VILNIUS UNIVERSITY

Mindaugas Juozapaitis

SYNTHESIS OF *PARAMYXOVIRIDAE* NUCLEOPROTEINS IN YEAST *SACCHAROMYCES CEREVISIAE* AND THEIR APPLICATION IN VIRAL DIAGNOSTICS

Summary of doctoral dissertation

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VILNIAUS UNIVERSITETAS

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PARAMYXOVIRIDAE ŠEIMOS VIRUSŲ NUKLEOKAPSIDĖS BALTYMŲ SINTEZĖ MIELĖSE *SACCHAROMYCES CEREVISIAE* IR JŲ PANAUDOJIMAS DIAGNOSTIKAI

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CONTENTS

INTRODUCTION

The *Paramyxoviridae* include some of the great and ubiquitous disease-causing viruses of humans and animals. Human respiratory syncytial virus (hRSV) and human parainfluenza virus types 1 and 3 (hPIV1 and hPIV3, respectively) are the most prevalent and infectious respiratory pathogens which belong to the family *Paramyxoviridae* (Chanock, 1957; Chanock et al., 1958). According to estimates of the World Health Organization, global hRSV disease burden is 64 million cases and 160.000 deaths every year, and infants together with young children are among those who are mostly affected. Parainfluenza viruses cause a spectrum of respiratory illnesses and the annual estimated rates of hospitalizations caused by these viruses are from 15.000 to 80.000 cases only in the US (World Health Organization 2009). Parainfluenza viruses and human respiratory syncytial virus have been associated with every kind of upper and lower respiratory tract illnesses which, in their symptoms, only slightly differ from respiratory diseases caused by other infectious pathogens. Consequently, diagnosis of the infectious agent causing the respiratory disease based only on clinical symptoms in most cases is impossible. For this a laboratory diagnosis is highly recommended (Hall, 2001; Henrickson, 2003).

Nipah, Hendra and Menangle viruses (NiV, HeV and MenV, respecively) join a growing list of recently identified paramyxoviruses for which bats have been implicated as the natural host (Chua et al., 1999; Philbey et al., 1998; Selvey and Sheridan, 1995). Latest reports have indicated that deadly outbreaks caused by these viruses are sparse and worldwide burden is not very significant (Eaton et al., 2006; Philbey et al., 2008). However, these newly identified paramyxoviruses change fast, infect wide host range including several terrestrial species in four mammalian orders, cause systemic infections affecting various organ systems also central nervous system, and NiV and HeV are deadly human pathogens (Eaton et al., 2006; Philbey et al., 2008). Therefore, it is likely that with the laps of time in the changing climate era these viruses may become wide spread infectious pathogens causing deadly outbreaks in humans and livestock. Since NiV, HeV and MenV were identified only recently, consequently, knowledge

about them is relatively scanty. It is little known about the biology, spread, virulence and pathology of these viruses, also no effective means of diagnostics, which would help a lot to fill existing knowledge gaps, are available by now (Wong, 2010) .

The study presented here describes the yeast-derived N proteins as useful tools for the development of new detection systems for the infections caused by respiratory and zoonotic paramyxoviruses.

Definite diagnosis of the *Paramyxoviridae* infections requires conventional laboratory methods which are skill-demanding and not specific enough (Hall, 2001; Henrickson, 2003; Lee et al., 1992). Paramyxovirus specific antibodies have been efficiently detected by binding to viral antigens in enzyme-linked immunosorbent assays (ELISAs). For this purpose whole virus particles, isolated from human cell cultures, have been used as antigens for a long time. Although whole-virus-based ELISA diagnostics offers a number of advantages, namely, low cost, simple handling and ability to test many samples at a time, however, these diagnostic tests are still not specific enough (Hall, 2001; Henrickson, 2003). Assays for immunity based on recombinant viral proteins may offer a number of advantages over whole-virus-based ELISAs, namely, simplified production, improved standardization, enhanced stability and no risk of virus distribution (Bouche et al., 1998). Though using recombinant viral proteins instead of whole virus particles the more narrow spectra of virus specific antibodies could be detected, the right choice of proper viral antigen may improve the specificity of the diagnostic test. It is well known that a C terminal part of N protein of various paramyxoviruses is very immunogenic and differs in amino acid sequence even among parainfluenza virus types (Karron and Collins, 2007). Therefore, using recombinant N proteins for ELISA based diagnostics of infections coused by paramyxoviruses it should be possible to detect virus specific antibodies in oral fluids and blood serum samples.

Host defence against most paramyxoviruses is mediated largely by humoral immunity to surface glycoproteins of virus particle. During the infection specific antibodies to surface glycoproteins are usually produced (Connors et al., 1991; Eaton et

al., 2006; Philbey et al., 2008; Ray et al., 1988). However, antigenic properties of viral surface glycoproteins change with time and specific antibodies to these proteins usually crossreact with the same proteins of closely related viruses (Lamb and Parks, 2007). Synthesis of recombinant viral glycoproteins in heterologous expression systems is complicated and usually the yield of product is very low (Fooks et al., 1993; Nayak et al., 1985). Also, the synthesis of measles and mumps viral glycoproteins in yeast *S.cerevisiae* proved to be unsuccessful (Čiplys et al., 2011, in press). The yeast expressed glycoproteins are not folded correctly and their antigenic properties do not resemble those of native viral proteins. The N protein of paramyxoviruses also induces a host humoral immune response, and N protein specific antibodies are produced in response to viral infection. Using N proteins, synthesized in yeast *P.pastoris* and *S.cerevisiae*, specific ELISA tests for the diagnosis of mumps and measles were developed. These tests offer a number of advantages over any other diagnostic tests, available to date and become a significant tool in control of mumps and measles epidemics (Samuel et al., 2002a; Samuel et al., 2003; Slibinskas et al., 2004; Slibinskas et al., 2003). Consequently, the yeast expressed N proteins could be useful tools for the development of new serology based diagnostic systems for the infections caused by respiratory and zoonotic paramyxoviruses.

The aims of this study. The purpose of the study presented here was to investigate synthesis of SeV, hPIV1, hPIV3, hRSV, NiV, HeV and MenV N proteins in yeast *S.cerevisiae*, to determine properties of recombinant N proteins and evaluate the feasibility to use them in diagnostics. Towards the goals of this study the following tasks have been formulated:

1. To synthesize SeV, hPIV1, hPIV3, hRSV, NiV, HeV and MenV N proteins in yeast *S.cerevisiae* expression system effectively.

2. To purify recombinant SeV, hPIV1, hPIV3, hRSV, NiV, HeV and MenV N proteins from yeast cells, and define their properties.

3. To determine the nature of interaction between recombinant N proteins of paramyxoviruses and nucleic acids of yeast cell, and investigate an inpact of the C terminal part of N protein to the stability of virus nucleocapsid like particles (vNLP).

4. To test recombinant hPIV1, hPIV3, hRSV, NiV, HeV and MenV N proteins for their suitability in diagnostics.

Scientific novelty. Definite diagnosis of the *Paramyxoviridae* infections requires conventional laboratory methods which are skill-demanding and not specific enough (Hall, 2001; Henrickson, 2003; Lee et al., 1992). Also, NiV, HeV and MenV were identified only recently, consequently, knowledge about them is relatively scanty. It is little known about the biology, spread, virulence and pathology of these viruses, also no effective means of diagnostics, which would help a lot to fill existing knowledge gaps, are available by now (Wong, 2010) . Therefore, in this study the synthesis of SeV, hPIV1, hPIV3, hRSV, NiV, HeV ir MenV N proteins in yeast *S.cerevisiae* was investigated and the use of recombinant N proteins as a tool for the development of serology based systems for viral diagnostics was proved.

Practical value. In this study it was confirmed that yeast expressed N proteins are useful tools for the developmment of reliable, fast and cheap diagnostic tests for hPIV1, hPIV3, hRSV, NiV, HeV ir MenV infections. The effective diagnostic tools for viral infections would help to idenify dangerous paramyxoviruses and provide additional means to investigate their biology, spread, virulence and pathology.

MATERIALS AND METHODS

Reagents, enzymes and kits

All reagents used in this work were the highest quality available. All enzymes, their reaction buffers and kits were provided by UAB „Fermentas“ (Lithuania) and used according to the manufacturers‘ recommendations.

Oligonucleotides

Primers used for synthesis of N genes (for expression in *S.cerevisiae*):

1. SeV *N* gene:
   5‘ – CAACTAGTACAATGGCTGGATTGTTGAGCACC
   3‘ – GTACTAGTTTAGATTCCTCCTACCCCAGCT
2. hPIV1 *N* gene:
   5‘ – AAACTAGTACAATGGCAGGTCTACTAAGTACT
   3‘ – AGTACTAGTTTAAATTCCTCCTATCCCTGCAGCCGC
3. hPIV3 *N* gene:
   5‘ – TATTCTAGAACAATGTTGAGCCTATTTGATACATTTA
   3‘ – TTGTCTAGATTAGTTGCTTCCAAATGCATTAAACAGA
4. hRSV *N* gene:
   5‘ – GCAACTAGTACAATGGCTCTTAGCAAAGTCAAGTTG
   3‘ – TTTACTAGTTTAAAGCTCTACATCATTATCT
5. NiV *N* gene:
   5‘ – AAAACTAGTACAATGAGTGATATCTTTGAAGAG
   3‘ – AAAACTAGTTTACACATCAGCTCTGACGAAATCT
6. HeV *N* gene:
   5‘ – AGAACTAGTACAATGAGTGATATATTTGACGAG
   3‘ – GATACTAGTTTACACGTCTGCTCTAACAAAG
7. MenV *N* gene:
   5‘ – TTACTAGTACAATGGCCACACTTTTAAGGAGC
   3‘ – ACATCTAGAAATGATACTTGGGC

8. *NiV-N*$_{(N1\text{-}399)}$ gene:

   5‘ – AAAACTAGTACAATGAGTGATATCTTTGAAGAG

   3‘ – AAAACTAGTTTATGTTTCCTGAACTGCAGCAGCG

9. *HeV-N*$_{(N1\text{-}399)}$ gene:

   5‘ – AGAACTAGTACAATGAGTGATATATTTGACGAG

   3‘ – GTACTAGTTTAGATTCCTCCTACCCCAGCT

10. *MenV-N*$_{(N1\text{-}399)}$ gene:

    5‘ – TTACTAGTACAATGGCCACACTTTTAAGGAGC

    3‘ – AGATCTAGATTAGGTGACTGCTGCTGACATATCC

11. *MenV-N*$_{(C6His)}$ gene:

    5‘ – TTACTAGTACAATGGCCACACTTTTAAGGAGC

    3‘ – GTTATTAGTAGTCTAGATTAGTGATGGTGATGGTGATGCATGTCAAGAT

12. *MenV-N*$_{(N1\text{-}400+C6His)}$ gene:

    5‘ – TTACTAGTACAATGGCCACACTTTTAAGGAGC

    3‘ – GTTATTAGTAGTCTAGATTAGTGATGGTGATGGTGATGTCTGGTGACTGC

13. *MenV-N*$_{(N1\text{-}430+C6His)}$ gene:

    5‘ – TTACTAGTACAATGGCCACACTTTTAAGGAGC

    3‘ – GTTATTAGTAGTCTAGATTAGTGATGGTGATGGTGATGATTCCCCCTTCTT

14. *MenV-N*$_{(N1\text{-}460+C6His)}$ gene:

    5‘ – TTACTAGTACAATGGCCACACTTTTAAGGAGC

    3‘– GTTATTAGTAGTCTAGATTAGTGATGGTGATGGTGATGGGGTAACGGACGA

15. *MenV-N*$_{(N1\text{-}490+C6His)}$ gene:

    5‘ – TTACTAGTACAATGGCCACACTTTTAAGGAGC

    3‘ – GTTATTAGTAGTCTAGATTAGTGATGGTGATGGTGATGCCGATCTCGGG

16. *N*$_{(MeV\text{-}N\text{-}N1\text{-}400+hRSV\text{-}N\text{-}C275\text{-}392)}$ gene:

    5‘ – TTACTAGTACAATGGCCACACTTTTAAGGAGC

    3‘ – TTTACTAGTTTAAAGCTCTACATCATTATCT

Bacterial, yeast strains and plasmids

Bacterial recombinants were screened in *Escherichia coli* DH5α (F^- *gyrA96* (Nal^r) *recA1 relA1 endA1 thi-1 hsdR17* ($r_k^- m_k^+$) *glnV44 deoR* Δ*(lacZYA-argF)U169* [ϕ80dΔ*(lacZ)M15*]) cells. *S.cerevisiae* strains *AH 22-214* (*MATa ura3 leu2 his3*), its derivative *214Δpep4* (*MATa ura3 leu2 his3* Δ*pep4*) and *FH4* (*MATa /α*) were used for expression. Plasmid *pSeV(+)-V-C365R* with wild type SeV *N* gene (strain Z) (GeneBank Accession No. M30202) was kindly provided by Dr. T. Sakaguchi from Graduate School of Biomedical Sciences, Hiroshima University, Japan. Plasmids *pFastBacHT-NiV*, *pFastBacHT-HeN* and *pCR4-TOPO-MenV-N7* with wild type NiV, HeV and MenV *N* genes, respectively, (GeneBank Accession No.: AF212302, AF017149 and AF326114, respectively) were kindly provided by Dr. W. P. Michalski from CSIRO Livestock Industries, Australian Animal Health Laboratory, Geelong, Australia. Plasmid pFGG3 (Slibinskas et al., 2004) was used as *S.cerevisiae* expression vector.

Bacterial, yeast media, transformation and growing conditions

E.coli strains were grown in LB medium at 37°C. Transformations of *E.coli* were carried out by the $CaCl_2$-heat shock method (Sambrook et al., 1989). *S.cerevisiae* strains were grown at 30°C in YEPD medium (Guthrie and Fink, 1991), for plates 2 % agar was added. *S.cerevisiae* competent cells were prepared and transformed with plasmid DNA by LiAc/ssDNA/PEG method (Gietz et al., 1995). For selection and growing of *S.cerevisiae* transformants YEPD medium was supplemented with 5 mM formaldehyde, for induction of recombinant gene expression YEPG medium (yeast extract 1 %, peptone 2 %, and galactose 3 %) was used.

DNA preparation and manipulation

PCR amplification, DNA hydrolysis with restriction endonucleases, dephosphorylation of DNA 5'-termini and DNA ligation was performed using UAB "Fermentas" enzymes and kits, according to manufacturers' recommendations. Plasmids were prepared by the alkaline lysis procedure (Birnboim and Doly, 1979). Recombinant plasmid construction and analysis, DNA electrophoresis in agarose gels were performed according to standard technique (Sambrook and Russell, 2001).

