Med Microbiol Immunol (2010) 199:117–121
DOI 10.1007/s00430-010-0143-4

ORIGINAL INVESTIGATION

Determination of serum antibodies against swine-origin influenza A virus H1N1/09 by immunofluorescence, haemagglutination inhibition, and by neutralization tests: how is the prevalence rate of protecting antibodies in humans?

Regina Allwinn · Janina Geiler · Annemarie Berger · J. Cinatl · H. W. Doerr

Received: 21 January 2010 / Published online: 17 February 2010

Abstract In April 2009, a new variant of influenza A virus, subtype H1N1v emerged in Mexico and spread all over the world producing the H1N1 pandemic in mankind after 1918–1920 and 1978/1979. Obviously there was no herd immunity against this new virus variant. Mainly young people, but less elderly were affected and presented severe and even lethal courses of disease. Since virus-specific antibodies are commonly regarded as markers of partial or complete immunoprotection, we performed antibody determinations in serum samples obtained from people before and after the pandemic has arrived in our region (Frankfurt/M., Germany). The assays were done by indirect immunofluorescence, by neutralization test, and by a haemagglutination inhibition test (HI), which was established in a practical modification for general and easy use. Among 145 individuals, of whom serum specimens had been drawn before the onset of pandemic, 19 revealed humoral immunity, i.e. titres of H1N1v neutralizing antibodies (at least 1:64). Eleven were older than 60 years, one belonged to the age group 40–59 years, three to the age group 20–39 years, and two to the age group 15–19 years. After the onset of pandemic in Frankfurt, serum specimens drawn from $n = 225$ randomly selected patients of our local university hospital were investigated for antibodies against H1N1v by HI, which is generally recommended for routine check of immunity. Twenty-eight individuals revealed the protecting antibody titre of at least 1:40. The age distribution had moved to mean age groups. The results fit to the incidence of influenza A/H1N1(09) disease, as confirmed by RT–PCR in patients admitted to our hospital, peaking in the younger age groups up to 30 years (second affected group: 30–40 years). While commonly used solid-phase antibody tests (like immunofluorescence) are not suitable to diagnose passed H1N1(09) infection and acquired immunity, this can be easily done by HI. Expecting the next waves of influenza A/H1N1v infections, HI testing may avoid vaccinations under special risk of severe or hidden adverse reactions.

Keywords H1N1 pandemic · Seroprevalence · Immunity

R. Allwinn · J. Geiler · A. Berger · J. Cinatl · H. W. Doerr (✉)
Institute of Medical Virology, University Hospital,
Frankfurt/M., Germany
e-mail: H.W.Doerr@em.uni-frankfurt.de;

R. Allwinn
allwinn@em.uni-frankfurt.de

Introduction

In April 2009, a new variant of influenza A virus (IAV) emerged in Mexico [21]. It was tracked to a virus which had developed in pigs as reassortant from genetic elements out of avian, human, and swine influenza A viruses [18, 21]. Its envelope spikes being responsible for virus-cell adsorption (haemagglutinin, which initiates infection) and for release of progeny virus out of infected cells (neuraminidase) were classified as H1 and N1. However, antigenetic differences to the seasonally circulating H1N1 viruses proved so high that no herd immunity against the new H1N1v was present in mankind. In the next months, swine-origin IAV H1N1v spread from Mexico all over the world producing the third (scientifically confirmed) influenza A/H1N1 pandemic in mankind after 1918–1920 and 1978/1979. Up to date, the current pandemic reveals to be so mild like 1978/1979. Whether a second wave of pandemic will be more dangerous and life threatening like 1918/1920 depends on the ongoing evolution of viral envelope antigens H1 and N1. Its potential has been recently elucidated (compiled in 11). In opposite to 1978/1979, it is mainly

young people who are affected by the current pandemic and present severe or even lethal courses of disease. A second peak of life-threatening respiratory infections is seen in people older than 65 years. This feature resembles the pandemic of 1918/1920. It indicates some immunoprotection in the elderly and correlates with antibody determinations in samples of non-infected people [6, 9]. Virus-specific antibodies are commonly regarded as markers of partial or complete immunoprotection depending on against which structural antigen of IAV they are directed.

Here, we present the results of antibody determination in serum samples obtained from people before and after the pandemic have arrived in our region. The serum samples were screened by indirect immunofluorescence test (for antibodies to influenza A virus), as well as by haemagglutination inhibition assay (HI) and by neutralization tests (for protecting antibodies to IAV H1N1v).

