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The development of a multiplex serological assay for avian influenza based on Luminex technology

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ABSTRACT

Avian influenza (AI) is an infectious disease in birds with enormous impact on the poultry sector. AI viruses are divided into different subtypes based on the antigenicity of their surface proteins haemagglutinin (HA) and neuraminidases (NA). In birds, 16 HA subtypes and 9 NA subtypes are detected in different combinations. Traditional serological methods for the subtyping of AI antibodies are labour-intensive and have to be performed for each HA and NA subtype separately. This study describes the development of a multiplex serological assay for subtyping AI antibodies in poultry sera using Luminex xMAP technology. This multiplex assay allows the detection of all AI serotypes in one single assay. For all HA and NA subtypes, recombinant proteins were purified and coupled to colour-coded magnetic bead sets. Using the Luminex MAGPIX device, binding of serum antibodies to the antigens on the bead sets is detected by fluorescent secondary antibodies, and the different bead sets are identified. The results of the multiplex assay were compared with that of the traditional singleplex assays. We show that serotyping using the novel multiplex serological assay is consistent with the results of the traditional assays in 97.8% of the reference sera and in 90.8% of the field sera. The assay has a higher sensitivity than the traditional assays, and requires a smaller sample volume. Therefore, the assay will allow complete AI-serotyping in small volumes of field sera, which will improve the monitoring of AI subtypes circulating in poultry significantly.

1. Introduction

Avian influenza (AI) is an infectious disease in birds caused by Influenza A viruses belonging to the family Orthomyxoviridae. Wild birds, primarily waterfowl, gulls and shorebirds, are the natural reservoir of the virus and generally show no clinical signs of infection [1]. Wild birds spread AI viruses (AIV) worldwide during migration [2–4]. Influenza A viruses are divided into different subtypes based on the antigenicity of their surface glycoproteins haemagglutinin (HA) and neuraminidases (NA) [1,5]. Nowadays, 16 different HA and 9 different NA subtypes are detected in birds, which can be found in different combinations [6]. Some HA subtypes are primarily detected in specific wild bird species, e.g. H13 subtypes are frequently found in gulls but rarely in other birds [7]. The evolution of AI viruses is rapid and unpredictable, which can lead to a sudden appearance of new virus strains with possible new characteristics and risks.

AI viruses can be transmitted to poultry by contact with wild birds or their excretions [8]. Most AIV are of low pathogenic avian influenza (LPAI) phenotype and do not cause severe clinical signs in poultry [1]. However, H5 and H7 subtypes can evolve from LPAI to highly pathogenic avian influenza (HPAI) viruses upon introduction into poultry [9]. The emergence of HPAI viruses results from the insertion of basic amino acids at the HA cleavage site [10]. This results in systemic replication of the virus, damaging vital organs and tissues, resulting in high mortality rates in poultry (up to 100% in a few days) [11]. Due to the risk of mutation of LPAI H5 and H7 to HPAI, the subsequent economic consequences and the impact on animal welfare, infections with both LPAI and HPAI H5 and H7 are notifiable [12]. Poultry flocks infected with H5 and H7 AIV are culled to prevent virus spreading [13].

In 2003, a large epidemic of HPAI H7N7 in poultry occurred in the Netherlands that had an enormous impact on the poultry sector. After this outbreak, the Dutch government implemented an active

Abbreviations: AI, avian influenza; AIV, avian influenza virus; Ch. IgY, chicken IgY; EtAm, Ethanolamine; HA, haemagglutinin; HI assay, haemagglutination inhibition assay; HAU, haemagglutination units; HPAI, highly pathogenic avian influenza; LPAI, low pathogenic avian influenza; MFI, median fluorescent intensity; NA, neuraminidase; NC, negative control; NP-protein, Nucleoprotein; PC, positive control

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surveillance program to monitor the introductions of LPAI viruses in poultry. In this program, all poultry flocks are screened for the presence of antibodies against AI, at least once a year, depending on the type of poultry and housing and the risk related to this [14]. The sera are first screened by ELISA for the presence of antibodies against all influenza A viruses. Positive sera samples are subsequently tested in the Haemagglutination Inhibition (HI) assay against H5 and H7 antigens to screen for these subtypes specifically. The HI assay is a classic laboratory procedure for the subtyping of antibodies of haemagglutinating viruses, e.g. AIV, and is based on the inhibition of the agglutination reaction by HA subtype-specific antisera [1]. When the sera are negative for H5 and H7, HI-assays against other antigen subtypes can be performed to identify the virus subtype. When H5 or H7 antibodies are detected, new samples will be taken at the farm for detection of AIV. Antibodies can still be detected in the sera for months after infection with AIV, when the virus is cleared. Only when the virus is detected, the appropriate measures are taken to prevent spreading of the virus. The NA subtype of AIV can be determined by the NA inhibition assay using 9 virus antigens representing the 9 NA subtypes or a NA specific ELISA.

