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Cross-neutralization between three mumps viruses & mapping of haemagglutinin-neuraminidase (HN) epitopes

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***Background & objectives*: The reports from the countries where mumps vaccine is given as routine immunization suggest differences in mumps virus neutralizing antibody titres when tested with vaccine and wild type viruses. Such reports are unavailable from countries like India where mumps vaccine is not included in routine immunization. We, therefore, undertook this study to understand the cross-neutralization activity of Indian mumps viruses.**

***Methods*: By using commercial mumps IgG enzyme immunoassay (EIA) and a rapid focus reduction neutralization test (FRNT), a panel of serum samples was tested. The panel consisted of 14 acute and 14 convalescent serum samples collected during a mumps outbreak and 18 archived serum samples. Two wild types (genotypes C and G) and Leningrad-Zagreb vaccine strain (genotype N) were used for the challenge experiments and FRNT titres were determined and further compared. The HN protein sequence of three mumps viruses was analyzed for the presence of key epitopes.**

***Results*: All serum samples effectively neutralized mumps virus wild types and a vaccine strain. However, significantly lower FRNT titres were noted to wild types than to vaccine strain ($P<0.05$). The comparison between EIA and FRNT results revealed 95.6 per cent agreement. No amino acid changes were seen in the epitopes in the Indian wild type strains. All potential N-linked glycosylation sites were observed in Indian strains.**

***Interpretation & conclusions*: Good cross-neutralization activity was observed for three mumps virus strains, however, higher level of FRNT titres was detected for mumps virus vaccine strain compared to Indian wild type isolates.**

Key words Cross-neutralization - India - mumps wild types - mumps Leningrad-Zagreb vaccine strain

Mumps virus (MuV) is a member of the *Paramyxoviridae* family, subfamily *Paramyxovirinae* and belongs to the genus *Rubulavirus*, which only infects humans[1]. MuV has a single-stranded, negative sense RNA genome consisting of 15,384 nucleotides. The mumps virus genome encodes two surface glycoproteins, fusion (F) and haemagglutinin-neuraminidase (HN); four core proteins, nucleoprotein (N), phospho (P), matrix (M) and large protein (L); and the membrane associated small hydrophobic (SH) protein[2]. Within the MuV genome, the most sequence variation is found in the *SH* gene, and this region has

been recommended for the genotypic classification[3]. A standard naming convention has been proposed for mumps virus genotypes that differentiated these into 12 genotypes, *i.e.* A-N, except for E or M[4]. However, serological tests with human serum indicate presence of a single serotype. Circulation of mumps virus genotype C has been reported from the States of Maharashtra and Tamil Nadu[5,6], and that of mumps genotype G from the States of Maharashtra and Punjab[5,7].

The HN is the major antigenic protein, known to elicit neutralizing antibodies, which may be critical for generating a protective host humoral immune response[8,9]. A study on mumps HN sequences showed antigenic divergence between vaccine (Leningrad-Zagreb, Urabe AM9 and Jeryl Lynn-5) and wild-type mumps (genotypes C, D and G) viruses[10]. We investigated a mumps outbreak in 2012 in an unimmunized population from Osmanabad, Maharashtra, India where circulation of two different mumps viruses (genotypes- C and G) was noted in nearby villages[5]. Therefore, a study was designed to understand the cross-neutralization activity of mumps viruses isolated from two villages. In addition, mumps Leningrad-Zagreb vaccine strain (MuV-LZ) was included in the cross-neutralization study.

