Significant impact of the Rh blood group and gender on influenza infection in Estonia

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Abstract. To get an insight into the epidemics of influenza caused by the pandemic A(H1N1)pdm09 virus in Estonia, titres of anti-H1N1 antibodies were determined from 530 blood donor sera and from 355 pig sera collected during the second post-pandemic year (2010–2011) in Estonia. The results indicate that A(H1N1)pdm09 was well spread among the Estonian population during the second year of spreading. The median antibody titres of A(H1N1)pdm09 were higher in the sample of Estonian men than women, but the reason for this is unclear. The impact of blood group, age, and gender on the titre of anti-H1N1 antibodies in human serum samples was studied. A significant difference was observed between the RhD donor groups: the RhD+ group had a higher antibody titre than the RhD– group. A significant influence of the RhCcEe system was observed, the Ccee combination promoting the highest antibody titre. These observations suggest that viral infections might exert substantial pressure on the evolution of human blood groups. Pig serum samples from half of the Estonian pig farms were tested, indicating that the A(H1N1)pdm09 virus had infected animals in two-thirds of the farms. Altogether, our study shows that a virus serologically similar to the A(H1N1)pdm09 virus was prevalent in Estonia in both human and pig populations during 2010–2011, and reveals important factors influencing the serum titre of antibodies to this virus.

Key words: influenza H1N1, pandemia, RhD, RhCcEe, anti-H1N1 antibodies, ELISA.

INTRODUCTION

The 2009 A(H1N1)pdm09 influenza pandemic was considered a global health threat due to a new reassortant influenza strain [1]. As other northern countries, Estonia experiences regular influenza outbreaks, which coincide with the cold season. Influenza A(H1N1)pdm09, which emerged in spring 2009 in North America [2], arrived in Estonia during October–December 2009, and active public vaccination started in February 2010 [3].

Most influenza surveillance systems are passive laboratory-based systems that capture only symptomatic patients seeking medical advice. Therefore, these systems are likely to underestimate the true infection rate. Alternatively, measurement of antibodies against A(H1N1)pdm09 can be used to assess the extent of population exposure to the virus [4]. The emergence of a novel influenza virus strain provided a unique opportunity to study the behaviour of the influenza strains in Estonia, and to better understand their differential effects across various population groups.

Although a possible connection between the ABO blood group and influenza infection has been extensively studied, the reports remain controversial. The susceptibility to a number of diseases has been linked with the ABO phenotype. Additionally, a few studies from the 1960s and 1970s reported significant relationships between the ABO blood group and influenza virus titre: blood
group A made a quick response [5] while the response of blood group B was slow against influenza type A [6]. Other studies indicate low titres of influenza [7,8] for blood group O and B [9], B alone [10], A [11], A and B [12], AB [5,12], or no difference by blood group [13]. In spite of this inconsistency, later studies indicate again that after circulation of influenza A viruses, the immune response differs between the blood groups [14]. However, no effect of the RhD/CE blood groups on the anti-influenza antibody titres has been reported to date.

The human Rh antigens, D, C, c, E, and e, are highly immunogenic and are frequently the cause of transfusion reactions and haemolytic disease of newborn babies. In humans, these antigens are coded by two genes, RHD and RHCE [15], which probably arose as a result of gene duplication. The Rh antigens are expressed as part of an ankyrin complex in the red blood cell (RBC) membrane as a heterotrimer consisting of one molecule of Rh-associated glycoprotein (RhAG) and two molecules of Rh proteins. The Rh proteins may be RhD (carrying the D antigen) or RhCE (carrying the C or c antigen and the E or e antigen) [16,17]. The absence of the Rh complex alters the RBC shape, increases its osmotic fragility, and shortens its lifespan, resulting in a haemolytic anaemia, which is usually mild in nature. These patients are at risk of adverse transfusion reactions because they may produce antibodies against several of the Rh antigens [18].