Expression and purification of virus nucleocapsid like particles from yeast

The expression of SeV, hPIV1, hPIV3, hRSV, NiV, HeV and MenV proteins in *S.cerevisiae* cells (Slibinskas et al., 2004), yeast transformants (strain *214Δpep4*) harbouring *pFGG3-SeV*, *pFGG3-hPIV1*, *pFGG3-hPIV3*, *pFGG3-hRSV*, *pFGG3-NiV*, *pFGG3-HeV* and *pFGG3-MenV* plasmids were inoculated into YEPD media supplemented with 5 mM formaldehyde, grown overnight and reinoculated into YEPG induction media and cultured at 28°C for 24 h. After 24 h induction the yeast cells were harvested by centrifugation and stored at −70°C. Five gram of each recombinant clone of yeast biomass were suspended in PBS containing 5 mM EDTA and 1 mM PMSF and after disruption by homogenisation in the presence of glass beads were used for purification. The N proteins were purified by successive ultracentrifugations through 30 % sucrose cushion (100.000 × g, 3 h) followed by ultracentrifugation in CsCl gradient ranging from 1.23 to 1.38 g/cm^3 (100.000 × g, 36 h) performed twice. Fractions containing N proteins were pooled and dialysed against PBS for EM and Western blot analysis (Slibinskas et al., 2004). The buoyant density of fractions was determined with a refractometer. Protein concentrations were determined by method of Bradford (1976).

SDS-PAGE and Western

SDS-PAGE was performed according to standart protocols (Ausubel, 1992). Western blotting was performed as described in Sambrook and Russell (2001). The blots were incubated with N protein specific antibodies. Horseradish peroxidase (HRP)-labelled anti-mouse, anti-rabbit and anti-goat IgG conjugates (Sigma, USA) for detection of specific antibody-binding were used. The blots were stained with the TMB substrate (Fermentas UAB, Vilnius, Lithuania).

Determination of expression level of recombinant proteins in yeast lysates

The percentage of yeast expressed N proteins in crude lysates was determined by scanning of immunostained protein bands. Analysis was conducted with the ImageQuant^{TM} TL software (GE Healthcare, JAV). At least three scans were used in the estimation of expressed protein.

Electron microscopy

After purification by CsCl centrifugation suspensions of vNLPs were placed on 400-mesh carbon coated palladium grids. Samples were stained with 2 % aqueous uranyl acetate solution and examined with the JEM-100S electron microscope.

Isolation and analysis of RNA

Total RNA was isolated from yeast by acid phenol method (Schmitt and Tipper 1990). Agarose-formaldehyde gel electrophoresis and Northern blotting were performed according to Sambrook and Russell (2001). For isolation of RNA from virus nucleocapsid like particles 100 µg of recombinant vNLPs, purified from yeast *S.cerevisiae*, were incubated in PBS, containing either 10 µg/ml of DNase I or 10 µg/ml RNase A, at 37°C for 20 min. Then tubes were transferred to ice bath and RNA was extracted and ethanol precipitated as in the case of total RNA isolation from yeast (Schmitt and Tipper 1990). Before agarose-formaldehide gel electrophoresis control samples were treated with either 10 µg/ml of DNase I or 10 µg/ml RNase A, at 37°C for 10 min. Then treated RNA samples were fractionated by electrophoresis in 0.8 % native agarose gel and transferred on nylon membrane exactly as in the case of Northern blotting, then hibridisation with ^{32}P labeled probes was performed according to standard protocols (Sambrook and Russell, 2001).

Mass spectrometric analysis of recombinant proteins

Protein samples (1 mg/ml) were digested with trypsin, chymotrypsin and endoproteinase Asp N (proteomics sequencing grade, Roche, Penzberg, Germany) at a ratio of 1:100 (w/w) for 16h at 37°C. Reversed-phase chromatography of digests was performed on a Vydac C18 column (3 µm, 0.15 mm × 150 mm, Grace Davison, Hesperia, CA, USA) using a Surveyor-MS HPLC system (Thermo, San Jose, CA, USA). Mobile-phase buffers were 0.2 % (v/v) formic acid in water (A) and 0.2 % (v/v) formic acid in acetonitrile (B), and were run at a flow rate of 1µl/min with a linear gradient of 5–95 % B over 20 min, held at 95 % B for 10 min and finally 95–5 % B over 2 min. The effluent from the column was connected directly to the nanospray ion source of a LCQ Classic quadrupole ion-trap mass spectrometer (Thermo). Nanospray ion source settings were

as follows: nanospray tip voltage 2.3 kV, capillary temperature 150°C, capillary voltage 24 V and tube lens offset 5 V. Tandem mass spectrometry scans (MS/MS) were performed with normalized collision energy of 35 %. Automated interpretation of the resulting peptide fragment spectra was performed using the Sequest algorithm (Eng et al., 1994) (Thermo) which cross-correlates raw MS/MS spectra of peptides with sequences from protein databases. Spectra were matched against either a “Hendra”, “Nipah” or “Menangle” subset of the NCBI non-redundant protein database. Peptide matches with a Sequest cross-correlation score (Xcorr) of >2.0 were used to confirm the amino acid sequence coverage of the N proteins. Molecular weight calculation of large peptide fragments was performed using the BIOMASS deconvolution algorithm from Bioworks Browser program 3.1 (Thermo). Sequence assignments were made by matching the calculated average masses with masses generated by *in silico* tryptic digests of N proteins using the MS-Digest program (http://prospector.ucsf.edu/msdigest.htm).

RESULTS

Synthesis of recombinant Sendai virus N protein in yeast *S.cerevisiae*

For cloning into the yeast expression vector, the SeV *N* gene was amplified from the plasmid *pSeV(+)-V-C365R*, containing cDNA of the SeV genome (Huang et al., 2000); GenBank acc. no. M30202). Primers used in the amplification of the SeV *N* gene included a *BcuI* site for subcloning into the yeast expression vector. The amplified PCR fragment encoding the SeV *N* gene was ligated into the *BcuI* site of the *pFGG3* yeast expression vector under the control of *GAL7* promoter (Slibinskas et al., 2004). The resulting *pFGG3-SeV-N* plasmid was used for the transformation of yeast *S.cerevisiae* strains *FH4*, *AH 22-214* and *214Δpep4*.

The procedure used in this study to synthesize the recombinant SeV N protein in yeast *S.cerevisiae* was similar to that described earlier (Samuel et al., 2002a). Briefly, yeast cells harboring the *pFGG3-SeV-N* expression plasmid were inoculated into YEPD medium containing 5 mM of formaldehyde and cultured for 24 h at 30°C. Thereafter, yeast cells were re-inoculated into YEPG medium containing 5 mM of formaldehyde and cultured further for 24 h at 30°C. After induction yeast cells were harvested by centrifugation, re-suspended in disruption buffer (DB – PBS, pH 7.2, 5 mM EDTA, 1 mM PMSF) and disrupted by homogenization in the presence of glass beads. The crude lysate was analyzed by SDS-PAGE and Western blot (WB) using hyperimmune rabbit sera generated by immunizing rabbits with purified SeV virions. After induction with galactose, the Coomassie brilliant blue stained SDS-PAGE of the crude lysate of *S.cerevisiae* cells harbouring the *pFGG-SeV-N* expression plasmid, revealed the presence of an additional protein band of approximately 60 kDa (Fig. 1, lanes 2), which is consistent with the molecular weight of the native SeV N protein reported elsewhere (Neubert et al., 1991; Sakaguchi et al., 1993). The identity of the detected additional protein band as the N protein of SeV was confirmed by WB using SeV specific hyperimmune rabbit sera (Fig. 1, Lane 5). No immunostaining was observed in induced yeast cells harbouring only the *pFGG3* expression plasmid without a gene insert (Fig. 1, Lane 4). The main problem to obtain large quantities of recombinant proteins might be

their instability in yeast cells. The SeV N protein which has been described here is stable in yeast. The analysis of yeast lysates obtained after the induction of heterologous protein synthesis did not reveal any significant degradation of the recombinant SeV N protein in WB. The absence of degradation products in crude lysates or only minute presence detected by WB have indicated that the recombinant SeV N protein is quite stable in yeast cells.

In order to identify the optimum conditions for the maximum synthesis of the SeV N protein, yeast cells harbouring the *pFGG-SeV-N* expression plasmid were collected at different time points after induction and disrupted. Then, crude yeast lysates were subjected to WB with SeV specific hyperimmune rabbit sera. The WB analysis revealed that the maximum level of the SeV N protein synthesis was achieved cultivating yeast for 24 h in the induction medium. Furthermore, the synthesis of the SeV N protein was increased almost by 25 % when the induction medium containing higher concentration of galactose (2.5 %) was used to induce the heterologous protein synthesis. Under the optimal conditions, the calculated maximal synthesis yield of the SeV N protein was approximately ~28 mg per liter of induced yeast culture.

To assess whether different yeast S.cerevisiae strains affect the synthesis level of the SeV N protein the pFGG-SeV-N expression plasmid was transformed into the wild-type (FH4), AH 22-214 and 214Δpep4 yeast strains. The *214 Δpep4* strain, if compared with wild-type or *AH 22-214* strains, lacks the *PEP4* locus encoding protease A. This protease activates the set of other cellular proteolytic enzymes, thereby making the *214 Δpep4* strain less proteolytically active. Synthesis of the Sev N protein was similar in all three strains tested. *S.cerevisiae* strain *214Δpep4*, lacking vacuolar peptidase A, was chosen for further experiments.

For the purification of the yeast-expressed SeV N protein, induced yeast cells harbouring the *pFGG-SeV-N* expression plasmid were collected, re-suspended in DB and disrupted by homogenization in the presence of glass beads. Then, the crude lysate was centrifuged at 10.000 x g for 20 min at 4°C, soluble protein fraction (SPF) separated and used for further purification. The yeast-expressed SeV N protein was purified by

successive ultracentrifugation through the 30 % sucrose cushion (100.000 × g for 3 h at 4°C) followed by two ultracentrifugations in the CsCl gradient ranging from 1.23 to 1.38 g/cm^3 (100,000 × g for 48 h at 4°C). Fractions containing N proteins were identified by SDS-PAGE. After the first centrifugation in the CsCl gradient fractions containing proteins with the molecular weight corresponding to the native SeV N protein (60 kDa) were pooled, diluted with 1.31 g/cm^3 CsCl and centrifuged again under the same conditions. Then, fractions were collected and those containing highest concentrations of the recombinant SeV N protein pooled and dialyzed against PBS for electron microscopy (EM). A buoyant density of the fractions was determined with a refractometer. The purified SeV N protein was stable after freezing or lyophilization.

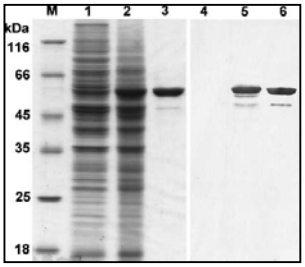

Fig.1. SDS-PAGE (lanes 1–3) andWestern blot (lanes 4–6) analysis of yeast lysates and samples of yeast expressed SeV N protein after ultracentrifugation in CsCl. Five micrograms of purified SeV N protein (lanes 3 and 6) or 20μg of yeast lysates (lanes 1, 2 and 4, 5) were separated in a 12% SDS-PAGE and stained with Coomassie brilliant blue. After separation proteins were analyzed by Western blot using hyperimmune rabbit sera generated by immunizing rabbits with purified virions. Lanes 1 and 4, *S.cerevisiae* [*pFGG3*] lysate; lanes 2 and 5, *S.cerevisiae* [*pFGG3-SeN*] lysate; lane 3 and 6, CsCl purified SeV N protein; M—protein molecular mass marker (Fermentas UAB) 116, 66, 45, 35, 25 and 18 kDa.

For electron microscopy analysis, suspensions of the SeV N protein purified by ultracentrifugation were placed on 400-mesh carbon coated palladium grids. Samples were stained with 2 % aqueous uranyl acetate solution and examined with a JEM-100S electron microscope. Electron microscopy of the purified SeV N protein revealed structures with typical “herring-bone” morphology: relatively rigid rods with repeated serration along the edges ~20 nm in diameter, with a central core of 4 nm, a helical pitch of ~5 nm and varying in length from 20 to 300 nm (Fig. 2). Ring structures representing single turns of the helical nucleocapsid were also observed. It is well

known, that yeast derived N proteins from other paramyxoviruses and the SeV N protein, expressed in animal and insect cells, also form virus nucleocapsid like particles (vNLPs) (Bhella et al., 2002; Samuel et al., 2002b; Slibinskas et al., 2003).

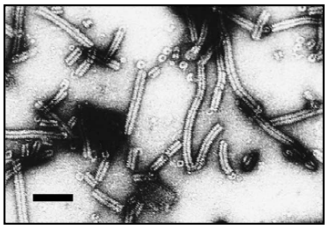

Fig.2. Electron micrographs of negatively stained SeV N protein isolated from S. cerevisiae. Scale-bar: 100 nm.

Northern blot hybridization of nucleic acids isolated from purified and nuclease treated yeast-derived SeV vNLPs confirmed presence of nucleic acids unaffected by DNase I treatment but sensitive to RNase A digestion. ^{32}P labeled total yeast cDNA derived from the recipient yeast strain harbouring the *pFGG3* expression vector was used in the hybridization assay (Fig. 3). The encapsidation appears to be relatively non-specific in terms of RNA sequence. The nucleic acids associated with yeast-derived SeV vNLPs were heterogeneous in size and ranged from 60 to 500 nt, however, after RNase A treatment of recombinant vNLPs all of them were partially degraded to 60-80 nt fragments.

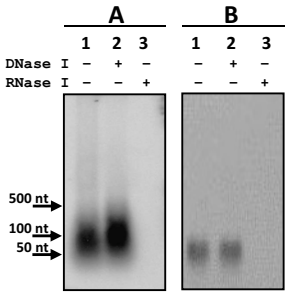

Fig.3. Northern blot hybridization of nucleic acids isolated from SeV N vNLPs. A – SeV N vNLPs treated with RNAse free DNAse I, 10 μg/ml. B – SeV N vNLPs treated with RNAse A, 10 μg/ml. Lane 1 — nucleic acids isolated from vNLPs; lane 2 — nucleic acids isolated from NLPs treated with RNAse free DNAse I, 10 μg/ml; lane 3 — nucleic acids isolated from vNLPs treated with RNAse A, 10 μg/ml. ^{32}P labelled total yeast cDNR from vector harbouring the recipient strain was used in the hybridization assay.

