Analyses of genotoxicity induced by dichloromethane and 1,2-dichloropropane, being responsible for occupational bile duct cancer, （ポスター）	後藤正憲、戸塚ゆ加里、土原一哉、三牧幸代、尾島英知、柴田龍弘、落合淳志、中釜斉	横浜（日本癌学会第73回大会）	2014年9月	国内
A multidisciplinary approach to explore the etiology of esophageal cancer in China（口演）	Totsuka Y, Lin Y, Kato M, Shibata T, Matsushima Y, Nakagama H	横浜（日本癌学会第73回大会）	2014年9月	国内
Identification of the location of DNA adducts within the genome（ポスター）	椎崎 一宏、 後藤 正憲、荒井 康仁、 濱 奈津子、柴田 龍弘、 中釜 斉、戸塚 ゆ加里,	横浜（日本癌学会第74回大会）	2014年9月	国内
職業性胆管癌及びジクロロプロパン曝露細胞の塩基置換シグネチャー解析（口演）	後藤正憲、三牧幸代、久保正二、土原一哉、中釜斉、戸塚ゆ加里	日本環境変異原学会第43回大会	2014年12月	国内
ゲノム中のDNA修飾の単分子検出方法の検討（ポスター）	椎崎 一宏、 後藤 正憲、濱 奈津子、荒井 康仁、柴田 龍弘、 中釜 斉、戸塚 ゆ加里	日本環境変異原学会第43回大会	2014年12月	国内
非遺伝毒性発がん物質、1,4-ジオキサン投与ラット肝臓におけるDNA付加体の網羅解析（ポスター）	秋元峻太朗、加藤 護、柴田龍弘、遠藤 治、魏 民、中釜 斉、鰐淵英機、福島昭治、戸塚ゆ加里	日本環境変異原学会第43回大会	2014年12月	国内
中国における食道癌発症要因の集学的アプローチによる解明（口演）	池田 茜、加藤 護、柴田龍弘、黒坂 功、林 櫻松、松島芳隆、遠藤 治、中釜 斉、戸塚ゆ加里	日本環境変異原学会第43回大会	2014年12月	国内

２．学会誌・雑誌等における論文掲載

掲載した論文（発表題目）	発表者氏名	発表した場所（学会誌・雑誌等名）	発表した時期	国内・外の別
Newly defined aberrant crypt foci as a marker for dysplasia in the rat colon.	Ochiai M, Hippo Y, Izumiya M, Watanabe M, Nakagama H	Cancer Sci. 105:943-950	2014年8月	国内
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学 会 等 発 表 実 績

委託業務題目「アジア地域にまん延している疾病に関する研究」
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担当責任者　稲垣　暢也（研究者氏名）

１．学会等における口頭・ポスター発表

発表した成果（発表題目、口頭・ポスター発表の別）	発表者氏名	発表した場所（学会等名）	発表した時期	国内・外の別
Efficacy and safety of trelagliptin, a novel once-weekly oral DPP-4 inhibitor: a phase 3, double-blind, non-inferiority study in Japanese type 2 diabetes mellitus patients /poster	Inagaki N, Onouchi H, Sano H, Kuroda S, Kaku S.K.	50th EASD Annual Meeting	2014.9	国外開催(国際会議)
Efficacy and Safety of Canagliflozin in Japanese Patients with Type 2 Diabetes Mellitus Inadequately Controlled with Diet and Exercise over 24 Weeks /poster	Inagaki N, Kondo K, Yoshinari T, Maruyama N, Takahashi N, Susuta Y, Kuki H.	ADA's 74th Scientific Sessions	2014.6	国外開催(国際会議)

２．学会誌・雑誌等における論文掲載

掲載した論文（発表題目）	発表者氏名	発表した場所（学会誌・雑誌等名）	発表した時期	国内・外の別
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Review

Antigenic Properties of N Protein of Hantavirus

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Abstract: *Hantavirus* causes two important rodent-borne viral zoonoses, hemorrhagic fever with renal syndrome (HFRS) in Eurasia and hantavirus pulmonary syndrome (HPS) in North and South America. Twenty-four species that represent sero- and genotypes have been registered within the genus *Hantavirus* by the International Committee on Taxonomy of Viruses (ICTV). Among the viral proteins, nucleocapsid (N) protein possesses an immunodominant antigen. The antigenicitiy of N protein is conserved compared with that of envelope glycoproteins. Therefore, N protein has been used for serological diagnoses and seroepidemiological studies. An understanding of the antigenic properties of N protein is important for the interpretation of results from serological tests using N antigen. N protein consists of about 430 amino acids and possesses various epitopes. The N-terminal quarter of N protein bears linear and immunodominant epitopes. However, a serotype-specific and multimerization-dependent antigenic site was found in the C-terminal half of N protein. In this paper, the structure, function, and antigenicity of N protein are reviewed.

Keywords: nucleocapsid; monoclonal antibody; epitope

1. Introduction

Species of the genus *Hantavirus* are classified in the family *Bunyaviridae.* Rodents, shrews, and bats are persistently infected with hantaviruses, whereas humans are infected accidentally, occasionally causing severe illness. Among rodent-borne hantaviruses, the causative agents of two important

zoonoses are known: hemorrhagic fever with renal syndrome (HFRS) and *Hantavirus* pulmonary syndrome (HPS) [1,2].

