臨床症状

ヒトにおける潜伏期間は2～12日で多くは不顕性感染に終わるが，チクングニヤ熱を発症すると，発熱，全身倦怠，リンパ節腫脹，頭痛，筋肉痛，一過性の発疹，亜急性の関節炎を呈する。また出血傾向（鼻出血・歯肉出血）や悪心・嘔吐をきたすこともある。関節炎は特に指関節，手根関節，趾関節，足関節に多発する。関節痛が数日～数カ月持続する場合，激しい関節痛および多発性腱滑膜炎をともなう慢性末梢性リューマチ様症状を呈し，日常生活に困難をともなう。おもな血液所見はリンパ球減少および血小板減少である。近年のレユニオン島での流行の特徴は，特に高齢者に多くの死亡例が報告されていることであるが，これらの例では，呼吸器不全，心代償不全，髄膜脳炎，劇症肝炎，腎不全等が報告されている[11]。

Ⅴ 診断と鑑別診断

チクングニヤウイルスの診断

チクングニヤウイルスの日本への侵入は現在まで起こっていないため，臨床診断には，チクングニヤウイルス侵淫地域を2週間以内に訪問した経歴があること，またはこの地域からの訪問者であること等の確認が重要である。チクングニヤ熱は他のウイルス性熱性疾患，特にデング熱・出血熱との鑑別を必要とすることから，チクングニヤ熱の確定診断には実験室診断が必須である（**表1**）。確定診断にはチクングニヤウイルスの病原体検査と血清学的検査が必須である。前者は血清からのウイルス分離あるいはウイルス遺伝子の検出を目的とし，後者はIgM捕捉ELISA（Enzyme-linked immunosorbent assay），中和抗体試験，HI（赤血球凝集抑制）試験，CF（補体結合反応）試験等による抗ウイルス抗体の検出を目的とする。ウイルス分離は急性期患者の血清，血漿を用いて乳のみマウスの脳内接種，動物および昆虫由来培養細胞への接種，蚊の胸部接種にて行う。ウイルス遺伝子の検出にはRT-PCR（逆転写ポリメラーゼ連鎖反応）法，リアルタイムPCR（TaqMan PCR）法を用いる。ウイルス遺伝子は患者の血清，血漿より精製し，RT-PCR法，リアルタイムPCR法に供試して陽性および陰性コントロールを基準に目的領域の特異的増幅を確認する。血清学的検査

ELISA (Enzyme-linked immunosorbent assay)
CF (補体結合反応)
HI (赤血球凝集抑制)
RT-PCR (逆転写ポリメラーゼ連鎖反応)

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表1　実験室診断の基準

○診断した医師の判断により，症状や所見から当該疾患が疑われ，かつ以下のいずれかの方法によって病原体診断や血清学的診断がなされたもの
・病原体の検出（例，血液等からのウイルス分離など）
・病原体の遺伝子の検出（例，PCR法など）
・病原体に対する抗体の検出
（例：血清中のチクングニヤウイルス特異的IgM抗体の検出，特異的IgG抗体価のペア血清での4倍以上の上昇など）

は抗ウイルス抗体の検出を目的とし，特異性の高いIgM抗体捕足ELISA，急性期（発病後5日以内）および回復期（発病後14日以上）のペア血清を用いた中和試験などを行う。プラーク法による中和試験は，アフリカミドリザル腎細胞由来のVero細胞を用いて攻撃ウイルスと各被検血清の中和反応を行い，プラーク低減法により患者・患畜の血清中の中和抗体価を算出する方法である。本検査方法はチクングニヤウイルスを使用するためにP3施設で行う。診断においては同じアルファウイルス属のウイルスによって発症するオニョニョン熱，ロスリバー熱，西部ウマ脳炎，東部ウマ脳炎，ベネズエラ脳炎またフラビウイルス属のウイルスによって発症するデング熱・出血熱が上げられる。デング熱・出血熱はその流行地域および臨床症状が重なるため，特に鑑別疾患として重要である。また熱帯地域で流行している他の発熱性疾患（マラリア，腸チフス，発疹チフス，インフルエンザ，レプトスピラ症）との鑑別も重要である。