Both HI and NA inhibition assays are labour-intensive and have to be performed for every HA and NA subtype separately. In addition, the volume of serum needed for the HI assay and the NA inhibition assay or ELISAs makes complete subtyping often impossible. In this study, we describe the development of a multiplex serological assay for subtyping of AI antibodies using Luminex xMAP technology. This novel assay allows the detection of antibodies against the 16 HA and 9 NA subtypes simultaneously in one single test. The assay is high-throughput and efficient in cost and time compared to the traditional assays for serotyping. Therefore, this novel multiplex assay will facilitate the serotyping of all AI introductions in the poultry sector, which can be used to improve control measures and surveillance strategies.

2. Material and methods

2.1. Selection of HA and NA antigens

Genetic differences between viruses of the same subtype may lead to antigenic differences. We performed a phylogenetic analysis for all HA and NA sequences present in the GISAID database [15]. Based on the phylogenetic trees we selected HA and NA sequences that are representative for a sequence cluster. Generally, two sequences of each tree were selected: one Eurasian and one North American sequence. For HA subtypes H3, H5, H6, H7, H9 and H10 the genetic variation is larger, so more than two representative sequences were chosen from multiple clusters. For H14 and H15 subtypes the genetic variation is limited, only one Eurasian sequence was selected. For every NA subtype, one Eurasian sequence was chosen. In total, 45 HA proteins and 9 NA proteins were selected as antigens to be included in the multiplex serological assay (Table A1).

2.2. Production of HA and NA antigens

The method for production of recombinant soluble multimeric HA and NA antigens was previously described [16,17]. Briefly, Human and Drosophila Melanogaster codon-optimized sequences encoding the soluble haemagglutinin ectodomain and the neuraminidase head domain of influenza virus were synthesized (GenScript, Leiden, The Netherlands) and cloned into the expression plasmid pCD5 [17] for expression in HEK293T cells. The HA gene was preceded by a sequence encoding a N-terminal CD5 signal peptide and followed by sequences encoding a C-terminal artificial GCN4 trimerization domain (GCN4-pII) [18] and a Strep-tag®II (IBA, Göttingen, Germany) for affinity purification. The NA gene also contains the N-terminal CD5 signal peptide and double Strep-tag, and an artificial GCN4 tetramerization domain (GCN4-pLI) [18].

The expression plasmids were transfected to HEK293T cells using X-tremeGene (Roche, Merck, Zwijndrecht, The Netherlands) and Optimem (1:3) (Gibco, Thermo Fisher Scientific, Landsmeer, The Netherlands) according to the manufacturer's protocol. HEK293T cells were grown in DMEM supplemented with GlutaMAX™-I (Gibco, Thermo Fisher Scientific, Landsmeer, The Netherlands), 5% foetal calf serum and 0.1% penicillin/streptomycin (Gibco, Thermo Fisher Scientific, Landsmeer, The Netherlands). Culture supernatants were harvested at 4 days post transfection, and HA and NA proteins were purified from the culture medium using Strep-Tactin affinity chromatography (Strep-Tactin/Sepharose, IBA, Göttingen, Germany). The concentration of purified protein was determined using a Nanodrop 1000 spectrophotometer (Isogen Life Sciences) according to the manufacturer's instructions.

2.3. Coupling of antigens to the bead sets

The purified HA and NA proteins were coupled to different colour-coded bead sets (MagPlex™-C magnetic carboxylated microspheres, Luminex, 's Hertogenbosch, the Netherlands), using standard EDC/NHS coupling [19]. Briefly, carboxylated bead sets were treated with EDC and NHS, creating semi-stable amine-reactive NHS esters. These esters react with primary amines of the proteins, forming covalent amide bonds between the beads and the proteins. For each bead set, 2.5 µg of each protein was coupled to 1.25×10^6 beads.