Synthesis of recombinant human parainfluenza virus 1 and 3 N proteins in yeast *S.cerevisiae*

Prototype strains of hPIV1 (C-35) and hPIV3 (C-243) were used to obtain the hPIV1 and hPIV3 *N* genes, respectively. Nucleic acids were subjected to a single step combined RT-PCR amplification reaction. For cloning into the yeast expression vector, the hPIV1 and hPIV3 *N* genes were re-amplified using specific primers, which included a *BcuI* site for sub-cloning into the yeast expression vector. After amplification, DNA bands corresponding to the hPIV1 and hPIV3 *N* genes were gel-purified and cloned into the *pBluescript II KS* plasmid for sequencing. The hPIV1 and hPIV3 *N* genes isolated from the *pBluescript II KS* were ligated into the *BcuI* site of the *pFGG3* yeast expression vector under the control of *GAL7* promoter. The resulting *pFGG3-hPIV1-N* and *pFGG3-hPIV3-N* plasmids, each separately, were used for transformation of yeast *S.cerevisiae* strain *214Δpep4*.

The procedure used for the expression of the hPIV1 and hPIV3 *N* genes in *S.cerevisiae* cells was similar to that described for the expression of the SeV *N* gene. After induction yeast cells harbouring the *pFGG3-hPIV1-N* and *pFGG3-hPIV3-N* expression plasmids, separately, were re-suspended in DB and disrupted by homogenization in the presence of glass beads. Synthesis of recombinant N proteins of hPIVs was analyzed by SDS-PAGE. The SDS-PAGE analysis of crude lysates of induced *S.cerevisiae* cells harbouring the *pFGG3-hPIV1-N* and *pFGG3-hPIV3-N* expression plasmids revealed the presence of an additional protein band of approximately 60 and 64 kDa, respectively (Fig. 4 A and B, lanes 2). The molecular weights of these bands are consistent with molecular weights of native N proteins of corresponding hPIVs reported in literature (Henrickson, 2003; Miyahara et al., 1992). No additional bands of the corresponding molecular size were observed in crude lysates of *S.cerevisiae* cells harbouring an empty vector (Fig. 4 A and B, lanes 1). The SDS-PAGE analysis revealed that both N proteins were expressed at high levels. The identity of the recombinant hPIV1 N protein was confirmed by WB with hyperimmune rabbit sera generated by

immunizing rabbit with purified SeV (closely related murine parainfluenza virus type 1 which N protein shares high amino acid sequence homology with hPIV1) virions (Neubert et al., 1991). The identity of the recombinant hPIV3 N protein was confirmed with commercial polyclonal goat anti-hPIV3 antibodies (Chemicon International, Cat. No. AB1070). Only minute presence of degradation products in crude lysates detected by WB had indicated that recombinant N proteins of hPIVs are quite stable in yeast cells.

In order to identify the optimum conditions for maximum synthesis level of hPIV1 and hPIV3 N proteins, yeast cells harbouring the *pFGG3-hPIV1-N* and *pFGG3-hPIV3-N* expression plasmids were collected at various time points after induction and disrupted. Then, crude yeast lysates were subjected to WB. The WB analysis revealed that maximum synthesis level of recombinant hPIV1 and hPIV3 N proteins were achieved cultivating yeast for 24 h in the induction medium. Furthermore, synthesis level of hPIV1 and hPIV3 N proteins were increased almost by 25 % when the induction medium containing higher concentration of galactose (2.5 %) was used to induce the heterologous protein synthesis. Under the optimal conditions calculated maximal synthesis yield of hPIV1 and hPIV1 N proteins were approximately ~20 and 16 mg per liter of induced yeast culture, respectively.

For the purification of yeast-synthesized hPIV1 and hPIV3 N proteins, induced yeast cells harbouring the pFGG3-hPIV1-N and pFGG3-hPIV3-N expression plasmids were collected, re-suspended in DB and disrupted by homogenization in the presence of glass beads. Then, SPF was separated and analyzed by WB with hPIV1 and hPIV1 specific antibodies, respectively. The WB analysis revealed that the yeast-synthesized hPIV1 N protein is soluble and only trace amounts of this protein were detected in the pellets of disrupted yeast cells. Contrary, almost 30 % of the yeast-expressed hPIV3 N protein was insoluble and found in pellets together with derbies of disrupted yeast cells. In order to dissolve insoluble fraction of the yeast-expressed hPIV3 N protein, various concentrations of detergents Tween^{20} and Triton X-100 in the DB were used to disrupt yeast cells. After disruption the SPF was separated and pellets together with the

SPF were analyzed by WB. The WB analysis revealed that almost all yeast-expressed hPIV3 N proteins had become soluble when treated with 2 % of Tween^{20} or 1 % of Triton X-100. For further purification of the yeast-expressed hPIV3 N protein all solutions used in purification procedures were supplemented with 2 % of detergent Tween^{20}.

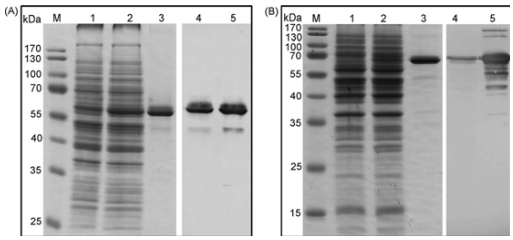

Fig.4. SDS-PAGE (lanes 1–3) and Western blot (lanes 4 and 5) analysis of yeast lysates and samples of yeast-expressed hPIV1 (A) and hPIV3 (B) N proteins after ultracentrifugation in CsCl. 5 μg of purified N protein (A and B, lanes 3 and 5) or 20 μg of yeast lysates (A and B, lanes 1, 2 and 4) were separated in a 12 % SDS-PAGE and stained with Coomassie brilliant blue. After separation proteins hPIV1 (A, lanes 4 and 5) was analyzed by Western blot using hyperimmune rabbit sera generated by immunizing rabbits with purified Sendai virions (Juozapaitis et al., 2005) and HPIV3 (B, lanes 4 and 5) was analyzed using goat anti-parainfluenza virus 2 and 3 polyclonal antibodies (Chemicon International, Cat. No. AB1070). Lanes 1: *Saccharomyces cerevisiae* [*pFGG3*] lysate; lanes 2 and 4: (A) *S.cerevisiae* [*pFGG3-hPIV1-N*] lysate, (B) *S.cerevisiae* [*pFGG3-hPIV3-N*] lysate; lane 3 and 5: CsCl purified hPIV1 (A) and hPIV3 (B) N proteins; M: protein molecular mass marker (Fermentas UAB) 170, 130, 100, 70, 55, 40, 35, 25 and 15 kDa.

The yeast-expressed N proteins of hPIVs were purified by successive ultracentrifugation through 30 % sucrose cushion followed by two ultracentrifugations in a CsCl gradient ranging from 1.23 to 1.38 g/ml^3. Fractions containing N proteins were identified by SDS-PAGE. After the first centrifugation in CsCl gradient fractions containing proteins with molecular weight corresponding to native hPIV1 or hPIV3 N proteins (60 and 64 kDa, respectively) were pooled, diluted with 1.31 g/ml^3 CsCl and repeatedly centrifuged in the CsCl gradient. Then, CsCl gradient fractions were collected and those containing recombinant N proteins were pooled and dialyzed against PBS for electron microscopy (EM). The buoyant density of the fractions was determined with a refractometer. In several preparative procedures, yield of purified recombinant N proteins of hPIV1 and hPIV3 were found to be 20 and 16 mg per liter of induced yeast culture, respectively. The recombinant N proteins were stable in CsCl solution at 4°C. Dialyzed samples were stored at −20°C in PBS containing 50 % glycerol. The obtained

yield of recombinant N proteins of hPIVs resembles to those observed previously for the yeast-expressed proteins of different paramyxoviruses (Razanskiene et al., 2004; Samuel et al., 2002b; Sasnauskas et al., 1999). Also, recombinant N proteins of hPIVs are stable and show no significant degradation when synthesized, purified or stored for a long period at −20°C.

Electron microscopy revealed that both recombinant N proteins of hPIVs form virus nucleocapside-like particles and rings which correspond to shorter nucleocapsids viewed along their axis (Fig. 5 A and B). In the case of the yeast-derived N protein of hPIV3, lengthy rods and the low abundance of ring structures suggest a relatively high intrinsic stability of nucleocapsids formed by the heterologically synthesized N protein (Fig. 5 B). Formation of vNLPs in yeast occurs in the absence of other viral proteins. If host cells proteins are involved in the formation of vNLPs, they appear to be conserved in both yeast and mammalian cells.

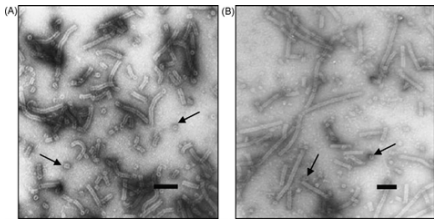

Fig.5. Electron micrographs of negatively stained hPIV1 (A) and hPIV3 (B) N proteins isolated from *S.cerevisiae*. Scale bar: 100 nm. Arrows indicate ring-like structures.

Synthesis of recombinant human respiratory syncytial virus N proteins in yeast *S.cerevisiae*

A human respiratory syncytial virus (hRSV) *N* gene was amplified from cDNA isolated from patients infected with the hRSV A2 strain (the major antigenic subgroup A) (GenBank Acc. no. M11486). The cDNA of the hRSV A2 strain was kindly provided by Dr. M. Coiras (*National Center of Microbiology, de Salud Carlos III Institute, Spain*). Primers used in the amplification of the hRSV *N* gene included a *SpeI* site for subcloning into the *pFGG3* yeast expression vector. The resulting *pFGG3-hRSV-N* plasmid was used for the transformation of yeast *S.cerevisiae* strain *214Δpep4*.

Synthesis of the recombinant hRSV N protein was analyzed in SDS-PAGE. The SDS-PAGE analysis of crude lysates of *S.cerevisiae* harbouring the *pFGG3-hRSV-N* expression plasmid, after induction with galactose revealed the presence of an additional 44 kDa protein band (Fig. 6 A, lane 2) consistent with the molecular weight of N protein of human respiratory syncytial virus reported in literature (Garcia-Barreno et al., 1996). No additional band of the corresponding molecular size was observed in crude lysates of *S.cerevisiae* cells harbouring the *pFGG3* expression vector (Fig. 6 A, lane 1). After centrifugation through 30 % sucrose cushion the recombinant hRSV N protein was found in pellets. Further, ultracentrifugation of the re-solubilized pellets in a CsCl gradient and analysis revealed the recombinant hRSV N protein in fractions with a buoyant density of 1.28–1.29 g/cm^3. The buoyant density ranging from 1.28 g/cm^3 to 1.31 g/cm^3 is characteristic for most virus-like particles or vNLPs, with or without nucleic acids (Myers et al., 1999). Fractions of this buoyant density contained the highly purified hRSV N protein with only minor amounts of contaminants as observed in SDS-PAGE. The SDS-PAGE analysis of the purified hRSV N protein revealed a band of 44 kDa (Fig. 6 A, lane 3), which was also observed in crude lysates (Fig. 6 A, lane 2). The identity of the recombinant hRSV N protein was confirmed by WB with hyperimmune sera generated by immunizing goat with purified hRSV virions (Fig. 6 B). Electron microscopy analysis revealed that the recombinant hRSV N protein form typical herring-bone and ring-like structures (Fig. 7). Similar vNLPs form yeast-derived N proteins of various paramyxoviruses (Juozapaitis et al., 2005). Yeast-derived hRSV vNLPs appeared to be loosely ordered and highly flexible helices. Weakly organized helical nucleocapsid is a characteristic feature of the genus *Pneumovirinea* (Bhella et al., 2002).

In several preparative procedures, the yield of the recombinant hRSV N was found to be 18 mg per liter of induced yeast culture. The yield obtained resembles that observed previously for N proteins of different other viruses (Slibinskas et al., 2004). The hRSV N protein was stable in CsCl at 4 °C. The recombinant hRSV vNLPs were stable after dialysis and long-term storage in 50% glycerol at –20 °C.

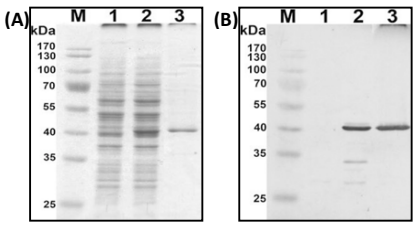

Fig.6. SDS-PAGE analysis of yeast lysates and samples of yeast expressed hRSV N protein after ultracentrifugation in CsCl. A – Comassie blue stained gel. B – Western blot using hyperimmune goat sera generated by immunizing goats with purified virions (Chemicon, Temecula, USA). Preparations loaded onto gels were as follows: lane 1 – 20 µg of yeast *S.cerevisiae* [*pFGG3*] lysate; lane 2 – 20 µg of yeast *S.cerevisiae* [*pFGG3-hRSV-N*] lysate; lane 3 – 1 µg of CsCl purified hRSV N protein; M — protein molecular mass marker (Fermentas UAB) 170, 130, 100, 70, 55, 40, 35 and 25 kDa.

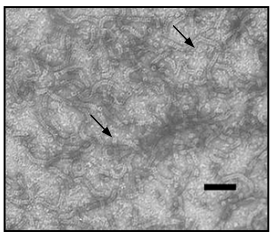

Fig.7. Electron micrographs of negatively stained hRSV N protein isolated from S. cerevisiae. Scale-bar: 100 nm. Arrows indicate ring-like structures.

Synthesis of recombinant henipah virus N proteins in yeast *S.cerevisiae*

For cloning into the yeast expression vector, the Nipah virus (NiV) and Hendra virus (HeV) *N* genes were amplified from the *pFastBacHT-NiV-N* (Chua et al., 2000, GenBank accession no. AF212302) and *pFastBacHT-HeV-N* (Wang et al., 2000, GenBank accession no. AF017149) plasmids containing cDNA of the NiV and HeV *N* genes, respectively. Primers used in the amplifications of the NiV and HeV *N* genes included a *SpeI* site for subcloning into the yeast expression vector. The resulting *pFGG3-NiV-N* and *pFGG3-HeV-N* plasmids, each separately, were used for transformation of yeast *S.cerevisiae* strain *214Δpep4*.