Material & Methods

This study was conducted in the WHO National Measles Reference Laboratory, National Institute of Virology (NIV), Pune, Mahrashtra, India, during December 2013-May 2014. A panel of 46 serum samples consisting of 14 acute and 14 convalescent serum samples collected during 2012 mumps outbreak from Osmanabad district, Maharashtra, India[5] and 18 stored serum samples referred for either measles laboratory diagnosis or measles immunity testing at NIV were included. The history of clinical mumps was not available for 18 stored serum samples. All subjects were likely unimmunized for mumps, since mumps vaccine is not used in universal immunization programme in India. Additionally, during investigation detailed information about the vaccination history was taken from the patient's parents or immunization records available at primary health centres. All samples were tested using commercial mumps IgG antibody enzyme immuno assay (EIA) (Siemens Healthcare Diagnostics Products GmbH, Germany). In addition, 14 acute and 14 convalescent samples were also tested by using commercial mumps IgM antibody EIA (Siemens Healthcare Diagnostics Products GmbH, Germany) and mumps focus reduction neutralization test (FRNT) standardized at NIV.

Mumps wild type and vaccine strains used in FRNT: A wild type mumps virus was isolated from a throat swab collected from an 11 yr old female patient presented with fever and bilateral parotitis from Apsinga village in Osmanabad district, India[5]. This patient showed presence of mumps IgM antibodies in serum sample and serologically confirmed as a mumps case. Mumps *SH* gene reverse transcriptase (RT)-PCR was performed as per the protocol described previously[5], and it revealed mumps virus genotype C (MuV-C) and sequence deposited in GenBank (KF305773) (*www.ncbi.nlm.nih.gov/genbank/*).

Another wild type mumps virus was isolated from throat swabs collected from a 6 yr old female presented with fever and bilateral parotitis from Sangavi, Pune (Unpublished data). This patient showed presence of mumps IgM antibodies in serum sample and serologically confirmed as a mumps case. Mumps *SH* gene RT-PCR revealed mumps virus genotype G (MuV-G) and sequence deposited in GenBank (JX 442438). Due to unavailability of mumps genotype G isolate from the Osmanabad outbreak, MuV Pune strain was used for the challenge experiment in FRNT.

Virus stocks were prepared in Vero cells and 0.5 ml aliquots were prepared by adjusting foetal bovine serum (FBS) concentration to 10 per cent. Aliquots were stored at -80°C. For each challenge experiment, a new aliquot was used.

Mumps Leningrad-Zagreb vaccine strain (MuV-LZ) was obtained from the Serum Institute of India (SII) Limited, Pune. Aliquots of 0.5 ml were prepared by adding extra 10 per cent FBS and stock vials were stored at -80°C.

Mumps focus reduction neutralization test (FRNT): Mumps FRNT was standardized with three mumps viruses as described previously[11]. A minor modification was made in FRNT protocol where the volume of primary/secondary antibody/substrate was reduced (50 µl); plates were overlaid with 0.8 per cent carboxy methyl cellulose (CMC) and washed manually. After development, blue stained foci were counted by eye and 50 per cent focus reduction neutralization titres were deduced using the Kärber formula[11]. A mumps neutralizing antibody titre >1:4 is considered as positive as described earlier[11]. The FRNT titres obtained by challenging with three viruses were $\log_{10}$ transformed and statistical analysis was undertaken.

Analysis of HN gene sequences of wild types and vaccine strains: The complete *HN* gene sequencing of mumps isolates (*i.e.* stock virus used for the challenge experiments in FRNT) were performed (GenBank accession numbers; KF843895 & KF738114). *HN* gene sequence of MuV-LZ was retrieved from GenBank (AY685920) and multiple alignments of nucleotide and amino acid sequences were undertaken using MEGA version 5 standard procedure. N-linked glycosylation sites were located in amino acid sequences with pattern N-X-[S or T], where X represents any amino acid, followed by a serine (S) or threonine (T) except a proline.

Statistical analysis: Descriptive statistics were reported for continuous variables. The titres were compared using Student t-test.

Results

Qualitative comparison between EIA and FRNT: Forty six serum samples were tested using commercial mumps IgG EIA and FRNT. Of these 46 serum samples, 37 (80.4%) were positive by mumps IgG EIA and 35 (76%) by FRNT using all three mumps virus strains *i.e.* MuV-C, MuV-G and MuV-LZ. Overall, a good concordance was noted between EIA and FRNT results with a concordance of 95.6 per cent (35 positive and 9 negative by both tests) and discordant results observed for two samples. These samples were positive in mumps IgG EIA but negative in FRNT.

