Asian Biomedicine Vol. 7 No. 2 April 2013; 155-167 DOI: 10.5372/1905-7415.0702.163

Original article

Electron micrographs of human and avian influenza viruses with high and low pathogenicity

Suda Louisirirotchanakul[a], Pornparn Rojanasang[a], Kleophant Thakerngpol[a], Naree Choosrichom[a], Kridsda Chaichoune[b], Phisanu Pooruk[a], Aphinya Namsai[a], Robert Webster[c], Pilaipan Puthavathana[a]
[a]Departments of Microbiology and Pathology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok 10700, [b]Faculty of Veterinary Science, Mahidol University, Nakhon Pathom 73170, Thailand, [c]St. Jude Children's Research Hospital, Tennessee, USA

Background: An outbreak of highly pathogenic avian influenza (HPAI) H5N1 virus in Thailand was first reported in 2004. To date, electron micrographs demonstrating the morphology of HPAI H5N1 virus particle are quite limited.
Objective: To demonstrate the morphology of HPAI H5N1 virus particles, avian influenza viruses with low pathogenicity, seasonal influenza viruses, and H5N1 structural components in infected cells. The M amino acid residues that might affect the viral morphology were also analyzed.
Methods: Electron micrographs of negatively-stained virus particles and positively-stained thin sections of the HPAI H5N1 virus infected cells were visualized under a transmission electron microscope. M amino acid sequences of the study viruses were retrieved from the GenBank database and aligned with those of reference strains with known morphology and residues that are unique for the morphological type of the virus particles.
Results: Morphologically, three forms of influenza virus particles, spherical, regular, and irregular rods, and long filamentous particles, were demonstrated. However, the spherical form was the most predominant morphological type and accounted for more than 80% of the virus populations examined. In addition, the viral entry and exit steps including incomplete particles in infected Madin–Darby canine kidney cells were visualized. Our analyses did not find any M amino acid residues that might influence the viral morphology.
Conclusion: Of all virus isolates studied, we demonstrated that the spherical particles were the major population observed regardless of virus subtype, host of origin, virus virulence, or passage history. Our study suggested that the morphology of influenza virus particles released, might not be strongly influenced by *M* gene polymorphism.

***Keywords*:** Electron micrographs, highly pathogenic avian influenza H5N1 virus, influenza virus, low pathogenic avian influenza virus, M amino acid sequence, virus morphology

The influenza A virus particle is comprised of an eight-segment RNA genome surrounded by a nucleocapsid and outermost envelope, which is underlined with matrix protein and covered with two kinds of surface glycoprotein projections: hemagglutinin (H) and neuraminidase (N) [1]. Influenza virus particles are pleomorphic and vary in size. The morphology seen under an electron microscope is classified into three forms, a spherical form with a diameter ranging from 80 to 120 nm, a rod-shaped form with a length ranging from 120 to 300 nm, and a filamentous form, which is normally longer than 300 nm [1-4].

Collective information from previous works suggests that morphology of influenza virus particles is influenced by two factors, type of host in which the viruses are propagated, either in cell culture or embryonated egg, and virus passage at investigation, either early or late passage [5, 6]. It has been reported that the filamentous form predominates among the virus population isolated from chick embryo or cell culture at early passages. By contrast, laboratory-adapted strains are predominantly spherical [5]. Alternatively, it has been reported that both filamentous and spherical forms appeared simultaneously in tissue culture [2, 6]. Moreover, it has been suggested that the spherical form was the

Correspondence to: Pilaipan Puthavathana, Department of Microbiology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok 10700, Thailand. E-mail: siput@mahidol.ac.th

final product from segmentation of filamentous particles [5]. Subsequent observation showed that the polarized cell type and the integrity of the actin microfilament network are important for the formation of filamentous particles [6]. It has been suggested that morphologic features of influenza viruses are under genetic control, not a result of host-induced modification [7-11]. It has also been suggested that the filament forming ability of the viruses is genetically stable under control of multiple genes, *H*, *N*, *M* (matrix), and *NP* (nucleoprotein) genes [8, 12]. Using reverse genetic techniques to analyze M1 amino acid sequences, Elleman and Barclay [9] suggested that three residues, Ala41, Arg95, and Ala218 might contribute to filamentous morphology whereas residues Arg95 and Glu204 were proposed by Bourmakina et al. [10]. Based on the cytoplasmic tail of M2 as reported by Rossman et al. [11], the residues in amphipathic helix (Phe47, Phe48, Iso51, Tyr52, Phe55) together with the Ser71, Met72, and Arg73 may contribute to the formation of filamentous morphology.

Based on the H and N antigens, influenza A viruses are further divided into 17 H and 9 N subtypes [13-16]. The first 16 subtypes are found in aquatic birds. Furthermore, most of them are of low pathogenicity, except for some isolates in H5 and H7 subtypes that are considered to be highly pathogenic avian influenza (HPAI) viruses [1, 13-15]. The seventeenth subtype was detected in bats by molecular techniques, but the attempts to isolate the virus did not succeed [16]. Influenza in the human population is attributed only to H1N1, H1N2, H2N2 (which existed from 1957 to 1968), and H3N2 subtypes [17, 18]. Nevertheless, a cross-species barrier of avian viruses to humans had been documented [19]. Transmission of HPAI H5N1 viruses from poultry to humans was first reported in 1997 and the disease subsided within that year [15]. The re-emergence of HPAI H5N1 outbreaks since 2003 has been even more catastrophic. It spread globally and involved more human cases. Furthermore, it had a higher fatality rate, at about 60% [http://www.who.int/influenza/human_animal_interface/EN_GIP_20120810CumulativeNumberH5N1cases.pdf].

