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Recombinants of influenza virus type B as potential live vaccine candidates: RNA characterization and evaluation in man

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SUMMARY

Two recombinants (R22 and R75) of the attenuated B/USSR/69 strain Brigit and the virulent B/Hong Kong/5/72 and one recombinant (R5) of Brigit and the virulent B/Hong Kong/8/73 were selected for genotypic and phenotypic characterization. All three recombinants had the growth property of the attenuated parent Brigit. Analysis of their RNA's by polyacrylamide gel electrophoresis revealed that, the strains R22 and R75 had derived all their genes from Brigit, those coding for haemagglutinin excepted. These recombinants were clinically evaluated and found to be attenuated and immunogenic. The recombinant R5 which derived, besides the gene coding for the haemagglutinin, several other genes from B/Hong Kong/8/73 was only partly attenuated since it induced influenza-like symptoms in one out of three volunteers.

It is concluded that the strain Brigit can be used as a donor of genes for the attenuation of the B/Hong Kong/5/72 virus and that recombinants of influenza type B can be identified, like influenza type A recombinants, by their RNA pattern.

INTRODUCTION

Antigenic hybrids of influenza type B viruses have been described (Tobita & Kilbourne, 1974) and genetic reassortment has been used to obtain attenuated strains with the serotype of the wild type virulent parent viruses (Beare *et al.* 1977*a*, *b*, Polazhaev & Aleksandrova, 1978). As stressed by Beare *et al.* (1977*a*) biological properties of the influenza type B viruses are unlikely to give a complete characterization of recombinants and to predict attenuation.

For instance properties such as distinct neuraminidase serotypes, plaque sizes and amantadine sensitivity which segregate during genetic crosses of influenza A viruses do not always differentiate influenza B viruses (Beare *et al.* 1977*a*, *b*, Appleyard, 1977).

Analysis of the RNA of influenza type A recombinant viruses, using various biochemical methods, has allowed the correlation of most of the viral biological properties with the genome fragments (Palese & Schulman, 1976, Scholtissek *et al.* 1976, Hay *et al.* 1977). Using such methods, suitable live vaccine candidates have

been selected among the recombinants of attenuated and wild type viruses (Florent *et al.* 1977, Hay *et al.* 1977, Florent *et al.* 1979).

A preliminary genetic map of the influenza virus type B has been reported (Racaniello & Palese, 1978).

In this paper we present a study of the RNA patterns of recombinants of wild type B/Hong Kong/5/72 and B/Hong Kong/8/73 viruses with the attenuated Brigit (A/USSR/69) strain and the properties of these strains in man.

MATERIALS AND METHODS

Viruses

B/USSR/69 was received from Dr A. A. Smorodintsev (All-Union Research Institute of Influenza, Ministry of Health, Leningrad, USSR) and the wild type viruses B/Hong Kong/5/72 and B/Hong Kong/8/73 were obtained from Dr G. C. Schild (National Institute for Biological Standards and Controls, London, UK).

Brigit, an attenuated virus (Peetermans, Lamy & Delem, 1975) was derived from B/USSR/69 by serial passages in embryonated eggs. Genetic reassortment between Brigit and each wild-type virus was performed following a general scheme established by Kilbourne & Murphy (1960) for type A influenza. Specific pathogen-free embryonated eggs were inoculated with pairs of viruses of similar infectious titers (about $10^{7.5}$ EID_{50} per egg). After 24 h incubation at 33 to 35 °C, the progeny was harvested and submitted to selective passages in the presence of rabbit immune anti-serum against Brigit and then to cloning passages at terminal dilutions.

The wild type parent of R22 and R75 was B/HK/5/72 strain and B/HK/8/73 for the R5.

Characterisation of the viruses

Conventional haemagglutination inhibition (HI) tests and standard neuraminidase inhibition (NI) tests (Aymard-Henry *et al.* 1973) were performed for the parent and recombinant strains using chicken and rabbit antisera.

Infectivity titration tests were performed comparatively in allantois-on-shell bits (AOS), as described by Fazekas de St Groth & White (1958) and in embryonated eggs. Haemagglutination (HA) titers of the supernatant medium were determined in AOS system.