Of the 14 acute serum samples, mumps IgM antibodies were detected in 13 and mumps IgG antibodies detected in 12 samples. However, 10 of 14 samples showed the presence of neutralizing antibodies by FRNT using MuV-C, MuV-G and MuV-LZ strains. In 14 convalescent samples, mumps IgM antibody was detected in 13 and mumps IgG antibody was detected in all 14 samples. As expected, all 14 convalescent serum samples showed presence of neutralizing antibodies in FRNT using all three mumps virus strains. Of the 18 stored samples, 11 showed positive results in mumps IgG EIA and in FRNT with all three mumps strains indicating past exposure to MuV.

Comparative neutralizing antibody titres to three mumps viruses: Forty six serum samples were tested in FRNT using three challenge viruses, *i.e.* MuV-C, MuV -G and MuV-LZ (Table). The quantitative correlations (R^2) of neutralizing antibody titres between MuV-LZ vs. MuV-C, MuV-LZ vs. MuV-G, and MuV-C vs. MuV-G were 0.91, 0.96 and 0.94, respectively. However, 21 samples showed more than two-fold difference in neutralizing antibody titres to MuV-C and MuV-LZ; 18 samples showed more than two-fold difference in titres to MuV-G and MuV-LZ. Two samples showed more than two-fold difference between FRNT titres to MuV-C and MuV-G strains.

When mean FRNT titres (GMT) to three-challenge viruses were statistically compared, MuV-LZ vaccine showed higher titres than wild types (MuV-C and MuV-G) in all the samples ($P<0.05$, paired t test). The mean (± SE) FRNT titres to MuV-C, MuV-G and MuV-LZ were 1.80±0.08, 1.83 ± 0.08) and 2.11 ± 0.08, respectively.

When individual groups of serum samples were compared for FRNT titres, 14 acute serum samples showed significantly higher FRNT titres to MuV-LZ than MuV-C and MuV-G but FRNT titre differences amongst wild types were not evident. Similar observations were noted for 14 convalescent samples and 18 stored serum samples.

Mapping of amino acid changes in HN gene: Majority of neutralizing epitopes are localized in the *HN* gene of mumps virus; therefore, sequences of wild type and vaccine strains were analyzed. Indian wild types showed eight potential N-linked glycosylation sites (at amino acid positions; 127, 284, 329, 400, 448, 464, 507 and 514). Overall, no changes were detected in known neutralizing epitopes in Indian wild type strains. Additional regions for escaping neutralizing antibodies as reported in the Croatian wild types[10] were also seen in Indian wild types (Figure).

Discussion

The comparison of mean FRNT titres for MuV-C, MuV-G and MuV-LZ showed higher titres for vaccine strain (MuV-LZ) compared to wild type viruses (MuV-C and MuV-G). This observation needs further study to confirm in individuals vaccinated with mumps Leningrad-Zagreb strain. Our study confirmed the findings from other countries that documented lower neutralization titres to wild-type mumps strains compared to the particular mumps vaccine strains, *i.e.* JL, Urabe, Hoshino and L-3[12-16].

Interestingly, 14 convalescent serum samples collected from two villages showed good cross-reactivity to both wild type (genotypes C and G) viruses and mumps LZ-vaccine virus (genotype N). A study conducted in USA among vaccinated individuals suggested neutralization activity to genetically diverse mumps virus strains (Jeryl Lynn/USA63, Urabe-AM9/

Table. Focus reduction neutralization test (FRNT) titres obtained against three mumps viruses