Like other viral members of the *Bunyaviridae* family, hantaviruses are enveloped RNA viruses that contain three-segmented negative-sense RNAs, designated S, M, and L based on the molecular weight of their virion. The S, M, and L RNA segments encode nucleocapsid protein (N), envelope glycoproteins (Gn and Gc), and RNA-dependent RNA polymerase (L) protein, respectively [3]. Nonstructural (NS) protein was detected in the S genome segment of some hantaviruses as a candidate interferon antagonist [4–6]. Among the structural proteins, N protein is the most abundant in the hantavirus virion and accumulates in the cytoplasm of infected cells. Since N protein is immunodominant, diverse recombinant N proteins produced by various expression systems have been applied as diagnostic antigens to detect hantavirus-specific antibody (US patent number: 5614193) [7–10].

In addition to its value as a diagnostic antigen, N protein exhibits RNA-binding activity [11–13] and multimerization [14], which are crucial functions for the encapsidation of the viral genome in the virion [15]. Furthermore, N protein has been suggested to play an important role in the initiation of transcription and translation of the hantavirus genome. Thus, it is a multifunctional protein that contributes to not only virus encapsulation and assembly but also to the translation and transcription of genomic RNA to complete the viral lifecycle.

Each species of *Hantavirus* appears to have a single predominant rodent, shrew, mole, or bat species that serves as its natural reservoir [16–20], probably because of the co-evolution of hantaviruses with their animal reservoirs. Rodent-borne hantaviruses comprise three large groups according to their host rodents: Murinae-, Arvicolinae-, and Sigmodontinae/Neotominae-associated. Due to the close association between rodent species and hantaviruses, their geographical distributions are the same. Therefore, Murinae- and Arvicolinae-associated hantaviruses, which are distributed in Eurasia, are called Old World hantaviruses. Alternatively, Sigmodontinae/Neotominae-associated hantaviruses are distributed in North and South American countries and are called New World hantaviruses [21]. Causative agents of HFRS and HPS are exclusive to rodents. Murinae-associated hantaviruses include causative agents of HFRS: Hantaan virus (HTNV), Seoul virus (SEOV), and Dobrava virus (DOBV). Thailand virus (THAIV), which is carried by *Bandicota indica,* is suspected of being pathogenic to humans in Asia [22]. Arvicolinae-associated hantaviruses are distributed throughout Eurasia, and Puumala virus (PUUV) is a causative agent of HFRS that has been called nephropathia epidemica (NE) in Northern Europe. Numerous Arvicorinae rodent-borne hantaviruses have been reported from both the Old World and New World. Among them, only PUUV is known to be pathogenic. The group of Sigmodontinae/Neotominae-associated hantaviruses, Sin Nombre virus (SNV), Andes virus (ANDV), Laguna Negra virus (LANV), and variable HPS-related hantaviruses were found in North and South America [23,24]. Hantavirus N protein shares a common antigenic site with each group.

Here, we review the antigenic properties of epitopes on hantavirus N protein, particularly in relation to the structure, virus species and function.

2. Hantavirus N Protein

2.1. Antigenic Profiling of N Protein Using Monoclonal Antibodies (MAbs) and Polyclonal Antibodies

The initial research using MAbs against N protein of HTNV, SEOV, and PUUV was reported by Ruo and coauthors [25]. Most clones against HTNV and SEOV were cross-reactive against two viruses but not cross-reactive with PUUV. Also, one serotype-specific clone against HTNV was established. Yoshimatsu *et al.* confirmed the binding region of MAbs produced by Ruo *et al.* All clones excluding the serotype-specific form were found to bind with the N-terminal part of N [26]. On the other hand, Lundkvist and coauthors reported MAbs against PUUV [27]. All clones against PUUV exhibited unique epitopes distinct from HTNV and SEOV. Among eleven clones, six were PUUV-specific. The clone 3H9 was a PUUV-specific antibody and its epitope was determined as amino acids (aa) 251 to 260 (VKPGTPAQEI) using the pepscan assay [28]. However, epitopes of other MAbs were not determined with the same assay [28]. Elgh *et al.* showed that most PUUV-specific MAbs bind to 100 aa of the N terminus of PUUV N [29]. MAbs against N protein of New World hantaviruses and against ANDV and Carizale virus (CARV) [30,31] were reported. In those studies, group-common, genus-common, and serotype-specific epitopes were found within the N-terminal region of N protein. MAbs against the N-terminal 120 aa of N of PUUV showed cross-reactivity against New World hantaviruses, and these antibodies were useful for detecting antigens in immunohistochemical assays [32]. These results obtained by using MAbs indicate that the N terminus of hantavirus N protein is immunodominant.