RNA preparation and polyacrylamide gel electrophoresis

Virus preparations were used to infect MDCK monolayers in small dishes (25 cm^2) at a multiplicity of infection (MOI) greater than ten. After adsorption, maintenance medium containing 2·5 mCi ^{32}P (as phosphoric acid) was added to each dish.

Fourteen h later cell supernatants were harvested and layered onto 30–60 % sucrose gradients. Centrifugation in an SW27 rotor (Beckman) at 25000 rev./min for 3 h resulted in visible virus bands which were removed into a syringe through the side of the tube. Purified virus was digested with proteinase K (500 μg/ml) in the presence of 1 % SDS and then extracted with equal volumes of phenol-chloroform. RNA was precipitated with ethanol, pelleted, and resuspended in

Loenings buffer (36 mM tris-phosphate pH 7·8, 1 mM EDTA) containing 20 % sucrose as previously described by Ritchey, Palese & Schulman (1976). Samples were applied to polyacrylamide gels of either 2·4 %, 2·6 % or 3·0 % monomer concentration (acrylamide: bis ratio was 30:1.725).

The gels contained 6M urea, Loenings buffer and 0·1 % TEMED. Electrophoresis was performed at 120 V for 15 h at 26 °C. Gels were dried on Whatman 'MM' filter paper and exposed to Kodak X-Omat X-ray film.

Clinical trials

Pilot studies were performed in a small number of healthy adult volunteers having a serum HI titer against B/Hong Kong/8/73 $\leqslant 8$. Placebo controls were not included.

The recombinants were administered intranasally as drops. They were presented as monodose, lyophilized vaccine pellets and all vaccine lots were tested for potency and safety by standard control tests.

The lyophilized viruses were reconstituted before use in 0·5 ml distilled water. The mean virus concentrations of the inocula were $10^{7.2}$ EID_{50} for R22, $10^{6.8}$, $10^{7.3}$ or $10^{7.5}$ EID_{50} for R75, and $10^{7.6}$ EID_{50} for R5.

Two doses of R22 were given at one week interval but only one dose of each of R75 and R5 was administered.

The studies of R22, R5 and R75 were performed in the beginning of 1974, mid 1974 and mid 1977 respectively; these trials were not closed since the wild strains B/HK/5/72 and B/HK/8/73 were the serotypes isolated during epidemics of Influenza B.

In all the trials the volunteers were examined by a physician before inoculation and on day 7 and 21 after. Volunteers having evidence of chronic cardiovascular, haematologic, respiratory diseases or allergy to egg white and neomycin were excluded. These studies were conducted after an exemption had been obtained from the regulatory body.

In the trials performed with R22 and R5, the symptoms were assessed by self observation and reported to a physician on days 3 and 7 after inoculation. In the trial with R75, the volunteers were provided with printed forms for the recording of symptoms and temperature (twice daily) from the day of inoculation to day 7. The following symptoms were recorded: rhinorrhoea, nasal stuffiness, hoarseness, sore throat, expectoration and headache. The volunteers were asked to grade these symptoms as mild, moderate or severe.

Nasal washings for virus isolation were collected on day 1 and 2 after inoculation of R75 and on day 1, 2, 3 and 4 after administration of R5. No virus isolation was attempted for R22.

An aliquot (0·2 ml) of each washing was inoculated in 10-11 day old embryonated eggs incubated at 35 °C; 72 h later, the presence of virus in the allantoic fluid was checked by haemagglutination.

Conventional HI tests were carried out to evaluate sero-conversion which was defined as a fourfold or greater rise in the titre of a serum sample collected three weeks after inoculation as compared to that of the sample taken before inoculation. Four to eight haemagglutination units of B/Hong Kong/8/73 were used as antigen in the HI test.