Electron micrographs of influenza virus particles have been presented by groups of investigators for longer than 50 years, and the studies have been mostly confined to human viruses [2-4]. Novel influenza subtypes have been discovered from time to time, assuming that the morphology of these influenza subtypes is similar to what has been previously shown. To date, electron micrographs demonstrating the morphology of avian influenza virus particles, especially HPAI H5N1 viruses are quite limited. Ultimately, viral morphology is one piece of basic information for characterizing a newly virus. With the higher resolution of current electron microscopes and the higher quality of reagents and equipment now available, it is of interest to further characterize the morphology of the H5N1 virus in terms of (1) comparative morphology with human influenza viruses and avian influenza viruses with low pathogenicity (LPAI), (2) the viral structures seen during replication in Madin–Darby canine kidney cells (MDCK), and (3) the amino acid residues in M1 and M2 proteins that might affect the virus morphology.

Materials and methods

The study viruses

The virus isolates employed in this study were derived from either human or poultry origin. We have listed the viruses studied that comprised nine HPAI H5N1 isolates (4 from humans and 5 avian), four LPAI isolates, and two human influenza isolates in **Table 1**. These viruses were isolated and propagated in MDCK cells or in 9-day-old embryonated chick eggs. MDCK cells were grown in Eagle's minimal essential medium (EMEM) (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS) (Hyclone, Logan, UT, USA), 200 U/ml penicillin, 20 μg/ml gentamycin, and 0.001 mg/ml Fungizone. Upon virus propagation, cells were maintained in EMEM supplemented with antibiotics, Fungizone and 1.5 μg/ml trypsin tosyl phenylalanyl chloromethyl ketone (TPCK, Sigma, St. Louis, MO), and no FBS supplement. These virus isolates were stored at –80°C until tested. The experiments on H5N1 viruses were performed in the biosafety laboratory level 3 (BSL-3) in the Faculty of Medicine Siriraj Hospital and the Faculty of Veterinary Science, Mahidol University. The isolates were collected and experiments were conducted with the approval of our institutional review boards.

Negative staining of the free virus particles

In order to destroy the viral infectivity, the virus particles in the culture supernatants or allantoic fluids were treated with 2% glutaraldehyde before sedimenting by high-speed centrifugation at 18,000*g* for 90 minutes. The viral pellets were negatively stained with phosphotungstic acid (PTA), and morphology of the stained virus particles were visualized under

Table 1. The study viruses and GenBank accession numbers of influenza M1 and M2 amino acid sequences

Virus name	Source*	Passage history**	GenBank accession No.	
			M1 protein	M2 protein
High pathogenic avian influenza (H5N1) viruses				
A/Thailand/1(KAN-1)/2004	MU	LLC-MK2/ MDCK 6	AAV35110	AAV35111
A/Thailand/676/2005	MU	MDCK 5	ABC72651	ABC72652
A/Laos/Nong Khai 1/2007	MU	MDCK 4	ACA64012	ACA64013
A/Thailand/NBL 1/2006	MU	MDCK 4	ACU46646	ACU46647
A/chicken/Thailand/ICRC-V143/2007	MU	MDCK 3/Egg 1	ABW89592	ABW89593
A/chicken/Thailand/ICRC-195/2007	MU	Egg 4	ACE73587	ACE73588
A/chicken/Thailand/ICRC-213/2007	MU	MDCK 3/Egg 1	ACF36779	ACF36780
A/duck/Thailand/ICRC-V629/2008	MU	Egg 3		
A/chicken/Thailand/ICRC-VS1069/2008	MU	Egg 2		
Human influenza viruses				
A/New Caledonia/20/1999 (H1N1)-like virus (Siriraj07/2000)	MU	MDCK 8	ABF21304	ABF21305
A/Fujian/411/2002 (H3N2)-like virus (Siriraj03/2004)	MU	MDCK 8	-	ABB71834
Low pathogenic avian influenza (LPAI) viruses				
A/aquatic bird/Hong Kong/D125/2002 (H1N1)	SJ	Egg 1/MDCK 3		
A/duck/Shan tou/1883/2001 (H3N8)	SJ	Egg X/MDCK 2		
A/duck/Jiangxi/6151/2003 (H5N3)	SJ	Egg 4	ABA12315	ABA12316
A/ostrich/Zimbabwe/222/1996 (H7N1)	SJ	Egg 2		
Reference strains				
A/Udorn/1972 (H3N2)	-	-	ABD79033	ABD79034
A/FW/1/1950 (H1N1)	-	-	CAA30888	CAA30889

*MU = Mahidol University, SJ = St. Jude Children's Research Hospital, **All H5N1 viruses belong to clade 1 except A/ Laos/Nong Khai 1/2007 which belongs to clade 2; Egg X = unknown passage in egg

a transmission electron microscope (JEM-1230; JEOL Co, Tokyo, Japan) [20]. Briefly, 10 µl of the virus suspension was absorbed on a grid (200 mesh; SPI supplies, West Chester, PA, USA) coated with Formvar–carbon for 1 minute. The excess virus suspension was drained off, followed by UV irradiation of both sides of the grid for 5 minutes. A drop of 1.6% PTA, pH 7.0 was then applied for 1 minute, and the excess fluid was removed prior to examining under a TEM.

Using electron micrographs at magnifications of 50,000□ and 80,000□, numbers of virus particles in 3 HPAI H5N1 virus isolates and one seasonal H1N1 virus isolate were determined. The numbers of particles present in 5 grid squares were counted for each virus isolate.

