Cross-neutralisation of antibodies elicited by an inactivated split-virion influenza A∕Vietnam∕1194∕2004 (H5N1) vaccine in healthy adults against H5N1 clade 2 strains

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Background Highly pathogenic avian influenza A H5N1 viruses are widespread in different parts of the world and have evolved into clade 1 and 2 lineages. Their continuing circulation represents serious pandemic threat, spurring human vaccine development efforts. Initial clinical trials tested vaccines prepared from clade 1 strains circulating in 2004.

Methods Post-vaccination sera from a phase I trial of an inactivated split-virion vaccine based on A/Vietnam/1194/ 2004/NIBRG14 (H5N1) were analysed *in vitro* for cross-reactivity against highly pathogenic, wild-type clade 2 H5N1 strains isolated from human cases, and their corresponding reverse genetics derived vaccine candidate strains.

Results Neutralisation of clade 1 and 2 wild-type and reverse-genetics viruses was seen, with highest titres observed for viruses most closely related to the vaccine strain. There was no consistent relationship between vaccine dose given, or presence of aluminium adjuvant and cross-neutralising antibody titre, possibly because of small sample size. Use of wild-type highly pathogenic strains compared with antigenically equivalent reverse-genetics viruses suggests presence of a higher level of cross-neutralising antibody.

Conclusion Vaccination with a clade 1 H5N1 virus elicited antibodies capable of neutralising diverse clade 2 H5N1 strains. This data underlines that while a close match between vaccine virus and circulating virus is important to achieve maximum protection, population priming with a 'pre-pandemic' vaccine may be beneficial for the protection of a naïve population. The data suggests that use of reverse-genetic viruses in neutralisation assays may underestimate the extent of cross-protective antibody present following H5N1 vaccination.

Key words Clade, cross-neutralisation, H5N1, influenza, pandemic, vaccine.

Introduction

Avian influenza A viruses of the H5N1 subtype have become endemic in poultry and wild birds in eastern Asia since the late 1990s and since 2005 have spread from Asia to Europe and Africa, infecting wild birds, domestic poultry and an increasing range of mammals including humans.[1] In 2006, human cases of H5N1 influenza resulted in a total of 79 fatalities and was reported for the first time in Turkey, Iraq, Azerbaijan, Egypt and Djibouti.[2–4] In 2007, case fatalities have already been confirmed by August in Indonesia, Egypt, Lao People's Democratic Republic, China, Nigeria and Cambodia.[4]

Phylogenetic and antigenic analysis of viruses collected since the human H5N1 outbreak in Hong Kong in 1997, which was caused by a virus strain belonging to what is now classified as clade 3 indicates that they have evolved into different clades.[5] Clade 1 and 2 viruses co-circulated for several months in animal reservoirs, although the westward spread of H5N1 in different reservoirs has been exclusively associated with clade 2. Clade 2 strains predominated in isolates collected worldwide from all animal species in 2006/2007 and clade 2 is now a very diverse group with at least three sublineages (subclades).[6,7]

Optimum choice of candidate vaccine strains for human pre-pandemic or pandemic vaccines requires an

understanding of the degree of cross-reactivity between antibodies elicited by particular selected strains.[8–11] An H5N1 vaccine for mass human vaccination should ideally elicit an immune response that is cross-protective against a range of H5N1 viruses, including newly emerged strains.[12–14]

We report the results of a cross-neutralisation study that tested the ability of human antibodies elicited by a clade 1 H5N1 vaccine to neutralise genetically and antigenically distant clade 2 strains.

Materials and methods

Viruses

Reactivity was tested against the following H5N1 reverse genetic vaccine candidate reference strains: A/Vietnam/1194/2004/NIBRG14, A/turkey/Turkey/1/2005/NIBRG23 and A/Indonesia/5/2005/PR8-IBCDC-RG2 (Figure 1). These viruses contained the haemagglutinin (HA) and

neuraminidase of their wild-type antigen donor strains and the internal genes of A/Puerto Rico/8/1934 (H1N1). The NIBRG14 and NIBRG23 strains were from the stocks at NIBSC (Potters Bar, UK); the Ind/5/05/RG2 strain was kindly provided by the CDC (Atlanta, GA, USA). Wild-type highly pathogenic A/Vietnam/1194/2004, A/turkey/Turkey/1/2005 and A/Indonesia/5/2005 were kindly provided by the WHO Collaborating Centre, London. Virus stocks for all viruses were propagated in the allantoic cavity of 9- to 11-day-old embryonated hens' eggs at 35–37°C. Wild-type viruses were harvested 24 hours post-inoculation, reverse genetic viruses 3 days post-inoculation and stored at −80°C.