The procedures used for expression of the *N* genes in *S.cerevisiae* cells and purification of the recombinant N proteins were described previously (Samuel et al., 2002b; Slibinskas et al., 2004). The synthesis of the recombinant N proteins was

analyzed by SDS-PAGE and WB. The SDS-PAGE analysis of crude lysates of *S.cerevisiae* cells harboring the *pFGG3-NiV-N* and *pFGG3-HeV-N* expression plasmids, after induction with galactose revealed the presence of an additional protein band of approximately 62 kDa (Fig. 8 A and B, lane 2) consistent with the molecular weight of native N proteins of henipah viruses reported in literature (Eshaghi et al., 2005; Wang et al., 2000). WB using a cross-reactive monoclonal antibody generated against Nipah virus N protein immunostained bands consistent with NiV and HeV N proteins in the galactose-induced yeast cells harbouring the *pFGG3-NiV-N* and *pFGG3-HeV-N* expression plasmids (Fig. 8 A and B, lane 5) and in the CsCl fractions containing purified NiV and HeV N proteins (Fig. 8 A and B, lane 6). Immunostaining was not observed in induced yeast cells harboring the *pFGG3* plasmid without a gene insert (Fig. 8 A and B, lane 4). In WB low molecular weight bands are also clearly visible (Fig. 8 A and B, lane 6), suggesting that some degradation of the NiV and HeV N proteins occurred during the synthesis and purification.

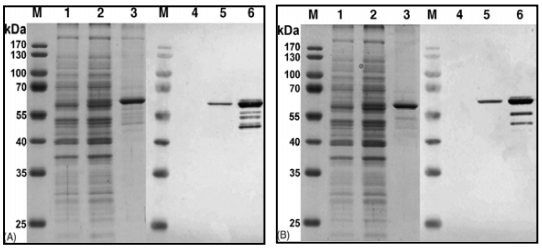

Fig.8. SDS-PAGE (lanes 1–3) and Western blot (lanes 4–6) analysis of yeast lysates and samples of yeast expressed NipV (A) and HenV (B) N proteins after ultracentrifugation in CsCl. 2 μg of purified N proteins (lanes 3 and 6) or 20 μg of yeast lysates (lanes 1, 2 and 4, 5) were separated in a 12% SDS-PAGE and stained with Coomassie brilliant blue. After separation proteins were analyzed by Western blot using mouse monoclonal antibody generated by immunizing mouse with purified Nipah N protein. (A) Lanes 1 and 4, *S.cerevisiae* [*pFGG3*] lysate; Lanes 2 and 5, *S.cerevisiae* [*pFGG3-NiV-N*] lysate; lanes 3 and 6, CsCl purified NiV N protein. (B) Lanes 1 and 4, *S.cerevisiae* [*pFGG3*] lysate; Lanes 2 and 5, *S.cerevisiae* [*pFGG3-HeV-N*] lysate; lanes 3 and 6, CsCl purified HeV N protein. M — protein molecular mass marker (Fermentas UAB) 170, 130, 100, 70, 55, 40, 35 and 25 kDa.

After centrifugation through a 30 % sucrose cushion the recombinant N proteins of both viruses were found in pellets (data not shown), which is consistent with their large size and multimeric organization. Ultracentrifugation of the solubilized pellets in a CsCl gradient and analysis revealed the recombinant N proteins in fractions with

buoyant densities of 1.28–1.30 g/cm^3. Fractions of this buoyant density contained the highly purified N proteins with only minor contaminants as observed in SDS-PAGE. The SDS-PAGE and WB analysis of the purified N proteins of henipah viruses after centrifugation in CsCl revealed bands of ~62 kDa (Fig. 8 A and B, lanes 3 and 6), which was also observed in crude lysates (Fig. 8 A and B, lanes 2 and 5). Both expressed recombinant N proteins formed nucleocapsid-like herringbone structures and rings, which correspond to shorter nucleocapsids viewed along their axis (Fig. 9 A and B). The abundance of ring structures suggests an intrinsic fragility in nucleocapsids formed by heterologically synthesized nucleocapsid proteins. The N protein structures of henipah viruses observed in yeast expression system were similar to those previously observed for nucleocapsids of other paramyxoviruses, expressed in different systems (Fooks et al., 1993; Rima et al., 1983; Slibinskas et al., 2004; Thorne and Dermott, 1977). In yeast expression system formation of vNLPs occurs in the absence of other viral proteins. If host cell proteins are involved in formation of vNLPs, they appear to be conserved in both yeast and mammalian cells. In repeated preparative procedures, yields of purified recombinant N proteins of NiV and HeV were 20 mg per one liter of induced yeast culture. The yields obtained were similar to those reported previously for proteins of other viruses generated in yeast expression system (Razanskiene et al., 2004; Samuel et al., 2002b; Sasnauskas et al., 1999). In comparison to other expression systems used for henipavirus N protein expression, the yeast system employed in this study was fairly stable with only minimal degradation observed in WB (Fig. 8 A and B, lane 6). The main differences in amino acid sequences of the two N proteins of henipavirus are situated in the C-terminal part of the protein (Fig. 10). The C-terminal part of N protein is usually hypervariable and has no major effect on the formation of vNLPs in different heterologous expression systems (Longhi et al., 2003). Expression in yeast of henipavirus N proteins (1–399 aa) with a C-terminal deletion have shown that truncated N proteins formed rods as efficiently as full length proteins, however, the solubility of such proteins was lower in comparison with full length constructs (Fig. 9 C and D).

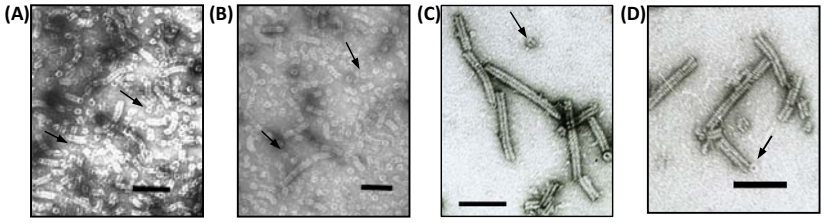

Fig.9. Electron micrographs of negatively stained NiV (A) and HeV (B) N proteins isolated from *S.cerevisiae*. Electron micrographs of negatively stained NiV (C) and HeV (D) $N_{(N1-399)}$ proteins isolated from *S.cerevisiae*. Scale bar: 100 nm. Arrows indicate ring-like structures.

```
                1                                                  50
NiV N     (1) MSDIFTEAASFRSYQSKLGRDGRASAATATLTTKIRIFVPATNSPELRWE
HeV N     (1) MSDIFDEAASFRSYQSKLGRDGRASAATATLTTKIRIFVPATNSPELRWE
               51                                                 100
NiV N    (51) LTLFALDVIRSPSAAESMKVGAAFTLISMYSERPGALIRSLLNDPDIEAV
HeV N    (51) LTLFALDVIRSPSAAESMKIGAAFTLISMYSERPGALIRSLLNDPDIEAV
              101                                                 150
NiV N   (101) IIDVGSMVNGIPVMERRGDKAQEEMEGLMRILKTARDSSKGKTPFVDSRA
HeV N   (101) IIDVGSMLNGIPVMERRGDKAQEEMEGLMRILKTARESSKGKTPFVDSRA
              151                                                 200
NiV N   (151) YGLRITDMSTLVSAVITIEAQIWILIAKAVTAPDTAEESETRRWAKYVQQ
HeV N   (151) YGLRITDMSTLVSAVITIEAQIWILIAKAVTAPDTAEESETRRWAKYVQQ
              201                                                 250
NiV N   (201) KRVNFFALTQQWLTEMRNLLSQSLSVRKFMVEILIEVKKGGSAKGRAVEI
HeV N   (201) KRVNFFALTQQWLTEMRNLLSQSLSVRKFMVEILMEVKKGGSAKGRAVEI
              251                                                 300
NiV N   (251) ISDIGNYVEETGMAGFFATIRFGLETRYPALALNEFQSDLNTIKSIMLLY
HeV N   (251) ISDIGNYVEETGMAGFFATIRFGLETRYPALALNEFQSDLNTIKGLMLLY
              301                                                 350
NiV N   (301) REIGPRAPYMVLLEESIQTKFAPGGYPLLWSFAMGVATTIDRSMGALNIN
HeV N   (301) REIGPRAPYMVLLEESIQTKFAPGGYPLLWSFAMGVATTIDRSMGALNIN
              351                                                 400
NiV N   (351) RGYLEPMYFRLGQKSARHHAGGIDQNMANRLGLSSDQVAELAAAVQETSA
HeV N   (351) RGYLEPMYFRLGQKSARHHAGGIDQNMANKLGLNSDQVAELAAAVQETSV
              401                                                 450
NiV N   (401) GRQESMVQAREAKFAAGGVLIGGSDQDIDEGEEPIEQSGRQSVTFKREMS
HeV N   (401) GRQDNMMQAREAKFAAGGVLVGGGEQDIDEEEEPIEHSGRQSVTFKREMS
              451                                                 500
NiV N   (451) LSSLANSVPSSSVSTSGGTRLTNSLLNLRSRLAAKAAKEAASSNATDDPA
HeV N   (451) MSSLADSVPSSSVSTSGGTRLTNSLLNLRSRLAAKAIKETAQ-SSSERN
              501                                                 531
NiV N   (501) ISNRTQGESEKNNQDLKPAQNDLDFVRADV
HeV N   (500) PNNRPQADSGRKDDQSPKPAQNDLDFVRADV
```

Fig.10. Alignment of amino acid sequences of nucleocapsid proteins of HeV (Wang et al., 2000; GenBank accession no. AF017149) and NiV (Chua et al., 2000; GenBank accession no. AF212302). Underlined sequences were confirmed by MS/MS analysis.

In order to confirm the sequence identity of the N proteins, enzymatic digests of nucleocapsids were performed using trypsin, chymotrypsin and endoproteinase-AspN to generate internal peptides from the recombinant N proteins for detailed LC MS/MS analysis. A composite of results from peptides identified from all three enzymes digests by cross-correlation (Sequest) analysis of MS/MS data was used to construct overall amino acid sequence coverage for each protein, and is displayed as underlined sequence in Fig. 10. Coverage for both proteins was in excess of 92%. MS/MS analysis

revealed that the amino terminal of both N proteins is modified by removal of methionine and that serine at position 2 is acetylated. A similar modification has been found in native N proteins of Menangle (Shiell et al., 2002) and Hendra viruses (B. Shiell, W. Michalski, personal communication).

Synthesis of recombinant Menangle virus N protein in yeast *S.cerevisiae*

For cloning into the yeast expression vector, an insert containing the full-length open reading frame (ORF) for the MenV N protein was amplified from the *pCR4-TOPO-MenV-N* plasmid (Bowden et al., 2001; GenBank accession no. AF326114). Primers used in the amplification of the ORF included an *XbaI* site for sub-cloning into the yeast expression vector. In addition, a truncated version of the MenV N ORF, which lacked the coding sequence for the C-terminal tail (MenV $N_{(N1-399)}$), was also amplified from the *pCR4-TOPO-MenV-N* plasmid for insertion into the yeast expression vector. The size of truncated protein was selected after comparison to measles N protein by using consensus secondary structure prediction server JPRED (Cuff et al., 1998, available online http://www.compbio.dundee.acuk/~www-jpred/). The resulting *pFGG3-MenV-N* and *pFGG3-MenV-*$N_{(N1-399)}$ plasmids, respectively, were used for transformation of yeast *S.cerevisiae* strain *214Δpep4*.

Expression of the recombinant MenV N protein in yeast was analyzed by SDS-PAGE. The SDS-PAGE analysis of crude lysates of *S.cerevisiae* cells harbouring *pFGG3-MenV-N* expression plasmid, after induction with galactose, revealed the presence of an additional protein band of approximately 68 kDa (Fig. 11 A, lane 2). No additional band of the corresponding molecular size was observed in crude lysates of S.cerevisiae cells harbouring *pFGG3* vector without an insert (Fig. 11 A, lane 1). The SDS-PAGE analysis also revealed that the molecular weight of the yeast-expressed N protein (~68 kDa) differed from that of native MenV N protein (~61 kDa) reported in literature (Shiell et al., 2002).

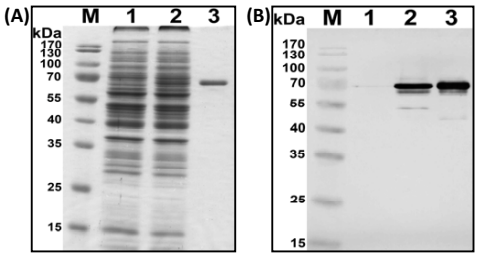

Fig.11. SDS-PAGE analysis of yeast lysates and samples of yeast expressed MenV N protein after ultracentrifugation in CsCl. A – Comassie blue stained gel. B – Western blot using using mouse monoclonal antibodies (MAb 2D1–B) generated by immunizing mice with purified recombinant full-length MenV N protein. Preparations loaded onto gels were as follows: lane 1 – 20 μg of yeast *S.cerevisiae* [*pFGG3*] lysate; lane 2 – 20 μg of yeast *S.cerevisiae* [*pFGG3-MenV-N*] lysate; lane 3 – 1 μg of CsCl purified MenV N protein; M — protein molecular mass marker (Fermentas UAB) 170, 130, 100, 70, 55, 40, 35, 25 and 15 kDa.

After centrifugation through a 30% sucrose cushion the recombinant MenV N protein was found in pellets (data not shown), which is consistent with their large size and multimeric organization. Ultracentrifugation of the solubilized pellets in a CsCl gradient revealed the recombinant N protein in fractions with buoyant densities of 1.30–1.32 g/cm^3. Fractions of this buoyant density contained highly purified N proteins, with only minor contaminants present, as observed by SDS-PAGE. The SDS-PAGE analysis of the CsCl-purified MenV N protein revealed a band of ~68 kDa (Fig. 11 A, lane 3), which was also observed in crude lysates (Fig. 11 A, lane 2). In repeated preparative procedures, the yield of the purified recombinant MenV N protein was ~10 mg per liter of induced yeast culture, which was similar to that reported previously for proteins of other viruses generated in yeast expression systems (Razanskiene et al., 2004; Samuel et al., 2002b; Sasnauskas et al., 1999).

The yeast-expressed MenV N protein formed nucleocapsid-like herringbone structures and rings, which corresponded to shorter nucleocapsids viewed along their axis (Fig. 12 A). The abundance of ring structures suggests an intrinsic fragility in nucleocapsids formed by the heterologically expressed nucleocapsid protein. The MenV N protein structures observed in the yeast expression system were similar to those previously observed for nucleocapsids of other paramyxoviruses expressed in different systems (Fooks et al., 1993; Rima et al., 1983; Slibinskas et al., 2004; Thorne and

Dermott, 1977). The expression of the MenV $N_{(N1\text{-}399)}$ construct demonstrated that this truncated N protein formed significantly longer and more dense nucleocapsid-like particles compared to full-length proteins and, as was also demonstrated by electron microscopy, the proportion of ring-like structures in the purified fractions of this protein was relatively low (Fig. 12 B).

