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Genetic reassortment in pandemic and interpandemic influenza viruses

A study of 122 viruses infecting humans

L.P. Shu[1], G.B. Sharp[1,2], Y.P. Lin[1,3], E.C.J. Claas[1,4], S.L. Krauss[1], K.F. Shortridge[3] & R.G. Webster[1]

[1] *Department of Virology & Molecular Biology, St. Jude Children's Research Hospital, Memphis, Tennessee, USA*;
[2] *Department of Hematology/Oncology, St. Jude Children's Research Hospital, Memphis, Tennessee, USA*;
[3] *Department of Microbiology, The University of Hong Kong, Pathology Building, Queen Mary Hospital Compound, Hong Kong*; [4] *Department of Virology and WHO National Influenza Centre, Erasmus University, Rotterdam, The Netherlands*

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Abstract. The human influenza pandemics of 1957 and 1968 were caused by reassortant viruses that possessed internal gene segments from avian and human strains. Whether genetic reassortment of human and avian influenza viruses occurs during interpandemic periods and how often humans are infected with such reassortants is not known. To provide this information, we used dot-blot hybridization, partial nucleotide sequencing and subsequent phylogenetic analysis to examine the 6 internal genes of 122 viruses isolated in humans between 1933 and 1992 primarily from Asia, Europe, and the Americas. The internal genes of A/New Jersey/11/76 isolated from a human fatality at Fort Dix, New Jersey in 1976 were found to be of porcine origin. Although none of the geographically and temporally diverse collection of 122 viruses was an avian-human or other reassortant, cognizance was made of the fact that there were two isolates from children from amongst 546 influenza A isolates obtained from The Netherlands from 1989–1994 which were influenza A reassortants containing genes of avian origin, viruses which have infected European pigs since 1983–1985. Thus, genetic reassortment between avian and human influenza strains does occur in the emergence of pandemic and interpandemic influenza A viruses. However, in the interpandemic periods the reassortants have no survival advantage, and the circulating interpandemic influenza viruses in humans do not appear to accumulate avian influenza virus genes.

Key words: Influenza A viruses, Genetic reassortment, Interspecies transmission, Pandemic

Introduction

Zoonotic infections of humans with influenza A viruses have been sporadically reported in the past. They are important because of their possible link to infrequent, but catastrophic, influenza A pandemics, six of which have occurred since 1889. Phylogenetic analysis suggests that the H1N1 pandemic virus of 1918 may have derived some or all of its genes from an avian virus [13, 15]. The polymerase B1 (PB1), hemagglutinin (HA), and neuraminidase (NA) genes of the H2N2 influenza virus causing the 1957 pandemic were of avian origin, the other 5 genes of this virus being of human origin [22, 35]. The PB1 and HA genes of the 1968 H3N2 pandemic strain were also of avian origin, its other genes being of human origin [22, 35]. The segmented nature of the genome and the random incorporation of 8 RNA segments in the virus allow genetic reassortment to occur when cells are doubly infected with two different parental viruses. Humans do not have receptors for avian viruses [1], and pigs, which do have receptors for both human and avian viruses, have been suggested as possible hosts for reassortment events (mixing vessels) for viruses of human and avian origin which allow viruses containing avian genes to be passed back to human populations, in some instances causing pandemics [34, 43]. Although direct transmission of influenza A viruses from avians to humans or via intermediaries other than the pig cannot be ruled out, no such transmissions of influenza A viruses have ever been documented.

Some reported episodes of virus transfer from pigs to humans have had benign results, but at least 11 deaths have been reported since a swine influenza virus was first isolated from a human in 1974 [4–6, 8, 10, 30, 31, 38, 39, 41]. Fatal cases have occurred in immunosuppressed persons, including a 13-year-old boy with Hodgkin's Disease in 1974 [39] and a 4-year-old girl with acute lymphoblastic leukemia in 1982 [31]. Fatal cases have also been reported in person who were less immunocompromised,

including a Wisconsin woman in 1988 who was exposed to sick pigs at an agricultural fair during her last trimester of pregnancy [26, 44], as well as in persons not immunocompromised, including a Maryland animal caretaker who died in 1991 after being exposed to sick pigs [45]. The most well known fatality occurred at Ft. Dix, New Jersey in 1976 with the death from pneumonia of one of five military recruits from whom H1N1 influenza virus of swine origin was isolated, an outbreak which prompted a national vaccination effort in the USA [12, 42]. A total of 230 recruits in this outbreak showed serologic evidence of infection with this virus [11, 21, 42]. In Texas in 1979 and 1980 two humans who had been exposed to pigs were non-fatally infected with swine H1N1 viruses [8]. We recently reported two cases where young children living in the different regions of The Netherlands were non-fatally infected with avian-human influenza A reassortant viruses (H3N2) that began circulating in European pigs between 1983 and 1985 [3, 7].