Staining of HPAI H5N1 virus infected MDCK cells

A MDCK cell monolayer was inoculated with the test virus, A/Thailand/1(KAN-1)/2004 (H5N1), at a multiplicity of infection of 1 for 24 hours at 37°C in a CO_2 incubator. The infected cells were pelleted before processing and embedding. Thin sections of the virus infected cells and an uninfected cell control were positively stained and examined under a TEM as previously described [21]. The cell pellets were prefixed with 4% glutaraldehyde in PBS for 30 minutes at 4°C, and followed by three washes with Millonig's phosphate buffer and postfixed with 2% phosphate buffered osmium tetroxide for 30 minutes at room temperature, and lastly by two washes in distilled water. Then, the cells were stained with 2% uranyl acetate aqueous solution for 20 minutes at room temperature as described after the protocol for rapid tissue processing [22]. The stained sample was dehydrated in a sequence of steps as follows: 70% ethyl alcohol for 90 seconds with two changes; 80% ethyl alcohol for 90 seconds with two changes; 90% ethyl alcohol for 3 minutes with two changes; 95% ethyl alcohol for 3 minutes with three changes; absolute ethanol for 3 minutes with three changes; and lastly, with propylene oxide for 3 minutes with

three changes. Then, the stained sample was infiltrated with a 50:50 mixture of propylene oxide and epoxy resin for 30 minutes at 37°C. Subsequently, the mixture solution was replaced with an epoxy resin solution and further incubated for 2 hours at 37°C. Finally, the sample was embedded in polypropylene capsules and polymerized in a hot air oven at 70°C overnight followed by sectioning with an ultramicrotome. The ultrathin sections was mounted onto copper support grids (200 mesh; SPI supplies) prior to nuclear staining with uranyl acetate for 30 minutes and followed by cytoplasmic staining with lead citrate for 15 minutes. The stained particles were examined under a TEM.

Genetic characterization of the study viruses

Our study analyzed the amino acid residues that might affect the virus morphology after those reported by Elleman and Barclay [9] and Bourmakina and Garcia-Sastre [10] for M1, and Rossman et al. [11] for M2. The M1 and M2 amino acid sequences of our study viruses together with the reference filamentous or spherical type particles were retrieved from the GenBank database (**Table 1**). The amino acid sequences were aligned using the programs BioEdit version 7.0.9.0 [23]. The HPAI H5N1 viruses in this study belong to clade 1, except A/Laos/Nong Khai 1/2007, which belongs to clade 2.3.4.

Results

Electron microscopy of influenza virus particles

Electron micrographs of human and avian HPAI H5N1 virus particles originating are shown in **Figures 1** and **2**, respectively. Additionally, micrographs of human H1N1 and H3N2 viruses as well as LPAI H3N8, H5N3, and H7N1 viruses are shown in **Figures 3** and **4**, respectively. These virus isolates were comprised of three morphological types of particles, spherical with a diameter of from 80 to 120 nm, regular and irregular rods with a length between 120 and 300 nm, and filaments with a length longer than 300 nm. Nevertheless, the majority of them were spherical particles. A multilayered-coil structure suggestive of the helical ribonucleocapsids could be seen inside a virus particle in this study **(Figure 3a).**

We determined the numbers of virus particles of each different morphological type in virus isolates using electron micrographs at a magnification of 50,000× or 80,000×, or both. To avoid inaccurate data when too few particles were present in the stained samples, the results were excluded when the number of particles was lower than 80. The counting was successful for three HPAI H5N1 virus isolates and one human H1N1 isolate that demonstrated approximately 80% to 90% of the virus particles observed were spherical, and the remaining were a mixed population of rod-shaped and filamentous particles **(Table 2)**. Unfortunately, numbers of the viral particles for LPAI viruses and human viruses were less than 80. Therefore, the numbers obtained were excluded from calculations. Nevertheless, most of particles seen were clearly spherical. Overall, we demonstrated that the majority of all influenza viruses in this study were spherical, while filamentous particles were the minority.

Electron microscopy of influenza virus infected MDCK cells

H5N1 virus infected MDCK cells and uninfected cell controls were examined using a positive staining technique. The morphology of the uninfected MDCK cell surface appendages, which might be mistaken for their appearance as the filamentous-like particles was demonstrated (**Figure 5a**). An infected cell with chromatin condensation suggesting cell necrosis (**Figure 5b**), invagination of the cell membrane for endocytosis of a virus particle (**Figure 5c**), budding virus particles (**Figures 5d** and **5e**), and released virus particles (**Figure 5f**) were seen. Spherical particles were the predominant form of HPAI H5N1 viruses in all stages of replication in MDCK cells.

Genetic characterization of the study viruses

We examined the amino acid residues that might influence the formation of filamentous or spherical particles according to previous groups of investigators. The residues comprised Ala41, Arg95, and Ala218 in M1 for filamentous particle formation and residues Val41, Lys95, and Thr218 for spherical particle formation. These residues were examined following data from Elleman and Barclay [9]. Residues Arg95 and Glu204 in M1 for filamentous particle formation were examined following data from Bourmakina and Garcia-Sastre [10]. Amino acid alignment demonstrated the presence of all amino acid residues that suggested filamentous morphology in almost all of our virus isolates that contained mainly the spherical type particles **(Figure 6** and **Table 3).** We also examined the residues in the M2 cytoplasmic tail, such as Phe47, Phe48, Iso51, Tyr52, Phe55, Ser71, Met72, and Arg73, which are related to the stability

of filamentous particles as demonstrated by Rossman et al. With the exception Phe55, these residues were also present in our virus isolates **(Figure 7).**

Our study suggested that there was no amino acid residue unique for a certain morphological type of viral particle. In the other words, our findings suggested that the morphology of virus particles is unlikely to be under genetic control.