Materials and methods

Serum sampling

Serum specimens were obtained from two sources. A sample was taken from $n = 145$ blood donors previously recruited to establish a serum survey of enterovirus 71 infection spread. These sera had been collected from healthy persons in the years 2007 and 2008 and stored by −20° C before testing [15]. A second sample was randomly taken from serum specimens of in- and outpatients of our hospital. They had been drawn from $n = 225$ individuals, not specifically suspected of a recent respiratory infection during November 2009 and were immediately tested.

Cells and virus

MDCK cells (Mardin Darby kidney cells; ATCC: CCL-34, Manassas, VA, USA) were grown at 37°C in minimal essential medium (MEM, Biochrom AG, Berlin, Germany) supplemented with 10% foetal bovine serum (FBS, Sigma–Aldrich Chemie GmbH, Munich, Germany), 100 IU/ml penicillin (Grünenthal GmbH, Aachen, Germany), and 100 µg/ml streptomycin (Sigma–Aldrich Chemie GmbH, München, Germany).

The S-OIV strain A/HH/01/2009 (H1N1) (obtained from M. Eickmann, Institut für Virologie, Marburg, Germany) was used. H1N1 virus stock was prepared by infecting MDCK cells at MOI 0.1. Three days p.i. supernatants of infected cultures were collected and stored as aliquots at −80°C. Virus titre was determined as 50% tissue culture infectious dose ($TCID_{50}$/ml) in confluent cells in 96-well microtitre plates (Greiner Bio-One, Frickenhausen, Germany).

Antibody determinations

The *indirect immunofluorescence test* (IFT) was performed by the use of a commercially available test system according to the guideline of the manufacturer (Labor Dr. Merk & Kollegen GmbH, Ochsenhausen, Germany). In principle, the serum antibodies react with influenza A virus particles coated to formularized erythrocytes fixed on a slide. Subsequently, immunofixed antibodies are detected by goat anti-human IgG conjugated to fluorescein isothiocyanate (FITC), as previously described [1].

The *haemagglutination inhibition test* (HI) for antibodies against the new H1N1/09 was performed according to the WHO standard protocol frequently used in diagnostic laboratories [7, 8, 23].

Reagents used for testing were standardized fresh red blood cells (RBC) of turkeys in Alsever's solution (Bundesinstitut für Risikobewertung, Alt-Marienfelde, Berlin, Germany), and the pandemic H1N1-Virus vaccine containing highly purified H1 from IAV H1N1 (A/California/7/2009 NYMC X-179A); GlaxoSmithKline Biologicals, Dresden) served as antigen. We yielded four HAU (haemagglutinating units) of virus antigen with the dilution of 1:128. Serum samples were inactivated with heat incubation by 56ºC and treated with receptor destroying enzyme (RDE, cholera filtrate, Sigma–Aldrich, Seelze, Germany) with overnight incubation at 37ºC. The serum–antigen mixture was incubated for 45 min at room temperature and lastly RBC's (0.5%) were added to each well. Plates were read promptly when the RBC control has completely settled. All specimens were tested in serial twofold dilutions, and a negative and high-positive control were used, including sera controls for each specimen. Titres below the detection limit of 1:10 were assigned to a value of 1:5, and 1:5120 was the end point titration. The HI titre was the highest dilution of serum that inhibited virus induced haemagglutination.

The *neutralization test* (NT) for antibodies to new H1N1/09 was performed using pandemic H1N1 virus (A/HH/01709) and MDCK (NBL-2) cells similar to assays with other viruses [8]. For neutralization of the virus, sera from healthy people were diluted in MEM (Ø FCS) in a twofold series to achieve dilution factors from 1:8 to 1:1024, incubated with the virus (1×10^4) TCID50/ml) at 33°C for 1 h and added to microtitre wells containing the host cells (1×10^4 cells per well, MEM Ø FCS, +2 µg/ml Trypsin). The neutralization assays were performed in triplicate, and cultures were inoculated at 37°C for 72 h. and cytopathogenic effect was observed. Additionally, staining of the cells against Influenza A nucleoprotein was performed. Because of preliminary investigations, a result was considered seropositive, if a serum neutralizes virus infectivity in dilution of at least 1:64.

Routine virus diagnostic service

Multiplex real-time PCR on seasonal IAV and IBV taken from nasal and pharyngeal swabs of patients, which have fallen ill with influenza like symptoms, is performed according a standard protocol, as previously described [1] using IAV and IBV matrix gene-specific primers and Taqman probes [22, B. Schweiger RKI personnel communication]. To detect swine-origin IAV, H1N1(2009)-specific primers and probes are used [13].