Sera samples

Human serum samples were from a phase I clinical safety and immunogenicity study of an inactivated, split-virion influenza A (H5N1) vaccine based on the A/Vietnam/
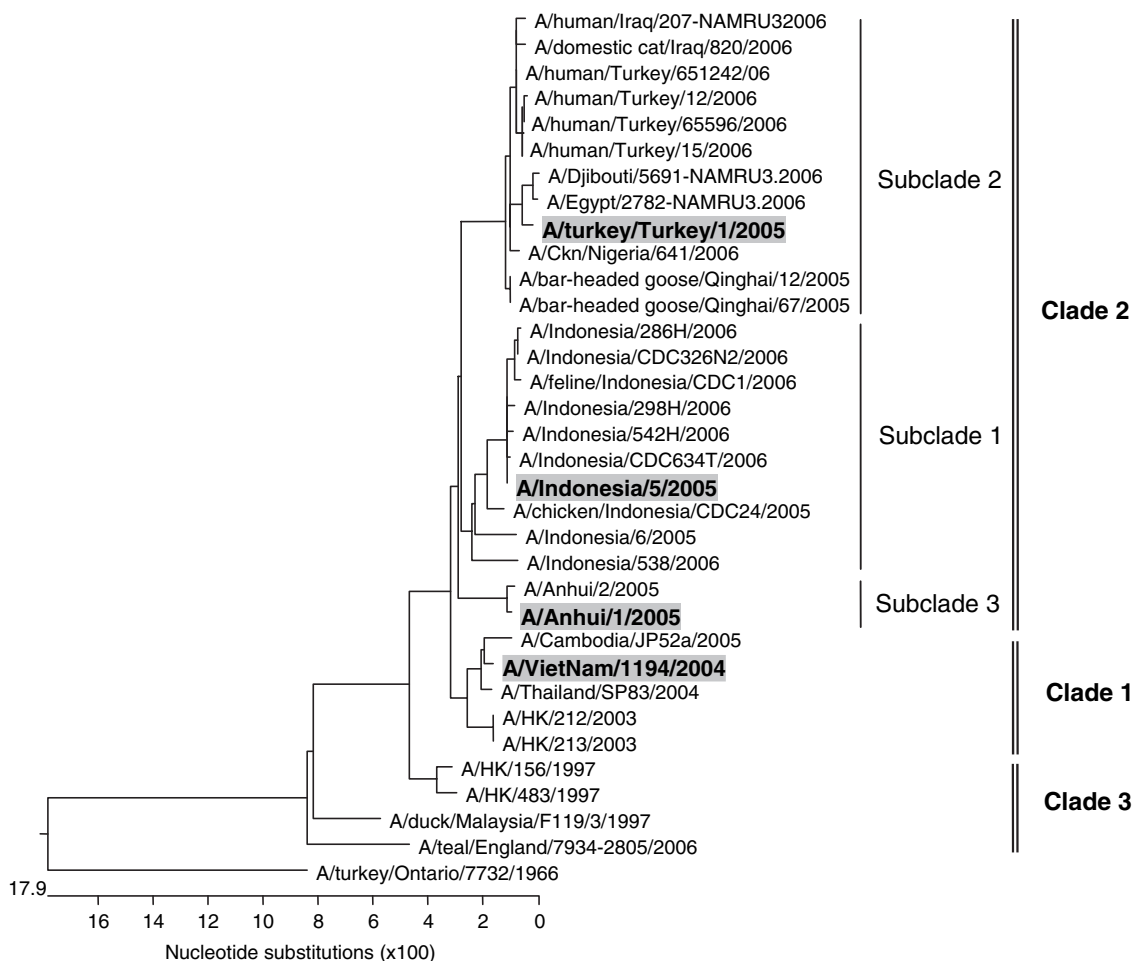