Table 1. *Characterization of Influenza type B recombinants R22, R75, R5: antigenic composition by HI and NI using various antisera*

	HI test				NI test*				
	B/HK/5/72							Brigit	
Virus strain	rabbit	chicken	B/HK/8/73 chicken	Brigit chicken	R75 chicken	B/HK/5/72 chicken	B/HK/8/73 chicken	chicken	chicken
R22	⩾ 4000	NT	16	< 8	NT	NT	NT	NT	NT
R75	2000	512	16	< 8	3·7*	3·3	NT	3·6	NT
R5	⩾ 4000	NT	64	< 8	NT	NT	NT	NT	NT
B/HK/5/72	⩾ 4000	1024	16	< 8	3·6	3·7	2·0	3·7	1·5
B/HK/8/73	⩾ 4000	NT	128	8	NT	NT	1·8	NT	1·4
Brigit	256	128	8	1024	3·3	3·4	1·6	3·4	1·9

* Log_{10} NI units/ml.

Table 2. *Characterization of influenza type B recombinants R22, R75, R5: growth properties*

Virus strain	Mean ratios of $EID_{50}/(AOS)ID_{50}$*	Mean HA titres on AOS†
R22	1·35 ± 0·35 (6)	3·1 ± 1·1 (55)
R75	2·74 ± 0·35 (5)	3·1 ± 1·1 (54)
R5	2·20 ± 0·29 (6)	3·3 ± 1·1 (90)
Brigit	2·35 ± 0·33 (10)	4·8 ± 1·0 (54)
B/HK/5/72	4·47 ± 0·75 (10)	1·3 ± 0·7 (58)
B/HK/8/73	3·96 ± 0·38 (5)	1·2 ± 0·4 (32)

* Geometric mean, in $\log_{10}$, ± standard deviation, of ratios obtained from (n) comparative titration tests in embryonated eggs and in allantois-on-shell membrane system.

† Geometric mean titres, $\log_2$, ± standard deviation, of haemagglutinin produced on (n) individual bits of allantois-on-shell membrane.

RESULTS

Characterization of the viruses

The recombinant strains R75, R22 and R5 have the haemagglutinin of their wild-type parent which can be easily distinguished from that of Brigit strain. In contrast, using the standard NI test with chicken antisera, similar NI titres were obtained for Brigit, B/Hong Kong/5/72 and B/Hong Kong/8/73 (Table 1).

Their growth characteristics in the AOS system was the marker property used to differentiate Brigit and the recombinants from the wild type viruses. Indeed, Brigit and the recombinants exhibited higher infectious titres in the AOS system than do the wild type strains B/HK/5/72 and B/HK/8/73, even when they have similar titres in embryonated eggs. The production of haemagglutinin in the AOS system is also higher for Brigit, R22, R75 and R5 than for B/HK/5/72 and B/HK/8/73 strains. The results are recorded in Table 2. They indicate that the ratios of infective titres obtained in eggs and in AOS cultures are lower than 3·0 $\log_{10}$ for Brigit and for the recombinants whereas the ratios for both wild type strains are close to 4·0 $\log_{10}$. The values obtained for B/HK/5/72 and B/HK/8/73 viruses are not significantly different from each other whereas they differ from those observed for Brigit and the three recombinants. R22 strain had significantly higher titres in AOS than Brigit, R75 and R5 (analysis of variance and t-test $P < 0·05$). Using the mean haemagglutination titres in AOS, three different patterns were observed. Brigit had a titre of 4·8 $\log_2$, B/HK/5/72 and B/HK/8/73 had titres of 1·2 $\log_2$ and the recombinants exhibited intermediate titres. The results of the statistical analysis are summarized in Table 3. Geometric means of infectivity ratios and haemagglutination titres of the six virus strains were compared by analysis of variance which revealed a significant F ratio. Then the t-test on paired means was applied, using the common variance and corresponding degree of freedom obtained in the analysis of variance.

Parent viruses Brigit, B/HK/5/72 and B/HK/8/73 were submitted to two successive passages in AOS in order to see if the wild types could be adapted to grow in AOS.

Brigit exhibited approximately the same titre (10^4–10^5 AOS_{ID50}) at each passage but the infectivity of B/HK/5/72 and B/HK/8/73 strains was lost in the first or the second passage.

