Journal of Clinical Virology 56 (2013) 323–330

Contents lists available at SciVerse ScienceDirect

Journal of Clinical Virology

journal homepage: www.elsevier.com/locate/jcv

Validation of the modified hemagglutination inhibition assay (mHAI), a robust and sensitive serological test for analysis of influenza virus-specific immune response

A. Morokutti[a,c], M. Redlberger-Fritz[b], S. Nakowitsch[a], B.M. Krenn[a], N. Wressnigg[a], A. Jungbauer[c], J. Romanova[a], T. Muster[a], T. Popow-Kraupp[b], B. Ferko[a,*]

[a] AVIR Green Hills Biotechnology AG, Forsthausgasse 11, A-1200 Vienna, Austria
[b] Department of Virology, Medical University of Vienna, Kinderspitalgasse 15, A-1095 Vienna, Austria
[c] Department of Biotechnology, University of Natural Resources and Life Sciences, Muthgasse 18, A-1190 Vienna, Austria

ARTICLE INFO

Article history:
Received 27 September 2012
Received in revised form
28 November 2012
Accepted 5 December 2012

Keywords:
Influenza serology
Bioassay validation
ICH guideline
GxP analysis
Immunogenicity
Vaccine efficacy/effectiveness

ABSTRACT

Background: The hemagglutination inhibition assay (HAI) is universally regarded as the gold standard in influenza virus serology. Nevertheless, difficulties in titre readouts are common and interlaboratory variations are frequently reported.
Objective: We developed and validated the modified HAI to facilitate reliable, accurate and reproducible analysis of sera derived from influenza vaccination studies.
Study design: Clinical and preclinical serum samples, NIBSC reference sera and seasonal influenza virus type A (H1N1 and H3N2) and type B antigens were employed to validate the mHAI. Moreover, pandemic virus strains (H5N1 and H1N1pdm09) were used to prove assay robustness.
Results: Utilisation of a 0.08% solution of stabilised human erythrocytes, assay buffer containing bovine serum albumin and microscopical plate readout are the major differences between the modified and standard HAI assay protocols.

Validation experiments revealed that the mHAI is linear, specific and up to eightfold more sensitive than the standard HAI. In 95.6% of all measurements mHAI titres were precisely measured irrespective of the assay day, run or operator. Moreover, 96.4% (H1N1) or 95.2% (H3N2 and B), respectively, of all serum samples were determined within one dilution step of the nominal values for spiked samples. Finally, the mHAI results remained unaffected by variations in virus antigens, erythrocytes, reagents, laboratory location, sample storage conditions or matrix components.
Conclusion: The modified HAI is easy to analyse, requires only a single source of erythrocytes and allows utilisation of numerous influenza virus antigens, also including virus strains which are difficult to handle by the standard HAI (e.g. H3N2, H5N1 and H1N1pdm09).

1. Background

Influenza viruses, amongst others, are able to agglutinate mammalian or avian red blood cells (RBCs).[1–3] RBCs carry sialic acids that are recognised as receptors by the influenza surface glycoprotein hemagglutinin, resulting in the hemagglutination of erythrocytes (hemagglutination assay, HA).[4–9] Virus-specific antibodies interfere with the interaction between virus particles and erythrocytes preventing hemagglutination (HAI).[10,11] Neuraminidase,[12–14] the second influenza surface glycoprotein, antagonises hemagglutination due to its sialidase activity but might also be involved in binding of RBCs, as indicated recently.[15]The HA and HAI are widely used methods, requiring only few and inexpensive reagents and equipment. For instance, the HA is utilised to determine the efficiency of virus production and purification,[16–18] to analyse virus titration assays when CPE is unambiguous and to adjust HAI virus antigens.[19] The HAI is applied for the diagnosis of influenza virus infections,[20–22] to determine vaccine immunogenicity[23,24] and for seasonal surveillance.[25–29] Regulatory authorities demand the calculation of seroprotection and seroconversion rates based on HAI data to demonstrate the efficacy and effectiveness of influenza

Abbreviations: CPE, cytopathic effect; GxP, GCP/GLP/GCLP; GCP, good clinical practise; GCLP, good clinical laboratory practise; GLP, good laboratory practise; GMT, geometric mean titre; HA, hemagglutination assay; HAI, hemagglutination inhibition assay; HAU, hemagglutination unit; ICH, International Conference on Harmonisation; IVR, influenza virus resource; m, modified; NIBSC, National Institute of Biological Standards and Controls; NYMC, New York Medical College; RBC, red blood cell; RDE, receptor destroying enzyme; SPF, specific pathogen free; WHO, World Health Organisation.

* Corresponding author. Tel.: +43 1 3199670; fax: +43 1 3196099.
E-mail address: b.ferko@greenhillsbiotech.com (B. Ferko).

1386-6532/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.jcv.2012.12.002

Table 1
Summary of the standard and the modified HAI protocols. Compared are the main parameters for both assay types, as applied in routine analysis of clinical and preclinical study samples as well as validation experiments. The standard HAI protocol from the WHO manual was modified by using a single source of erythrocytes (human) at a reduced concentration (0.08%), an assay buffer supplemented with 1% BSA and a microscope for analysis due to the low amounts of RBCs (examples for common differences in standard HAI protocols are summarised by Wood et al.[36]).