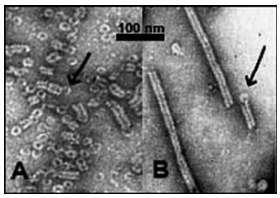

Fig.12. Electron micrographs of negatively stained MenV N (A) and $N_{(N1\text{-}399)}$ (B) proteins isolated from *S.cerevisiae*. Scale bar: 100 nm. Arrows indicate ring-like structures.

```
                1                                                  50
MenV N    (1)  MSSVFRAFELFTLEQEQNEHGNDIELPPETLRTNIKVCTLNNQEPQARHD
               51                                                 100
MenV N   (51)  MMCFCLRLIASNSARAAHKTGAILTLLSLPTAMMQNHLRIADRSPDADIE
              101                                                 150
MenV N (101)   RLEIDGFEPGTFRLRANARTPMTNGEVTALNLMAQDLPDTYSNDTPFLNP
              151                                                 200
MenV N (151)   NTETEQCDEMEQFLNAIYSVLVQVWVTVCKCMTAHDQPTGSDERRLAKYQ
              201                                                 250
MenV N (201)   QQGRLDQRYALQPELRRQIQTCIRSSLTIRQFLTHELQTARKQGAITGKY
              251                                                 300
MenV N (251)   YAMVGDIGKYIDNAGMSAFFMTMRFALGTKWPPLALAAFSGELLKLKSLM
              301                                                 350
MenV N (301)   QLYRGLGDRARYMALLEMSEMMEFAPANYPLCYSYAMGIGSVQDPMMRNY
              351                                                 400
MenV N (351)   TFARPFLNPAYFQLGVETANRQQGSVDKAMAAELGLTEDEKRDMSAAVTR
              401                                                 450
MenV N (401)   LTTGRGGNQAQELINVMGARQGRDQGRRGNRDYDVVEENEETESDSDNDE
              451                                                 500
MenV N (451)   EQEIQNRPLPPIPQMPQNIDWEVRLAEIERRNQQMAARDRPQAVVTADVH
              501             519
MenV N (501)   QEPVDARVDEQDMLLDLDM
```

Fig.13. Sequence confirmation (underlined) of recombinant MenV N protein by LC-MS/MS analysis of various enzymatic digests.

In order to confirm the sequence identity of the full-length recombinant N protein, enzymatic digests were performed using trypsin, chymotrypsin and endoproteinase-AspN to generate internal peptides for detailed LC-MS/MS analysis. A composite of results from peptides identified from digests with all three enzymes by cross correlation (Sequest) analysis of MS/MS data was used to construct overall amino acid sequence coverage for the N protein, and is displayed as underlined sequence in Fig. 13. Sequence coverage was in excess of 87 % and included extensive coverage of both the

amino and carboxy termini. The MS/MS analysis also revealed that the amino terminal is modified by removal of methionine and that serine at position 2 is acetylated. This modification has also been found in native N proteins of Menangle (Shiell et al., 2002) and Hendra (B. Shiell, W. Michalski, personal communication) viruses. Regions of sequence where no coverage was obtained by LC-MS/MS analysis generally corresponded to small peptide fragments that were below the mass/charge range of the instrument.

Immunoreactivity of yeast-expressed hPIV1 and hPIV3 N proteins with human sera

The immunoreactivity of the recombinant hPIV1 and hPIV3 N proteins was tested in an indirect ELISA format using serum samples collected from hPIV-infected patients. In five cases paired serum samples were available. In two of them, seroconversion to the homologue type from negative to positive (case 3, due to hPIV3), and from low positive to positive (case 2, due to hPIV1) was detected. Case 1 (with a positive identification of hPIV1) and case 4 showed positive results for both hPIV1 and hPIV3, both in acute and convalescent sample. Case 5 showed no antibodies to hPIV1 and hPIV3. In the cases with single serum sample and typification available, the serological study confirmed the diagnosis (cases 6 and 8). The remaining cases were classified as probable hPIV3, due to the reactivity to this virus. The observed cross-reactivity of hPIV1 and hPIV3 N proteins with serum antibodies (as in case 1 with confirmed hPIV1 infection) is explained by a high level of homology between hPIV1 and hPIV3 N protein sequences. Six serum specimens equivocal (+/−) for hPIV-IgG in commercial ELISA system were found to be positive in vNLP-based anti-hPIV1/3 IgG assays. Thus, low levels of anti-hPIV IgG developed at an early stage of the infection were not detectable by a commercial assay but were detectable in vNLP-based assays. The discrepancy between vNLP-based and commercial test might be explained by the fact that commercial kit is based on the use of total viral proteins with a relatively low content of viral nucleocapsids. It is well documented that most paramyxoviruses induce a strong and long-lasting antibody response directed against nucleocapsid proteins (Pumpens et

al., 2002; Samuel et al., 2002b). To confirm the specificity of NLP-based assays, control serum samples (negative and positive for HPIV-IgG, respectively) from the commercial kit were analyzed. The negative control was non-reactive, and the positive control was strongly reactive in vNLP-based ELISAs (Table 1). The reactivity of vNLPs with serum antibodies suggests that recombinant hPIV1 and hPIV3 N proteins show antigenic similarity with viral nucleocapsids and mimic infectious virus in the presentation of surface-exposed immunodominant epitopes. To confirm the reactivity of serum antibodies with recombinant hPIV1 and hPIV3 N proteins, Western blot analysis with hPIV-IgG-positive serum samples was performed. Protein bands corresponding to the MW of hPIV1 and hPIV3 N proteins (60 and 64 kDa, respectively) specifically immunostained with serum hPIV-IgG were detected (Fig. 14, lanes 2 and 3). These data demonstrate that recombinant yeast-derived vNLPs are specifically recognized by serum antibodies developed during hPIV1/hPIV3 infection and therefore might be suitable for the development of serological tests for the detection of hPIV1/hPIV3-specific antibodies.

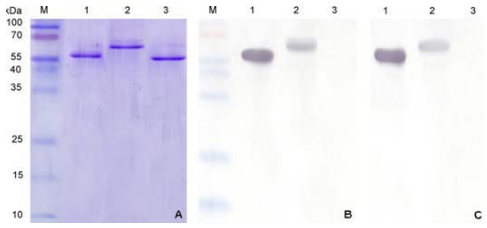

Fig.14. Immunoreactivity of recombinant hPIV1 and hPIV3 N proteins with serum specimens #1-c (B) and #1-a (C) from a patient with confirmed hPIV1 infection (Table 1). SDS-PAGE (A) and Western blots (B and C) analysis of purified hPIV1 N protein (lanes 1), hPIV3 N protein (lanes 2) and yeast-expressed lyssavirus(CVS) N protein (negative control, lanes 3; Kucinskaite et al., 2007). Recombinant proteins were separated in a 12% SDS-PAGE (A–C) and stained with Coomassie brilliant blue (A). M: protein molecular mass marker (Fermentas UAB) .

Table 1. Immunoreactivity of recombinant yeast-derived hPIV1 and hPIV3 vNLPs with serum antibodies of patients with confirmed HPIV infection.

Case[a]	IBL-hPGV1/2/3 IgG ELISA kit		hPGV1 N IgG ELISA		hPGV3 N IgG ELISA		hPIV type	Serological classification
1-a	0.32	+/-	3.39	+++	3.32	+++	hPGV1	
1-c	2.16	+++	3.29	+++	3.19	+++		Cross-reactivity
2-a	0.10	-	0.39	+/-	0.43	+/-		
2-c	0.46	+/-	2.97	+++	0.55	+		hPGV1
3-a	0.09	-	0.22	-	0.29	-		
3-c	0.33	+/-	0.23	-	2.35	+++		hPGV3
4-a	0.31	+/-	0.82	+	2.73	+++		
4-c	0.42	+/-	1.21	++	2.45	++		Cross-reactivity
5-a	0.14	-	0.07	-	0.10	-		
5-c	0.13	-	0.07	-	0.10	-		Negative
6-a	0.58	+	0.35	+/-	0.79	+	hPGV3	hPGV3
7-a	0.36	+/-	0.29	-	0.98	+		hPGV3
8-c	0.16	-	2.56	+++	0.44	+/-	hPGV1	hPGV1
9-c	0.09	-	0.29	-	0.47	+/-		Probable hPGV3
10-c	0.10	-	0.39	-	0.48	+/-		Probable hPGV3
11-c	0.12	-	0.25	-	0.49	+/-		Probable hPGV3
Control serum samples from IBL-hPGV1/2/3 IgG ELISA kit								
Positive	2.39	+++	2.93	+++	3.06	+++		
Negative	0.06	-	0.08	-	0.08	-		

+++: OD value, >1.5; ++: OD value, 1.0-1.5; +: OD value, 0.5-1.0; OD value, 0.3-0.5; -: OD value, <0.3.

[a] a: acute sample; c: convalescent sample.

Immunoreactivity of yeast-expressed hRSV N protein with human sera

The immunoreactivity of the recombinant hRSV N protein was tested in an indirect ELISA format using 61 serum samples collected from hospitalized pediatric patients with respiratory illness. At the beginning all collected serum samples were tested in direct immunofluorescence assay with commercially available anti-hRSV antibodies (Chemicon, JAV). Two of them showed positive results for hRSV. To confirm the specificity of vNLP-based assays, control serum samples (negative and positive for hRSV-IgG, respectively) from the commercial kit were analyzed. The negative control was non-reactive, and the positive control was strongly reactive in vNLP-based ELISAs. Thirty three serum specimens positive for hRSV-IgG in commercial ELISA system were found to be positive in vNLP-based anti-hRSV IgG assays. Thus, three serum specimens tested negative by the commercial ELISA kit were positive in vNLP-based assays and one

was false negative. The reactivity of vNLPs with serum antibodies suggests that the recombinant hRSV N protein shows antigenic similarity with viral nucleocapsids and mimic infectious virus in the presentation of surface-exposed immunodominant epitopes.

Immunoreactivity of yeast-expressed henipavirus N proteins

Immunoreactivity of recombinant proteins was tested with a set of sera derived from humans, fruit bats and horses infected with HeV, and from humans, fruit bats and pigs infected with NiV (Fig. 15). The sera were sourced from the Australian Animal Health Laboratory Serum Bank and some of them were obtained during the outbreaks of HeV in Queensland, Australia and of NiV in Nipah, Malaysia. Both recombinant proteins, in nanogram quantities, were detected by corresponding positive sera, indicating that yeast produced recombinant N proteins are suitable for antibody detection in different species. However, it should be pointed out that further optimization is required to reduce the background reactivity with negative sera of some species, particularly bat and pig, before the tests can be applied in outbreak investigation and/or serological surveillance. Due to the highly conserved nature of henipavirus N proteins, serological tests based on recombinant N proteins are not expected to be capable of differentiation between antibodies against HeV and NiV. This is evident from the data presented in Fig. 15.

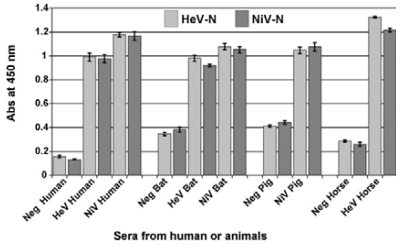

Fig.15. Immunodetection of HeV and NiV N proteins with sera derived from humans and fruit bats infected with HeV or NiV, from horses infected with HeV and pigs infected with NiV. Error bars represent ±S.D. from three independent measurements. All sera were from the Australian Animal Health Laboratory Serum Bank.

Immunoreactivity of yeast-expressed menangle virus N protein

The immunoreactivity of the full-length recombinant MenV N protein was tested in an indirect ELISA format using paired (naive and convalescent) sera derived from experimentally infected pigs. Clear differentiation between naïve and convalescent sera was evident for each pig tested using nanogram quantities of MenV N (see Fig. 16), indicating that this recombinant protein is suited to the development and standardization of serological tests for the detection of MenV-specific antibodies.

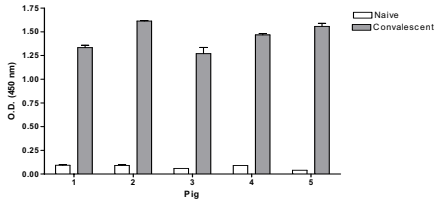

Fig.16. Immunoreactivity of yeast-expressed MenV N protein in an indirect ELISA format. All sera tested were from experimentally infected pigs. Naïve and convalescent sera were collected prior to and three weeks post infection with MenV, respectively. Optical density (O.D.) values are the means of triplicate samples and error bars indicate standard deviations.

Generation of monoclonal antibodies against yeast-expressed hPIV1, hPIV3, NiV and MenV N proteins

Recombinant hPIV1, hPIV3, NiV and MenV N proteins expressed in yeast cells and self-assembled into virus nucleocapsid-like particles were used here as immunogens for production of MAbs. The generated MAbs reacted strongly with corresponding recombinant N proteins by WB. The MAbs did not show any crossreactivity with irrelevant antigens — yeast-expressed N proteins of heterologous virus and proteins of crude yeast lysate. Their specificity of the MAbs was assessed in detail and summarized in table 2.