Table 3. *Statistical analysis of the infectivity and haemagglutinin produced by the strains tested*

	R75	R5	BRIGIT	B/HK/5/72	B/HK/8/73
R22	I; -	I; -	I; HA	I; HA	I; HA
R75		-; -	-; HA	I; HA	I; HA
R5			-; HA	I; HA	I; HA
Brigit				I; HA	I; HA
B/HK/5/72					-; -

Strains were compared by analysis of variance followed by *t*-test. I (Infectivity) indicates significantly different ($P < 0.05$) ratios $EID_{50}/(AOS)ID_{50}$; HA (haemagglutinin) indicates significantly different ($P < 0.05$) HA titres and - indicates no significant difference ($P > 0.05$).

Table 4. *Characterization of the influenza type B recombinants R22, R75 and R5 on the basis of the derivation of their RNA segments*

	RNA segment							
Virus	1	2	3	4	5 (HA)	6 (NA)	7	8
R22	*B	B	B	B	†H_{72}	B	B	B
R75	B	B	B	B	H_{72}	B	B	B
R5	‡H_{73}	B	B	H_{73}	H_{73}	?	?	H_{73}

* B = RNA segment derived from Brigit.
† H_{72} = RNA segment derived from B/Hong Kong/5/72 virus.
‡ H_{73} = RNA segment derived from B/Hong Kong/8/73 virus.

Analysis of the RNAs

Plate 1 shows viral RNAs separated on 2·4, 2·6 and 3·0% polyacrylamide gels (A, C, B). These different gel concentrations are necessary in order to obtain migration differences among all RNA segments of the two parent viruses.

The 2·4% gel shown in Plate 1A gave the best differentiation between RNA 8 of B/Hong Kong/5/72 and Brigit viruses. The pattern in this gel indicates that both recombinants R22 and R75 inherited RNA 8 from Brigit (indicated by solid arrows).

The 3·0% gel shown in Plate 1B demonstrated migration differences between RNA segments 4, 5, 6 and 7 of the parent viruses. Examination of this gel indicates that both recombinants R22 and R75 derived RNAs 4, 6 (NA gene; Ueda *et al.* 1978 and unpublished results), and 7 from Brigit (indicated by solid arrows). On the other hand, RNA 5 (HA gene; Ueda *et al.* 1978 and Racaniello & Palese, 1978) of R22 and R75 is inherited from the B/Hong Kong/5/72 virus parent (indicated in the Plate by open arrows).

Finally, the separation of RNA segments on a 2·6% gel is shown in Figure 1C. This gel differentiates RNAs 1, 2 and 3 of the parent viruses. The results show that, both R22 and R75 derive RNAs 1, 2 and 3 from Brigit (indicated by solid arrows). A summary of these findings together with the derivation of the RNA segments of recombinant R5 is shown in Table 4.

Recombinants R22 and R75 derived all genes from Brigit except for the HA gene (RNA 5) which was given by the B/Hong Kong/5/72 virus. Recombinant R5 has not been completely characterized. It has inherited RNA 2 and 3 from Brigit and

RNA 1, 4, 5 and 8 from B/Hong Kong/8/73; the origin of RNA 6 and 7 has not yet been determined.

Clinical evaluation of the recombinant strains

Only the results obtained in volunteers having a prevaccination HI titre $\leqslant 8$ against B/Hong Kong/8/73 are reported here (Table 5).

Nine volunteers were inoculated with 2 doses (one week apart) each containing $10^{7.2}$ EID_{50} of R22. The volunteers did not report any symptoms to the physician for a period of 7 days after inoculation and they did not complain of any adverse effects on day 28. Virus isolation was not attempted in this trial. Six out of 9 volunteers seroconverted with a fourfold or greater increase in HI titre.

Forty-three volunteers received one dose of R75, 19 at a virus concentration of $10^{7.3}$ or $10^{7.5}$ EID_{50} and 24 at a virus concentration of $10^{6.8}$ EID_{50}. Reactions as assessed by self observation for 7 days and noted on printed forms were absent or mild after both doses, except for one volunteer receiving $10^{6.8}$ EID_{50}. This volunteer reported moderate nasal stuffiness, rhinorrhoea, pharyngitis and cough from the day after inoculation till day 7. On day 28 none of the volunteers reported any more adverse effects to the investigator.

