DOI:10.1111/j.1750-2659.2009.00118.x
www.blackwellpublishing.com/influenza

Original Article

Phenotypic characteristics of novel swine-origin influenza A/California/07/2009 (H1N1) virus

Irina Kiseleva, Natalie Larionova, Vasily Kuznetsov, Larisa Rudenko

Institute of Experimental Medicine RAMS, St. Petersburg, Russia
Correspondence: Irina Kiseleva, Institute of Experimental Medicine, 12 Acad. Pavlov Street, 197376 St. Petersburg, Russia. E-mail: irina.v.kiseleva@mail.ru

Accepted 17 August 2009. Published online 8 December 2009.

Background The 2009 novel A(H1N1) virus appears to be of swine origin. This strain causing the current outbreaks is a new virus that has not been seen previously either in humans or animals. We have previously reported that viruses causing pandemics or large outbreaks were able to grow at a temperature above the normal physiological range (temperature resistance, *non-ts* phenotype), were found to be inhibitor resistant and restricted in replication at suboptimal temperature (sensitivity to grow at low temperature, *non-ca* phenotype). In this study, we performed phenotypic analysis of novel A(H1N1) virus to evaluate its pandemic potential and its suitability for use in developing a live attenuated influenza vaccine.

Objectives The goal of this study is to identify phenotypic properties of novel A(H1N1) influenza virus.

Methods A/California/07/2009 (H1N1) swine-origin influenza virus was studied in comparison with some influenza A viruses isolated in different years with respect to their ability to grow at non-permissive temperatures. We also analyzed its sensitivity to gamma-inhibitors of animal sera and its ability to agglutinate chicken, human and guinea pig erythrocytes.

Results Swine-origin A/California/07/2009 (H1N1) virus was found to be *non-ts* and inhibitor resistant and was not able to grow at 25°C (*non-ca*). We did not find any difference in the ability of the hemagglutinin of A/California/07/2009 (H1N1) virus to bind to erythrocytes of different origin.

Conclusion The novel swine-origin A(H1N1) virus displays a phenotype typical of the past pandemic and epidemic viruses. This finding suggests that this virus might be a good wild type parental prototype for live vaccine for potential use for controlling pandemic influenza.

Keywords Cold-adaptation, inhibitor and temperature sensitivity, novel A(H1N1) influenza virus.

Please cite this paper as: Kiseleva *et al.* (2009) Phenotypic characteristics of novel swine-origin influenza A/California/07/2009 (H1N1) virus. Influenza and Other Respiratory Viruses 4(1), 1–5.

Introduction

The ability (*non-ts* phenotype, temperature resistance) or inability (*ts* phenotype, temperature sensitivity) to grow at elevated temperature is an important characteristics of wild type viruses. Previously, it has been shown that the temperature sensitive characteristics of past influenza viruses exhibited a cyclical pattern.[1] New antigenic shift/drift variants causing pandemics or large epidemics typically displayed a *non-ts* phenotype while at the end of the circulation *non-ts* strains were replaced with antigenically related but temperature sensitive (*ts*) strains[2] which changed later to novel *non-ts* viruses possessing different antigenic properties. It appears that the *ts* phenotype of circulating strains depends on the viruses being antigenically novel for humans. It seems that analysis of the temperature sensitive phenotype may help to evaluate the pandemic potential of circulating viruses and predict appearance of new epidemic or pandemic strain.

The 2009 novel A(H1N1) virus which is causing infection among humans was shown to be genetically related to recent swine influenza viruses, but doesn't have the genetic make-up previously detected among viruses infecting humans or animals.[3,4] It appears to be more contagious than typical seasonal flu.[5] Today the novel influenza A(H1N1) virus is causing pandemic activity. On 11 June 2009, WHO announced that the level of influenza alert was raised from phase 5 to 6 and now we are in the first stages of the pandemic.[6] Influenza vaccination is the primary method for preventing influenza. Live, attenuated cold-adapted reassortant influenza vaccines are currently licensed in the Russian Federation and the United States and appear to be safe and efficacious and might possibly provide broader immune responses than inactivated vaccines.