Results

Serum specimens drawn from 145 healthy blood donors before the onset of pandemic influenza A/H1N1 (2009) were investigated on neutralizing antibody activity. $n = 19$ delivered seropositive results (1:>32) distributed to seven age groups of the individuals tested (Table 1).

Predominantly individuals aged more than 60 revealed neutralizing antibodies (11 among 30). The NT-positive sera had been tested also seropositive by IFT (data not shown). Age distribution of NT-positive person reciprocally correlates with the incidence of new H1N1/09, which was detected by RT–PCR in nasal or pharyngeal swabs from $n = 301$ H1N1-positive patients affected by the current pandemic to December 2009, as seen in the routine virus diagnostic service of our hospital. Most of these influenza patients were found in the age group 10–29 years ($n = 136$ out of 301 seen in Table 2).

After the arrival of the new influenza pandemic this winter season, the routine serodiagnostic service of our hospital was extended. Beside of influenza virus type-specific IFT, an HI test was established to detect serum antibodies against the new variant of influenza A virus subtype H1N1/09. During November 2009, among 225 selected adult patients not specifically suspected of a respiratory infection, 63 were found seropositive, while 157 yielded a negative result. Five serum specimens showed an unspecific test reaction. With two exceptions, each HI-seropositive case was seen also positive by IFT. The HI antibody titres of the 63 seropositive individuals covered a broad range peaking with low titres of 1:10 and 1:20.

The HI results, distributed in five age groups of patients, are displayed in Table 3. In individuals younger than 30 years, only two HAI-seropositive cases were seen. The high antibody titres of 1:320 and 1:1280 indicate a passed H1N1/09 infection, since vaccination was excluded in all patients. The titre distribution of the patients in the other age groups was found similar to each other ranging between 1:5 and 1:320. Most seropositive individuals were seen in the age group of 40–49 years ($n = 23$).

A serum antibody titre of at least 1:40 is considered to provide immunity. Among 225 patients, 27 individuals (about 12%) presented a protecting antibody titre, 6 out of 14 in age group of 30–39, 9 out of 23 in age group of 40–49, 3 out of 6 in age group of 50–59, and 3 out of 13 in age group of >60 years old patients.

Table 1 Age distribution of healthy blood donors tested for neutralizing serum antibodies against IAV/H1N1 (09)

Age group (years)	Number of individuals tested for neutralizing serum antibodies	Positive (1:>32)
1–4	20	1
5–9	20	1
10–14	20	0
15–19	20	2
20–39	10	3
40–59	25	1
>60	30	11
	145	19

Table 2 Age and sex distribution of IAV/H1N1 (09) cases confirmed by RT–PCR in the routine diagnostic service, University hospital, Frankfurt/M., May–December 2009

Age group (years)	Case number	Male	Female	NA
<10	**68**	39	18	11
10–29	**136**	69	54	13
30–39	**31**	19	12	0
40–49	**24**	17	6	1
50–59	**15**	6	9	0
>60	**13**	11	2	0
NA	**14**	NA	NA	NA
Σ	**301**			

Most cases were detected since October 2009. Number of patients = 301

NA no data on age or sex available

Table 3 Titre and age distribution of tested positive for serum antibodies to new IAV/H1N1 (09) by HI (NA = no data on age available), among a randomly selected collection of $n = 225$ individuals

Age group (years)	Distribution of reciprocal antibody titres									
	5	10	20	40	80	160	320	640	1,280	∑
20–29	0	0	0	0	0	0	1	0	1	**2**
30–39	1	2	5	3	1	1	1	0	0	**14**
40–49	3	6	5	1	5	2	0	1	0	**23**
50–59	0	1	2	0	3	0	0	0	0	**6**
>60	0	6	4	1	1	1	0	0	0	**13**
NA	0	1	0	2	1	1	0	0	0	**5**
∑	**4**	**16**	**16**	**7**	**11**	**5**	**2**	**1**	**1**	**63**