Nasal washings for virus isolation were collected from 12 subjects having received the highest dose, on day 1 and day 2 after vaccination. These nasal washings were inoculated in embryonated eggs and the allantoic fluids containing virus were characterized for their haemagglutinin serotype and their growth in AOS. Virus was recovered from 5 out of 12 volunteers. This virus had the same HI titre as R75 against a specific antiserum and exhibited a growth in the AOS system within the statistical limits of R75 for that marker.

Eight out of the 19 volunteers inoculated with the highest dose seroconverted and 8 out of 24 receiving $10^{6.8}$ EID_{50}. The volunteer who reported moderate symptoms had an increase in HI titre from < 8 to 8.

One dose containing $10^{7.6}$ EID_{50} of R5 was inoculated into three volunteers. One of them had influenza-like symptoms from day 1 to 4 after administration and temperature rises above 37·5 °C. This volunteer excreted the virus for at least four days and seroconverted. Another volunteer excreting the virus for two days had a fourfold rise in serum HI titre but had no symptoms. The last volunteer excreted the virus for at least four days but did not seroconvert and had no symptoms, apart from an allergic reaction to neomycin. This volunteer was not aware of this allergy before inoculation.

Virus isolates exhibited the haemagglutinin of R5 as tested by HI and yields in AOS were within the statistical limits of this marker for R5.

DISCUSSION

Recombination of wild type influenza B viruses with an attenuated strain allowed the selection of recombinants which exhibited differing reactogenicity in man.

These recombinants had the haemagglutinin serotype of the wild type parents but, as shown by RNA analysis, the neuraminidase (NA) was derived from the attenuated parent. There is a high level of cross-reactivity between the NA of

Table 5. *Responses of volunteers having preinoculation HI titres of* $\leqslant 8$ *to the administration of R22, R75 and R5*

Strain	Dose (Log_{10} EID_{50})	Number of administration	*Virus shedding	Clinical reactions				†Seroconversion rate
				Severe	moderate	mild	nil	
R22	7·2	2	not tested	0	0	0	9	6/9
R75	7·3–7·5	1	5/12	0	0	6	13	8/19
	6·8	1	not tested	0	1	4	19	8/24
R5	7·6	1	3/3	1	0	0	2	2/3

* Number positive/number tested. † Four-fold or greater rise in HI titre.

influenza B viruses and therefore their identification by the NI test is difficult (Beare *et al.* 1977*a*, *b*). Growth properties in AOS cultures have proved a useful and stable genetic marker of the recombinants.

Analysis of the RNA's of influenza viruses is now possible and has allowed the identification of all the RNA segments of two recombinants (R22 and R75). They have all the genes (except that coding for HA) from the attenuated strain, whereas the third recombinant (R5) has at least two genes from the attenuated parent (two segments were not identified). Preliminary evaluation in man has shown that R22 and R75 are fully attenuated and suitable vaccine candidates. Their innocuity has been confirmed in other trials that were placebo-controlled (Peetermans, Lamy & Delem, 1975, Spencer, Cherry & Powell, 1977). The reactogenicity and immunogenicity of the two strains were similar, but R75 was chosen as the vaccine strain because it gave higher yields in eggs. The third recombinant R5 was still virulent when tested in volunteers. One could speculate that this strain was not attenuated because it has several genes of the wild parent (at least four) and might still have the genetic constellation of virulence.

This study demonstrates that the gene constellation for virulence of B/Hong Kong/5/72 can be changed by genetic reassortment with the attenuated strain Brigit thus producing attenuated recombinants. Biochemical characterization of the genome of influenza type B recombinants was found, as for influenza type A, to be a promising method for the selection of live vaccine candidates.

The analysis of RNA for characterization of the recombinants has been performed by Dr V. R. Racaniello and Dr P. Palese at the Mount Sinai Medical Center. Obviously it is a major contribution to this study and we are very much indebted to them.