Although a large number of persons showed evidence of infection in the Ft. Dix outbreak, most reported infections of humans with viruses originating in pigs are associated with very limited person-to-person transmission of these viruses. In the Texas outbreak, a virus transmitted from a pig to a human was also transmitted to the roommate of one of the patients [8]. There is serological evidence that the H1N1 swine virus which killed the woman who had attended the Wisconsin agricultural fair was also transmitted to 19 children exhibiting pigs there [44]. In the recent outbreak in The Netherlands, the infected children were not directly exposed to pigs, but the father of one child was, while the father of the other was a teacher in a rural area who had serological evidence of infection with the reassortant virus [7]. Thus, the two reassortants are likely to have been transmitted person-to-person to the children from their fathers.

The major goal of this study was to determine if there were other incidences when influenza A viruses originating in other species, especially avians, had been transmitted to humans and to thereby determine the importance of the fact that pandemic strains have genes of avian origin. In past years reference laboratories of the World Health Organization have conducted extensive antigenic analysis of the external hemagglutinin and neuraminidase genes of influenza A viruses to establish the extent of antigenic drift and to determine what changes in vaccine strains are needed. Thus, we would know if past virus strains, for which reference samples are available, had hemagglutinin and neuraminidase genes of non-human origin. Since little attention has been paid to the internal genes of these viruses, our study focused on these genes, which encode 8 of the virus' 10 proteins. To determine the species of origin for as many different influenza A viruses as possible, we drew upon the resources of three virus repositories, one in the United States and two in Asia, analyzing isolates collected around the world. This provided an opportunity for examination of the internal genes of the A/New Jersey/11/76 (H1N1) virus isolated from the fatal case at Fort Dix in 1976. We used methods which allow large numbers of viruses to be studied: do blot hybridization [46] combined with phylogenetic analysis of gene sequences [13, 14] and were able to determine the species of origin for the 6 internal genes of 122 viruses isolated in humans throughout the world between 1933 and 1992. About half of these viruses were isolated in China and Hong Kong where recent pandemics were first detected.

Materials and methods

Virus strains. A total of 122 different human influenza viruses were maintained in repositories at St. Jude Children's Research Hospital, Memphis, TN, USA; Nanjing Medical College, Jiangsu Province, People's Republic of China; and the Department of Health, Hong Kong. These viruses included examples of human H1N1, H2N2, and H3N2 viruses isolated from different geographic areas of the world between 1933 and 1992. Sixty-four (52%) isolates were from Asia with the majority of Asian samples coming from Hong Kong and China (Table 1). Forty-two (34%) isolates were from the United States; other sources of samples were Australia, New Zealand, Russia, and the United Kingdom (Table 1).

Identification of host of gene origin. We conducted dot-blot hybridization of the six internal genes of each of the 122 viruses to determine their host of origin using a method described previously [46]. Briefly, we extracted viral RNA from allantoic fluid containing human influenza viruses. To synthesize the cDNAs of all RNA segments, we used reverse transcriptase (Life Sciences, St. Petersburg, FL) and a 12-base oligonucleotide primer (5'AGCAAAAGCAGG) common to all RNA segments of influenza A viruses. The cDNAs were amplified by the polymerase chain reaction (PCR) using pairs of forward and reverse primers that are specific for the six internal genes: nucleoprotein (NP), matrix (M), non-structural (NS) and the three polymerase genes (PA, PB1, and PB2). For convenience we are classifying the M gene, which encodes both M1 and M2 proteins, as an internal gene even though the latter is a surface protein. The PCR products were denatured and then cross-linked by ultraviolet light (Stratalinker, Stratagene, La Jolla, CA) to nylon membranes (Zeta Probe, BioRad, Richmond, CA) for dot-blot hybridization. The design and detailed location for all primers and probes have been reported previously [46]. Three probes that were specific for influenza viruses