Serum ID	IgG EIA result	MuV-C titre	MuV-C result	MuV-G titre	MuV-G result	MuV-LZ titer	MuV-LZ result
A-1	POS	15.8	POS	12.3	POS	8.9	POS
A-2	POS	50.3	POS	56.3	POS	59.8	POS
A-3	POS	77.1	POS	62.7	POS	133.1	POS
A-4	POS	188.7	POS	189.0	POS	506.9	POS
A-5	POS	49.7	POS	37.8	POS	123.5	POS
A-6	POS	97.8	POS	50.5	POS	212.9	POS
A-7	POS	123.2	POS	186.1	POS	284.7	POS
A-8	POS	446.6	POS	403.6	POS	>1:512	POS
A-9	POS	>1:512	POS	315.3	POS	325.3	POS
A-10	POS	90.3	POS	99.1	POS	151.9	POS
A-11	POS	40.9	POS	40.3	POS	42.6	POS
A-12	POS	147.2	POS	97.9	POS	260.7	POS
A-13	POS	90.3	POS	139.8	POS	208.0	POS
A-14	POS	8.3	POS	19.7	POS	20.0	POS
C-1	POS	160.9	POS	103.8	POS	290.3	POS
C-2	POS	323.9	POS	466.4	POS	>1:512	POS
C-3	POS	77.3	POS	73.7	POS	196.3	POS
C-4	POS	28.9	POS	36.6	POS	100.0	POS
C-5	POS	<1:4	NEG	<1:4	NEG	<1:4	NEG
C-6	POS	<1:4	NEG	<1:4	NEG	<1:4	NEG
C-7	NEG	<1:4	NEG	<1:4	NEG	<1:4	NEG
C-8	POS	10.8	POS	9.5	POS	21.0	POS
C-9	POS	14.1	POS	9.0	POS	9.6	POS
C-10	POS	9.8	POS	8.3	POS	17.1	POS
C-11	POS	65.9	POS	21.7	POS	41.5	POS
C-12	POS	21.0	POS	18.3	POS	45.3	POS
C-13	POS	228.5	POS	106.7	POS	161.2	POS
C-14	NEG	<1:4	NEG	<1:4	NEG	<1:4	NEG
S-1	NEG	<1:4	NEG	<1:4	NEG	<1:4	NEG
S-2	NEG	<1:4	NEG	<1:4	NEG	<1:4	NEG
S-3	NEG	<1:4	NEG	<1:4	NEG	<1:4	NEG
S-4	NEG	<1:4	NEG	<1:4	NEG	<1:4	NEG
S-5	POS	17.3	POS	22.1	POS	51.8	POS
S-6	POS	66.5	POS	104.2	POS	301.8	POS
S-7	POS	254.0	POS	322.0	POS	>1:512	POS
S-8	POS	62.1	POS	294.3	POS	476.6	POS

Contd...

Serum ID	IgG EIA result	MuV-C titre	MuV-C result	MuV-G titre	MuV-G result	MuV-LZ titer	MuV-LZ result
S-9	POS	86.4	POS	117.5	POS	396.3	POS
S-10	POS	22.9	POS	37.9	POS	138.7	POS
S-11	POS	34.6	POS	85.8	POS	139.6	POS
S-12	NEG	<1:4	NEG	<1:4	NEG	<1:4	NEG
S-13	NEG	<1:4	NEG	<1:4	NEG	<1:4	NEG
S-14	POS	169.7	POS	267.7	POS	438.9	POS
S-15	POS	63.8	POS	69.6	POS	150.9	POS
S-16	POS	45.2	POS	72.6	POS	191.8	POS
S-17	POS	58.5	POS	65.8	POS	202.3	POS
S-18	NEG	<1:4	NEG	<1:4	NEG	<1:4	NEG

A, acute; C, convalescent; S, stored

JPN73, Enders/USA45, Odate-1/JPN, Iowa-G/USA06, Lo1/UK88 and 88-1961/USA88) and indicated no evidence of mumps immune escape[16]. In the present study similar finding was observed in unvaccinated population from India.

Previous report showed that antibodies induced by immunization with Jeryl Lynn mumps vaccine effectively neutralized heterologous virus strains (genotype G) and higher neutralizing antibody titres were observed to Jeryl Lynn compared to genotype G virus[15]. We observed that serum samples collected from naturally infected or unimmunized population from India effectively neutralized mumps genotype C, genotype G and genotype N vaccine strain. However, higher neutralizing antibody titres to mumps vaccine strain were noted.