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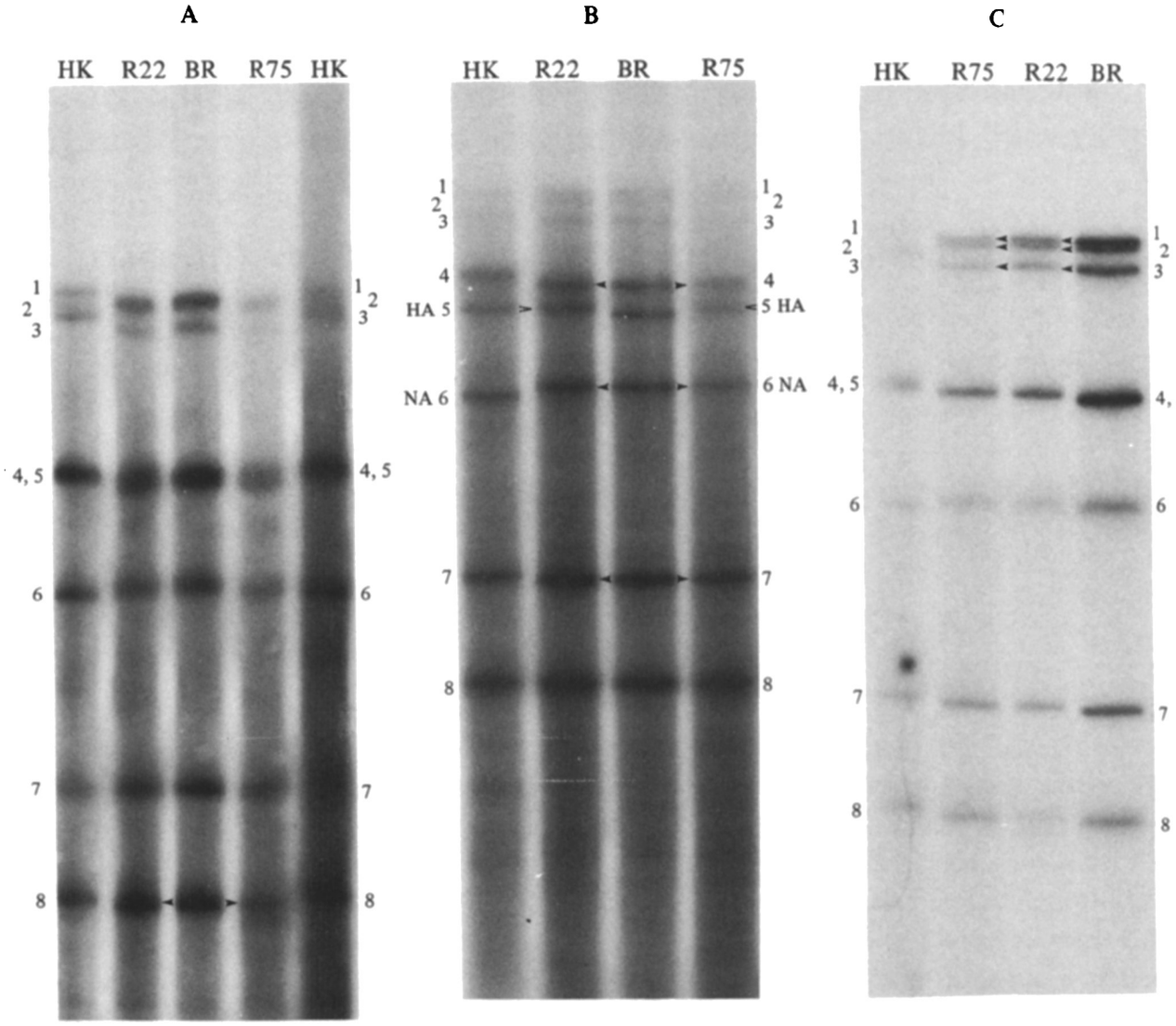
EXPLANATION OF PLATE 1

Separation of RNAs of different influenza B viruses on polyacrylamide gels. The concentration of polyacrylamide was 2·4 % (A), 3·0 % (B) and 2·6 % (C). HK denotes the RNAs of B/Hong Kong/5/72, BR those of Brigit and R22 and R75 those of two recombinants. Solid arrows on A, B and C indicate that the RNA segment of the recombinants was derived from Brigit and open arrows on B indicate that the RNA segment was derived from B/Hong Kong/5/72. For further intepretation of the patterns see text.



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