To localize the epitopes recognized by the generated MenV N specific MAbs, their reactivity was assessed with truncated forms of MenV N protein lacking 29–119 aa residues at the C-terminus. For this, genes coding his-tagged intact MenV N protein and its carboxy (C)-terminal truncations (lacking 29, 59, 89 and 119 C-terminal aa residues) were amplified from the *pFGG3-MenV-N* plasmid, which contains the full-length MenV

N gene. In order to fuse a his-tag at the C-terminus of the amplified genes, all reverse primers contained a sequence coding for 6 histidine residues. Primers used in the amplifications also included an *XbaI* site for subcloning into the yeast expression vector. Amplified genes were cloned into a unique *BcuI* site of the pFGG3 yeast expression vector under the control of the *GAL7* promoter. Cloned sequences were verified by DNA sequencing. The procedures used for transformation of yeast *S.cerevisiae* strain *214Δpep4* with the plasmids and expression of cloned genes were described previously (Slibinskas et al., 2004). Expression of his-tagged intact MenV N protein and its C-terminal truncations was confirmed by WB analysis of crude cell lysates with MAb against His-4 (Qiagen) (Fig. 17 A, lanes 2-6). To localize the epitopes recognized by the MAbs, their reactivity with truncated forms of MenV N protein lacking 29–119 aa residues at the C-terminus was analysed by western blot (Fig. 17 B-D). As a first step in examining the specificity of the MAbs, they were tested for their reactivity with a truncated version of the MenV N protein comprising aa 1–400 (Fig. 17 B-D, lane 6). The MAbs did not react with this protein, which suggested that the epitope was located at the C-terminus of the MenV N protein. Therefore, three additional truncated MenV N proteins lacking 29–89 C-terminal aa residues were constructed for fine epitope mapping. The MAbs did not react with truncated MenV N protein comprising aa 1–430 (Fig. 17 B-D, lane 5). In contrast, all MAbs reacted with truncated MenV N protein comprising aa 1–490 (Fig. 17 B-D, lane 3). The MAbs differed in their reactivity with truncated MenV N protein representing aa 1–460 (Fig. 17 B-D, lane 4). Two MAbs (clones 2D1 and 4G11) were reactive with this truncated protein, whereas one MAb (clone 10G8) was non-reactive. These data suggest that the epitopes of MAbs 2D1 and 4G11 are localized between aa 430 and 460 of the MenV N protein. The epitope of MAb 10G8 is localized downstream from this region, between aa 460 and 490 of the MenV N protein.

Table 2. Specificity of MAbs generated against yeast-expressed hPIV1, hPIV3, NiV and MenV N proteins

Rec. N protein	MAb	Reactivity in WB				Reactivity with proteins of crude yeast lysate in WB
		hPGV1 N	hPGV3 N	SeV N	hRSV N	
hPGV1 N	7F5	+	-	-	-	-
hPGV3 N	5B6	+/-	+	-	-	-
	9F2	-	+	-	-	-
	9G10	+/-	+	-	-	-
Rec. N protein	MAb	Reactivity in WB				Reactivity with proteins of crude yeast lysate in WB
		NiV N	HeV N	MenV N	TiV[1] N	
NiV N	1G3	+	+	-	-	-
	10A12	+	+	-	-	-
MenV	2D1	-	-	+	-	-
	4G11	-	-	+	-	-
	10G8	-	-	+	+	-

Rec. N protein	MAb	Reactivity in WB		Reactivity in IHCA[2]		
		NiV N without C-part	MenV N without C-part	MenV[3]	MenV (–)[4]	TiV[5]
NiV N	1G3	+	-			
	10A12	+	-			
MenV	2D1	-	-	+	-	-
	4G11	-	-	+	-	-
	10G8	-	-	+	-	-

Rec. – recombinant; WB – Western blot; IHCA – Immunohistochemical assay

1 – Tioman virus is a new paramixovirus discovered in several bat species in Malaysia (Chua et al., 2001). This virus is closely related to Menangle virus (Chua et al., 2002).

2 – This assay was performed in CSIRO Livestock Industries, Australian Animal Health Laboratory.

3 – Brain of MenV-infected stillborn piglet.

4 – Normal uninfected pig brain.

5 – Lymph node of TioV-infected pig.

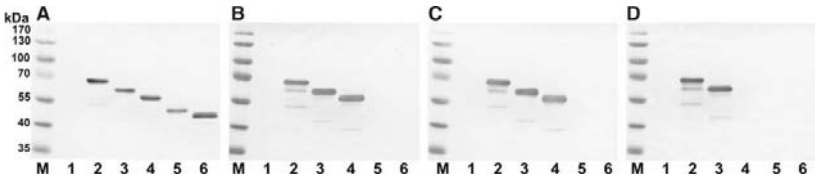

Fig.17. Reactivity of MAbs with truncated forms of MenV N protein comprising aa 1–490, 1–460, 1–430 and 1–400 by western blot. (a) anti-His 4 antibody (Qiagen); (b–d) MAbs: clone 2D1 (b), clone 4G11 (c) and clone 10G8 (d). Lane ''M'', prestained protein molecular mass markers; lane 1, the lysate of yeast cells transformed with expression vector pFGG3 (mock control); lane 2, full-length histagged MenV N protein; lane 3, truncated protein (aa 1–490); lane 4, truncated protein (aa 1–460); lane 5, truncated protein (aa 1–430); lane 6, truncated protein (aa 1–400).

DISCUSSION

The *Paramyxoviridae* include some of the great and ubiquitous disease-causing viruses of humans and animals. The best known members of this virus family are parainfluenza viruses (hPIV) and human respiratory syncytial virus (hRSV). These paramyxoviruses have been associated with every kind of upper and lower respiratory tract illnesses which, in their symptoms, only slightly differ from respiratory diseases caused by other infectious pathogens. Consequently, diagnosis of the infectious agent causing the respiratory disease based only on clinical symptoms in most cases is impossible. For this a laboratory diagnosis is highly recommended (Hall, 2001; Henrickson, 2003).

Nipah, Hendra and Menangle viruses (NiV, HeV and MenV, respecively) are newly identified paramyxoviruses which change fast, infect wide host range including several terrestrial species in four mammalian orders, cause systemic infections affecting various organ systems also central nervous system, and NiV and HeV are deadly human pathogens (Eaton et al., 2006; Philbey et al., 2008). Since NiV, HeV and MenV were identified only recently, consequently, knowledge about them is relatively scanty. It is little known about the biology, spread, virulence and pathology of these viruses, also no effective means of diagnostics, which would help a lot to fill existing knowledge gaps, are available by now (Wong, 2010) .

The purpose of the study presented here was to investigate the synthesis of nucleocapsid (N) proteins of respiratory (SeV, hPIV1, hPIV3 and hRSV) and recently discovered zoonotic (NiV, HeV and MenV) paramyxoviruses in yeast *S.cerevisiae*, to determine properties of the recombinant proteins and evaluate their feasibility in viral diagnostics. ELISA assays for viral diagnostics based on recombinant viral proteins may offer a number of advantages over whole-virus-based ELISAs, namely, simplified production, improved standardization, enhanced stability and no risk of virus distribution (Bouche et al., 1998). Though using recombinant viral proteins instead of whole virus particles the more narrow spectra of virus specific antibodies could be detected, the right choice of proper viral antigen may improve the specificity of the

diagnostic tests. It is well known that the C terminal part of the N protein of various paramyxoviruses is very immunogenic and differs in amino acid sequence even among parainfluenza virus types (Karron and Collins, 2007). Therefore, using recombinant N proteins for ELISA based diagnostics of infections caused by paramyxoviruses it should be possible to detect virus specific antibodies in oral fluids and blood serum samples. It is also known that using N proteins, synthesized in yeast *P.pastoris* and *S.cerevisiae*, specific ELISA tests for the diagnosis of mumps and measles were developed. These tests offer a number of advantages over any other diagnostic tests, available to date and become a significant tool in control of mumps and measles epidemics (Samuel et al., 2002a; Samuel et al., 2003; Slibinskas et al., 2004; Slibinskas et al., 2003). Therefore, using recombinant N proteins for ELISA based diagnostics of infections caused by paramyxoviruses it should be possible to detect virus specific antibodies in oral fluids and blood serum samples. The study presented here proves the feasibility of yeast-synthesized N proteins of respiratory and zoonotic paramyxoviruses for the development of ELISA based viral diagnostics.

In this study the N proteins of SeV, hPIV1, hPIV3, hRSV, NiV, HeV and MenV were successfully synthesized in yeast *S.cerevisiae*. The study revealed that the N proteins are expressed at high levels. The synthesis yield of SeV, hPIV1, hPIV3, hRSV, NiV, HeV and MenV N proteins was 11, 3.4, 1.4, 3.2, 5.3, 5.3 and 1.8 % of total protein, respectively. Usually heterologous proteins expressed in yeast *S.cerevisiae* yield maximum of 1–5 % of total protein, even with a strong promoter (Valenzuela et al., 1982; Gellissen and Hollenberg, 1999; Dargeviciute et al., 2002; Sasnauskas et al., 2002). The synthesis yield of N proteins was not as high as those of several heterologous proteins, such as measles virus nucleoprotein and the core protein of hepatitis B virus, which were expressed in yeast *S.cerevisiae* up to 20 % and 40 % of total protein, respectively. However, the synthesis level of the N proteins described in this study was as high as those of many heterologous proteins expressed in yeast *S.cerevisiae* and fell in the range from 1 % up to 5 % of total protein. In addition, the maximum expression of the N proteins in yeast was achieved cultivating the

transformed yeast strains in the induction medium for 24 h. Also, increased galactose concentration from 1.5 to 2.5 % in the induction medium led to the increase of the recombinant N proteins synthesis levels almost up to 25 %. The yield of the hPIV1 N protein from *S.cerevisiae* was at least two-fold greater than that of the hPIV3 N protein and about three-fold lower than the yield of the Sendai virus N protein. Hence, it is surprising why the expression efficiency of very closely related N proteins, which are very similar in their amino acid sequence, is so different in yeast *S.cerevisiae*. The yield of heterologous proteins may depend on many factors, such as, stability of mRNA, stability of heterologous protein, "toxicity" of heterologous protein for the host cell, ability to form stable substructures like vNLP, which do not interfere with cellular processes and protein quality control (Kaufman, 1999; Wickner et al., 1999).

The expression analysis of the SeV N protein in three different yeast *S.cerevisiae* strains (the wild-type (*FH4*), *AH 22-214* and *214Δpep4*, respectively) revealed no significant differences. The *214 Δpep4* strain, if compared with wild-type or *AH 22-214* strains, lacks the *PEP4* locus encoding protease A. This protease activates the set of other cellular proteolytic enzymes, thereby making the *214 Δpep4* strain less proteolytically active. It was supposed that using this strain, lacking vacuolar peptidase A, might slow down the hydrolysis of the yeast-expressed N proteins during the purification process. Consequently, the *214 Δpep4* strain was chosen to synthesize paramyxovirus N proteins described in this study.

Mainly the problem to obtain large quantities of a foreign protein might concern its instability inside the yeast cell. The N proteins of paramyxoviruses, which have been described here, are stable in yeast; analysis of yeast lysates obtained after induction of heterologous protein synthesis did not reveal any significant degradation of the recombinant N proteins in Western blots. The absence of degradation products in crude yeast lysates or only minute presence detected by WB has indicated that non-purified recombinant N proteins are quite stable in yeast cells.

The identity of the recombinant yeast-expressed SeV, hPIV1, hPIV3 and hRSV N proteins was confirmed by WB with specific antibodies. Whereas the specific antibodies

for NiV, HeV and MenV N proteins were not available, the identity of the full-length recombinant NiV, HeV and MenV N proteins was confirmed by MS/MS analysis. The MS/MS analysis also revealed that the amino terminal of the NiV, HeV and MenV N proteins is modified by removal of methionine and that serine at position 2 is acetylated. A similar modification has been found in native N proteins of Menangle (Shiell et al., 2002) and Hendra viruses (B. Shiell, W. Michalski, personal communication). This observation suggests that cellular machinery responsible for the post-translational modifications of the paramyxovirus N proteins is conserved in higher and lower eukaryotes.

Many virus surface and nucleocapsid proteins when expressed in animal host in the absence of other viral gene products have the intrinsic capacity to self-assemble into virus like-particles and nucleocapsid-like particles (vNLPs), respectively (Pumpens and Grens, 2002). We aimed to develop an efficient expression system suitable for generation of SeV, hPIV1, hPIV3, hRSV, NiV, HeV and MenV vNLPs and determine, whether the SeV, hPIV1, hPIV3, hRSV, NiV, HeV and MenV N proteins form vNLPs in yeast without the assistance of other viral gene products and RNA. Ultracentrifugation of the yeast-expressed SeV, hPIV1, hPIV3, hRSV, NiV, HeV and MenV N proteins in CsCl gradient and fraction analysis revealed buoyant density of 1.29-1.31 g/cm^3, which is characteristic for most vNLPs with or without nucleic acids (Thorne and Dermott, 1976; Robbins et al., 1980). Electron microscopy of the purified recombinant N protein revealed herring-bone structures similar to native SeV, hPIV1, hPIV3, hRSV, NiV, HeV and MenV nucleocapsids (Eaton et al., 2006; Heggeness et al., 1981; Henrickson, 2003; Garcia-Barreno et al., 1996; Miyahara et al., 1992). Formation of vNLPs occurs in the absence of other viral proteins in the yeast *S.cerevisiae* expression system. If host cells proteins are involved in formation of vNLPs, they appear to be conserved in both yeast and mammalian cells.

It was shown in this study that the yeast-expressed SeV, hPIV1 and hPIV3 N proteins assemble into long helical structures and the abundance of the ring-like structures in the purified samples is relatively low. It suggests that the yeast generated

SeV, hPIV1 and hPIV3 vNLPs are rigid helical rods and do not fall apart into ring like structures even after two consecutive round of ultracentrifugation in CsCl gradient. Electron microscopy analysis revealed that the recombinant hRSV N protein forms typical herring-bone and ring-like structures, too. But yeast-derived hRSV vNLPs appeared to be loosely ordered and highly flexible helices. Weakly organized helical nucleocapsid is a characteristic feature of the genus *Pneumovirinea* (Bhella et al., 2002). Contrary, in the purified samples of the yeast-expressed NiV, HeV and MenV N proteins only few vNLPs and large number of the ring like structures were observed. This might mean either yeast-expressed NiV, HeV and MenV N proteins are not able to assemble into long helices or yeast generated NiV, HeV and MenV vNLPs are not stable and fall apart into ring like structures when purified in CsCl gradient. Recently, a three-dimensional crystal structure of a decameric ribonucleoprotein complex of the hRSV N protein bound to RNA was resolved (Tawar et al., 2009). The resolved structure suggests that N proteins interact laterally in the ring with their counterparts from the adjacent subunits but do not make tight contacts. The interacting surfaces are highly hydrated, primarily involving intermittent van der Waals contacts. The ring is stabilized by the RNA belt and by the N chain that results from the insertion of the N arm (residues 1 to 35) of one subunit into the compact fold of the adjacent one. This lateral connectivity helps explain the observed malleability of the N-N interactions in the flexible, yet very stable, RSV nucleocapsid. The C arm lies above the C-terminal domain in the ring, occupying the space that would be between consecutive turns of the helical nucleocapsid. We assumed that the C-terminal part of the full length NiV, HeV and MenV N proteins synthesized in yeast may either impede the formation of long helical nuclocapsids or decrease their stability when they are formed. The expression in yeast of the NiV, HeV and MenV N proteins (1–399 aa) with a C-terminal deletion have shown that these truncated N proteins formed significantly longer and more dense vNLPs compared to the full-length proteins and the proportion of ring-like structures in purified fractions of these proteins was relatively low. We also speculate that the stability of yeast generated vNLPs of different paramyxoviruse may be due to their

ability to encapsidate non-specifically yeast cell RNA which might stabilize relatively fragile helical structure. It seems that yeast generated SeV, hPIV1, hPIV3 and hRSV vNLPs are able to interact with cellular RNA quite efficiently while NiV, HeV and MenV vNLPs are not.