In a previous report we showed that during the 2009–2010 influenza A(H1N1)pdm09 season both humans and pigs were infected with the H1N1 virus, often having no clinical signs, which may indicate that an attenuated virus spread in the Estonian population with some rare changes in the amino acid composition of the H1N1 haemagglutinin [3]. In this work we determined the titres of antibodies against the H1N1 influenza virus in a collection of sera from blood donors and pigs, collected during the second post-pandemic year 2010–2011. Our aim was to estimate based on titres for influenza A(H1N1)pdm09 antibodies the frequency of asymptomatic infections. We also analysed correlations between antibody titres and a number of factors potentially influencing the response to the virus.

**MATERIALS AND METHODS**

**Ethics statement**

Blood samples used in the current study were obtained from healthy donors in accordance with the principles of Helsinki Declaration of 1975 and subsequent amendments by the World Medical Assembly. Permission No. 181/T-1 was issued to Sirje Rüütel Boudinot on 20 April 2009 by the Ethics Review Committee on Human Research of the University of Tartu, Estonia.

Pig blood samples were collected by the Estonian Veterinary and Food Board in the frame of the Estonian Infectious Animal Disease Control Programme, founded in 1999. This governmental agency carries out its tasks under the Ministry of Agriculture of the Estonian Government and operates in accordance with Estonian Acts of Law. Samples collected in the frame of the same programme before the onset of the H1N1-2009 epidemic were used as controls. No samples from pigs or humans were collected specifically for this study. None of the authors of this study was involved in the blood collection from pigs or humans. The human blood donors provided written informed consent for their samples to be also used for research purposes, following the rules of the Estonian Blood Centre. Human blood samples collected before 2008 in the same way were used as negative controls. All human samples were de-identified.

**Blood samples**

Sera of 530 donors were collected in five days between the 1st and the 5th of August 2011 in the North Estonian Blood Centre, Tallinn, Estonia. In addition to blood donors, we collected more data about two volunteers’ samples (V1 and V2), neither of who had a medical history of A(H1N1)pdm09. Samples from volunteer V1 were taken on 20 September 2010 and 1 March 2011. The blood sample from volunteer V2 was taken on 3 December 2010. Volunteer V2 had been vaccinated against seasonal influenza (H1N1, H3N2, and influenza B) in January 2009 and against A(H1N1)pdm09 in February 2010.

Also, blood samples were collected from 355 pigs from 18 herds from different counties in Estonia (Nos 1 and 14 from Saaremaa; No. 2 from Järva ma; Nos 3–5, 10, 11, and 18 from Lääne-Viruma; Nos 6 and 12 from Harjumaa; No. 7 from Põlvamaa; No. 8 from Tartumaa; Nos 9, 13, and 17 from Jõgevamaa; Nos 15 and 16 from Viljandimaa). Blood samples were taken during June–November 2011. At least 5% of the pigs were tested in each farm.

The positive control sample used in this study was collected in December 2010 from a man who was diagnosed for A(H1N1)pdm09 using the laboratory test. An additional negative control sample was collected in 2008 from a young man 2 months after confirmed seasonal influenza (showed high titres of anti-H3N2 antibodies) [3]. The negative control sample used in the pig study was collected before the beginning of the pandemic in 2008.

For blood tests, 4 mL of venous blood was coagulated during 2 h at room temperature and centrifuged for 10 min at 1300 g. During the collection period the samples were stored at 4°C. The age, sex, and blood group were recorded. No medical history or information about vaccinations (except volunteers V1 and V2) is...
known. Blood samples were transported to the Department of Gene Technology, Tallinn University of Technology, where sera were isolated and stored at 4°C.

**Enzyme-linked immunosorbent assay (ELISA)**

A commercially available vaccine – inactivated influenza virus produced in cell culture on Vero cells (Celvapan, H1N1 IV Pandemic09 developed by Baxter) – was diluted in 50 mM sodium carbonate, pH 9.6, with final concentration of antigen 3.75 μg/mL and used to coat Nunc Maxi-Sorp Immuno Plates, 50 μ per well. The plates were incubated overnight at 4°C and washed three times with 0.05% Tween 20 in deionized H2O between every step. Non-specific binding sites were blocked with 200 μL of 2% casein (Tere AS, Estonia) in phosphate buffered saline (PBS) and incubated for 1 h at room temperature.