Figure 1. Phylogenetic tree of partial haemagglutinin (HA) sequences of influenza A H5 viruses. Viruses emphasised with grey background represent candidates for vaccine reference viruses.
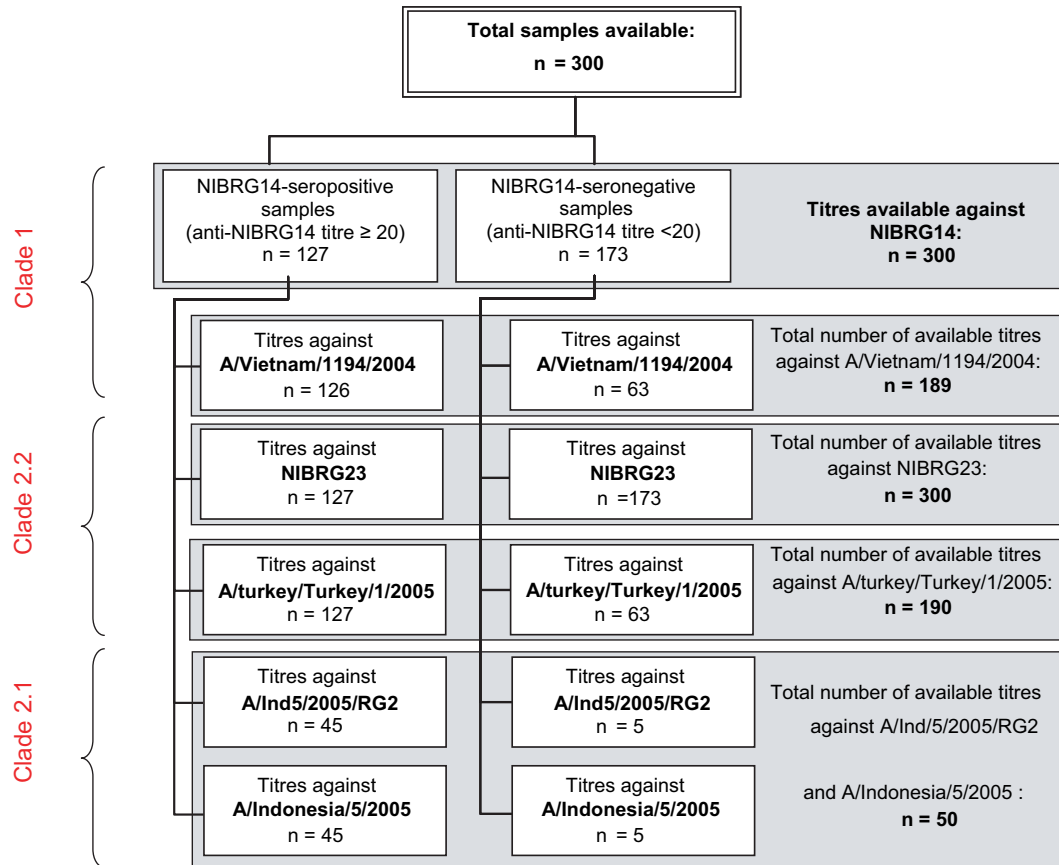
Figure 2. Sample size flow chart: number of neutralisation titres available against various clade 1 and 2 H5N1 strains. Serum was obtained 21 days after two vaccinations with different formulations of an inactivated split-virion NIBRG14 influenza vaccine. Differences between sample numbers in subsets and available titres are due to validation issues for some titre values, which had to be excluded from the analysis.

1194/2004/NIBRG14 reference strain.[15] The 300 study subjects received two intramuscular injections, 21 days apart, of 7·5, 15 or 30 μg of HA, with or without an aluminium hydroxide adjuvant (AlOH). Serum was obtained on D0, D21 and D42.

All D42 samples were analysed for the ability to neutralise the vaccine strain and NIBRG23, a clade 2 subclade 2 vaccine strain (Figures 1 and 2). A first sample subset was constituted to test the response against the A/Vietnam/1194/2004 and A/turkey/Turkey/1/2005 wild-type viruses. This subset of sera was selected to minimise the testing required at Containment Level 4 laboratory (CL4) and therefore included all 127 available samples with neutralising titres ≥20 against the NIBRG14 ('NIBRG14-seropositive' samples) and only a limited number (n = 63) of seronegative samples. A second, smaller subset of D42 sera were examined for neutralising antibodies against both, the wild-type A/Indonesia/5/05 virus and its reverse genetics vaccine strain. This subset comprised sera from 20 subjects with the highest neutralising titres against NIBRG14 (≥89, ≤479; geometric mean titre (GMT) = 155), 25 subjects with

an intermediate titre (≥32, ≤72; GMT = 48) and five NIBRG14-seronegative subjects (titres <20). This second subset was limited in sample size as only very few samples of the original complete set contained enough residual material to be tested with both viruses, resulting in small discrepancies in the number of titres available against the various virus strains.