Ⅵ　チクングニヤウイルスに対する治療と予防

一般にチクングニヤウイルス感染症に対する特異的治療法は確立されていないため，対症療法が中心である。フランスのグループの報告によれば，抗炎症剤および鎮痛薬の処方によっても関節痛および関節の硬直の回復には顕著な効果は認められず，回復に数カ月の時間をともなった症例が報告されている[12]。またインターフェロンαとリバビリンの併用による抗ウイルス効果が*in vitro*において報告されているが，臨床的応用の報告はない。さらにチクングニヤウイルスに対する弱毒生ワクチンがこれまでに米国で開発されたが，実用化されていない。したがって，チクングニヤウイルスの侵淫地域においては，チクングニヤウイルスに感染するリスクを減らすため蚊に刺されることを避けることが重要である。蚊による吸血を避けるためには，① 蚊との接触を防ぐため肌の露出をさけること，② N,N-diethyl-3-methylbenzamide（DEET：ディート）等を含む虫除け剤を適切に使用すること，③ 蚊の吸血時間帯である夕方から夜明けにかけては外での活動を避け，やむをえない場合は忌避剤を使用すること等が重要である。虫除け剤は過剰に使用する必要はなく，露出した肌や衣服の上から全身に行き渡るように使用し，衣服の下には使用しない。また長期滞在者は，蚊の防除・駆除および蚊の繁殖を抑制することが必要である。そのためには家屋における蚊の侵入を防ぐスクリーン（網）等を設置し，必要のないときは窓や戸を閉じる等の対策をとる。また，媒介蚊の発生源となる溜まり水の除去，屋内の空き缶，花瓶，水瓶等の適切な管理を行うことも重要である。地域社会においては，蚊の繁殖可能な古タイヤ，空き缶などの廃棄物の除去，屋外イベントでの忌避剤使用の奨励などが必要となる。日本のようにまだ国内感染の認められない地域においても，万が一ウイルスが侵入した場合に備え，蚊との接触防止と駆除が重要である。2005年にはスリランカから日本への輸入症例が発生したことからも，流行地域に渡航する場合は特に注意を必要とすることが再確認されるべきである。チクングニヤウイルス流行地での滞在によりチクングニヤウイルスの感染が疑われた場合は，すみやかに信頼できる医療機関を受診する必要がある。

DEET（N,N-diethyl-3-methylbenzamide；ディート）

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Ⅶ 感染症法における取り扱い

チクングニヤ熱は,「感染症の予防及び感染症の患者に対する医療に関する法律」(感染症法) および検疫法に指定されていないが, デング熱・出血熱 (四類感染症) の鑑別疾患として特に重要である。これらの疾患については国立感染症研究所において実験室内診断が実施可能であり, チクングニヤ熱を疑う患者を診察した医師は最寄りの保健所あるいは国立感染症研究所に相談する必要がある。

Ⅷ おわりに

急速な輸送手段の発達とネッタイシマ蚊, ヒトスジシマ蚊の分布拡大, 熱帯雨林地域への人口拡張により, 世界の熱帯・亜熱帯地域においてチクングニヤ熱の流行は今後もしばらく続くことが予想される。さらに2006年にはわが国において初めて輸入症例も報告された。チクングニヤ熱の治療法は確立されておらず, チクングニヤウイルスの動向には, ヒト, 蚊, 気候, 環境等の要因が複雑に関わり, その状況の予測は困難であるためチクングニヤウイルスのわが国への侵入は予断を許さない。したがって, 日本においては輸入症例に対する医療ならびに保険衛生関係者の迅速な対応が求められており, 特に関節痛あるいは筋肉痛をともなう発熱疾患を示す熱帯・亜熱帯地域からの帰国者を診察した医師は, デングウイルスとともに本疾病を考慮に入れる必要がある。現在, 医療関係者, 公衆衛生関係者や科学者等の努力によりチクングニヤウイルスに関する知見が得られており, 国内においても国立感染症研究所等いくつかのウェブページ等を通じてチクングニヤウイルスの情報が提供されている (一例として http://www.nih.go.jp/vir1/NVL/NVL.html)。ワクチンが実用化されていない現在, 旅行先におけるチクングニヤ熱の流行状況を把握し, 蚊対策を十分考慮することおよび, 医療機関, 地域住民, 行政, 研究機関の一層の協力体制確立も重要である。

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注目されるウイルス

チクングニヤウイルス

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要旨 チクングニヤウイルス感染症は近年，世界の熱帯・亜熱帯地域を中心に流行が認められる蚊媒介性の再興感染症である．2007年にはイタリアにおいても流行が確認され，温帯地域における初めての流行として注目された．イタリアのチクングニヤ熱流行は日本にも生息するヒトスジシマカにより媒介された．日本でもこれまで5例のチクングニヤ熱輸入症例が報告されていることから，日本に侵入する可能性は否定できない．チクングニヤウイルスに対するワクチンは実用化していないため媒介蚊対策や流行状況の把握等に基づく対策が重要である．

チクングニヤウイルス（chikungunya virus：CHIKV）はアフリカ，南アジア，東南アジアの熱帯および亜熱帯地域で流行しているチクングニヤ熱の原因ウイルスである（図1）[1]．チクングニヤ熱は発熱，関節痛，筋肉痛，発疹を伴う急性疾患である．「チクングニヤ」とはマコンデ語で「屈む」あるいは「ねじ曲げる」を意味するが，これはチクングニヤ熱の激しい関節痛を伴う臨床症状に由来する．近年チクングニヤ熱はインド洋諸島を中心に大流行し，その流行はインド，東南アジアへ拡大した．インドの流行では少なくとも140万人の患者が報告されている．チクングニヤ熱の輸入症例も米国，ヨーロッパ，オーストラリア，香港，台湾等で報告されている．日本においてもこれまでに5例の輸入症例が報告された．都市部における媒介蚊はネッタイシマカ（*Aedes aegypti*）およびヒトスジシマカ（*A. albopictus*）である．ヒトスジシマカはヨーロッパ，米国，東アジアに広く分布している．またイタリアで初めて報告されたチクングニヤ熱の流行はヒトスジシマカによって媒介された．これは温帯地域での初めての流行であり，チクングニヤウイルスが媒介蚊の存在するいずれの地域にも侵入する可能性を示唆したため大きな注目を集めた．