WHO announced that the majority of the novel influenza A(H1N1) isolates are antigenically and genetically related to the A/California/07/2009 (H1N1) virus and recommended that vaccines preparing against the novel influenza A(H1N1) virus, have to contain the A/California/07/2009 (H1N1)-like virus.[7]

As the pandemic virus is available, a rapid evaluation of it's characteristics is needed. For the rapid and successful development of live, attenuated reassortant influenza vaccine, wild type parental virus has to be phenotypically and antigenically different from master donor virus. In this study, we performed an analysis of the phenotypic properties of the novel A(H1N1) virus to evaluate its pandemic potential by phenotypic analysis and characterize this virus as a prototype wild type parent for possible live, attenuated cold-adapted reassortant influenza pandemic vaccine.

Methods

Viruses

A/California/07/2009 (H1N1) virus was obtained from CDC (Atlanta, GA), CDC ID number 2009712112. A/Leningrad/134/17/57 (H2N2), the cold-adapted (*ca*) master donor virus that is used to make Russian live attenuated influenza virus (intranasal) vaccine and eight strains of type A influenza virus isolated in different years – A/Singapore/1/57 (H2N2), A/Hong Kong/1/68 (H3N2), A/Beijing/262/95 (H1N1), A/Perth/13/95 (H1N1), A/New Caledonia/20/99 (H1N1), A/Malaysia/01/04 (H3N2), A/Brisbane/59/2007 (H1N1), A/Brisbane/10/2007 (H3N2) – were also chosen for this study.

Hemagglutination test was performed at room temperature with 1% suspension of chicken, human 0(I) Rh+ and guinea pig red blood cells (RBCs) by a routine technique.[8]

Determining *ts* and *ca* phenotype

The capacity to grow at optimum (33°C), elevated (40°C) and low (25°C) temperatures was studied for A/California/07/2009 (H1N1) influenza virus in comparison with viruses isolated in different years and was determined by titration in eggs. *Ts* phenotype was expressed as a reduction of virus titer at 40°C from the titer at permissive temperature (33°C). *Ca* phenotype was expressed as a reduction of virus titer at 25°C from the titer at 33°C. The log EID_{50}/ml calculation was based on the Reed and Muench method.[9] Viruses were considered as *non-ts* if log EID_{50}/ml at 33°C – log EID_{50}/ml at 40°C ≤ 3·0 log EID_{50}/ml. Viruses were considered as *non-ca* if log EID_{50}/ml at 33°C – log EID_{50}/ml at 25°C ≥ 5·0 log EID_{50}/ml. The cold-adapted and temperature sensitive A/Leningrad/134/17/57 (H2N2) master donor virus for Russian live, cold-adapted reassortant influenza vaccine was used as a positive control of *ts* and *ca* markers.

Hemagglutination inhibition (HAI) test was performed using standard techniques as described[10] in 96-well microtiter plates at room temperature with 1% suspension of human 0(I) Rh+ red blood cells.

Sensitivity to serum inhibitors

For analysis of sensitivity of influenza viruses to non-specific inhibitors, normal (non-immune) horse serum (BioloT, St. Petersburg, Russia), normal rabbit and guinea pig sera (Laboratory Animals Farm ''Rappolovo'', St. Petersburg, Russia) were used. Sera were heat inactivated for 10 min at 80°C to eliminate temperature sensitive inhibitors and used for HAI assay with four hemagglutinating units of tested viruses and for neutralization by animal sera.

Results and discussion

Determining *ts* and *ca* phenotype of influenza A viruses

Our previous data provided evidence of the dominance of the *ts* viruses in circulation prior to the appearance of new antigenic variants in the human population. In contrast, the phenotype of antigenically new viruses was temperature resistant. During circulations they were replaced with antigenically related but temperature sensitive strains which turned later to *non-ts* viruses possessed different antigenic properties.[1] In this study, we demonstrated that influenza A viruses from recent years such as A/Malaysia/01/04 (H3N2), A/Brisbane/59/2007 (H1N1) or A/Brisbane/10/2007 (H3N2) were temperature sensitive (Table 1). According to these data we assumed that novel *non-ts* influenza virus with pandemic/epidemic potential may appeared in the near future. Results of the phenotypic analysis of the 2009 novel A(H1N1) virus may be considered as direct experimental support for this hypothesis. We demonstrated that *ts* and *ca* characteristics of A/California/07/2009 (H1N1) virus are similar to those of past pandemic and epidemic viruses such as A/Singapore/1/57 (H2N2), A/Hong Kong/1/68 (H3N2), A/Beijing/262/95 (H1N1), A/Perth/13/95 (H1N1), A/New Caledonia/20/99 (H1N1) (Table 1).