Northern blot hybridization confirmed the presence of nucleic acids in yeast-derived SeV vNLPs. The encapsidation appears to be relatively non-specific in terms of RNA sequence. The nucleic acids associated with yeast-derived SeV vNLPs were heterogeneous in size and ranged from 60 to 500 nt, however, after RNase A treatment of recombinant vNLPs all of them were partially degraded to 60-80 nt fragments.

The immunoreactivity of the recombinant yeast-derived hPIV1, hPIV3 and hRSV vNLPs was tested with hPIV1, hPIV3 and hRSV-specific IgG antibodies from human sera. The reactivity of the yeast generated hPIV1, hPIV3 and hRSV NLPs with serum antibodies suggests that recombinant hPIV1, hPIV3 and hRSV N proteins show antigenic similarity with viral nucleocapsids and mimic infectious virus in the presentation of surface-exposed immunodominant epitopes. These data demonstrate that recombinant yeast-derived vNLPs are specifically recognized by serum antibodies developed during hPIV1/hPIV3/hRSV infection and therefore might be suitable for the development of serological tests for the detection of hPIV1/hPIV3/hRSV -specific antibodies. The Immunoreactivity of the recombinant NiV, HeV and MenV N proteins was tested with a set of sera derived from humans, fruit bats, horses infected with HeV, and from humans, fruit bats and pigs infected with NiV or MenV. The sera were sourced from the Australian Animal Health Laboratory Serum Bank and some of them were obtained during the outbreaks of HeV in Queensland, Australia and of NiV in Nipah, Malaysia. The immunoreactivity of the recombinant MenV N protein was tested in an indirect ELISA format using paired (naive and convalescent) sera derived from experimentally infected pigs. All three recombinant N proteins, in nanogram quantities, were detected by corresponding positive sera, indicating that yeast produced recombinant NiV, HeV and MenV N proteins are suitable for antibody detection in different species. However, it should be pointed out that further optimization is required to reduce the background

reactivity with negative sera of some species, particularly bat and pig, before the tests can be applied in outbreak investigation and/or serological surveillance. Due to the highly conserved nature of henipavirus N proteins, serological tests based on recombinant N proteins are not expected to be capable of differentiation between antibodies against HeV and NiV.

The N protein is the most abundant structural protein of paramyxoviruses and the essential component of viral nucleocapsid. Therefore, N-protein-specific antibodies are a suitable tool for virus detection in infected tissues. To generate hPIV1, hPIV3, NiV and MenV N-specific MAbs, the recombinant yeast-expressed hPIV1, hPIV3, NiV and MenV N protein were used as immunogens. Following immunizations with the recombinant yeast-derived N proteins, corresponding N specific MAbs of the IgG class were produced, and their specificity was assessed in detail. In summary, the newly generated hPIV1, hPIV3, NiV and MenV N-specific MAbs may be useful for studying the biology, spread, virulence and pathology of these viruses and facilitate the development of laboratory diagnostic tools for hPIV1, hPIV3, NiV and MenV N infections.

In this study we have demonstrated, that yeast *S.cerevisiae* is an excellent host for a high-level production of proteins SeV, hPIV1, hPIV3, hRSV, NiV, HeV and MenV N proteins as vNLPs. The hPIV1, hPIV3, hRSV, NiV, HeV and MenV vNLPs described above represent useful tools for the development of new virus detection systems and demonstrate the effectiveness of yeast as a host for generation of recombinant proteins organised in complex structures like human virus NLPs.

CONCLUSIONS

1. SeV, hPIV1, hPIV3, hRSV, NiV, HeV ir MenV N proteins form vNLPs in yeast *S.cerevisiae*. The average yields of SeV, hPIV1, hPIV3, hRSV, NiV, HeV and MenV N proteins were 1.2, 0.7, 0.5, 0.9, 0.8, 0.8, ir 0.3 mg per gram of wet biomass from *S.cerevisiae*, respectively.

2. SeV, hPIV1, hPIV3, hRSV, NiV, HeV and MenV N proteins in yeast *S.cerevisiae*, retain antigenic properties of native viral analogs.

3. C terminal part of recombinant NiV, HeV and MenV N proteins destabilize yeast-derived vNLPs.

4. Recombinant SeV N protein packages non-specific mRNA into vNLPs in yeast *S.cerevisiae* cells. The encapsidated RNA is partially resistant to an RNAse A treatment.

5. Yeast-derived hPIV1, hPIV3, hRSV, NiV, HeV and MenV N proteins are suitable tools for the development of diagnostics for the corresponding viral infections.

PUBLICATIONS

The data presented in this dissertation is published in these articles:

Juozapaitis M, Slibinskas R, Staniulis J, Sakaguchi T, Sasnauskas K. 2005. Generation of Sendai virus nucleocapsid-like particles in yeast. *Virus Res* 108:221-4

Juozapaitis M, Coiras M, Staniulis J, Sasnauskas K. 2006. Synthesis of human respiratory syncytial virus nucleoprotein in yeast *Saccharomyces cerevisiae*. *Biologija* 3:79-82

Juozapaitis M, Serva A, Kučinskaite I, Žvirblienė A, Slibinskas R, et al., 2007. Generation of menangle virus nucleocapsid-like particles in yeast Saccharomyces cerevisiae. *J Biotechnol* 130:441-7

Juozapaitis M, Serva A, Žvirblienė A, Slibinskas R, Staniulis J, et al., 2007. Generation of henipavirus nucleocapsid proteins in yeast Saccharomyces cerevisiae. *Virus Res* 124:95-102

Juozapaitis M, Žvirblienė A, Kučinskaite I, Šėžaitė I, Slibinskas R, et al., 2008. Synthesis of recombinant human parainfluenza virus 1 and 3 nucleocapsid proteins in yeast Saccharomyces cerevisiae. *Virus Res* 133:178-86

Žvirblienė A, Šėžaitė I, Plečkaitytė M, Kučinskaitė-Kodzė I, **Juozapaitis M**, Sasnauskas K. 2009. Mapping of an antigenic site on the nucleocapsid protein of human parainfluenza virus type 3. *Viral Immunol* 22:181-8

Žvirblienė A, Kučinskaitė-Kodzė I, **Juozapaitis M**, Lasickienė R, Gritėnaitė D, et al., 2010. Novel monoclonal antibodies against Menangle virus nucleocapsid protein. *Arch Virol* 155:13-8

The articles unrelated to the data presented in the dissertation:

Juozapaitis M, Antoniukas L. 2007. [Influenza virus]. *Medicina (Kaunas)* 43:919-29

Kučinskaitė I, **Juozapaitis M**, Serva A, Žvirblienė A, Johnson N, et al., 2007. Antigenic characterisation of yeast-expressed lyssavirus nucleoproteins. *Virus Genes* 35:521-9

Petraitytė R, Tamošiūnas PL, **Juozapaitis M**, Žvirblienė A, Sasnauskas K, et al., 2009. Generation of Tioman virus nucleocapsid-like particles in yeast Saccharomyces cerevisiae. *Virus Res* 145:92-6

Wunderlich K, **Juozapaitis M**, Manz B, Mayer D, Gotz V, et al., 2010. Limited compatibility of polymerase subunit interactions in influenza A and B viruses. *J Biol Chem* 285:16704-12

Wunderlich K, **Juozapaitis M**, Ranadheera C, Kessler U, Martin A, et al., 2011. Identification of high-affinity PB1-derived peptides with enhanced affinity to the PA protein of influenza A virus polymerase. *Antimicrob Agents Chemother* 55:696-702

CONFERENCE ABSTRACTS

Juozapaitis M, Coires M, Sasnauskas K. Synthesis of recombinant human respiratory syncytial virus nucleoprotein in yeast. XIII International Conference on Negative Strand Viruses, June 17-22, 2006, Salamanca, Spain.

Juozapaitis M, Coiras M, Staniulis J, Sasnauskas K. Žmonių kvėpavimo sincitinio viruso nukleokapsidės baltymo sintezė mielėse S.cerevisiae. Lietuvos Biochemikų draugija. Biochemija: mokslas ir žinių visuomenė. IX-asis suvažiavimas – konferencija. 2006 m. birželio mėn 16-18, Tolieja.

Sasnauskas K, Gedvilaite A, Slibinskas R, **Juozapaitis M**, Žvirbliene A, Ulrich R. Generation of viral proteins as virus-like particles in yeast. 7th ScanBalt Forum and Biomaterials Days 2008, September 24-26, Vilnius.

Žvirblienė A, Šėžaitė I, Plačkaitytė M, Kučinskaitė-Kodzė I, **Juozapaitis M**, Sasnauskas K. Mapping of an antigenic site on the nucleocapsid protein of human parainfluenza virus type 3. XIV International Conference on Negative Strand Viruses, June 20-25, 2010, Brugge, Belgium.

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REFERENCES

Ausubel JH. 1992. Industrial ecology: reflections on a colloquium. *Proc Natl Acad Sci U S A* 89:879-84

Bhella D, Ralph A, Murphy LB, Yeo RP. 2002. Significant differences in nucleocapsid morphology within the Paramyxoviridae. *J Gen Virol* 83:1831-9

Birnboim HC, Doly J. 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res* 7:1513-23

Bouche F, Ammerlaan W, Fournier P, Schneider F, Muller CP. 1998. A simplified immunoassay based on measles virus recombinant hemagglutinin protein for testing the immune status of vaccinees. *J Virol Methods* 74:77-87

Bowden TR, Westenberg M, Wang LF, Eaton BT, Boyle DB. 2001. Molecular characterization of Menangle virus, a novel paramyxovirus which infects pigs, fruit bats, and humans. *Virology* 283:358-73

Chanock RM. 1957. Recovery of a new type of myxovirus from infants with croup. *Ann N Y Acad Sci* 67:287-95

Chanock RM, Parrott RH, Cook K, Andrews BE, Bell JA, et al. 1958. Newly recognized myxoviruses from children with respiratory disease. *N Engl J Med* 258:207-13

Chua KB, Bellini WJ, Rota PA, Harcourt BH, Tamin A, et al. 2000. Nipah virus: a recently emergent deadly paramyxovirus. *Science* 288:1432-5

Chua KB, Goh KJ, Wong KT, Kamarulzaman A, Tan PS, et al. 1999. Fatal encephalitis due to Nipah virus among pig-farmers in Malaysia. *Lancet* 354:1257-9

Chua KB, Wang LF, Lam SK, Crameri G, Yu M, et al. 2001. Tioman virus, a novel paramyxovirus isolated from fruit bats in Malaysia. *Virology* 283:215-29

Chua KB, Wang LF, Lam SK, Eaton BT. 2002. Full length genome sequence of Tioman virus, a novel paramyxovirus in the genus Rubulavirus isolated from fruit bats in Malaysia. *Arch Virol* 147:1323-48

Connors M, Collins PL, Firestone CY, Murphy BR. 1991. Respiratory syncytial virus (RSV) F, G, M2 (22K), and N proteins each induce resistance to RSV challenge, but

resistance induced by M2 and N proteins is relatively short-lived. *J Virol* 65:1634-7

Cuff JA, Clamp ME, Siddiqui AS, Finlay M, Barton GJ. 1998. JPred: a consensus secondary structure prediction server. *Bioinformatics* 14:892-3

Dargeviciute A, Brus Sjolander K, Sasnauskas K, Kruger DH, Meisel H, et al. 2002. Yeast-expressed Puumala hantavirus nucleocapsid protein induces protection in a bank vole model. *Vaccine* 20:3523-31

Eaton BT, Broder CC, Middleton D, Wang LF. 2006. Hendra and Nipah viruses: different and dangerous. *Nat Rev Microbiol* 4:23-35

Eshaghi M, Tan WS, Ong ST, Yusoff K. 2005. Purification and characterization of Nipah virus nucleocapsid protein produced in insect cells. *J Clin Microbiol* 43:3172-7

Fooks AR, Stephenson JR, Warnes A, Dowsett AB, Rima BK, Wilkinson GW. 1993. Measles virus nucleocapsid protein expressed in insect cells assembles into nucleocapsid-like structures. *J Gen Virol* 74 (Pt 7):1439-44

Garcia-Barreno B, Delgado T, Melero JA. 1996. Identification of protein regions involved in the interaction of human respiratory syncytial virus phosphoprotein and nucleoprotein: significance for nucleocapsid assembly and formation of cytoplasmic inclusions. *J Virol* 70:801-8

Gellissen G, Hollenberg CP. 1997. Application of yeasts in gene expression studies: a comparison of Saccharomyces cerevisiae, Hansenula polymorpha and Kluyveromyces lactis -- a review. *Gene* 190:87-97

Gietz RD, Schiestl RH, Willems AR, Woods RA. 1995. Studies on the transformation of intact yeast cells by the LiAc/SS-DNA/PEG procedure. *Yeast* 11:355-60

Guthrie C, Fink G. Guide to yeast genetics and molecular biology. Methods in Enzymology. Academic Press, San Diego. 1991.