Immunogenicity assays

Neutralising antibody titres were determined using a previously described microneutralisation (MN) assay[15] and used sheep and post-infection ferret antisera to both the vaccine strain and wild-type strains as positive controls. Serum samples were tested in duplicate, separately at initial dilutions of 1:20. Sera with titres of 20 or greater were considered positive. MN assays using wild-type H5N1 viruses were carried out at CL4 and those using reverse genetic viruses at CL3.

Data analysis

Seroneutralisation titres below the 1:20 limit of detection were given a value of 1:10. Samples with a titre >640 were

assigned a value of 640. Samples were tested in duplicate in two assays with the mean of the two results used. The proportion of results with titres ≥1:20 was compared between study groups using chi-squared tests. For those samples with titres ≥1:20 for NIBRG14 GMTs with 95% CIs were calculated for each neutralising assay within those samples that were above the 1:20 limit of detection. Frequency distributions were also plotted according to the percentage of samples in the titre ranges of <20, 20–49, 50–99 and ≥100. Spearmans' rank correlation was calculated for comparison of each tested strain with all others and scatter plots produced to compare the wild-type and non-wild-type strains.

Results

In a significant number of subjects antibodies elicited by the clade 1 vaccine NIBRG14 were seen to neutralise clade 2 wild-type and vaccine strains, although the degree of this cross-neutralisation varied markedly between strains (Table 1). The HA of the A/Vietnam/1194/2004 virus, which belongs to clade 1, differs from the HA's of the A/Indonesia/5/2005, belonging to clade 2·1 by 3·2% homology and the A/turkey/Turkey/1/2005, a clade 2·2 strain by 3·4% homology. The amino acid divergence between A/Indonesia/5/2005 and A/turkey/Turkey/1/2005 HA was found to be 4·2%.

Homologous reactivity; clade 1 viruses

The percentage of subjects with antibody titres ≥20 against NIBRG14 varied significantly ($P = 0.008$) between the dose/formulation groups because of an increasing trend with dose within the adjuvanted group (24% to 42% to 61%) but not within the no adjuvant group (Table 1). The measured response to the wild-type parental virus, A/Vietnam/1194/2004, was higher than to the vaccine strain (Table 1) with 98% of the NIBRG14-seropositive samples able to neutralise the wild-type parental virus. Within the 63 NIBRG14-seronegative samples tested against wild-type A/Vietnam/1194/2004, 65% were also able to neutralise the virus and there was a significant effect of dose/formulation group ($P = 0.007$) because of a trend with increasing HA dose within the adjuvanted group. Within the NIB-RG14 positive samples GMTs were significantly higher ($P < 0.001$) for A/Vietnam/1194/2004 than NIBRG14 (88 versus 54, Table 2).

Cross-reactivity; clade 2 viruses

As seen with the serum neutralisation with clade 1 viruses there was no dose dependence in the proportion of samples with detectable neutralising activity towards clade 2 viruses, in the absence of adjuvant but an increasing trend was seen in the adjuvanted groups (Table 1). This was statistically significant ($P < 0.02$) for NIBRG23 and A/turkey/Tur-

key/1/2005 but numbers were too small to see a significant trend for Ind/5/05/RG2 and A/Indonesia/5/2005. As seen with clade 1 viruses, when comparing responses between adjuvanted and non-adjuvanted formulation groups, the proportion of responders appeared lower at lower doses with adjuvant than without, and higher at 30 μg with adjuvant (Table 1).

Clade 2·2

Of the 127 NIBRG14-seropositive samples, 35% were also able to cross-neutralise the clade 2·2 NIBRG23 candidate vaccine strain. The proportion of cross-neutralising sera rose to 80% in experiments using its parental wild-type A/turkey/Turkey/1/2005 strain. However, although a greater proportion of sera were seen to cross-neutralised the wild-type A/turkey/Turkey/1/2005 strain, in contrast to the clade 1 viruses the geometric mean titres within the seropositives for this subclade was similar (61 versus 59, Table 2).

Clade 2·1

Of the 45 NIBRG14-seropositive samples tested, three were able to neutralise the Ind/5/05/RG2 candidate vaccine strain. Moreover, these positive responses were only seen in samples with high antibody titres against the clade 1 vaccine virus (titres >100).