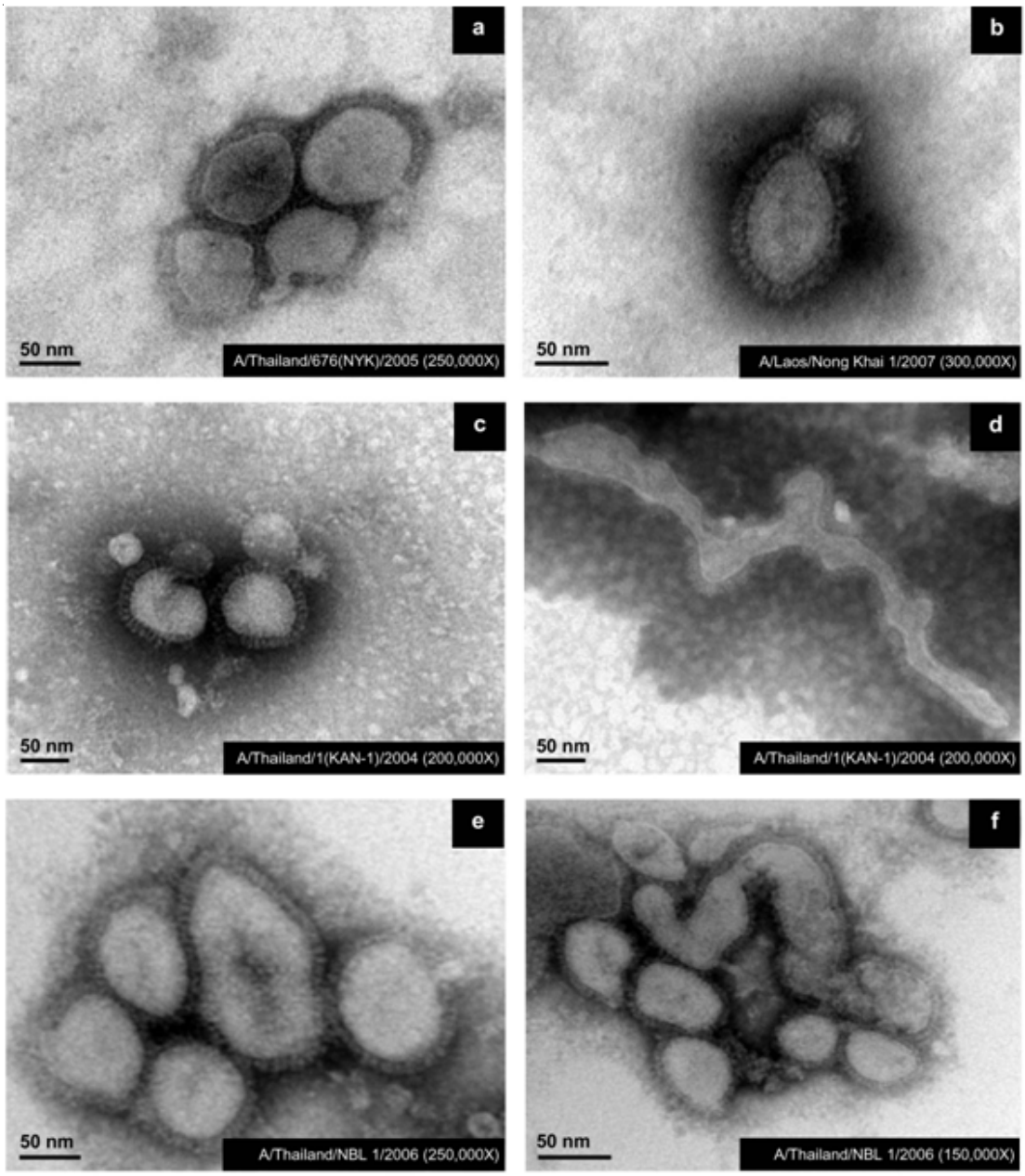

Figure 1. Electron micrographs of HPAI H5N1 isolates from humans: A/Thailand/676(NYK)/2005 (**a**); A/Laos/Nong khai 1/2007 (**b**); A/Thailand/1(KAN-1)/2004 (**c**, **d**); and A/Thailand/NBL 1/2006 (**e**, **f**). Note the pleomorphic morphology of spherical and filamentous particles.