Serum samples and controls were diluted in PBS up to 1:64 000. The dilution 1:16 000 was selected to calculate individual titres within the linear range of all dilution curves and was selected to adjust individual titres so that on average most of them would fall to the middle of the linear part of the dilution curve. These dilutions were added to plates in duplicates (each 50 μL) and incubated overnight at 4°C. The secondary antibody (50 μL; DAKO Rabbit Anti-Human IgA, IgG, IgM, Kappa, Lambda/HRP; DAKO Rabbit anti-pig antibody, 162.5 pg/mL) was added to each well and incubated for 1 h at room temperature. Freshly mixed peroxidase substrate reagent (1 mM tetramethylbenzidine and 2.3 mM H2O2 in 0.1 M potassium citrate buffer, pH 4.5) was added to the plates and incubated for 20 min at room temperature. To stop the reaction, 50 μL of 1 M H2SO4 was added to each well. Optical densities were detected by reading the plates on an ELISA plate reader (Thermo Scientific Multiskan FC, Germany) at the wavelength of 450 nm. For statistical analysis, the average of the duplicates was used.

The ELISA method used in this study to examine presence of antibodies to A(H1N1)pdm09 has been previously validated with a haemagglutination inhibition (HI) assay and another ELISA where a trivalent influenza vaccine was used (H1N1-A-Brisbane 07, H3N2-A-Brisbane 07, and B-Brisbane 08) to exclude the prevalence of antibodies to this subtype [3].

**RESULTS**

The main results of our study can be summarized as follows.

- The proportion of A(H1N1)pdm09 seropositive samples among blood donors in Estonia in 2010–2011 was high.

The 530 samples with no record of clinical infection at the sampling time or during previous two months collected from Estonian blood donors in August 2011 and tested by ELISA indicated that A(H1N1)pdm09 was well spread among the Estonian population during the second year of spreading (Figs 1 and 2). This suggests that the blood donors had been infected within the last four months. As it is not possible to establish retroactively whether the blood donors had had a clinical influenza infection within four months before the sampling, it was established that a sample taken from volunteer V1 in September 2010 was negative while it had become highly positive in March 2011 in the absence of any clinical signs of viral infection (Fig. 2). Moreover,
Fig. 2. Optical densities (OD) of 1:16,000 serum dilutions from volunteers V1 and V2, showing reaction to Celvapan A(H1N1)pdm09 antigens. A sample was regarded as positive to A(H1N1)pdm09 when 1:16,000 dilution gave twice higher OD values than the negative control (human serum collected before the H1N1 pandemic 2009–2010). The last two columns represent OD values for the positive and negative controls. Error bars show the standard error of duplicates.

Fig. 3. Optical densities (OD) of 1:16,000 serum dilutions from 18 Estonian pig herds showing reaction to Celvapan A(H1N1)pdm09 antigens. Herd No. 16 is the largest in Estonia and has a section with elevated requirements for hygiene isolated from the remaining facility (No. 16A).

- The influenza A(H1N1)pdm09 virus was also largely spread within the Estonian domestic pig population. It is generally accepted that, in contrast to the pig farms in neighbouring countries, there was no H1N1 influenza in Estonian pig farms in 2009 [20,21]. High prevalence of the virus within human blood donors prompted us to undertake a comprehensive survey of antibody titres to Influenza A(H1N1)pdm09 in a collection of pig sera from 18 Estonian farms sampled between June and November 2011. The pig herds studied showed clear differences in A(H1N1)pdm09 titre distribution: a few herds showed only low titres (Nos 5, 6, 8, 12, and 18), while the rest had a broad distribution of A(H1N1)pdm09 titres, suggesting that A(H1N1)pdm09 was well spread among the Estonian domestic pig population (Fig. 3). From herd 16, two sets of samples were taken from a clean ‘separated breeding sows section’ zone (No. 16A) and from a conventional zone (No. 16B). Sera from the clean separated breeding sows section had distinctly low antibody titres while the titres from the pigs from other areas of this farm varied significantly (Fig. 3). Hence, our results show that contrary to the common opinion, the A(H1N1)pdm09 virus had infected a substantial part of the Estonian pig population.