Discussion

Influenza A virus infection is worldwide present. A serum survey, which was established in on IAV type-specific IFT results in the routine diagnostic service of our hospital during the years 1996–2000, revealed that antibody prevalence in the population rapidly rises from infants to 10 years old children, who reveal a fraction of 70% seropositivity, later on declining to 60% in the elderly [1]. This serum survey has recently been confirmed by another German group using a type-specific antibody ELISA [17]. After emergency of the new influenza A pandemic, which was caused by a swine-origin IAV H1N1 variant, the question was frequently asked, whether and in which fraction of population a pre-existing immunoprotection against this antigenetically very different new IAV might be assumed, since obviously not everybody was susceptible to this new virus. Although new H1N1/09 virus variant revealed high infectivity, the reproduction index stayed rather low, i.e. one virus carrier transmitted the agent only to one or two other individuals [11] The spread of this new influenza focused on younger people indicating that there exists at least partial immunity in the population. It is generally accepted that immunoprotection against influenza is preferentially mediated by antibodies. Cell-mediated immunity (which is not only variant-, but type-specific) may shorten virus shedding during influenza [11]. It was shown by different groups that elderly people show cross-protecting serum antibody activity [6, 9]. This finding is confirmed by our pilot tests on the prevalence of neutralizing antibodies (Table 1) and "explains" why incidence of infection is rather rare in elderly people (Table 2).

Interpretation of our data against the pandemic influenza A (H1N1) virus is complicated by the lack of recognized immune correlates. The insensitivity of HI assays to some avian haemagglutinin has required that microneutralization assays, haemagglutination inhibition assays involving horse erythrocytes or single radial haemolysis are used [12, 16, 19]. Recently, antibody responses likely to be associated with protection against H1N1 in two vaccination trials were assayed using HI and NT [3, 5]. The results demonstrated that both assays yielded similar results. In concordance, our observations suggest that HI and NT provide comparable sensitivity to detect persons with protective immunity against H1N1 pandemic virus.

After the real onset of the new influenza pandemic in our region in October 2009, we tested 225 randomly selected in- and outpatients of our hospital for serum antibodies against the new variant H1N1/09 virus by the use of HI. Sixty-three of them were found seropositive with a cut-off antibody titre of 1:5, and 59 serum samples had an antibody titre $\geq$1:10. 27 individuals presented a titre of 1:40 or more (12%), which is commonly considered to provide immunoprotection and which has to be reached after vaccination [3, 5, 10, 12, 14, 20]. Although there was no anamnesis of a recent respiratory infection, some of those people might have passed the infection of swine-origin IAV.

Conclusion

After the first wave of this current influenza pandemic has gone and after the first campaign of vaccination has been launched, much more people will probably present protecting serum antibodies. The message of our pilot investigation is as follows: in the future, simple HI antibody testing should be considered in case of difficult indication of vaccination [4, 5]. Adverse reactions are well known and not completely ruled out. Hidden hazards of influenza vaccination might be realized even only after months or years, as recently outlined by Bhakdi et al. [2]. The more people are found already IAV/H1N1/09 seropositive with an antibody titre of 1:>20, the more vaccinations in difficult cases can be saved.

Acknowledgments We thank Cornelia Rühl and Safia-Zahoua Ouazar for excellent technical assistance, and Ute Kauk for kind advice.

References

1. Allwinn R, Preiser W, Rabenau H, Buxbaum S, Stürmer M, Doerr HW (2002) Laboratory diagnosis of influenza–virology or serology? Med Microbiol Immunol 191(3–4):157–160
2. Bhakdi S, Lackner K, Doerr HW (2009) Possible hidden hazards of mass vaccination against new influenza A/H1N1: have the cardiovascular risks been adequately weighed? Med Microbiol Immunol 98:205–209
3. Clark TW, Pareek M, Hoschler K, Dillon H, Nicholson KG, Groth N, Stephenson I (2009) Trial of 2009 influenza A (H1N1) monovalent MF59-adjuvanted vaccine. N Engl J Med 361(25): 2424–2435
4. Doerr HW, Allwinn R, Cinatl J (2009) Against the new H1N1 Influenza (swine Influenza): vaccinate or don't vaccinate (all)? That is currently the question. Infection 37(5):379–380
5. Greenberg ME, Lai MH, Hartel GF, Wichems CH, Gittleson C, Bennet J, Dawson G, Hu W, Leggio C, Washington D, Basser RL (2009) Response to a monovalent 2009 influenza A (H1N1) vaccine. N Engl J Med 361(25):2405–2413
6. Hancock K, Veguilla V, Lu X, Zhong W, Butler EN, Sun H, Liu F, Dong L, DeVos JR, Gargiullo PM, Brammer TL, Cox NJ, Tumpey TM, Katz JM (2009) Cross-reactive antibody responses to the 2009 pandemic H1N1 influenza virus. N Engl J Med 361(20):1945–1952
7. Hirst GK (1942) The quantitative determination of influenza virus and antibodies by means of red cell agglutination. J Exp Med 75(1):49–64
8. Kendal AP, Pereira MS, Skehell JJ (1982) eds. Center for Disease Control, Concepts and procedures for laboratory-based influenza surveillance. Atlanta
9. Ledford H (2009) Old seasonal flu antibodies target swine flu virus. Nature News online. (http://www.nature.com/news/2009/090521/full/news.2009.503.html)