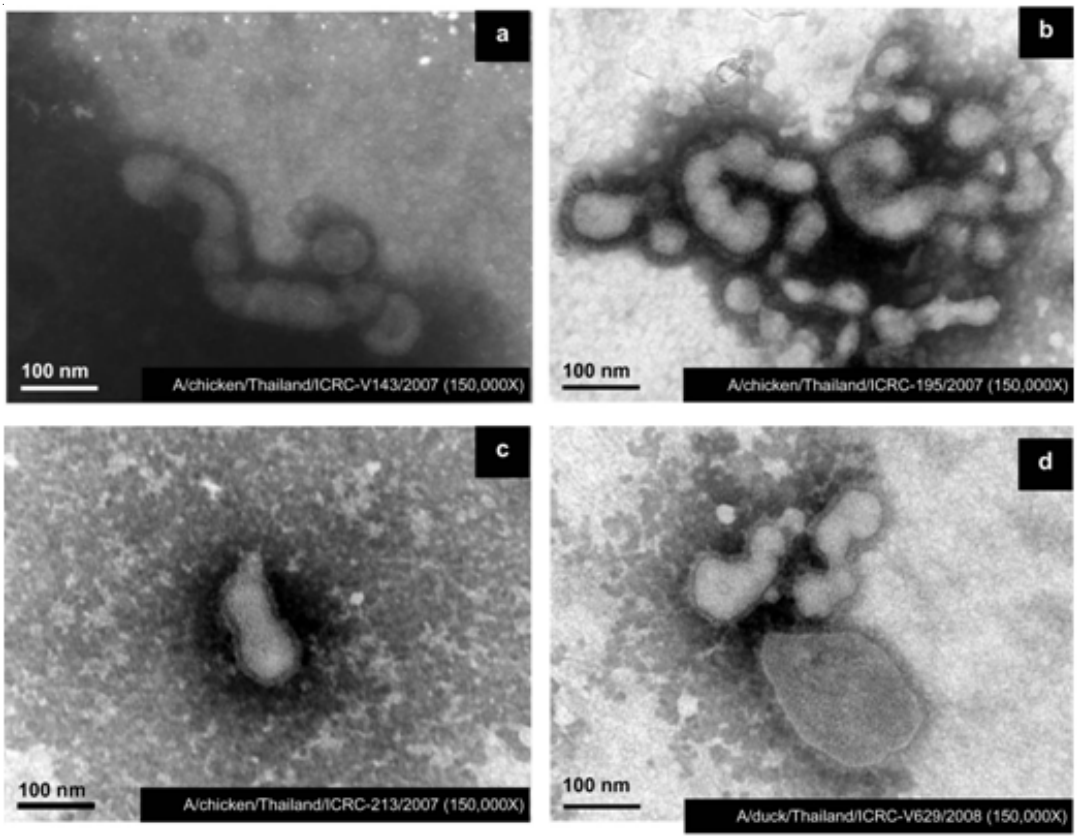

Figure 2. Electron micrographs of HPAI H5N1 isolates from animals: A/Chicken/Thailand/ICRC-V143/2007 (**a**); A/Chicken/Thailand/ICRC-195/2007 (**b**); A/Chicken/Thailand/ICRC-213/2007 (**c**); and A/Duck/Thailand/ICRC-V629/2008 (**d**).

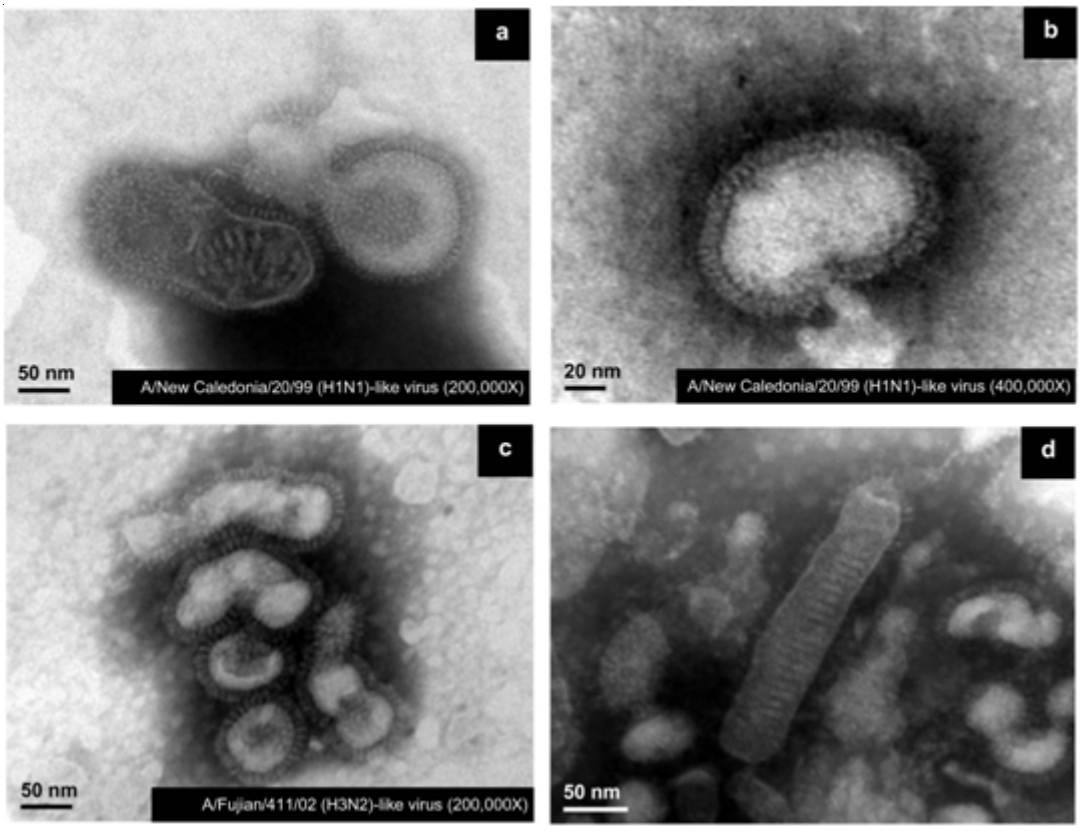

Figure 3. Electron micrographs of human influenza viruses: A/New Caledonia/20/1999 (H1N1)-like virus (**a**, **b**); and A/Fujian/411/2002 (H3N2)-like virus (**c**, **d**). A multilayered-coil structure of a virus is demonstrated inside a spherical (**a**) and a rod (**d**) particle.