HN protein is a major target for humoral immune response in mumps virus infection as it elicits neutralizing antibodies[8,9]. The amino acid positions 265-288, 329-340 and 352-360 of HN protein have been reported to evoke immune response and responsible for virulence[8,9,17]. Indian mumps virus isolates did

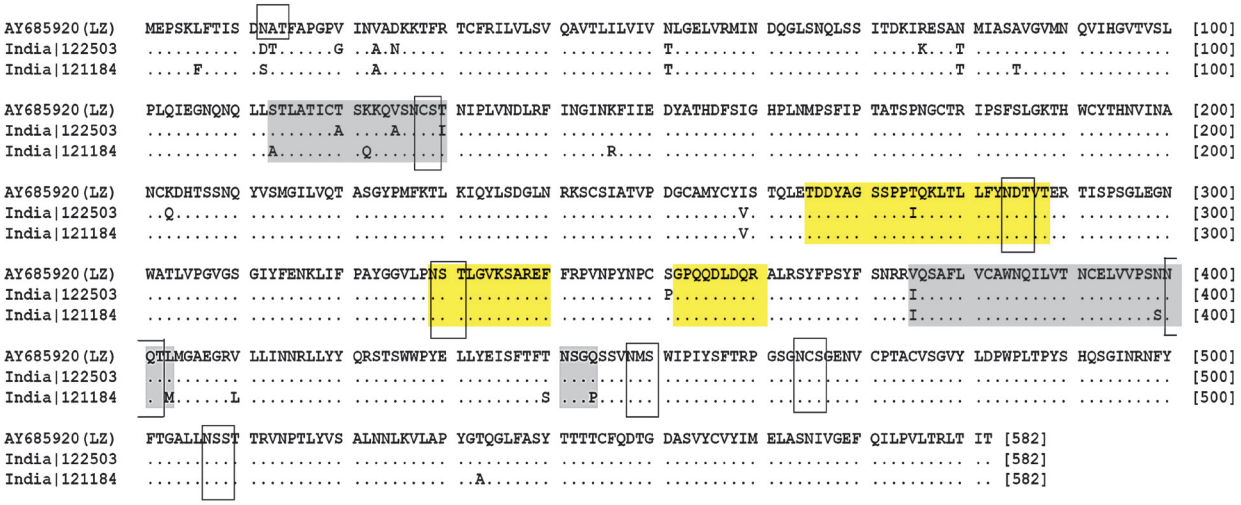

Figure. Deduced amino acid sequence of HN of mumps viruses isolated from India and LZ vaccine strain. Potential glycosylation sites are marked by rectangles. Regions highlighted in yellow colour indicate epitopes. Gray coloured regions are involved in viral escape from neutralizing antibodies as suggested in earlier study[10].

not show any change at these amino acid positions. The region 329-340 is reported to have the ability to induce neutralizing antibodies not only to attenuated virus strains but also to wild types[6]. This region is well conserved in Indian wild types and vaccine virus LZ. The known motifs *viz*. leucine-zipper, neuraminidase (240-NRKSCS-245) and receptor-binding site of haemagglutinin (405-GAEGRV-410) were reported as conserved in mumps viruses[18]. All potential N-linked glycosylation sites were observed in Indian wild type viruses except at position 12. Mutation of N to D/S at position 12 resulted in loss of a potential glycosylation site in both Indian wild type strains. As reported earlier, the gain or loss of a carbohydrate could affect neutralization epitopes, reduce accessibility, and may facilitate immune escape[19]. This may be the possible reason for differences in neutralizing antibody titres against three challenge viruses.

Overall, good cross-neutralization activity was observed between three mumps viruses. However, MuV-LZ strain showed higher levels of neutralizing antibody titres than wild types isolates from India.

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***Conflicts of Interest*:** None.

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