The assay includes two internal controls. Positive control beads were prepared by coupling 0.5 µg Chicken IgY (Chicken IgY polyclonal-Isotype control, Abcam, Cambridge, UK) to one bead set. The purpose of this control bead is to verify the addition of both test serum and secondary antibody to the assay. Negative control beads were prepared by coupling Ethanolamine [20] to a bead set. The purpose of this latter control is to monitor the background signal in the assay.

2.4. Development of the multiplex serological assay

Each protein was individually coupled to a bead set, after which the sets were mixed to create a multiplex assay. The HA and NA proteins were divided over six different multiplex assays (called 'multiplexes'), that allowed us to use only 11 different bead sets. The set-up of the assay is shown in Table 1. By testing a sample in all six multiplexes, the serum was investigated for antibodies against all 54 AIV proteins (= antigens).

Multiplexes and 2 µl of serum were both diluted 200x in sample buffer (PBS + Tween 20 0.05%, 10% PRIblocker (PrimeDiagnostics, Wageningen, The Netherlands)). Each diluted bead mixture contains approximately 1000 beads per used bead set. Diluted bead mixture and serum were added to the wells (1:1) of a 96 well plate (Corning® Thermowell PCR 96 well plates, Merck, Zwijndrecht, The Netherlands) and incubated in the dark for 1 h at room temperature while shaking (600 rpm) at a VWR Microplate Shaker (VWR International, Amsterdam, the Netherlands). The supernatant was removed using a Dynamag-96 magnet (Thermo Fisher Scientific, Landsmeer, the Netherlands) to hold the paramagnetic beads. After washing with washing buffer (PBS + Tween 20 0.05%), the beads were incubated with anti-IgY chicken Phycoerythrin conjugate (1:1000) (Goat anti-chicken IgG (H + L)-PE, Southern Biotechnology, Birmingham, USA). After 1 h incubation and a last wash, the beads were resuspended in washing buffer and shaken for 30 s. Analysis was performed using the MAGPIX device of Luminex and the Luminex® xPONENT® for MAGPIX® software version 4.2. Results were reported as median fluorescent intensity (MFI), analysing a minimum of 100 beads per bead set.

2.5. Selection of reference sera

Sera generated by experimental infection of chickens in a laboratory setting with known subtypes of AIV were used as reference sera. In total, 43 reference sera were selected for initial testing in the multiplex serological assay. From these, a subset of 16 sera was selected for a

Table 1
The set-up of the multiplex serological assay. The assay is divided in six 'multiplexes' to reduce the number of required bead sets. The codes of the proteins correspond with the codes mentioned in Table A1 (H1.2 means the second H1 bead set). Symbols: - : bead set was not used in this multiplex; Ch. IgY: chicken IgY (positive control bead set); EtAm: ethanolamine (negative control bead set).

Bead sets	Multiplex 1	Multiplex 2	Multiplex 3	Multiplex 4	Multiplex 5	Multiplex 6
MC10018-01	H1.2	N1	H5.1	H3.3	H4.2	H12.2
MC10021-01	H2.1	N2	H5.2	H1.1	H6.2	H14.1
MC10035-01	H3.1	N3	H5.3	H2.2	H6.4	H15.1
MC10044-01	H5.4	N4	H13.1	H3.2	H7.2	H16.1
MC10048-01	H6.3	N5	H5.5	H4.1	H8.2	–
MC10052-01	H7.3	N6	H5.6	H6.1	H9.2	–
MC10055-01	H9.5	N7	H5.7	H7.1	H9.3	–
MC10061-01	H10.2	N8	H5.8	H8.1	H9.4	–
MC10067-01	–	N9	H5.9	H9.1	H10.3	–
MC10075-01	H12.1	H11.1	H13.2	H10.1	H11.2	–
MC10078-01	H16.2	–	–	–	Ch. IgY	EtAm

standard control serum panel. This panel represents all 16 different HA subtypes and all 9 NA subtypes (Table A2). In addition, a panel of 15 sera was selected containing antibodies against other avian (respiratory) viruses, i.e. avian leukosis virus, adenovirus (EDS), reticuloendotheliosis virus, avian encephalomyelitis virus, Marek disease virus, gallid herpesvirus 1 (ILT), infectious bursal disease virus, infectious bronchitis virus, reovirus, avian metapneumovirus (TRT), avian nephritis virus and avian avulavirus 1, 2, 3 and 7. These sera were generated by experimental infection of chickens, and were tested as negative controls.