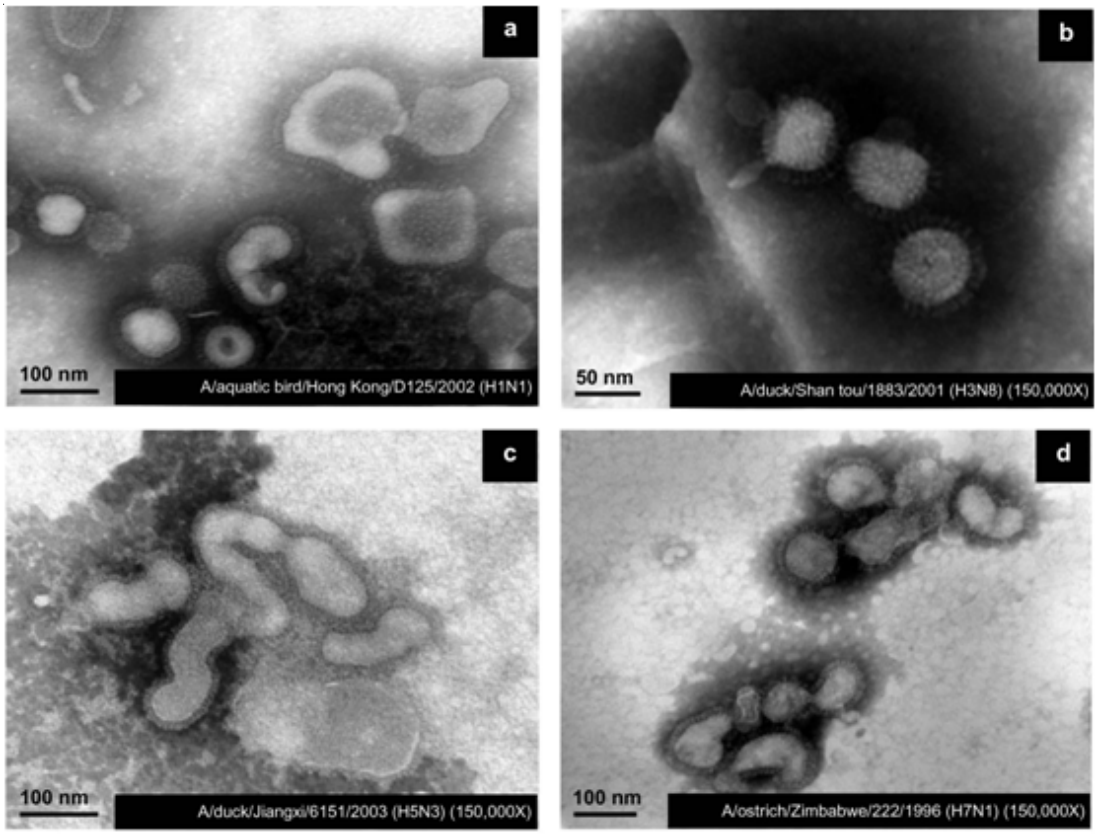

Figure 4. Electron micrographs of LPAI isolates from animals: A/aquatic bird/Hong Kong/D125/2002 (H1N1) (**a**); A/duck/Shantou/1883/2001 (H3N8) (**b**); A/duck/Jiangxi/6151/2003 (H5N3) (**c**); and A/ostrich/Zimbabwe/222/1996 (H7N1) (**d**).

Table 2. Morphological types of HPAI H5N1 and H1N1 viruses

Morphological type	Total No.	Spherical forms	Rod and filamentous forms
A/Thailand/1 (KAN-1)/2004 (H5N1)	87	79 (90.8%)	8 (9.2%)
A/Thailand/676/2005 (H5N1)	233	223 (95.7%)	10 (4.3%)
A/Laos/ Nong Khai 1/2007 (H5N1)	176	147 (83.5%)	29 (16.5%)
A/New Caledonia/20/1999 (H1N1)-like virus (Siriraj07/2000)	125	113 (90.6%)	12 (9.4%)