Determining the ability of influenza A viruses to agglutinate red blood cells of different origin

The HA protein binds to a sialic acid-containing receptor on the red blood cell surface. The ability of virus strains, necessary for vaccine production, to absorb differrent types of red blood cells must be monitored closely. It is known that some current influenza A(H1N1) viruses have lost the ability to agglutinate chicken red blood cells. For example, some A(H1N1) viruses (A/Aichi/4/92) isolated in Japan during the 1991–1992 season agglutinated chicken RBCs,

Table 1. Restriction of growth of human influenza A viruses at different temperatures

Virus	Virus titer at 33°C, log EID_{50}/ml	Mean log reduction of virus titer* (EID_{50}/ml) at:		Phenotype
		33°C/40°C	33°C/25°C	
A/Singapore/1/57 (H2N2)**	8·2	1·8	6·1	*non-ts, non-ca*
A/Hong Kong/1/68 (H3N2)**	7·7	1·7	7·1	*non-ts, non-ca*
A/Beijing/262/95 (H1N1)***	8·0	3·0	5·0	*non-ts, non-ca*
A/Perth/13/95 (H1N1)***	8·5	3·0	5·0	*non-ts, non-ca*
A/New Caledonia/20/99 (H1N1)***	8·8	3·0	6·5	*non-ts, non-ca*
A/Malaysia/01/04 (H3N2)***	7·3	7·3	6·1	*ts, non-ca*
A/Brisbane/59/07 (H1N1)***	9·2	9·2	5·5	*ts, non-ca*
A/Brisbane/10/07 (H3N2)***	7·2	7·2	7·2	*ts, non-ca*
A/California/07/2009 (H1N1)**	8·0	1·2	6·0	*non-ts, non-ca*
A/Leningrad/134/17/57 (H2N2)†	9·0	7·0	2·5	*ts, ca*

*From the titer at permissive temperature (33°C).
**Pandemic virus.
***Epidemic virus.
†A/Leningrad/134/17/57 (H2N2) master donor virus was used as a positive control of *ts* and *ca* markers.

while other viruses (A/Aichi/24/92) did not.[11] We have studied the ability to agglutinate red blood cells of different origin of some influenza A(H1N1) viruses which were in use at different years as parental viruses for vaccine development. We did not find any significant difference in the ability of the HA of A/Beijing/262/95, A/New Caledonia/20/99 or A/Perth/13/95 influenza viruses to bind chicken, human or guinea pig erythrocytes – they demonstrated equal capability to agglutinate all tested types of RBCs (Table 2). The HA protein of novel A/California/07/2009 (H1N1) virus also recognized all tested red blood cells equally. The fact that novel A(H1N1) virus was able to agglutinate RBCs from different animal species efficiently and didn't display preferential tropism made it suitable for vaccine development process.

Table 2. Hemagglutination of erythrocytes from different animal species of human influenza A(H1N1) viruses

Virus	Hemagglutination with erythrocytes from*		
	Chicken	Human	Guinea pig
A/Beijing/262/95 (H1N1)	512	512	512
A/Perth/13/95 (H1N1)	128	128	128
A/New Caledonia/20/99 (H1N1)	1024	1024	1024
A/California/07/2009 (H1N1)	64	128	128

*Hemagglutination assay was performed with 1% suspension of chicken, human 0(I) Rh+, and guinea pig erythrocytes.

Determining inhibitor sensitive phenotype of influenza A viruses

The receptor-binding activity of influenza viruses can be inhibited by non-specific inhibitors present in the sera of animals.[12–14] Normal (non-immune) animal sera contain the temperature stable glycoprotein gamma-inhibitors

Table 3. Sensitivity of human influenza A viruses to hemagglutinin inhibition (HAI) by horse, rabbit and guinea pig sera

Virus	HAI titer* by serum from:			Inhibitor sensitivity
	Guinea pig	Horse	Rabbit	
A/Singapore/1/57 (H2N2)	<10	<10	nd	Resistant
A/Malaysia/01/04 (H3N2)	1280	2560	640	Sensitive
A/Beijing/262/95 (H1N1)	<10	<10	nd	Resistant
A/Perth/13/95 (H1N1)	<10	<10	nd	Resistant
A/New Caledonia/20/99 (H1N1)	<10	<10	<10	Resistant
A/California/07/2009 (H1N1)	<10	<10	<10	Resistant

nd, not determined.
*HAI tests were performed using standard techniques in 96-well microtiter plates with non-immune horse, rabbit and guinea pig sera and 1% suspension of human 0(I)Rh+ erythrocytes. HAI titer is expressed as the reciprocal of the highest dilution of animal sera causing inhibition of 1% suspension of human erythrocytes agglutination by 4 hemagglutinating units of virus. Sera were diluted 1:10 and heat inactivated 10 min at 80°C.