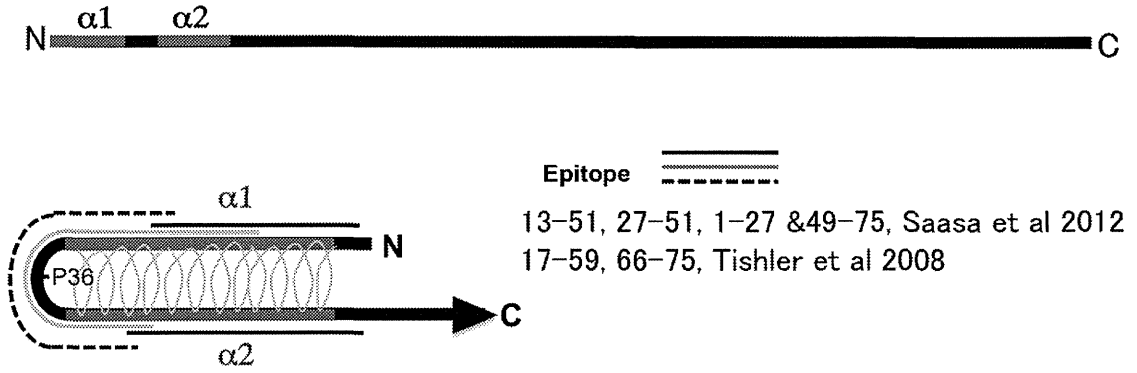
Thottapalayam virus (TPMV) is a prototype virus of shrew-borne hantavirus and is the most distinct form of rodent-borne hantaviruses [33]. Shigel *et al.* reported MAbs against TPMV [34]. All four clones were directed against the N-terminal region. These results indicate that, even in TPMV, the N terminus of N protein is immunodominant. Furthermore, the N-terminal region showed strong reactivity in a Western blot assay on staining by MAbs and polyclonal antisera. This indicated that the epitope found in the N terminus is linear and immundominant [34]. Similarly, antibody epitopes induced in HFRS and HPS patient sera and rodent-borne hantavirus-infected animals using a synthetic peptide antigen and/or truncated antigens were investigated. The results indicated that linear and immunodominant epitopes of N protein are also present in the N-terminal region even in these polyclonal antibodies [28,34–39]. Based on the results of epitope-mapping studies with MAb observations, MAbs against N protein were mostly produced against the N-terminal region of N protein of Old World, New World, and shrew-borne hantaviruses. These results reveal that the basic structure of N protein may be common among viruses within the genus *Hantavirus*.

2.2. Deduced Structure of the N-Terminal Region of N Protein

As shown in Figure 1, secondary structure prediction based on the deduced aa sequence, two α-helices in the N-terminal region (α1 and α2), was reported by Alfadhli *et al.* [40,41]. At present, the intramolecular coiled-coil structure is a model of the N-terminal region favored by several researchers [42,43]. A coiled-coil structure of aa 1 to 74 based on crystal structure analysis [42] and NMR [44] was reported. Therefore, aa 1-74 produced two long helices (α1 and α2) that intertwine into a coiled-coil domain [44]. A schema of 100 aa of the N terminus of N protein is presented in the figure.

The aa 1-74 produced the antiparallel coiled-coil structure of α1 and α2 helices shown in Figure 1A. The conserved proline at the 36th position was a vertex of the structure [42,44]. Based on the model, Tischler and Saasa proposed an antigenic model of the N-terminal region [30,31]. They found several overlapping epitopes in the N-terminal region by employing a competitive binding assay of MAbs. These MAbs showed various antigen-binding profiles including group-common, genus-common, and serotype-specific profiles. However, the N-terminal region seems to have only group-common epitopes and not to have serotype-specific or genus-common epitopes in actual infection. The N-terminal 100 amino acid antigens showed a group-common binding profile and low cross-reactivities with patient sera. Sera from HFRS patients infected with PUUV showed extremely low or no cross-reactivities against HTNV and DOBV antigens and slight cross-reactivity against SNV antigen [38,39]. The N-terminal part of N of ANDV and PUUV showed low cross-reactivities between Sigmodontinae/Neotominae-borne and Microtinae-borne hantaviruses [45]. These observations indicate that the antigenic region shown with black and solid lines (1-27 & 49-75) mainly induced antibodies in patient and animal sera during actual infection. The mutant N protein lacking aa 1-35 (α1-deletion mutant) completely lost its antigenicity [26]. Using MAb binding to the N-terminal 100 aa of HTNV N protein (such as GBO4, ECO2, and ECO1), it was not possible to determine their epitopes by a peptide-scanning assay employing a previously described peptide-scanning assay [46]. These results also indicate that the coiled-coil structure of the N terminus was the main structure involved in the antigenicity of N.

Figure 1. Schema of the structure of the N terminus of hantavirus N and epitope mapping. Two α helices α1 and α2, of the N-terminal region are shown [42]. The model of the intramolecular antiparallel coiled-coil structure has gained much support. Regarding the structure, several epitopes were reported as genus-common, type-common, or a type-specific epitopes [30,31]. The turn of this structure was proline at the 36th position.

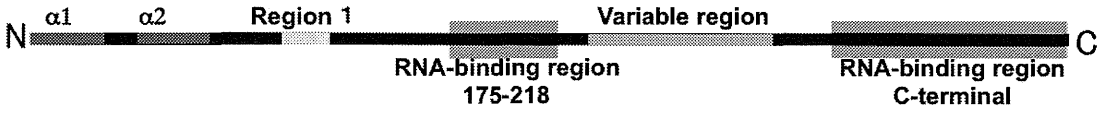


2.3. Deduced Structure of the Central Part of N Protein

As shown in Figure 2, another report also showed that the central region of N protein was responsible for RNA-binding activity [12,13]. N protein may bind to hantaviral genomic RNA selectively rather than to general RNA; however, this has yet to be confirmed [11,47,48]. Yoshimatsu and coauthors reported that Region I (aa 100-125) was highly conserved among hantaviruses, and they identified an important region assisting in N-N homotypic interaction [49]. A variable region of N

(aa 230-302) was identified in the central part of N protein (175-218) [50,51]. This region may be involved in the serotype-specific epitope of N protein.