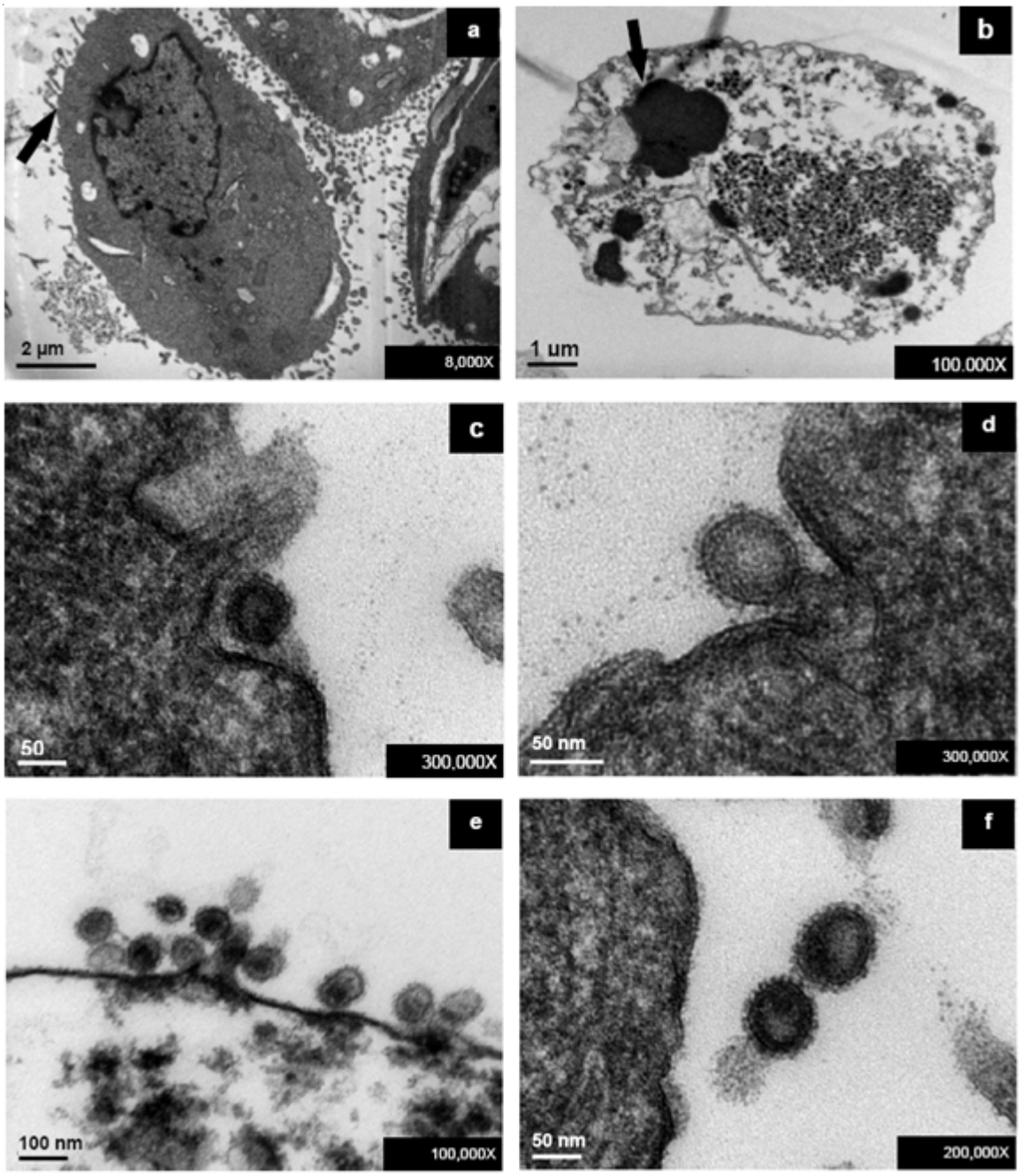

Figure 5. Electron micrographs of A/Thailand/1(KAN-1)/2004 virus infected MDCK cells: An uninfected cell showing surface appendages that might cause misleading as the budding filamentous particles (as indicated by arrow) (**a**); an infected cell showing chromatin condense suggesting of cell necrosis (as indicated by arrow) (**b**);_Invagination of a virus particle on the cell surface at entry step (**c**); Budding of a virus particle_from an infected cell (**d**); virus particles releasing from the surface of an infected cells (**e**); and free virions (**f**).

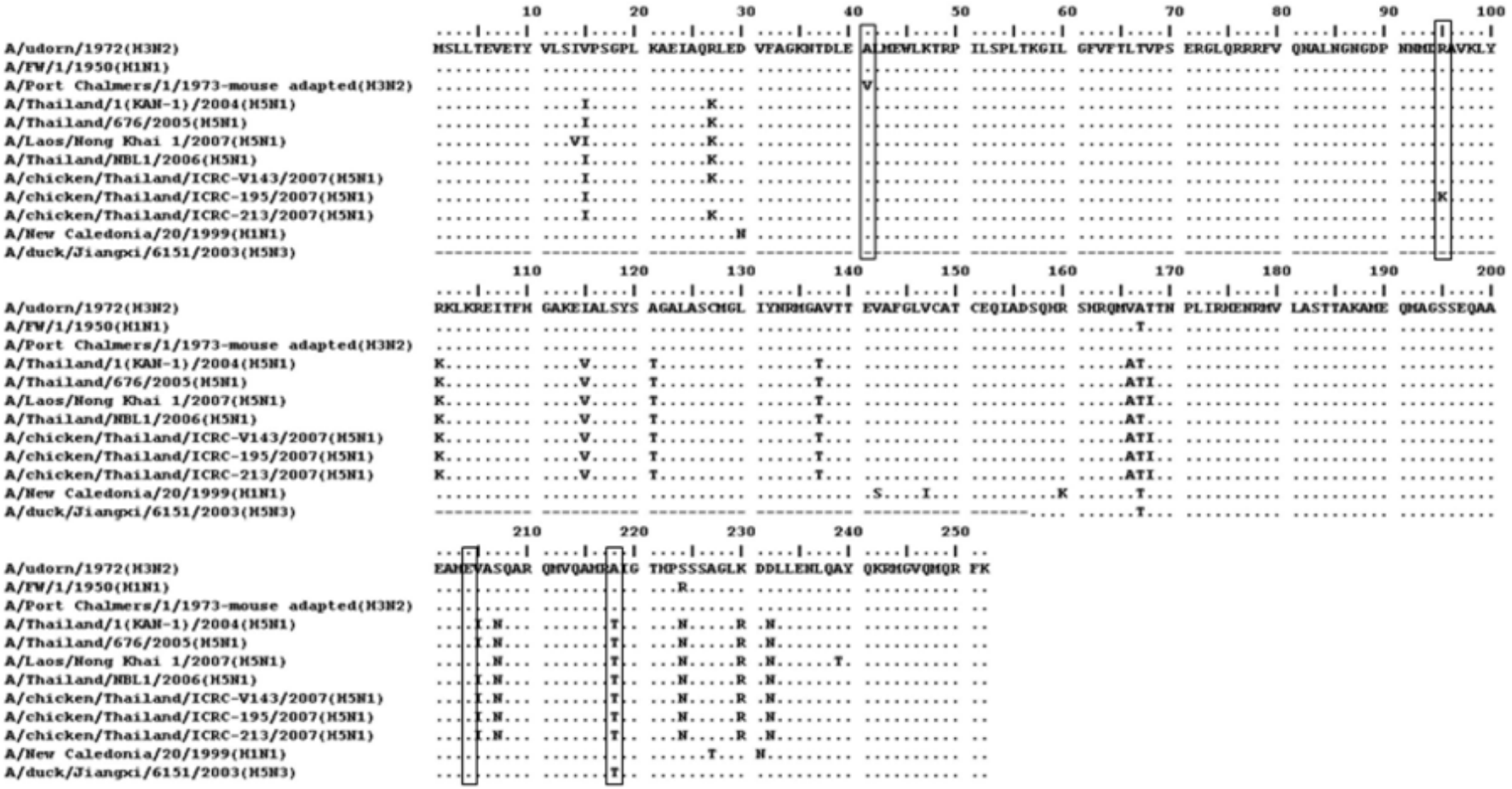

Figure 6. Amino acid sequence alignment of the influenza M1 protein. Boxes indicate the amino acid residues Ala41, Arg95, Glu204, and Ala218 that might affect the filamentous particle morphology as previously described by the other investigators._Nevertheless, these residues are found in our virus isolates that contain spherical particles as the major population.