Table 1. Human influenza viruses analyzed in this study according to region where sample collected and era of subtype dominance

Era of subtype dominance	Number of viruses analyzed	Geographic region where viruses isolated				Subtype		
		The Americas[a]	Asia	Australia, New Zealand	Europe[b]	H1N1	N2N2	N3N2
1933–1956 (H1N1)	12	7	4	0	1	12	0	0
1957–1967 (H2N2)	9	0	8	0	1	1[c]	8	0
1968–1976 (H3N2)	24	10	13	0	1	1[d]	0	23
1977–1992 (H3N2/H1N1)	77	26	39	4	8	17	0	60
Total	122	43	64	4	11	31	8	83

[a] One isolate from Brazil is included; the rest are from the United States.
[b] Category includes 3 samples from Russia, 7 from the UK.
[c] A/Luoyang/3/57, the last recorded H1N1 virus from China before the 1977 pandemic.
[d] A/New Jersey/11/76, isolate form the Fort Dix outbreak in 1976.

isolated from human, swine, and avian species, as well as a probe specific to a common conserved domain found in influenza viruses from all hosts (control), were used to identify the origin of each gene. Probes were end-labeled with digoxigenin (Boehringer, Indianapolis, IN) and hybridized to a DNA template. After hybridization the probe binding was detected by a colorimetic reaction with nitroblue tetrazolium and X-phosphate (Boehringer Mannheim, Indianapolis, IN).

Sequence analysis. Genes that showed cross-hybridization with more than one probe were partially sequenced by the fmol™DNA Sequencing System (Promega, Madison, WI). The PCR products were purified by Geneclean (Promega, Madison, WI); the primers were end-labeled with [$^{32}\gamma$P] ATP by incubation at 37 °C for 10 min and then heated at 100 °C for 2 min to eliminate kinase activity. The extension/termination reactions were performed in a Programmable Thermal Controller with incubation at 95 °C for 2 min, followed by denaturation at 95 °C for 30 sec, annealing at 42 °C for 30 sec and extension at 70 °C for 1 min. There were 30 cycles of amplification. Three microliters of each reaction mixture were loaded on a sequencing gel after addition of the stop solution and heating at 100 °C for 2 min.

Phylogenetic analysis. The gene sequences were analyzed with the FastDB (IntelliGenetics) program to find out the best matching sequence homology from GenBank. Gene alignment was done with the IntelliGenetics program, and phylogenetic analysis of sequence data was performed with PAUP (Phylogenetic Analysis Using Parsimony) software, version 2.4 (David Swofford, Illinois Natural History Survey, Champaign, IL), which relies on maximum parsimony to generate phylogenetic relationships.

Results

Dot-blot hybridization. To determine the host of origin of the internal gene segments in the human viruses studied, we used dot-blot hybridization with specific probes to human, avian, and swine viruses. We studied a total of 122 viruses in all, characterizing a total of 732 genes (Table 2). The internal genes for most of these viruses reacted specifically with the human virus probe. The exception was the swine-like virus A/New Jersey/11/76 (H1N1), the virus which caused the Ft. Dix, New Jersey outbreak. The P genes of this virus, PB2, PB1, and PA, cross-reacted with both the swine and human virus probes (Table 2), and two of the other 3 internal genes of this virus, NP and M, reacted only with the swine virus probes and not with the human probes (Table 2). The sixth gene, NS, reacted only with the human virus probe (Table 2). Seven other viruses were found to contain PB1 genes which reacted with both the human and avian virus probes, and to determine the host of origin of these PB1 genes, it was necessary that partial sequencing be performed.

Sequence analysis. As shown in Table 3, we determined more than 20% of the sequence of each of the genes in question. Of the 7 human influenza viruses with PB1 genes that gave equivocal results in the dot-blot analysis, each showed highest homology with

Table 2. Characterization of human influenza viruses by dot blot hybridization

Gene	No. studied	Host of origin determined by dot blot hybridization			
		Human	Swine	Avian	Cross hybridization*
PB2	121	121	0	0	
PB1	121	114	0	0	7 (H/A)
PA	121	121	0	0	
NP	121	121	0	0	
M	121	121	0	0	
NS	121	121	0	0	
One swine-like human virus (A/New Jersey/11/76)					
PB2	1	0	0	0	1 (H/S)
PB1	1	0	0	0	1 (H/S)
PA	1	0	0	0	1 (H/S)
NP	1	0	1	0	
M	1	0	1	0	
NS	1	1	0	0	

* H – human virus probe; A – avian virus probe; S – swine virus probe; H/A – indicate cross-hybridization with human and avian virus probes; H/S – indicate cross-hybridization with human and swine virus probes.