Parameter	Standard HAI	Modified HAI
Serum dilution	1:8	1:8
RDE-treatment and heat inactivation	Overnight @ 37 °C; 45 min @ 56 °C	Overnight @ 37 °C; 45 min @ 56 °C
Erythrocyte source	Human or animal (e.g. chicken, horse)	Human (stabilised)
Erythrocyte concentration[a]	0.8% (human) or 0.5% (animal)	0.08%
Microtitre plates	U- (human) or V-(animal) bottom	V-bottom
Assay buffer	DPBS	DPBS + 1% BSA
Assay volume[b]	25 μl	25 μl
Virus antigen (HAU/25 μl)	4	4
First incubation (after mixing of serum and virus antigen)	30 min	45 min
Second incubation (after addition of erythrocytes)	60 min	75 min
Assay temperature	RT (or +4 °C, in case of virus antigens with high neuraminidase activity)	RT
Assay plate readout	By eye, 60° tilted plate	Microscope (40-fold magnification)
Endpoint determination for seropositivity	Settled erythrocytes and leakage pattern	Clear to irregular shaped erythrocyte dot
Reference material	Varies with virus antigen and erythrocyte source	NIBSC sheep serum

[a] Diluted in the respective assay buffer (DPBS for HAI or 1% BSA in DPBS for mHAI).
[b] The same amount of 25 μl for serum, assay buffer, assay antigen and RBC solution.

vaccines.[30,31] Although a general HAI protocol is provided by the WHO and the assay was qualified in the past, laboratories have developed their own versions.[3,19,32] This leads to considerable variations in interlaboratory comparison studies.[33–37]

In this paper we describe the validation of the mHAI, a robust and sensitive variant of the standard HAI with great potential for GxP analytics.

2. Objectives

Subjective readouts due to unspecific interferences, inappropriate erythrocytes and assay protocol modifications lead to serious problems in routine HAI testing. The standard HAI was optimised to increased robustness, thereby generating reliable and reproducible data, and the resulting modified assay version (mHAI) was validated.

3. Study design

3.1. Viruses

Viruses used in this study differ in origin and internal gene composition.

Hen's egg-derived influenza viruses containing wildtype NS1 were obtained as PR8-based reassortants from NIBSC and propagated in the allantoic cavity of embryonated SPF chicken eggs[38,39]: A/Brisbane/59/2007 IVR-148 (H1N1, 07/344), A/Brisbane/10/2007 IVR-147 (H3N2, 07/246), A/California/07/2009 NYMC X-179A (H1N1pdm, 11/112) and B/Florida/04/2006 (B, 08/138).

Vero cell culture-derived 6:2 delNS1 reassortants[40–42]: A/Brisbane/59/2007 (H1N1-BN), A/Vietnam/1203/2004 (H5N1-VN) and A/Indonesia/05/2005 (H5N1-IN) were obtained by reverse genetics.[38,43,44] The highly pathogenic polybasic cleavage site of the hemagglutinin in H5N1 reassortants was genetically removed.[45]

3.2. Serum samples

Human sera were obtained from a clinical trial with an intranasal delNS1 trivalent influenza vaccine (EudraCT 2010-023815-33). Ferret sera were obtained from preclinical studies. NIBSC hyperimmune sheep anti-hemagglutinin sera (reference serum) were used as controls and for spiking: anti-A/Brisbane/59/2007 (10/120), anti-A/Brisbane/10/2007 (08/246), anti-B/Florida/04/2006 (07/356) and pooled human sera from reconvalescent individuals (WHO International Standards): anti-A/Vietnam/1203/2004 (07/150) and anti-A/California/07/2009 (09/194).

3.3. Sample processing

All sera were diluted 1:4 with the receptor destroying neuraminidase (RDE II, Denka Seiken) from *Vibrio Cholerae*, incubated overnight at 37 °C and subsequently heat-inactivated at 56 °C for 45 min.

3.4. Erythrocytes

Different types of erythrocytes were used: (i) stabilised human erythrocytes (Siemens Healthcare Diagnostics Products), (ii) donated human serum ($0^{Rh\ neg.}$) and (iii) chicken whole blood. RBCs purified from human serum and chicken blood were diluted in the respective assay buffer (DPBS for HAI or 1% BSA in DPBS for mHAI) to the desired concentration.[19]

3.5. Equipment

Calibrated pipettes (Biohit) and U- (HAI) or V- (HAI, mHAI) bottom microtitre plates (Sterilin) were used. A light microscope with connected reflex camera (Olympus) was utilised for plate readout. The Scepter™ Automated Handheld Cell Counter (Millipore) was applied to determine the cell number of purified RBCs.

3.6. Standard and modified HAI

The standard HAI was performed according to the WHO manual, for the here described mHAI the WHO protocol was further optimised (Table 1).[19] Virus antigen was adjusted to 4 HAU/25 μl by performing a modified HA. Therefore, 50 μl undiluted virus antigen was serially diluted 1:2 with 25 μl assay buffer in V-shaped 96-well microtitre plates. Then 25 μl 0.08% stabilised human erythrocytes and 25 μl assay buffer were added. After incubation for 75 ± 10 min at room temperature (RT) the plates were analysed with a light microscope at 40-fold magnification. The mHA titre is the reciprocal of the last dilution of virus antigen that causes hemagglutination (Fig. 2C). To perform the mHAI, 25 μl RDE-treated serum samples were diluted 1:2 with assay buffer and serially twofold diluted with 25 μl assay buffer. Then, 25 μl adjusted virus antigen solution was added and incubated for 45 ± 5 min at RT. After addition of 25 μl