Hall CB. 2001. Respiratory syncytial virus and parainfluenza virus. *N Engl J Med* 344:1917-28

Henrickson KJ. 2003. Parainfluenza viruses. *Clin Microbiol Rev* 16:242-64

Huang C, Kiyotani K, Fujii Y, Fukuhara N, Kato A, et al. 2000. Involvement of the zinc-binding capacity of Sendai virus V protein in viral pathogenesis. *J Virol* 74:7834-41

Juozapaitis M, Slibinskas R, Staniulis J, Sakaguchi T, Sasnauskas K. 2005. Generation of Sendai virus nucleocapsid-like particles in yeast. *Virus Res* 108:221-4

Karron RA, Collins PL. Parainfluenza Viruses. In: Knipe DM, Howley PM, Griffin DE, Lamb RA, Straus SE, Martin MA, Roizman B, eds. Fields Virology. 5th ed. Philadelphia: Wolters Kluwer/Lippincott Williams and Wilkins. 2007. 1:1497-1526

Kaufman RJ. 1999. Stress signaling from the lumen of the endoplasmic reticulum: coordination of gene transcriptional and translational controls. *Genes Dev* 13:1211-33

Lamb RA, Parks GD. Paramyxoviridae: the viruses and their replication. In: Knipe DM, Howley PM, Griffin DE, Lamb RA, Straus SE, Martin MA, Roizman B, eds. Fields Virology. 5th ed. Philadelphia: Wolters Kluwer/Lippincott Williams and Wilkins. 2007. 1:1449-1496

Lee SH, Boutilier JE, MacDonald MA, Forward KR. 1992. Enhanced detection of respiratory viruses using the shell vial technique and monoclonal antibodies. *J Virol Methods* 39:39-46

Longhi S, Receveur-Brechot V, Karlin D, Johansson K, Darbon H, et al. 2003. The C-terminal domain of the measles virus nucleoprotein is intrinsically disordered and folds upon binding to the C-terminal moiety of the phosphoprotein. *J Biol Chem* 278:18638-48

Miyahara K, Kitada S, Yoshimoto M, Matsumura H, Kawano M, et al. 1992. Molecular evolution of human paramyxoviruses. Nucleotide sequence analyses of the human parainfluenza type 1 virus NP and M protein genes and construction of phylogenetic trees for all the human paramyxoviruses. *Arch Virol* 124:255-68

Myers TM, Smallwood S, Moyer SA. 1999. Identification of nucleocapsid protein residues required for Sendai virus nucleocapsid formation and genome replication. *J Gen Virol* 80 (Pt 6):1383-91

Nayak DP, Davis AR, McQueen NL, Bos TJ, Jabbar MA, et al. 1985. Biological and immunological properties of haemagglutinin and neuraminidase expressed from cloned cDNAs in prokaryotic and eukaryotic cells. *Vaccine* 3:165-71

Neubert WJ, Eckerskorn C, Homann HE. 1991. Sendai virus NP gene codes for a 524 amino acid NP protein. *Virus Genes* 5:25-32

Philbey AW, Kirkland PD, Ross AD, Davis RJ, Gleeson AB, et al. 1998. An apparently new virus (family Paramyxoviridae) infectious for pigs, humans, and fruit bats. *Emerg Infect Dis* 4:269-71

Philbey AW, Kirkland PD, Ross AD, Field HE, Srivastava M, et al. 2008. Infection with Menangle virus in flying foxes (Pteropus spp.) in Australia. *Aust Vet J* 86:449-54

Pumpens P, Grens E, Nassal M. 2002. Molecular epidemiology and immunology of hepatitis B virus infection - an update. *Intervirology* 45:218-32

Ray R, Glaze BJ, Compans RW. 1988. Role of individual glycoproteins of human parainfluenza virus type 3 in the induction of a protective immune response. *J Virol* 62:783-7

Razanskiene A, Schmidt J, Geldmacher A, Ritzi A, Niedrig M, et al. 2004. High yields of stable and highly pure nucleocapsid proteins of different hantaviruses can be generated in the yeast Saccharomyces cerevisiae. *J Biotechnol* 111:319-33

Rima BK, Roberts MW, Martin SJ. 1983. Comparison of morbillivirus proteins by limited proteolysis. *Med Microbiol Immunol* 171:203-13

Sakaguchi T, Takao S, Kiyotani K, Fujii Y, Nakayama T, Yoshida T. 1993. Expression of the HN, F, NP and M proteins of Sendai virus by recombinant vaccinia viruses and their contribution to protective immunity against Sendai virus infections in mice. *J Gen Virol* 74 (Pt 3):479-84

Sambrook J, Fritsch EF, Maniatis T. Molecular Cloning. A Laboratory Manual. Cold Spring Harbor Laboratory Press, New York. 1989.

Sambrook J, Russell DW. Molecular Cloning. A Laboratory Manual. Cold Spring Harbor Laboratory Press, New York. 2001.

Samuel D, Sasnauskas K, Jin L, Beard S, Zvirbliene A, et al. 2002a. High level expression of recombinant mumps nucleoprotein in Saccharomyces cerevisiae and its evaluation in mumps IgM serology. *J Med Virol* 66:123-30

Samuel D, Sasnauskas K, Jin L, Gedvilaite A, Slibinskas R, et al. 2003. Development of a measles specific IgM ELISA for use with serum and oral fluid samples using recombinant measles nucleoprotein produced in Saccharomyces cerevisiae. *J Clin Virol* 28:121-9

Samuel RC, Kashyap RP, Krawiec M, Watson WH, Neilson RH. 2002b. Synthesis, structure, and reactivity of some sterically hindered (Silylamino)phosphines. *Inorg Chem* 41:7113-24

Sasnauskas K, Bulavaite A, Hale A, Jin L, Knowles WA, et al. 2002. Generation of recombinant virus-like particles of human and non-human polyomaviruses in yeast Saccharomyces cerevisiae. *Intervirology* 45:308-17

Sasnauskas K, Buzaite O, Vogel F, Jandrig B, Razanskas R, et al. 1999. Yeast cells allow high-level expression and formation of polyomavirus-like particles. *Biol Chem* 380:381-6

Schmitt MJ, Tipper DJ. 1990. K28, a unique double-stranded RNA killer virus of Saccharomyces cerevisiae. *Mol Cell Biol* 10:4807-15

Selvey L, Sheridan J. 1995. Outbreak of Severe Respiratory Disease in Humans and Horses Due to a Previously Unrecognized Paramyxovirus. *J Travel Med* 2:275

Shiell BJ, Beddome G, Michalski WP. 2002. Mass spectrometric identification and characterisation of the nucleocapsid protein of Menangle virus. *J Virol Methods* 102:27-35

Slibinskas R, Samuel D, Gedvilaite A, Staniulis J, Sasnauskas K. 2004. Synthesis of the measles virus nucleoprotein in yeast Pichia pastoris and Saccharomyces cerevisiae. *J Biotechnol* 107:115-24

Slibinskas R, Zvirbliene A, Gedvilaite A, Samuel D, Jin L, et al. 2003. Synthesis of mumps virus nucleocapsid protein in yeast Pichia pastoris. *J Biotechnol* 103:43-9

Tawar RG, Duquerroy S, Vonrhein C, Varela PF, Damier-Piolle L, et al. 2009. Crystal structure of a nucleocapsid-like nucleoprotein-RNA complex of respiratory syncytial virus. Science 326:1279-83

Thorne HV, Dermott E. 1977. Y-forms as possible intermediates in the replication of measles virus nucleocapsids. *Nature* 268:345-7

Valenzuela P, Medina A, Rutter WJ, Ammerer G, Hall BD. 1982. Synthesis and assembly of hepatitis B virus surface antigen particles in yeast. *Nature* 298:347-50

Wang LF, Yu M, Hansson E, Pritchard LI, Shiell B, et al. 2000. The exceptionally large genome of Hendra virus: support for creation of a new genus within the family Paramyxoviridae. *J Virol* 74:9972-9

Wickner S, Maurizi MR, Gottesman S. 1999. Posttranslational quality control: folding, refolding, and degrading proteins. *Science* 286:1888-93

Wong KT. 2010. Emerging epidemic viral encephalitides with a special focus on henipaviruses. *Acta Neuropathol* 120:317-25

PARAMYXOVIRIDAE ŠEIMOS VIRUSŲ NUKLEOKAPSIDĖS BALTYMŲ SINTEZĖ MIELĖSE *SACCHAROMYCES CEREVISIAE* IR JŲ PANAUDOJIMAS DIAGNOSTIKAI

REZIUMĖ

Paramyxoviridea šeimai priklauso plačiai paplitę virusai, sukeliantys pavojingas žmogaus ir gyvūnų ligas. Vieni iš pavojingiausių šios šeimos atstovų, priklausančių *Pneumovirus* bei *Respirovirus* gentims, yra kvėpavimo takus pažeidžiantys žmogaus respiracinis sincitinis virusas (hRSV) bei 1 ir 3 serotipų žmogaus paragripo virusai (hPIV1 ir hPIV3). Nipah, Hendra ir Menangle virusai (NiV, HeV ir MenV) yra neseniai atrasti *Paramyxoviridae* šeimai priklausantys šikšnosparnių platinami virusai. NiV, HeV ir MenV žalingų infekcijų protrūkiai yra negausūs ir pasauliniu mastu iki šiol šių virusų padaryta žala nėra labai didelė (Eaton ir kt., 2006). Tačiau šie virusai sparčiai kinta, yra platinami įvairių rūšių gyvūnų, sukelia sisteminę infekciją, kurios metu yra pažeidžiamos įvairios organų sistemos, tarp jų ir centrinė nervų sistema, o NiV ir HeV yra mirtinai pavojingi žmogui, todėl yra manoma, kad laikui bėgant ir kintant klimatui šie šikšnosparnių platinami virusai turi nemažą potencialą tapti plačiai paplitusiais, pavojingais virusais (Philbey ir kt., 2008).

Kadangi hPIV ir hRSV bei kitų kvėpavimo takus infekuojančių virusų ir šią organizmo sistemą pažeidžiančių bakterijų sukeliamos kvėpavimo takų ligos nežymiai skiriasi savo simptomais, todėl nustatyti ligos priežastį, remiantis vien klinikiniais ligos požymiais, dažnai būna neįmanoma. Norint tiksliai nustatyti kvėpavimo takų ligą sukėlusį virusą, rekomenduojama naudoti molekulinės diagnostikos priemones (Henrickson, 2003). Iki pat šiandien kvėpavimo takus pažeidžiančių virusų infekcijų diagnostikai naudojami metodai yra sudėtingi ir nepakankamai specifiški. Kadangi NiV, HeV ir MenV buvo atrasti palyginti neseniai, kol kas nėra sukurta efektyvių šių virusų infekcijų diagnostikai skirtų priemonių, kurios žymiai palengvintų šių virusų biologijos, paplitimo, patogenezės ir patologijos tyrimus (Wong, 2010). Pastaruoju metu ypatingas dėmesys kreipiamas efektyvių šių paramikso virusų diagnostikos būdų paieškai, todėl tokie tyrimai yra savalaikiai ir svarbūs. Šiame darbe atliktas hPIV1, hPIV3, hRSV, NiV,

HeV ir MenV N baltymų sintezės mielėse *S.cerevisiae* tyrimas, apibūdintos rekombinantinių N baltymų savybės ir galimybės panaudoti juos diagnostikoje.

SeV, hPIV1, hPIV3, hRSV, NiV, HeV ir MenV N baltymus koduojantys genai klonuoti mielių *S.cerevisiae* raiškos vektoriuje *pFGG3* (Slibinskas ir kt., 2004), po galaktoze įjungiamo *GAL7* promotoriaus sekos. Sukonstruotomis plazmidėmis buvo transformuotos mielės *S.cerevisiae*, kamienas *214Δpep4*. Plazmidėmis transformuotose mielėse tirta SeV, hPIV1, hPIV3, hRSV, NiV, HeV ir MenV N baltymų sintezė. Nustatyta, kad mielėse *S.cerevisiae* įmanoma gauti didelius tirtų N baltymų kiekius (~ nuo 2 iki 10 % nuo bendro ląstelės baltymų kiekio). Mielėse susintetinti N baltymai buvo išgryninti juos centrifuguojant per tankų sacharozės tirpalo sluoksnį ir CsCl tankio gradiente. Elektroniniu mikroskopu (EM) nustatyta, kad mielėse susintetinti SeV, hPIV1, hPIV3, hRSV, NiV, HeV ir MenV N baltymai formuoja virusų nukleokapsidę primenančias daleles (vNPD), kurios yra panašios į darinius, aptiktus EM stebint išgrynintus N baltymus, išskirtus iš įvairių paramikso virusų dalelių (Bhella ir kt., 2004). Nustatyta, kad rekombinantiniai N baltymai yra stabilūs ir nėra gausiai hidrolizuojami sintezės mielėse *S.cerevisiae* bei gryninimo metu, be to, šių baltymų formuojami dariniai yra patvarūs ir gali būti ilgą laiką saugomi užšaldyti 50 % glicerolio tirpale arba juos liofilizavus. Įsitikinta, kad rekombinantiniai SeV N baltymai pakuoja mielių ląstelės RNR, kuri vNPD sudėtyje yra iš dalies atspari RNasės A poveikiui, o rekombinantinių NiV, HeV ir MenV N baltymų C dalis destabilizuoja šių baltymų formuojamas virusų nukleokapsidę primenančias daleles.

Mielėse *S.cerevisiae* susintetinti hPIV1, hPIV3, hRSV, NiV, HeV ir MenV N baltymai pasižymi antigeninėmis savybėmis, būdingomis iš šių virusų išskirtiems baltymams. Tiesioginės imunofermentinės analizės (IFA) metodu įsitikinta, kad rekombinantiniai hPIV ir hRSV N baltymai reaguoja su IgG klasės antikūnais, hPIV ir hRSV užsikrėtusių žmonių kraujo serumuose. Atlikus IFA, buvo nustatyta, kad mielėse *S.cerevisiae* susintetinti NiV, HeV ir MenV N baltymai reaguoja su atitinkamais virusais užsikrėtusių gyvūnų ir žmogaus kraujo serumais. Įsitikinus, kad mielėse *S.cerevisiae* susintetintų paramikso virusų N baltymų struktūra ir antigeninės savybės yra panašios į analogiškų

virusų natyvių N baltymų struktūrą ir antigenines savybes, šie baltymai buvo panaudoti mAk gauti. Naudojant rekombinantinius hPIV1, hPIV3, NiV ir MenV N baltymus, gauti šiems baltymams specifiniai mAk, kurie tinka šių virusų diagnostikai (Juozapaitis ir kt., 2007; Žvirblienė ir kt., 2010; Žvirblienė ir kt., 2009).

Apibendrinant galima teigti, kad mielės *S.cerevisiae* puikiai tinka paramikso virusų N baltymų sintezei. Mielėse susintetinti SeV, hPIV1, hPIV3, hRSV, NiV, HeV ir MenV N baltymai savo savybėmis yra panašūs į natyvius analogiškų virusų N baltymus: formuoja virusų nukleokapsidę primenančias daleles, sąveikauja su ribonukleorūgštimis ir turi natyviems virusų N baltymams būdingas po-transliacines modifikacijas (Shiell ir kt., 2002). Be to, rekombinantinių hPIV1, hPIV3, hRSV, NiV, HeV ir MenV N baltymų antigeninės savybės yra labai panašios į natyvių analogiškų virusų N baltymų antigenines savybes. Taip pat įsitikinta, kad šiame darbe mielėse *S.cerevisiae* susintetinti hPIV1, hPIV3, hRSV, NiV, HeV ir MenV N baltymai tinka šių virusų infekcijų serologinei diagnostikai.

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