As observed with the other strains, the proportion of cross-neutralising samples was markedly higher when the wild-type A/Indonesia/5/2005 strain was used: 29/45. The geometric means within the positives for both viruses were similar (35 versus 48, Table 2), but lower than those detected against clade 2·2 viruses.

Discussion

Protection from seasonal circulating human influenza A (H1N1 and H3N2) correlates with the detection of neutralising antibodies to the circulating strain.[16] This is usually measured using haemagglutination inhibition (HI) antibody titres with an antibody titre of 40 considered to provide a serological correlate of protection from infection.[17] In the case of H5 influenza, serological correlates of protection have not been determined, but the use of neutralising antibody is considered an important approach to determine the presence of protective immunity.[15,18–20]

We looked at the ability of antibodies induced by vaccination with a reverse genetics H5N1 vaccine strain to neutralise viral infectivity of diverse strains of highly pathogenic H5N1.

The differences in both, number of seropositives and GMT values (Tables 1 and 2) between serum sets revealed that antibodies discriminated between viruses from the different clades and subclades: the highest GMT values were

Table 1. Seroneutralisation results: proportion of samples with seroneutralisation activity (neutralisation antibody titre ≥20) against wild-type and reverse genetics-derived clade 1 and 2 reference strains among samples that were seropositive or seronegative to the clade 1 vaccine strain (post-dose 2)

| Virus neutralised | Titres available for | NIBRG14 vaccine formulation groups: haemagglutinin dosage and adjuvant content | | | | | | | | All combined |
| | | No adjuvant | | | | With Al³⁺ adjuvant | | | | |
		7·5 µg	15 µg	30 µg	All no adjuvant	7·5 µg + Al	15 µg + Al	30 µg + Al	All + Al	
NIBRG14, clade 1	300 post-dose 2 samples (full analysis set)	45% (22/49)	36% (18/50)	46% (23/50)	42% (63/149)	24% (12/50)	42% (21/50)	61% (31/51)	42% (64/151)	42% (127/300)
A/Vietnam/1194/2004, clade 1	126 NIBRG14 seropositive samples (1st subset)	91% (20/22)	100% (17/17)	100% (23/23)	97% (60/62)	100% (12/12)	100% (21/21)	100% (31/31)	100% (64/64)	98% (124/126)
	63 NIBRG14 seronegative samples (1st subset)	57% (4/7)	56% (5/9)	87% (13/15)	71% (22/31)	20% (2/10)	67% (8/12)	90% (9/10)	59% (19/32)	65% (41/63)
NIBRG23, clade 2·1	300 post-dose 2 samples (full analysis set)	20% (10/49)	18% (9/50)	24% (12/50)	21% (31/149)	6% (3/50)	10% (5/50)	29% (15/51)	15% (23/151)	18% (54/300)
	127 NIBRG14 seropositive samples	41% (9/22)	39% (7/18)	39% (9/23)	40% (25/63)	17% (2/10)	19% (4/21)	42% (13/31)	30% (19/64)	35% (44/127)
	173 NIBRG14 seronegative samples	4% (1/26)	6% (2/32)	11% (3/27)	7% (6/80)	3% (1/38)	3% (1/29)	10% (2/20)	5% (4/87)	6% (10/173)
A/turkey/Turkey/1/2005, clade 2·1	127 NIBRG14 seropositive samples (1st subset)	64% (14/22)	89% (16/18)	87% (20/23)	79% (50/63)	58% (7/12)	76% (16/21)	90% (28/31)	80% (51/64)	80% (101/127)
	63 NIBRG14 seronegative samples (1st subset)	14% (1/7)	56% (5/9)	67% (10/15)	52% (16/31)	20% (2/10)	50% (6/12)	60% (6/10)	44% (14/32)	48% (30/63)
A/Ind/5/05/RG2, clade 2·2	45 NIBRG14 seropositive samples (2nd subset)	0% (0/9)	11% (1/9)	13% (1/8)	8% (2/24)	0% (0/5)	0% (0/5)	9% (1/11)	5% (1/21)	7% (3/45)
	5 NIBRG14 seronegative samples (2nd subset)	0% (0/2)	–	0% (0/1)	0% (0/3)	0% (0/1)	0% (0/1)	–	0% (0/2)	0% (0/5)
A/Indonesia/5/2005, clade 2·2	45 NIBRG14 seropositive samples (2nd subset)	57% (4/7)	78% (7/9)	63% (5/8)	67% (16/24)	40% (2/5)	40% (2/5)	82% (9/11)	62% (13/21)	64% (29/45)
	5 NIBRG14 seronegative samples (2nd subset)	0% (0/2)	–	0% (0/1)	0% (0/3)	0% (0/1)	0% (0/1)	–	0% (0/2)	0% (0/5)