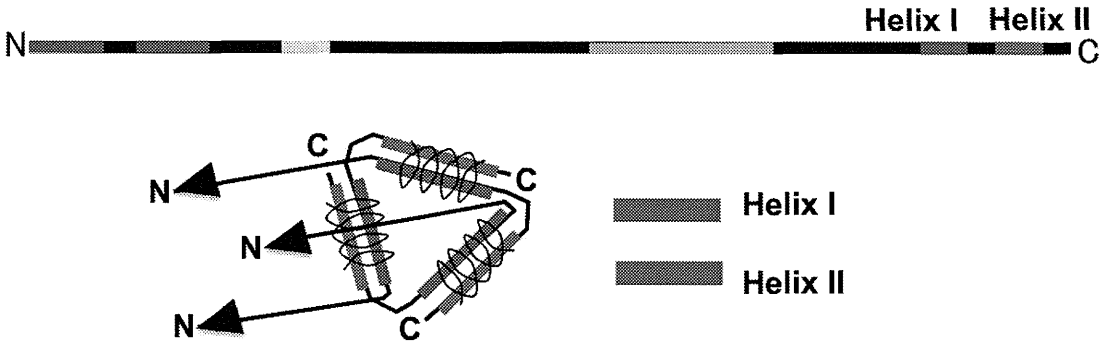
Figure 2. Schema of the central region of hantavirus N. Region 1 (aa 100-12) assists in N-N homotypic interaction [49]. RNA-binding regions involving aa 175-218 [12] and 100 aa of the C terminus [11] are shown.



2.4. Deduced Structure of the C-Terminal Region of N Protein

Two additional alpha-helical structures in the C-terminal region, Helix I and Helix II, were predicted by secondary structure analysis by Kaukinen *et al.* [52]. As shown in Figure 3, Helix I and Helix II contribute to the interaction with other N proteins through the intermolecular coiled-coil structure [52]. With deletion of Helix II from entire N, homotypic interaction did not occur in a yeast two-hybrid assay. On the other hand, N lacking Helix II showed interaction with entire N in the same assay [49]. These results also supported intermolecular interaction between Helix I and Helix II.

Figure 3. Interaction of the hantavirus N by using the C-terminal part. The coiled coil of Helix I (around aa 381-384) and that of Helix II (around aa 413-414) are parallel, with α helices aligned in the same direction [52]. Three molecules formed trimerized N throughout the three intermolecular coiled-coil structures.

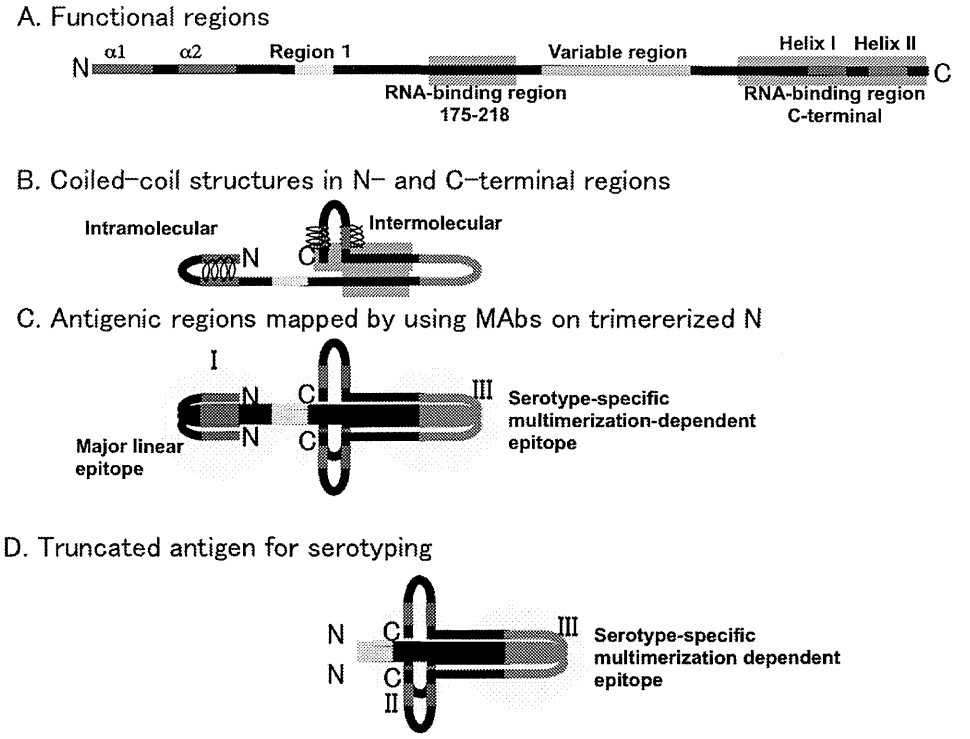


2.5. Deduced Structure of Trimerized N Protein

Mir and coauthors reported that N protein formed a trimer [53]. Based on their observations, a trimerized N protein is presented in Figure 4. In Figure 4A,B, a schema of N protein including coiled-coil interactions in the N and C terminal regions is presented. From epitope mapping of N protein using the competitive binding assay of MAbs, N- and C-terminal regions were in close proximity. Further, a serotype-specific epitope on N protein, which was expected to be located in the central region of N, was found as a projection from the N terminus [26]. According to these observations, an outline of N protein is proposed. In this schema, two RNA-binding regions are in close proximity. These two regions of N protein may cooperatively contribute to RNA binding. In