- Estonian men showed significantly higher titres of anti-A(H1N1)pdm09 antibodies than women. Since the majority of the sera from blood donors contained antibodies against the A(H1N1)pdm09 influenza virus indicating a relatively recent infection, we investigated the impact of different factors on the titre distribution. Distributions of antibody titres for all groups studied were analysed and found to follow a chi-squared probability density function.

a sample taken in December 2010 from volunteer V2, who had been vaccinated against seasonal influenza (H1N1, H3N2, and influenza B) in January 2009 and against A(H1N1)pdm09 in February 2010, was clearly negative (Fig. 2).
Estonian men had higher titres of anti-A(H1N1)pdm09 antibodies than women \((p < 0.001)\), which is also reflected in the fact that the sample median was about 10% higher in men (Fig. 4). The difference between the genders is clearly visible on histogram graphs (Fig. 5).

- No significant impact of age or ABO blood groups on anti-A(H1N1)pdm09 antibody titre distribution was observed.

The samples from blood donors were divided into eight age groups: 18–25 years old (180 samples), 26–30 (94 samples), 31–35 (73 samples), 36–40 (55 samples), 41–45 (44 samples), 46–50 (31 samples), 51–60 (42 samples), and 61–65 (11 samples). When the age parameter was considered, it could be seen that the antibody titre variation was the highest for the youngest donor group and decreased with age (Fig. 6). However, differences between the groups are statistically nonsignificant.

Differences in anti-A(H1N1)pdm09 antibody titres were also analysed for the possible influence of the ABO blood group system. Sizes of the blood groups followed roughly their distribution in the population (O group 179, A 197, B 98, AB 56 donors). The results showed that the O blood group had the highest titre of anti-A(H1N1)pdm09 antibodies, although the differences were statistically nonsignificant (Fig. 7).

- The Rh blood groups had a significant impact on the titres of anti-A(H1N1)pdm09 antibodies.

The titres of antibodies against A(H1N1)pdm09 were also analysed according to the Rh blood group system. The Rh proteins are transmembrane proteins located in the RBC membrane; they have very similar

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**Fig. 4.** Gender distribution of anti-A(H1N1)pdm09 antibody titres. Antibody titres are shown as optical densities (OD) of ELISA analysis of 1:16 000 serum dilutions showing reaction to Celvapan A(H1N1)pdm09 antigens. Significance levels were set at \(p < 0.001\) and are indicated by ***.

**Fig. 5.** Frequency of various optical density (OD) ranges for female (a) and male (b) donors. The OD \(\times 16\,000\) values of samples are divided into groups, each consisting of values of 25 samples.

**Fig. 6.** Age distribution of anti-A(H1N1)pdm09 antibody titres. Antibody titres are shown as optical densities (OD) of ELISA analysis of 1:16 000 serum dilutions showing reaction to Celvapan A(H1N1)pdm09 antigens.
sequences, and are encoded by two different genes (RHD and RHCE) (Fig. 8a). RhD is dominant over Rhd, which in fact corresponds to a deletion [22]. In RHCE, a number of point mutations determine well-known polymorphisms, for example, C/c (Cys16Trp, Ile60Leu, Ser68Pro and Ser103Pro) or E/e (Pro226Ala) [23].

We first assessed the impact of the RHD blood group on the anti-A(H1N1)pdm09 antibody titre (121 donors were RhD−, 409 RhD+). The difference between antibody titres was statistically significant ($p < 0.05$), with a median slightly higher for the RhD+ group and a higher proportion of RhD+ sera above the third quartile (Fig. 8b).