Table 3. Partial sequence analysis of gene segments to establish their host of origin

Viruses	Genes	Dot blot	Nucleotides analyzed	Virus with highest homology	Homology (%)
A/Beijing/7/53 (H1N1)	PB1	H/A	985–1483	A/Beijing/11/56 (H1N1)	99.4
A/Baylor/10788/82 (H1N1)	PB1	H/A	1534–2231	A/Beijing/11/56 (H1N1)	91.6
A/New York/2924/86 (H1N1)	PB1	H/A	1513–2247	A/Beijing/11/56 (H1N1)	94.6
A/Shanghai/202/57 (H2N2)	PB1	H/A	971–1445	A/Singapore/1/57 (H2N2)	99.8
A/Aichi/2/68 (H3N2)	PB1	H/A	970–1429	NT/60/68 (H3N2)	97.8
A/Memphis/1/71 (H3N2)	PB1	H/A	949–1482	NT/60/68 (H3N2)	99.4
A/Leningrad/360/86 (H3N2)	PB1	H/A	1731–1922 2050–2231	A/Memphis/8/88 (H3N2)	97.3
A/New Jersey/11/76 (H1N1)	PB2	H/S	997–1593	Swine/Tennessee/26/77 (H1N1)	98.0
A/New Jersey/11/76 (H1N1)	PB1	H/S	984–1478	Swine/Ontario/2/81 (H1N1)	96.2
A/New Jersey/11/76 (H1N1)	PA	H/S	54–543	Swine/Tennessee/26/77 (H1N1)	96.1
A/New Jersey/11/76 (H1N1)	NP	S	1189–1413	Swine/Italy/437/76 (H1N1)	98.2
A/New Jersey/1/75 (H1N1)	M	S	128–472	Swine/Tennessee/26/77 (H1N1)	98.8
A/New Jersey/11/76 (H1N1)	NS	H	155–350	Swine/Tennessee/26/77 (H1N1)	94.9

human strains. In most cases the homology ranged from 97.8 to 99.8% with previously characterized human PB1 genes. Two H1N1 strains (A/New York/2924/86 and A/Baylor/10788/82) showed lower levels of homology, 94.6 and 91.6%, reflecting the small number of PB1 gene sequences from H1N1 virus that are available in GenBank for comparison. We partially sequenced each of the 6 internal genes of A/New Jersey/11/76 (H1N1) to determine their host of origin. The PB2, PB1, PA, NP and M genes showed 96 to 98% homology with known swine sequences: the NS1 gene of A/New Jersey/11/76 (H1N1), which reacted only with the human probe, was outside this range, showing 94.9% homology with Sw/Tennessee/26/77 (H1N1).

Phylogenetic analysis. To further validate the host of origin of the PB1 genes, we analyzed 8 of the partial sequences together with 16 published sequences [22] (Figure 1). Four major lineages were apparent on the phylogenetic tree: (1) human H2N2 and H3N2 viruses, (2) avian viruses, (3) equine viruses, and (4) swine and human H1N1 viruses. The 7 PB1 genes