Figure 4C, a schema of trimerized N is presented [54]. Antigenic regions previously designated as I, II, and III [26] were overlaid on the trimerized model. Yoshimatsu *et al.* showed that antigenic regions I and III were immunodominant in actual infection. MAb binding to regions I and III competed with patient sera and infected rodent sera. The antigenicity of region I was reproducible by both *E. coli* and a baculovirus expression system. On the other hand, the antigenicity of region III was reproducible only by the baculovirus expression system [26]. Two HTNV-specific MAb clones, BDO1 [25] and C24B4 [26], were able to bind to N protein expressed by the baculovirus vector. However, recombinant N protein expressed by *E. coli* [26] and the yeast expression system (unpublished observation) showed low binding activity with these HTNV-specific MAbs. To express serotype-specific epitopes among hantavirus N, the selection of an appropriate expression system is important. In Figure 4D, the concept of serotyping antigen based on truncation of N antigen is shown. Morii *et al.* proposed serotyping antigens designed with deletion of group-common and major linear epitopes from N antigen [55].

Figure 4. A schema of trimerized hantavirus N. **A.** Functional regions of N were plotted on the primary structure of hantavirus N shown in Figure 1, Figure 2, and Figure 3. **B.** Interactions in the N-terminal and C-terminal part of hantavirus N. **C.** Antigenic regions mapped by poly- and monoclonal antibodies: From the competitive binding assay, two major antigenic regions were found in HTNV N. One was the N terminus (antigenic region I) and the other was the C terminus (antigenic region III). The central region was not a major antigenic site. Serotype-specific epitopes were found in the edge region and as discontinuous epitopes [26]. **D.** Concept of serotyping antigen based on truncation of N antigen. By deletion of group-common and major linear epitopes, serotyping antigens were designed [55].



2.6. Variety of Trimerization of N Protein Depending on the Viruses and Vectors

Multimerization of N is involved in the antigenicity of N. Most of the serotype-specific epitopes appear to be multimerization-dependent [49]. Authentic hantavirus N proteins in the virion should be multimerized. Native HTNV N proteins in inoculated Vero E6 cells were also detected as multimerized N proteins by competitive sandwich ELISA, as previously described [49]. However, the entire N protein of HTNV expressed in an *E. coli* vector system as described previously was detected as a monomer [26]. On the other hand, recombinant N proteins of SNV, HTNV, and SEOV showed an N-N interaction in yeast and mammalian two-hybrid assays [40,49]. These results indicate that the multimerization of recombinant N protein varied depending on the expression system. On the other hand, recombinant N proteins using the baculovirus expression system were more complicated. As shown in Table 1, all of the recombinant entire N proteins of SNV, ANDV, and LNV were monomeric. Hantavirus N proteins expressed in the baculovirus vector were detected as multimers. Although 155-429 aa of SEOV and DOBV N proteins were detected as multimers, 155-429 aa of HTNV were detected as a monomer. These results indicate that the region of N protein required for multimerization varies among hantaviruses in the baculovirus expression system. They also suggest that the reproducibility of N protein multimerization is dependent on the virus species. Furthermore, as shown in the results for New World hantaviruses except BCCV in Table 1, N-terminal region of the N protein inhibited N-N homotypic interaction.

Table 1. Homotypic interactions of recombinant and truncated N proteins.

Region (aa)	HTNV	SEOV	DOBV	THAIV	PUUV	SNV	ANDV	LANV	BCCV	CARV
1-429 (Entire)	M*	M*	M	M	M	S/M*	S	S	M	S
50-429	M*	M*	M	M	M	S	S*	S*	M	ND
100-429	M*	M*	M	ND	M	M	M	M	M	M
155-429	S	M	M	ND	M	S	ND	ND	ND	ND

Multimerization of recombinant and truncated N proteins expressed by the baculovirus vector were examined by competitive binding assay [49,51,56]. M, multimerized N; S, monomeric N; ND, not done. * Furthermore, interactions between entire and/or truncated N proteins were confirmed using the yeast or mammalian two-hybrid assay [49]. BCCV, Black Creek Canal virus.