Regarding RhC/c RhE/e blood groups, six allelic combinations were represented in donors: ccee, ccEe, ccEE, Ccee, CcEe and CCEE. Statistically significant differences for antibody titres were observed (Fig. 8c). Surprisingly, the highest median values were found for C/c heterozygous donors, either with Ccee or CcEe combinations. There was no clear correspondence between the impact of RhD+ vs RhD− phenotype and the C/c E/e polymorphisms on the antibody titres, suggesting that influences of the RhD and RhCE genes are independent.
DISCUSSION

Using an ELISA test to screen blood samples for the presence of anti-A(H1N1)pdm09 antibodies we established that a large proportion of Estonian blood donors had high titres of anti-A(H1N1)pdm09 antibodies in 2010–2011, a year after the pandemic began in Estonia. Considering that the sampling was carried out in August 2011 and the serum antibody level is typically high for about 4 months after infection [24,25], the high antibody rates observed in many donors show that donors had been infected during spring 2011. In Estonia, there were 85 000 officially diagnosed cases in 2010–2011 [26], which corresponds approximately to 6% of the Estonian population. The high proportion of Estonian blood donors with a high titre of anti-A(H1N1)pdm09 antibodies in 2011 suggests that the proportion of asymptomatic infections had been underestimated. The highest disease rate, 19.5%, was found in children aged 0–14, while people aged 15–65 (including blood donors) had a disease rate of around 3.9% [27,28]. The last outbreak of seasonal H1N1 influenza occurred in the USSR, including at that time Estonia, in 1977 [29]. Even if it was not statistically significant, we indeed observed a trend of higher rate of high antibody titres within the young donors, compared to the rates in older people, who may have been exposed to a H1N1 virus in 1977.

Pig serum samples from half of the Estonian pig farms were tested, showing that the A(H1N1)pdm09 virus had infected animals in two-thirds of the tested farms. There were no clinical signs of influenza in Estonian pig herds during the A(H1N1)pdm09 outbreak in Estonia nor the following year. However, sub-clinical respiratory diseases were common during that period [30]. A veterinarian followed the health status of all tested pigs. Hence, the absence of signs of influenza is reliable and indicates that the A(H1N1)pdm09 virus had infected animals. In fact, the last laboratory-confirmed case of influenza in pigs in Estonia dates from 1957 [31], and it was considered for a while that the H1N1 had not spread among pig farms.

Our results show that the male blood donors had about 10% higher anti-A(H1N1)pdm09 antibody titres than female donors. While the reported male–female differences in the incidence of infection vary with age in different countries, a higher incidence of infection with A(H1N1)pdm09 in young women than young men of comparable age was observed [32,33]. In Asia, the majority of A(H1N1)pdm09 cases were reported in men (57.1%) [34,35] and men harboured the A(H1N1)pdm09 virus in pharyngeal and nasopharyngeal samples for a longer time compared to females [36]. Although the relationship between the anti-H1N1 antibody titres and the severity of infection in patients is not a simple one, these parameters may represent an important factor in host–virus interactions, and further studies should clarify the importance of this observation.

Regarding blood groups, we did not find significant correlation between anti-H1N1 antibody titres and the ABO blood groups. In contrast, the Rh blood group system had a clear impact on the antibody titres: (1) RhD+ donors had a higher antibody titre than the RhD– donors; (2) surprisingly, heterozygous RhCc had also higher titres compared to RhCC or RhCc.

The Rh antigens are expressed as part of an ankryin-associated protein complex in the erythrocyte membrane, which is important to maintain the RBC shape and osmotic pressure and affects its lifespan. The components of this complex are encoded by the genes RHD, RHCE, and Rh-associated glycoprotein (RhAG) [15,37]. The RhD+ individuals express the D protein, while RhD– do not and are homozygous for the Rhd haplotype, which in fact corresponds to a RhD deletion. In this context, the effect of the heterozygocity of the C polymorphism appears very difficult to understand. However, since RHD and RHCE genes are tightly linked on the genome [16], the correlation of Cc allelic combination with higher antibody titres may be just a consequence of an effect of the RHD expression. Indeed, the RhD+ correspond to DD, Dd, or dD combinations of haplotypes; while C and c are each preferentially linked to a given D allele, a C/c (or c/C) combination, corresponding to a Dd or dd combination at the neighbour locus, would be frequently observed in RHD positive donors. Hence, we suggest that the effect of C/c heterozygocity on antibody titre may be explained by the expression of the D protein, although other explanations are certainly possible.