Table 2. Comparison of percentages positive and GMTs within the NIBRG14 positive sera against each candidate reverse genetics vaccine and wild-type viruses

Virus neutralised	Whole subset		Overlapping set*	
	Positive/total (%)	GMT (95% CI) within positives	Positive/total (%)	GMT (95% C I) within positives
NIBRG14	127/127 (100%**)	54 (48–61)	45/45 (100%**)	82 (66–101)
A/Vietnam/1194/2004	124/126 (98%)	88 (79–98)	45/45 (100%)	117 (96–142)
NIBRG23	44/127 (35%)	59 (47–74)	18/45 (40%)	62 (43–90)
A/Turkey/Turkey/1/2005	101/127 (80%)	61 (54–69)	36/45 (80%)	70 (56–87)
A/Ind/5/05/RG2	3/45 (7%)	48 (6–405)	3/45 (7%)	48 (6–405)
A/Indonesia/5/2005	29/45 (64%)	35 (29–42)	29/45 (64%)	35 (29–42)

*Sample set of 45 NIBRG14 seropositive samples that were analysed with all six viruses.
**NIBRG14 positive by definition.

elicited against the homologous clade 1 vaccine virus and its parental strain. Using heterologous strains, the highest cross-reactive GMT was determined with the A/turkey/Turkey/1/2005 wild-type and its candidate vaccine derivative. Comparing a small subset of 45 NIBRG14 seropositive samples which were analysed against all six viruses, we found GMT values for the neutralisation of A/Indonesia/5/2005, a clade 2 subclade 1 virus, was about twofold lower (comparison between both, wild-type strains and the appropriate reverse genetics strains), indicating that sera were generally less reactive to this virus. This is consistent with the antigenic and genetic distinctiveness of these viruses from the vaccine strain. Our findings are in keeping with those using reciprocal HI analysis with post-infection ferret sera against a range of H5N1 viruses, which demonstrated an eightfold drop in HI antibody titre when clade 2 viruses such as A/turkey/Turkey/1/2005 and A/Indonesia/5/2005 were tested with serum raised against A/Vietnam/1194/2004.[21] The observed discrimination within clade 2 is also consistent with results derived by reciprocal HI analysis using post-infection ferret antisera. Sera raised against a range of clade 2 viruses showed clear discrimination between subclades 1 and 2 when divergent clade 2 viruses were used in the HI tests.[21]

Generally, we observed higher GMT values when analysing the neutralisation of wild-type strains compared with their reverse genetics derivatives. Furthermore, a substantial proportion (65%) of the subset of NIBRG14-seronegative samples was also able to neutralise A/Vietnam/1194/2004 wild-type virus, most notable among samples from subjects vaccinated with the two 30 μg formulations (Table 1).

This observation was accompanied with higher titres of cross-neutralising antibodies, although the later was not as marked with the clade 2 viruses. We attribute this to a greater sensitivity of detection of neutralising antibodies in assays using wild-type viruses. The growth kinetics of wild-type viruses and reverse genetics viruses in tissue culture is significantly different (data not shown). Wild-type viruses are highly cytolytic at very low multiplicity of infection meaning that destruction of cells occurs very rapidly and at very low virus dose. Whilst the reverse genetic and wild-type viruses have been analysed using standardised protocols, the actual input virus challenge doses for the assessment of neutralising antibody do vary slightly, and are likely to account for these differences, emphasising the difficulty in standardising neutralising antibody titres between laboratories, and underlining the requirement for international antibody standards for H5 and standardised assay protocols, as has been identified in recent International collaborative studies comparing neutralisation assay protocols.[22] Differences in biological characteristics between strains may lead to significant differences in interpretation of immunogenicity and cross-neutralisation provided by vaccination. It is possible that reverse-genetic viruses used in neutralisation assays may underestimate the amount of cross-reactivity and antibody elicited post-vaccination. Further detailed analysis comparing properties of wild-type viruses and their reverse-genetic homologues are necessary to confirm this suggestion, and may be important in determining whether wild-type viruses should be used in future H5 vaccine evaluations. However, comparing the GMT ratios and titre distributions (Table 2, Figure 3) derived from either the wild-type, or the reverse genetic virus analyses, we find the trend in the level of cross-neutralisation between different clades/subclades conserved.