Figure 7. Amino acid sequence alignment of influenza M2 protein. Boxes indicate the amino acid residues Phe47, Phe48, Iso51, Tyr52, Ser71, Met72, and Arg73 that might affect the filamentous particle morphology as previously described by other investigators. Nevertheless, these residues are found in our virus isolates that contain spherical particles as the predominant type.

Table 3. Panel of amino acid residues in M1 that might affect influenza virus morphology

Virus	Amino acid residues in M1 at			
	41	95	204	218
A/Udorn/301/1972 (H3N2) [filament]*, **	Ala	Arg	Glu	Ala/Val
A/Victoria/3/1975 (H3N2) [filament]*	Ala	Arg	Glu	Ala
A/WSN/1933 (H1N1) [spherical]*,**	Val	Lys	Asp	Thr
A/Puerto Rico/8/1934 (H1N1) [spherical]*	Val	Lys	Glu	Thr
A/Thailand/1(KAN-1)/2004 (H5N1)	Ala	Arg	Glu	Thr
A/Thailand/676/2005 (H5N1)	Ala	Arg	Glu	Thr
A/Laos/Nong Khai 1/2007 (H5N1)	Ala	Arg	Glu	Thr
A/Thailand/NBL 1/2006 (H5N1)	Ala	Arg	Glu	Thr
A/chicken/Thailand/ICRC-V143/2007 (H5N1)	Ala	Arg	Glu	Thr
A/chicken/Thailand/ICRC-195/2007 (H5N1)	Ala	Lys	Glu	Thr
A/chicken/Thailand/ICRC-213/2007(H5N1)	Ala	Arg	Glu	Thr
A/New Caledonia/20/1999 (H1N1)-like virus (Siriraj07/2000)	Ala	Arg	Glu	Ala
A/duck/Jiangxi/6151/2003 (H5N3)	NA†	NA	Glu	Thr

*Elleman and Barclay [9]: Ala41, Arg95 and Ala218 for filamentous particle formation and Val41, Lys95 and Thr218 for spherical particle formation;
**Bourmakina and Garcia-Sastre [10]: Arg95 and Glu204 for filamentous particle formation
†NA = Not applicable (only partial sequence available)

Discussion

Based on electron microscopy, this study demonstrated three morphological types of the virus particles, spherical, rod, and filamentous forms for all of the influenza viruses studied, irrespective of their human or avian host of origin or of their low or high pathogenicity. However, spherical particles were the predominant form, whereas filamentous particles were a minor form, accounted for approximately 4% to 16.5% of all particles. A previous group of investigators [5] reported that the long filamentous form was predominantly found in early passages of the viruses grown in embryonated eggs, and that the filamentous form could transform into the spherical form after four to seven sub-passages. Unfortunately, we did not have an opportunity to study virus isolates at earlier passages because of inadequate numbers of the virus particles for electron microscopic examination. Nevertheless, almost all of our virus isolates were younger than eight sub-passages. On the other hand, our finding was supported by an electron microscopic study of HPAI H5N1 viruses from the 1997 influenza outbreak in Hong Kong, in which spherical virions were predominant among the mixed virus population after a few sub-passages in embryonated eggs, namely, 65% for the viruses isolated from chicken and 84% of those isolated from humans [24]. Discordant findings between different groups of investigators had been also observed with the 2009 pandemic A/H1N1 virus. While Itoh et al. [25] demonstrated the filamentous shaped particles predominantly; the spherical virions were the predominant form demonstrated for the 2009 pandemic virus by the Centers for Disease Control and Prevention [http://www.cdc.gov/h1n1flu/images.htm].

Bruce et al. [26] showed that TEM could demonstrate the morphogenesis of the spherical particles and the long filament viruses extruding from infected cells present in a positively stained thin section. However, scanning electron microscopy provided better visualization because of the larger area of the plasma membrane examined. In this study, TEM was employed to demonstrate the virion at entry and exit from the infected cells. We observed a multilayered-coil structure of the virus is similar to that reported by Clader et al. [27]. In addition, we could identify surface pili on the uninfected MDCK cell membrane, which may be mistaken for filamentous particles [3], using the high resolution and magnification of TEM.

We examined four amino acid residues (Ala41, Arg95, Glu204, and Ala218) in M1 that were predictive of filamentous features together with four amino acid residues (Val41, Lys95, Asp204, and Thr218) in M1 that were suggestive of spherical morphology based on previous reports [9-11]. We demonstrated that Ala41, Arg95, Glu204, and Thr218 were found in

almost all of our viruses, which contained mainly the spherical particles, irrespective of the virus subtype, host of origin, or virulence. Moreover, residues in the cytoplasmic tail of M2 (Phe47, Phe48, Iso51, Tyr52, Ser71, Met72, and Arg73), which suggest stability of filamentous particles [11], were also found in all of our virus isolates. Therefore, our study suggests that the morphology of influenza virus particles is unlikely to be under the control of viral genetics. Our study was limited by the lack of opportunity to explore whether the morphology is influenced by the number of virus passages.

Acknowledgements

This study was supported by a Thailand Research Fund for Senior Research Scholars, a Mahidol University Research Grant, and the Centers for Disease Control and Prevention (CDC), USA. This study was a part of the "Avian Influenza Surveillance in Thailand-Studies at Human-Animal Interface" project supported by Cooperative Agreement Number 1U19CI000399-01 from Centers for Disease Control and Prevention. Its contents are solely the responsibility of the authors and do not necessarily represent the official views of CDC. PR was from Srinakharinwirot University. The authors would like to express their sincere thanks to Mekkla Thompson and Steve Durako, Westat, USA for their kind coordination, and to Dr. Dianne Gross and Dr. Jacquerine Katz, CDC for their kind supervision of the project. There is no conflict of interest that might influence the outcome of this study.

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