2.7. Association of Cellular Components with N

Interactions among hantavirus N proteins were reported as above. In addition to homotypic interaction of N, cellular proteins associated with N were reported. Although interactions of N protein and small ubiquitin-like modifier-1 (SUMO-1) and its E3 ligase Ubc9 and PIAS were reported, the region responsible for the interaction was the C-terminal region of N [57,58]. Because Nedd4-like ubiquitin ligase E3 was found to be associated with the budding of viral particles from the cellular membrane in the case of Lassa virus, filoviruses, and retroviruses [59–62], contribution of sumoylation-related enzymes to the budding process of hantavirus virus particles was expected. The

binding of N and SUMO-1-related protein seemed to be multimerization-dependent [57]. Mir *et al.* also showed that multimerized N bound to the viral RNA panhandle [53]. These observations suggested that multimerized N might recruit a novel association with a cellular component. On the other hand, Cheng *et al.* reported interaction of N with the ribosomal protein L19 [63,64], and Ramanathan *et al.* showed N traffic in microtubules to the ER-Golgi intermediate compartment (ERGIC) [65,66]. Although regions responsible for N protein binding with L19 or ERGIC remain unclear, it is thought that these interactions participate in viral particle formation. An indirect interaction between N protein and the actin filament or Gn and Gc proteins may also be associated with the assembly of viral particles [67–70].

3. Conclusions

Hantavirus N protein is a major antigenic protein. Therefore, it has been important for serological diagnosis. At first glance, it appears simple. However, it has various epitopes such as genus-common, group-common, and serotype-specific epitopes. In addition to the cross-reactivity, both linear and discontinuous epitopes were found from analyses using monoclonal and polyclonal antibodies. Furthermore, the structure that constituted those epitopes has become clear. The N-terminus of N, which was constituted by an antiparallel coiled-coil structure, was found to be immunodominant. On the other hand, the C-terminal half of N, constituted by a parallel intermolecular coiled-coil structure, possessed serotype-specific and multimerization-dependent epitopes. The novel structure of N protein after multimerization might add a novel association with cellular proteins and/or RNA derived from host cells. Finally, reproducibility of epitopes of recombinant antigens is dependent on the expression system and viral species. By understanding the relation of the structure and antigenicity of N protein, a better diagnostic system will be constructed and interpretation of the results of serological tests will be more correct and informative.

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Conflicts of Interest

The authors declare no conflict of interest.

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Distinct genetic characteristics of Sri Lankan *Rattus* and *Bandicota* (Murinae, Rodentia) inferred from mitochondrial and nuclear markers

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We examined genetic variation in black rats (the *Rattus rattus* complex) from Kandy District, Sri Lanka using mitochondrial cytochrome *b* (*cytb*, 1140 bp) and nuclear melanocortin 1 receptor (*Mc1r*, 954 bp) gene sequences together with database sequences. We confirmed the existence of two divergent mitochondrial lineages in Sri Lankan black rats, with genetic distance of 2.2% and estimated divergence time of 0.3 million years ago. Because one lineage is unique to the island and the other is closely related to *R. rattus* populations on the Indian subcontinent, two migration events of *R. rattus* from the subcontinent are inferred, one ancient and one recent. *Mc1r* analyses revealed 12 haplotypes among the Sri Lankan black rats. A median-joining network together with other available sequences separated the 12 haplotypes into two groups, one unique to the island and the other related to previously reported *R. rattus* sequences. Notably, most individuals possessed various combinations of both haplotype groups which had no association with the *cytb* clades. These results imply that old and new *R. rattus* lineages are now intermingled as a result of hybridization in Sri Lanka. Specimens of the lesser bandicoot rat (*Bandicota bengalensis*) collected from Sri Lanka (n = 24) were shown to have no genetic variability in the *cytb* sequence. Our results indicate that the two most abundant groups of commensal rats in Sri Lanka, black rats and lesser bandicoot rats, are the product of contrasting evolutionary histories on different timescales.

Key words: *Bandicota bengalensis*, cytochrome *b*, melanocortin 1 receptor, *Rattus rattus*, Sri Lanka

INTRODUCTION

The island nation of Sri Lanka harbors particularly high levels of species diversity and endemism among terrestrial mammals and, together with the Western Ghats of southwestern India with which it shares many faunistic elements, has been designated as one of the world's biodiversity hotspots (Myers et al., 2000). Diversity is especially high among rats and mice of the subfamily Murinae, with 22 species having been recorded (Carleton and Musser, 2005; Bambaradeniya, 2006). However, this total includes a significant number of species that live as human commensals, and the status of these populations as either native or introduced is often ambiguous (Carleton and Musser, 2005; Bambaradeniya, 2006).

Two of the more ubiquitous and destructive of the Sri Lankan commensal rodents are black rats (members of the *Rattus rattus* Complex *sensu* Aplin et al., 2011) and bandicoot rats of the genus *Bandicota*. Both of these groups are thought to have undergone major range expan-

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sions in prehistoric to recent times, but both are also likely to have originated somewhere on the Indian subcontinent or surrounding region (Aplin et al., 2003; Carleton and Musser, 2005). Elucidation of their evolutionary history is a prerequisite for studies of mammalian biodiversity in Sri Lanka.