Using both, the A/turkey/Turkey/1/2005 reverse genetics and wild-type strains we observed a significant number of samples which were, seronegative against NIBRG14 but positive with NIBRG23 or the A/turkey/Turkey/1/2005 wild-type (6% and 48% of NIBRG14 seronegative samples).
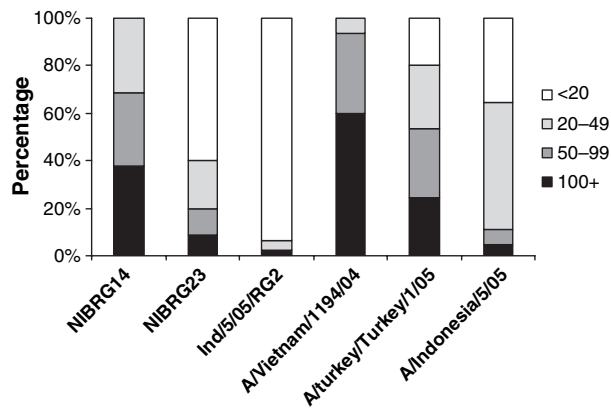
Figure 3. Titre distribution: analysis of neutralisation titres against all six viruses within 45 NIBRG14 seropositives samples.

Increased reactivity with wild-type virus might be attributed to the technical difference in assay performance described above. Importantly, we did not find this reactivity in bleeds prior to the vaccination and it appears to be a vaccine related effect, but the reason for this is unclear at present.

Overall, we did not find the use of the adjuvant aluminium hydroxide advantageous for achieving higher levels of cross-neutralisation, except at the highest antigenic dosage, and dose effect appeared less pronounced without adjuvant. This is in contrast to findings of an earlier study using an A/Duck/Singapore/1997 (H5N3) vaccine which suggested that the presence of an adjuvant was essential for the induction of antibodies capable of cross-neutralising a range of 1997–2004 human H5 viruses.[23] However, the adjuvant used in this earlier study – the oil/water emulsion type adjuvant MF59[24] – is known to act in a different manner to the gel-type adjuvant aluminium hydroxide used in our study, which is thought to promote predominantly a depot effect in the injection site.[25] Thus, the differences in vaccine formulations might explain the differences in study findings as different types of adjuvant will induce qualitatively different type of immune response.[26]

In the present study, we found evidence in human volunteers of cross-neutralisation of viruses from recent H5 outbreaks after using a split-virion vaccine made from a reverse genetics virus belonging to an older clade 1 strain. The protective effect of inactivated influenza vaccines is thought to be based on the induction of neutralising antibodies mainly to the viral HA. The measurement of neutralising antibodies therefore represents a valuable tool in predicting the actual protection of the vaccinated subject.[16] However, little is known about the correlation of neutralisation and HI titres and their respective correlation with immune protection against highly virulent wild-type influenza strains. Analysis of immune responses using either reverse-genetic or wild-type viruses may lead to some dif-

ferences in conclusions regarding correlation of antibody titre with protection, emphasising the need for detailed studies to extend our knowledge regarding correlates of immune protection, including in experimental animal models.

So far, studies in animals using both the ferret and the mouse model have shown that protection from heterologous viruses can be achieved in animals with low or undetectable antibody titres in HI and neutralisation assays.[11,27,28] However, vaccines used in these animal challenge models were either monovalent or bivalent whole virus or live-attenuated vaccines. Hence, the outcome cannot easily be extrapolated to the protection provided by a split-virion vaccine.[29] Nevertheless, the results of the animal studies show that H5 vaccines have the potential to induce cross-protective immunity. They also illustrate the necessity of further effort to define clear correlates of protection and link the results of human clinical trials with the appropriate animal challenge data.[8]

Both our human serological and animal model data as well as theoretical biomathematical[30] modelling support the approach of developing and stockpiling pre-pandemic vaccines in anticipation, that such vaccines will be able to provide substantial clinical protection, and prevent mortality even if the HA is not ideally antigenically matched to a circulating pandemic strain.

Conflicts of interest and source of funding

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