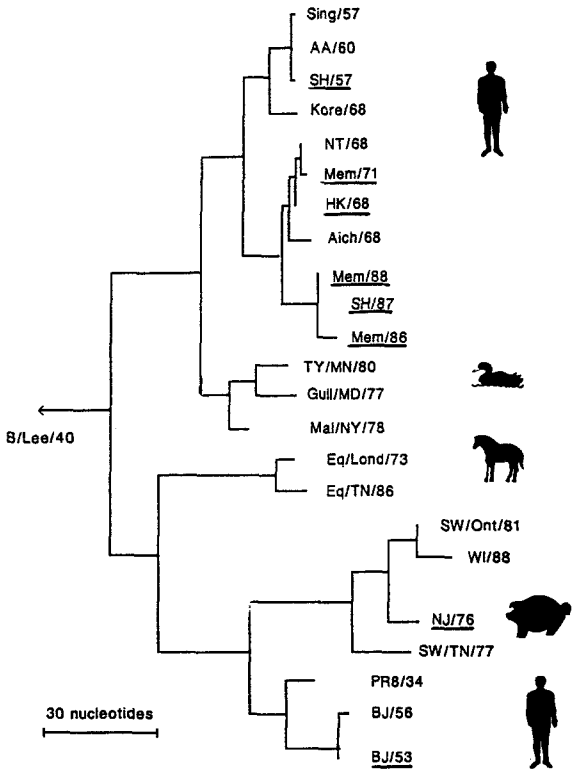

Figure 1. Phylogenetic tree of human influenza A viruses in which PB1 gene nucleotide sequences are rooted to the PB1 sequences of B/Lee/40. PAUP software, which relies on a maximum parsimony algorithm to find the shortest trees, was used in the sequence analysis. Horizonal distances are proportional to the minimum number of nucleotide differences required to join nodes. Vertical lines are for spacing branches and labels. The underlined sequences were determined in the present study; others were from a previous report by Kawaoka et al. [22].

of human viruses giving equivocal results in the dot-blot analysis were part of the human H2N2 and H3N2 lineage or the swine and human H1N1 lineage (underlined on Figure 1). The swine-like human virus, A/New Jersey/11/76 (H1N1), belonged to swine sublineages close to A/Sw/Ontario/2/81, confirming its origin in pigs.

Discussion

Our results show that only one of the 122 viruses studied, the virus isolated at Ft. Dix, New Jersey, contained genes of other than human origin. Nearly half of these viruses were isolated in China, the hypothetical epicenter of pandemic influenza [36]. Although isolates from the 1889, 1900, and 1918 pandemics are not available and it is not known if they contained genes of avian origin, we know that the 1957 and 1968 pandemic strains did [22, 35] and, based on indirect evidence, that the 1918 pandemic strain may also have contained such genes [13, 15]. The 1977 pandemic strain was a reintroduction of a previous pandemic strain [27]. The emergence in nature of influenza A virus reassortants is well documented, especially between different virus subtypes in birds [9, 18], as well as between viruses found in swine and avians [3, 20, 46]. Moreover, reassortants have been detected between H1N1 and H3N2 viruses infecting humans [2, 16, 25, 47, 48]. Thus, genetic reassortment between avian and human influenza strains does occur in the emergence of pandemic and interpandemic influenza A viruses. However, in the interpandemic periods the reassortants have no survival advantage and the circulating interpandemic influenza viruses in humans do not appear to accumulate avian influenza virus genes.

Despite the novelty of the virus causing the 1976 epidemic at Ft. Dix, New Jersey [A/New Jersey/11/76 (H1N1)], at the outset of this study, characterization had only been conducted of the HA and NA antigens of this virus and of the NP gene of another virus isolated during this outbreak [A/New Jersey/8/76 (H1N1)]. Here we show that all of the internal genes of A/New Jersey/11/76 have highest homology with those of H1N1 swine viruses. Our findings for the NP gene of this virus are consistent with the earlier finding of a swine origin for the NP gene of A/New Jersey/8/76 [13]. The phylogenetic tree of the PB1 gene shows that the swine-like human influenza virus is located in the swine phylogenetic lineage, hence, all eight genes of the virus were transmitted from pigs to humans. Although the virus was transmitted to humans and showed limited spread, it failed to become a pandemic strain and did not generate reassortants that became established in humans [12].

There is evidence that pigs may be fairly frequently infected with influenza A viruses circulating in humans [24, 28, 29, 37, 40]. Studies of influenza

viruses isolated in pigs in China between 1976–82 showed that 29 of 32 H3N2 viruses contained genes exclusively of human origin, the other three H3N2 viruses containing genes of both porcine and human origin [37]. Infection of a pig with a human/swine influenza reassortant occurred in Japan in 1978 [40], and a number of instances where pigs were infected with human H3N2 and H1N1 influenza viruses have been previously reported in Asia [24, 28, 29]. Thus, it is clear that H1N1 and H3N2 strains and human/avian reassortants can be transmitted from humans to pigs.