The phylogeny and phylogeography of black rats have attracted much recent attention (Pagès et al., 2010; Aplin et al., 2011) and these studies have provided a rich context for examining the affinities of Sri Lankan populations. In contrast, members of the genus *Bandicota* (usually called bandicoot rats) have been largely neglected in genetic studies other than as outgroups to the closely related *Rattus* (e.g., Pagès et al., 2010; Aplin et al., 2011). Members of both genera are known to be reservoirs for the pathogenic agents of major infectious diseases including hemorrhagic fever with renal syndrome (HFRS), hepatitis E, scrub typhus, rickettsial pox, leishmaniasis and leptospirosis (Meerburg et al., 2009; Aplin et al., 2011; Li et al., 2011), several of which have been reported in Sri Lanka (Vitarana et al., 1988; Gamage et al., 2011a, 2011b). Since these pathogens are often host species-specific (Bharti et al., 2003; Bi et al., 2008), a firm taxonomic and phylogenetic understanding of the rodent hosts is essential for understanding their evolutionary history, natural ecology and zoonotic behavior. To date, this foundation has been entirely lacking for infectious disease research in Sri Lanka.

In this study, we attempted to elucidate the phylogeographic histories and phylogenetic positions of *R. rattus* and *Bandicota bengalensis* from Sri Lanka using mitochondrial cytochrome *b* (*cytb*) gene sequences. In addition, for *R. rattus*, we used the nuclear gene marker melanocortin 1 receptor (*Mc1r*) to examine the hybridization history of genetically divergent populations within Sri Lanka. The results of our study provide new insights into the evolutionary history of *Rattus* and *Bandicota* in Sri Lanka.

MATERIALS AND METHODS

Sample collection Samples were collected for epidemiological study of leptospirosis during 2009 and 2010 in rural areas of Yatinuwara and Udunuwara Divisional Secretariats (DS), Kandy District, Sri Lanka (Fig. 1) (Gamage et al., 2011a). Rats were collected in houses in Yatinuwara and Udunuwara DS, and bandicoot rats were collected from farms in Yatinuwara DS. We obtained lung tissue samples from a total of 21 black rats *R. rattus* (*specimen code*: R01-21) and 24 lesser bandicoot rats *B. bengalensis* (*specimen code*: B01-24). All of the *R. rattus* collected were agouti in dorsal coloration and no melanistic specimens were encountered. Samples were stored at –80°C until laboratory analysis.

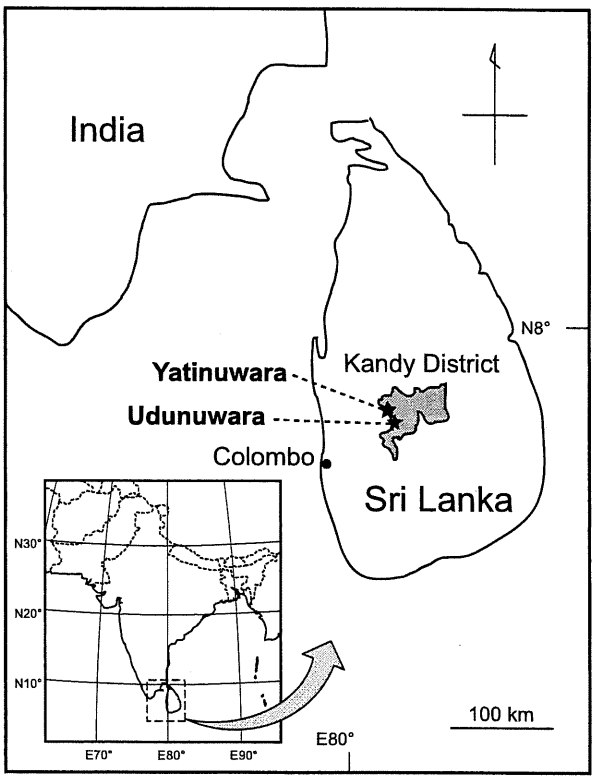

Fig. 1. Sampling localities. Specimens of *Rattus* and *Bandicota* were collected from Yatinuwara and Udunuwara Divisional Secreatriats, Kandy District, Sri Lanka.

DNA isolation, amplification and sequencing DNA was extracted from tissues using a DNeasy Blood & Tissue Kit (QIAGEN, Valencia, CA) according to the manufacturer's instructions. Two DNA fragments containing the N- and C-terminal halves of the mitochondrial *cytb* coding region (1140 bp) were amplified using the polymerase chain reaction (PCR) with the primer pairs L14115 (Yasuda et al., 2005) and H655A (5'-TGTGTAGTATGGGTGGAATGG-3') and L497A (5'-CCTAGTAGAATGAATCTGAGG-3') and H15300 (Yasuda et al., 2005), respectively. H655A and L497A were modified primers of H-15401 and L-15423 (Shinohara et al., 2004). Likewise, two DNA fragments containing the N- and C-terminal halves of the nuclear *Mc1r* coding region (954 bp) of *R. rattus* specimens were amplified with the primer pairs 5'*Mc1r* (–52) and 3'*Mc1r* (+504) and 5'*Mc1r* (+131) and 3'*Mc1r* (+1025), respectively (Shimada et al., 2009). Each PCR mix contained 0.5 U of Platinum *Taq* DNA Polymerase High Fidelity (Invitrogen) with specific buffer, 0.5 mM dNTP mix, 0.4 μM of each primer (8 pmol/reaction), 2 mM $MgSO_4$ and 0.1–0.5 μg of template total genomic DNA in a final volume of 20 μl. The thermal cycling parameters for the PCR of *cytb* were as follows: 94°C for 1 min and 35 cycles of 94°C for 20 sec, 50°C for 30 sec and 68°C for 60 sec, followed by a final extension of 68°C for