10. Liang XF, Wang HQ, Wang JZ, Fang HH, Wu J, Zhu FC, Li RC, Xia SL, Zhao YL, Li FJ, Yan SH, Yin WD, An K, Feng DJ, Cui XL, Qi FC, Ju CJ, Zhang YH, Guo ZJ, Chen PY, Chen Z, Yan KM, Wang Y (2010) Safety and immunogenicity of 2009 pandemic influenza A H1N1 vaccines in China: a multicentre, double-blind, randomised, placebo-controlled trial. Lancet 375:56–66
11. Michaelis M, Doerr HW, Cinatl J Jr (2009) Novel swine-origin influenza A virus in humans: another pandemic knocking at the door. Med Microbiol Immunol 198(3):175–183
12. Nicholson KG, Colegate AC, Podda A et al (2001) Safety and antigenicity of non adjuvanted and MF59-adjuvanted influenza A/Duck/Singapore/97 (H5N3) vaccine: a randomised trial of two potential vaccines against H5N1 influenza. Lancet 357:1937–1943
13. Panning M, Eickmann M, Landt O, Monazahian M, Olschläger S, Baumgarte S, Reischl U, Wenzel JJ, Niller HH, Günther S, Hollmann B, Huzly D, Drexler JF, Helmer A, Becker S, Matz B, Eis-Hübinger A, Drosten C (2009) Detection of influenza A(H1N1)v virus by real-time RT-PCR. Euro Surveill 14(36)
14. Plennevaux E, Sheldon E, Blatter M, Reeves-Hoché MK, Denis M (2010) Immune response after a single vaccination against 2009 influenza A H1N1 in USA: a preliminary report of two randomised controlled phase 2 trials. Lancet 375:40–48
15. Rabenau HF, Richter M, Doerr HW (2010) Hand, foot and mouth disease: seroprevalence of Coxsackie A16 and Enterovirus 71 in Germany. Med Microbiol Immunol 199:45–51
16. Rowe T, Abernathy RA, Hu-Primmer J et al (1999) Detection of antibody to avian influenza A (H5N1) virus in human serum by using a combination of serologic assays. J Clin Microbiol 37:937–943
17. Sauerbrei A, Schmidt-Ott R, Hoyer H, Wutzler P (2009) Seroprevalence of influenza A and B in German infants and adolescents. Med Microbiol Immunol 198(2):93–101
18. Shinde V, Bridges CB, Uyeki TM, Shu B, Balish A, Xu X, Lindstrom S, Gubareva LV, Deyde V, Garten RJ, Harris M, Gerber S, Vagasky S, Smith F, Pascoe N, Martin K, Dufficy D, Ritger K, Conover C, Quinlisk P, Klimov A, Bresee JS, Finelli L (2009) Triple-reassortant swine influenza A (H1) in humans in the United States, 2005–2009. N Engl J Med 360(25):2616–2625
19. Stephenson I, Wood JM, Nicholson KG, Zambon MC (2003) Sialic acid receptor specificity on erythrocytes affects detection of antibody to avian influenza haemagglutinin. J Med Virol 70:391–398
20. Vajo Z, Tamas F, Sinka L, Jankovics I (2010) Safety and immunogenicity of a 2009 pandemic influenza A H1N1 vaccine when administered alone or simultaneously with the seasonal influenza vaccine for the 2009–10 influenza season: a multicentre, randomised controlled trial. Lancet 375:49–55
21. Virus Investigation Team, Dawood FS, Jain S, Finelli L, Shaw MW, Lindstrom S, Garten RJ, Gubareva LV, Xu X, Bridges CB, Uyeki TM (2009) Emergence of a novel swine-origin influenza A (H1N1) virus in humans. Novel Swine-Origin Influenza A (H1N1). N Engl J Med 360:2605–2615
22. Ward CL, Dempsey MH, Ring CJ, Kempson RE, Zhang L, Gor D, Snowden BW, Tisdale M (2004) Design and performance testing of quantitative real time PCR assays for influenza A and B viral load measurement. J Clin Virol 29(3):179–188
23. WHO laboratory manual. http://www.wpro.who.int/internet/resources.ashx/CSR/Publications/manual+on+animal+ai+diagnosis+and+surveillance.pdf