which inhibit hemagglutinating activity of some influenza viruses. In the current study, the sensitivity of A/California/07/2009 (H1N1) virus to gamma-inhibitors of horse, rabbit and guinea pig sera were compared with inhibitor sensitivity of some past and current influenza A viruses was investigated. No inhibitor activity of horse, rabbit and guinea pig sera was detected in HAI test both for A/Singapore/1/57 (H2N2) or A/Hong Kong/1/68 (H3N2) past pandemic strains and seasonal or current pandemic A(H1N1) viruses tested. In contrast, A/Malaysia/01/04 (H3N2) virus was found to be highly sensitive to inhibitors (Table 3).

Analogous results to those observed with the HAI assays were obtained with respect to neutralization by animal sera. The pattern of A/California/07/2009 (H1N1) virus sensitivity to neutralization by horse, guinea pig and rabbit sera was studied (Table 4). A/Malaysia/01/04 (H3N2) virus was taken as a positive control to determine sensitivity to neutralization by animal sera. A/New Caledonia/20/99 (H1N1) was used as a serum resistant control virus.

A virus was considered to be resistant to neutralization by serum if there was <2 log EID_{50}/ml difference between growth of the virus in the presence and in the absence of serum. A virus was designated sensitive to neutralization by serum if there was more than 5 log EID_{50}/ml difference between growth of the virus in the presence and in the absence of serum.

Inhibitor sensitive A/Malaysia/01/04 (H3N2) virus had 5·5 log EID_{50}/ml reduction in titers when grown in the presence of rabbit serum and 5·8 log EID_{50}/ml in the presence of horse serum respectively. A/New Caledonia/20/99 (H1N1) virus was resistant to all animal sera tested in the neutralization assay (log reduction of virus titer was 0·1–0·5 log EID_{50}/ml). The novel swine-origin A/California/07/2009 (H1N1) virus growing in the presence of rabbit serum displayed clear inhibitor resistant phenotype (log reduction of virus titer was 0·8 log EID_{50}/ml). Also this virus did not display any significant differences in titers when grown in the absence or in the presence of horse and guinea pig sera (0·5 and 0·2 log EID_{50}/ml reduction respectively). Thus, these data confirmed the high inhibitor resistant phenotype of the novel A (H1N1) influenza virus demonstrated in this study by HAI test.

Little is known about inhibitor resistancy of pandemic or epidemic viruses. It doesn't seem that pandemic viruses have to be always inhibitor resistant or sensitive. But in the majority of cases epidemic/pandemic viruses are *non-ts, non-ca,* and inhibitor resistant with some exceptions like A/Malaysia/01/04 (H3N2) which is *non-ca*, but *ts* and inhibitor sensitive. We also registered the appearance of inhibitor sensitive H1N1 viruses and inhibitor resistant H3N2 viruses in inter-epidemic seasons (data not published). Inhibitor studies are important to evaluate the phenotypic characteristics of any antigenically new influenza virus, especially from the LAIV development point of view. Theoretically, inhibitor sensitive vaccine strains could be potentially less effective in comparison with inhibitor resistant strains. Also using inhibitor sensitive circulating viruses as wild type parents for LAIV development may cause difficulties in the process of reassortment because of non-specific inhibition of reassortants possessed wild type HA by serum raised against master donor virus.

Conclusion

Accordingly, it was confirmed that the analyzed phenotypic characteristics of novel swine-origin A/California/07/2009 (H1N1) strain are similar to those of the past pandemic influenza viruses, such as A/Singapore/1/57 (H2N2) or A/Hong Kong/1/68 (H3N2). The A/California/07/2009 (H1N1) influenza strain is serum and temperature resistant and sensitive to grow at low temperature and may be considered as of a good prototype wild type parent for live attenuated cold-adapted reassortant vaccine for potential use for controlling pandemic influenza.