■チクングニヤウイルスの性状

チクングニヤウイルスはトガウイルス科アルファウイルス属セムリキ森林ウイルス血清型群に分類されるエンベロープを被った直径約70nmの球状ウイルスである（図2）[2]．遺伝子は11,805塩基の一本鎖RNAであり，5'端にキャップ構造を3'端にポリAを有する（図3）．ゲノムRNAは2つのオープンリーディングフレームからなる[2]．5'端側の2/3の領域はnsP1，nsP2，nsP3，nsP4の4つの非構造蛋白質をコードする．残りの3'端の1/3の領域はCP，E3，E2，6K，E1の5つの構造蛋白質をコードする．構造蛋白質領域はキャ

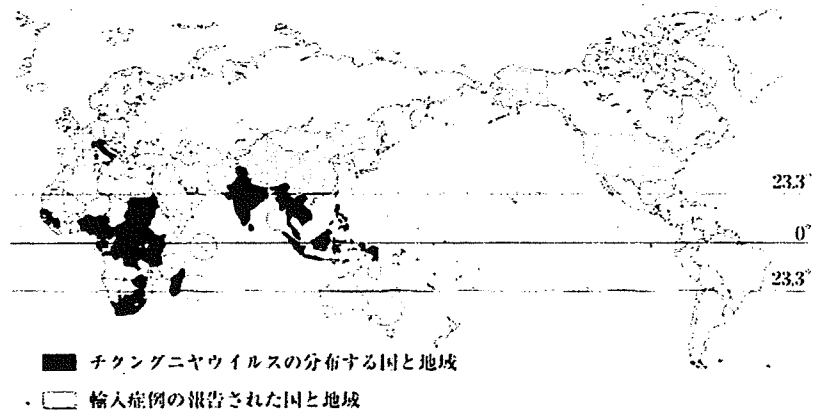

図1　チクングニヤウイルスの分布
チクングニヤウイルスはサハラ砂漠以南のアフリカ，南アジア，東南アジアに分布する

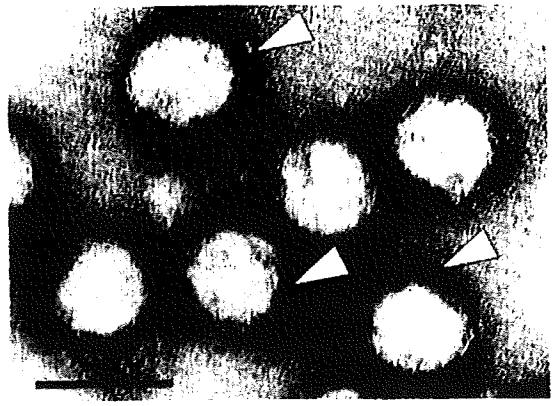

図2　チクングニヤウイルスの電子顕微鏡像
チクングニヤウイルスS27株を30～60%のショ糖密度勾配で精製し電子顕微鏡で観察した．矢頭はウイルス粒子を示す（bar＝100nm）

ップ構造とポリA構造を有する26SのサブゲノミックmRNAよりポリ蛋白質として翻訳される．チクングニヤウイルスのエンベロープにはウイルスの2つの糖蛋白質E1，E2が存在する．それぞれの分子量は約50kDaである．エンベロープは約30kDaのCP蛋白質からなる直径約40nmのヌクレオキャプシッド（コア）を被っている．非構造蛋白質はウイルス遺伝子の複製とサブゲノミックRNAの翻訳に必要である．

■チクングニヤウイルスの疫学的状況

1. アフリカおよびインド洋諸島

チクングニヤウイルスはサハラ以南のアフリカに広く分布している[3]．1952～1953年にタンザニアの熱性疾患患者から初めてウイルスが分離されて以来，60～80年代にかけてコートジボワール，ナイジェリア，セネガル等で繰り返し分離された．1999～2007年にコンゴ民主共和国，カメルーン，ガボンにおいてチクングニヤ熱が再興し，コンゴでは推計5万人の患者が報告された．

インド洋南西諸島では2004～2006年の間に推定50万人のチクングニヤ熱患者が報告されている．2004年にケニア沿岸のラム島で13,500人（全人口の75%）の患者が報告され，さらに2005年1～5月にかけてコモロ諸島およびモーリシャスに流行が拡大した．グランデ・コモロ島では人口の63%にあたる推計215,000人の患者と5,202人の確定診断患者が報告された．2005年末にはマヨッテ（推計2,833人），モーリシャス（推計6,000人，確定1,200人），セイシェル（推計8,818人），マダガスカル，レユニオン等の他の島々に流行が拡散した．最も流行の激しかったレユニオン島では推計244,000人（全人口の34%）の患者が2005年3月28日から2006年4月16日の間に