Since 1974 enough human infections with influenza A viruses containing genes of porcine origin have been reported in patients who usually have had recent contact with pigs that we know that such viruses can also be passed from pigs to humans. The transmission of swine H1N1 influenza viruses to humans at Fort Dix, New Jersey; Wisconsin and Maryland which were virtually identical to viruses isolated in pigs [19, 32, 45], and the apparent transmission of H3N2 human/avian reassortant viruses from pigs to humans in the Netherlands which were also very similar to a virus isolated from a pig in this country demonstrates that humans can be infected with viruses circulating in pigs which are of swine or of swine and avian origin [7].

Acknowledging that pig-to-human transmission of influenza A viruses occurs, the question becomes, how often are such viruses transmitted person-to-person within human populations? Our finding that only one of the geographically and temporally diverse collection of influenza A viruses isolated since 1933 contained genes of non-human origin suggests that such viruses do not circulate well, once introduced to a human population. This conclusion is also supported by the documented instances of pig-to-human transmission of influenza A viruses, because, with the exception of the Ft. Dix outbreak, such infections have resulted in very limited spread of these viruses to other humans. In the Dutch study, although it seems likely that each reassortant virus was spread from a father to his child (and perhaps from a student to his teacher, one of the fathers), there was no evidence of other person-to-person infections with these reassortants [7]. These viruses were probably not spread further, because in The Netherlands, as in the rest of the world, there was then and continues to be a high level of immunity to the H3N2 virus in the population. Similarly, person-to-person spread of viruses originating in swine has probably also been limited in past outbreaks because of sufficiently high population levels of immunity to the hemagglutinin and neuraminidase genes of these viruses. The limited ability of viruses with genes originating in non-human species to be passed person-to-person means that only a small proportion of influenza A viruses in circulation at any time are likely to contain such genes. However, the presence of avian genes in previous pandemic strains unequivocally demonstrates that not all influenza A viruses containing avian genes lack the capacity for person-to-person transmission, and when such transmission is possible, the results can be catastrophic.

How frequently are influenza A viruses transmitted from pigs to humans? The Netherlands study is the only study where influenza A infections have been systematically surveyed to determine how many involve viruses with non-human genes [7]. Those investigators surveyed what was for the most part a randomly collected sample of 546 influenza A infections occurring over a 5-year period, detecting two infections occurring 50 miles and two months apart that involved viruses containing avian genes (personal communication, Dr Eric Claas). These two avian-gene-positive viruses accounted for 0.3% of the 546 samples, 3.6% of the samples analyzed the year they were found, and 8.1% of that year's samples from children under age 4 years (personal communication, Dr Eric Claas). The reassortants are different enough that they appear to have evolved and been maintained in two geographically separate pig populations [7]. In light of those results, the number of documented swine-to-human transmissions of influenza A viruses detected without surveillance studies, and the likelihood of missing such infections without surveillance, it seems likely that non-fatal pig-to-human transmission of influenza A viruses occurs fairly often but goes undetected. Since such infections can also be fatal, persons in contact with pigs should be cautious, especially when pigs show symptoms of illness. When infections such as these do occur, the viruses involved should be carefully studied to determine if they have pandemic potential.

How often do influenza A viruses transmitted from pigs to humans contain avian genes, the virus composition associated with pandemics? While a virus with avian genes has circulated in European pigs since 1979 [17, 33], we found no evidence of avian genes in 73 porcine isolates collected in the United States from 1976 to 1990 [46] or in 104 porcine isolates collected at random in Southern China and Southeast Asia from 1976 to 1982 [37]. Thus, we would speculate that, while an influenza A virus containing avian genes would probably be readily passed from pigs to humans as other influenza A viruses have been transmitted, pigs appear to only rarely harbor viruses with such pandemic potential. Nevertheless, we should be on the lookout for pigs infected with viruses of avian origin to which the human population is not immune, since if viruses with pandemic potential infect pigs they may be likely to be passed on to humans, and the results of this could be catastrophic. The demonstration that pigs can be experimentally infected with avian influenza viruses representing the 13 HA subtypes available for study (H14 was not tested) is a salient reminder of this possibility [23].

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Address for correspondence: R.G. Webster, Department of Virology & Molecular Biology, St. Jude's Children's Research Hospital, 332 N. Lauderdale, P.O. Box 318, Memphis, TN 38101, USA
Phone: (901) 495 3400; Fax: (901) 523 2622