Table 1. Specimen list of cytochrome *b* gene sequences from DDBJ/EMBL/GenBank

species or lineage	country	accession	reference
RrC LI	Japan	AB211039	Chinen et al., 2005
	India	HM217367	Pagès et al., 2010
	India	GQ891573	Tollenaere et al., 2010
	India	GQ891572	Tollenaere et al., 2010
	India	GQ891569	Tollenaere et al., 2010
	India	GQ891570	Tollenaere et al., 2010
	India	GQ891571	Tollenaere et al., 2010
	Indonesia	AB033702	Suzuki et al., 2000
	Madagascar	HM217368	Pagès et al., 2010
	New Zealand	EU273707	Robins et al., 2008
	Oman	HM217366	Pagès et al., 2010
	Oman	GQ891576	Tollenaere et al., 2010
	Oman	GQ891575	Tollenaere et al., 2010
	Oman	GQ891574	Tollenaere et al., 2010
	Oman	GQ891577	Tollenaere et al., 2010
	Oman	GQ891578	Tollenaere et al., 2010
	South Africa	HQ157803	Bastos et al., 2011
	Tanzania	HM217365	Pagès et al., 2010
	Yemen	GQ891581	Tollenaere et al., 2010
	Yemen	GQ891582	Tollenaere et al., 2010
RrC LII		AB211040	Chinen et al., 2005
		HM217426	Pagès et al., 2010
RrC LIII		JN675599	Aplin et al., 2011
		JN675600	Aplin et al., 2011
		JN675601	Aplin et al., 2011
RrC LIV		HM217389	Pagès et al., 2010
		HM217372	Pagès et al., 2010
RrC LV		HM217421	Pagès et al., 2010
		HM217443	Pagès et al., 2010
		HM217454	Pagès et al., 2010
RrC LVI		HM217391	Pagès et al., 2010
		EF186492	Robins et al., 2007
		EF186514	Robins et al., 2007
R. argentiventer		AB033701	Suzuki et al., 2000
		HM217362	Pagès et al., 2010
		HM217364	Pagès et al., 2010
R. hoffmanni		EF186442	Robins et al., 2007
		EF186443	Robins et al., 2007
R. andamanensis		HM217396	Pagès et al., 2010
		HM217403	Pagès et al., 2010
R. exulans		HM217437	Pagès et al., 2010
		HM217470	Pagès et al., 2010
		HM217472	Pagès et al., 2010
R. norvegicus		AB033713	Suzuki et al., 2000
		HM217370	Pagès et al., 2010
		HM217429	Pagès et al., 2010
R. nitidus		HM217478	Pagès et al., 2010
		HM217474	Pagès et al., 2010
		HM217479	Pagès et al., 2010
B. indica		HM217390	Pagès et al., 2010
		HM217408	Pagès et al., 2010
		HM217425	Pagès et al., 2010
		HM217376	Pagès et al., 2010
		HM217378	Pagès et al., 2010
		HM217380	Pagès et al., 2010
		HM217386	Pagès et al., 2010
		HM217435	Pagès et al., 2010
		HM217447	Pagès et al., 2010
		HM217469	Pagès et al., 2010
		HM217476	Pagès et al., 2010
B. bengalensis		AM408336	Michaux et al., 2007
B. savilei		HM217427	Pagès et al., 2010
		HM217385	Pagès et al., 2010
		HM217387	Pagès et al., 2010
		HM217455	Pagès et al., 2010

5 min. Those for the PCR of *Mc1r* were as described by Shimada et al. (2009). All PCR products were purified using MicroSpin™ S-400 HR Columns (GE Healthcare) and were sequenced according to the manufacturer's instructions using a Big Dye Terminator v3.1 Cycle Sequencing Kit (ABI) and an ABI 3100 automated sequencer with two PCR primers. The sequence data obtained in this study were deposited in DDBJ/EMBL/GenBank with accession nos. AB762700-AB762765.

Phylogenetic analysis Phylogenetic analysis of the full *cytb* sequence dataset (Table 1) was conducted using four methods: neighbor joining (NJ) (Saitou and Nei, 1987), maximum parsimony (MP) (Swofford and Olsen, 1990), maximum likelihood (ML) (Felsenstein, 1981) and Bayesian inference (BI) (Huelsenbeck et al., 2001). For the ML method, the best-fit nucleotide-substitution model and parameters were determined using the AIC criterion (Posada and Buckley, 2004), as implemented in MEGA 5.1 (Tamura et al., 2011). The NJ and MP methods were implemented using MEGA 5.1, and the ML method was implemented using PHYML 3.0 (Guindon et al., 2005). Maximum composite likelihood distance and GTR + G distance were used for NJ and ML analyses, respectively. Bootstrap values (Felsenstein, 1985) were estimated for the NJ, MP and ML trees by resampling 10,000 iterations. For BI, the best-fit nucleotide-substitution model and parameters were determined using the AIC criterion as implemented in MrModeltest version 2.3 (Nylander, 2004), and the HKY + I + G model was selected. Trees were generated by the Metropolis-coupled Markov-chain Monte Carlo algorithm using MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003). Each run consisted of four simultaneous chains, one cold and three incrementally heated, starting from a random tree.