Table 4. Differential neutralization of human influenza A viruses by horse, guinea pig and rabbit sera

Virus	Virus titer, log EID_{50}/ml with serum*				Mean log reduction of virus titer**		
	No serum	Rabbit	Guinea pig	Horse	Rabbit	Guinea pig	Horse
A/California/07/2009 (H1N1)	8·0	7·2	7·8	7·5	0·8	0·2	0·5
A/Malaysia/01/04 (H3N2)	7·3	1·8	nd	1·5	5·5	nd	5·8
A/New Caledonia/20/99 (H1N1)	8·5	8·4	8·0	8·3	0·1	0·5	0·2

nd, not determined.
*Serial 10-fold dilutions of the viruses were made in animal serum, diluted 1:10 and heat inactivated 10 min at 80°C (or PBS) at +4°C for 30 min and then inoculated into chicken eggs. Viral growth in eggs was detected using the HA assay with 1% human red blood cells.
**From the titer without serum.

References

1 Rudenko LG, Kiseleva IV, Larionova NV *et al.* Analysis of some factors influencing immunogenicity of live cold–adapted reassortant influenza vaccines; in Kawaoka Y (ed.): Proceedings of Options for the Control of Influenza V. Okinawa, Japan, 6–9 October, 2003. Amsterdam: Elsever Sciense, 2004: 542–546.

2 Polezhaev FI, Aleksandrova GI. Isolation of temperature–sensitive strains of the influenza virus in the epidemic caused by the A/Victoria virus in 1975–1976. Vopr Virusol 1979; 4:430. [Article in Russian].

3 Novel Swine–Origin Influenza A(H1N1) Virus Investigation Team. Emergence of a novel swine-origin influenza A(H1N1) virus in humans. N Engl J Med 2009. Available from http://content.nejm.org/cgi/reprint/NEJMoa0903810v1.pdf (Accessed 20 November 2009)

4 Garten RJ, Davis CT, Russel CA *et al.* Antigenic and genetic characteristics of swine–origin 2009 A(H1N1) influenza viruses circulating in humans. Science express 22 May 2009. Available from http://www.sciencemag.org/cgi/rapidpdf/1176225.pdf (Accessed 20 November 2009)

5 World Health Organization. Assessing the severity of an influenza pandemic. 2009. Available from http://www.who.int/csr/disease/swineflu/assess/disease_swineflu_assess_20090511/en/index.html (Accessed 20 November 2009)

6 World Health Organization. Statement by WHO Director–General, Dr Margaret Chan 11 June 2009. World now at the start of 2009 influenza pandemic. Available from http://www.who.int/mediacentre/news/statements/2009/h1n1_pandemic_phase6_20090611/en/index.html (Accessed 20 November 2009)

7 World Health Organization. Characteristics of the emergent influenza A(H1N1) viruses and recommendations for vaccine development. Available from http://www.who.int/csr/resources/publications/swineflu/vaccine_recommendations/en/index.html (Accessed 20 November 2009)

8 Dowdle WR, Kendal AP, Noble GR. Influenza viruses; in Lennette E, Schimidt N (eds): Diagnostic Procedures for Viral, Rickettsial and Chlamydial Infections, 1st edn. Washington, DC: American Public Health Association, 1979, 603–605.

9 Reed L, Muench H. A simple method of estimating fifty per cent endpoints. Am J Hyg 1938; 27:493–497.

10 Palmer DF, Coleman MT, Dowdle WR, Schild GC. Advanced laboratory techniques for influenza diagnosis. Immunol Ser 1975; 6:51–52.

11 Morishita T, Nobusawa E, Nakajima K, Nakajima S. Studies on the molecular basis for loss of the ability of recent influenza A(H1N1) virus strains to agglutinate chicken erythrocytes. J Gen Virol 1996; 77:2499–2506.

12 Gottschalk A, Belyavin G, Biddle F. Glycoproteins as influenza virus hemagglutinin inhibitors and as cellular virus receptors; in Gottschalk A (ed.): Glycoproteins. Their Composition, Structure and Function, Part A. Amsterdam, The Netherlands, Elsevier Publishing Co, 1972, 1082–1096.

13 Matrosovich M, Gao P, Kawaoka Y. Molecular mechanisms of serum resistance of human influenza H3N2 virus and their involvement in virus adaptation in a new host. J Virol 1998; 72:6373–6380.

14 Ryan–Poirier K, Suzuki Y, Bean WJ *et al.* Changes in H3 influenza A virus receptor specificity during replication in humans. Virus Res 1998; 56:169–176.