How RhD expression affects the antibody response against influenza infection remains unknown. However, the effect suggested by the correlation observed might have a significant importance on the host–virus interactions. Indeed, the rate of antibodies against the virus reached during the adaptive response likely influences the modalities and the kinetics of the virus clearance. Thus, viral infections could be one of the factors exerting evolutionary pressures promoting human blood group diversity. It is known that viruses have shaped the evolution of blood groups by other mechanisms; for example, blood group antigens act as attachment factors of rabbit haemorrhagic disease virus infection in a virus strain-dependent manner [38] and individuals lacking ABH antigens in epithelia are resistant to Norovirus infection [38,39]. Moreover, receptors for influenza virus on RBC membrane surface have been found, which are part of M and N blood group antigens [40]. We would like to emphasize that comparing these observations in humans and pigs may not be appropriate, since pigs have a single RH gene, no polymorphism in the coding region of the gene has been identified and therefore, it does not appear to represent a blood group antigen for pigs [41]. Additionally, we could not see any significant difference between pig gender and their anti-H1N1 antibody titres.
CONCLUSIONS

The high proportion of Estonian donors having high titre of anti-A(H1N1)pdm09 antibodies in 2011 suggests that the proportion of asymptomatic infections has been underestimated. The influenza A(H1N1)pdm09 virus is also largely spread within the Estonian domestic pig population: the testing of pig serum samples from half of the Estonian pig farms showed that the A(H1N1)pdm09 virus had infected animals in two-thirds of the tested farms.

We can summarize that both the blood group and gender had a significant impact on the influenza A(H1N1)pdm2009 infection within Estonian blood donors. Our results show that male blood donors had higher titres than female blood donors (which is reflected in the fact that the sample median of men was about 10% higher). Statistically significant differences were also observed between the RhD donor groups, as well as in the RhCcEe system: the RhD+ group had a higher antibody titre than the RhD– group, and the CcEe combination had a higher titre than the other combinations. Therefore we hypothesize that yearly influenza infections can be one of the factors behind the evolutionary pressure that has resulted in a diversity of groups in human blood.

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Raamatutud välja välja viiruse pandeemia

Seal oli üheaegsel nakatumisel mitme viiruse alatüübiga. 2009. aasta juuniks oli viirus levinud üle maailma 30 riiki


Rh veregruppide ja soo mõju gripi levikule Eestis

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Käesoleva töö käigus uuriti H1N1pandemic09 levikut Eestis aastail 2010–2011. 530 doonori vereseerumi analüüs ELISA-meetodil näitas jätkuvalt suurt seropositiivsust H1N1pandemic09 viiruse suhtes. H1N1pandemic09 viiruse vastaste antikehade tiitri variaablis oli suurem noorad doonoritel, mis lubab oletada, et vanematel oli tänu vanematele kokkupuudetele gripiviiruse A alatüüpidega osa alati H1N1pandemic09 gripitüve vastu. Sugudevahisel võrdluses leiti statistiliselt oluline erinevus: uuritud meestel (210 positiivset 254-st) oli antikehade tiitri H1N1pandemic09 viiruse vastu umbes 10% kõrgem kui naisdoonoritel (195 positiivset 276-st). Ka RhD veregruppide analüüsil leiti statistiliselt oluline erinevus: RhD+ veregrupiga doonoreil oli H1N1pandemic09 viirusevastaste antikehade tiitri umbes 10% kõrgem kui RhD– doonoritel. Statistiliselt oluline erinevus pandeemse gripi tiitris leiti RhCcEc veregruppide võrdluses, kus Cce variant näitas suurimat tiitrit. See tähelepanek võib viidata gripiviiruse olulisusele inimese veregruppide evolutsioonis.