2.6. Selection of field sera

Sera from poultry flocks testing positive for AI antibodies in the active surveillance program in the Netherlands were selected. A panel of field sera was generated based on subtype and volume of sera available. This resulted in a panel of 87 field serum samples, collected between 2015 and 2017, originating from 13 chicken flocks.

2.7. HI assay and NA ELISA

HA results of the multiplex serological assay were compared with the results of the HI assay, the traditional assay for HA subtyping, which was performed according to the methods described in the OIE manual using eight haemagglutination units (HAU) of virus [21]. This test is based on the visible agglutination of erythrocytes (haemagglutination) when the HA subtype specific antibody binds to an antigen with the same HA subtype. Sera were tested in a twofold dilution series to determine the titre, which is expressed as the reciprocal value of the highest dilution that can prevent complete agglutination. To reduce auto-agglutination caused by nonspecific inhibition of haemagglutination, all field sera were diluted 1:1 with 20% chicken erythrocytes. After 30 min incubation at 4 °C, the erythrocytes were removed by centrifugation (1 min, 5000 rpm) [1]. A sample was considered positive when a titre was observed.

For AIV different subtypes can be distinguished based on genetics and serology. However, some subtypes are genetically more closely related [22], which cause some cross-reactivity in the HI assay between the different HA subtypes. In addition, false positive reactions caused by steric inhibition of an antigen which has the same (homologous) neuraminidase subtype as the antibody specificity of the serum, can be observed [1,21].

The NA results of the multiplex serological assay were compared with an in-house NA ELISA (unpublished, G. Koch). For this indirect sandwich ELISA, pre-incubated antigen and serum were added to an ELISA plate coated with llama antibodies against the NA antigen [23]. Antigens which were not blocked by the pre-incubated serum bind to the coating antibodies. To detect the captured antigens, a biotinylated secondary antibody was used, together with Streptavidin-horseradish peroxidase. The NA ELISA was performed for each NA subtype separately. The Cohen's kappa inter-rater agreement was calculated for the results of the reference sera (n = 506), negative sera (n = 6) and the sera with antibodies for other avian viruses (n = 15) in the multiplex serological assay and the traditional assays [24].

2.8. Calculation of results

For each sample, specific cut-offs were calculated for HA and NA separately. For HA bead sets, the 40 lowest MFI signals were regarded as 'background' (45 HA bead sets in total). The cut-off for HA was calculated using the average of these 40 bead sets $+5 \times$ standard deviation. For NA, the same type of calculation was performed using the bead sets with the seven lowest signals as background (nine NA bead sets in total). Bead sets with MFI values above the cut-off indicate the subtype of the serum. When MFI signals above the cut-off were obtained for multiple subtypes, the highest MFI signal is used to assign the subtype. When these subtypes were genetically closely related, it was called a cross-reaction. Samples were considered negative if all bead sets, the signal of the positive control bead set excluded, gave signals below 200 MFI, which was determined by 1) testing defined negative sera (n = 6) and 2) observing negative beads in positive samples. The assay is considered valid if the positive control bead set gives a signal $\geq$ 2000 MFI and the negative control bead $\leq$ 200 MFI.

3. Results

3.1. Development of the multiplex serological assay

In this study, a multiplex serological assay for fast and efficient detection of AI antibodies in poultry sera was developed. Representative HA and NA proteins for all known AI subtypes in birds were selected, resulting in 45 HA and 9 NA proteins. The recombinant proteins were produced, and coupled to bead sets. Sera were tested by incubation with the bead sets. After washing, secondary antibodies were added to provide a fluorescent signal. The binding of antibodies to the bead sets was detected using the Luminex MAGPIX device, which measured the fluorescent signals (in MFI). Finally, the subtype of HA and NA was determined after correction for background signals.

3.2. Characterization of reference sera using traditional methods

All 43 reference sera that were selected for this study were first characterized using HI assays against 38 different antigens. This also revealed cross-reactivity between genetically closely related subtypes. The antigens used in this study are derived of AI viruses of different subtypes that were isolated previously from poultry or wild birds. Titres were observed when the sera contained antibodies against a virus with a HA subtype identical to the antigen used in the HI test (Table A3).

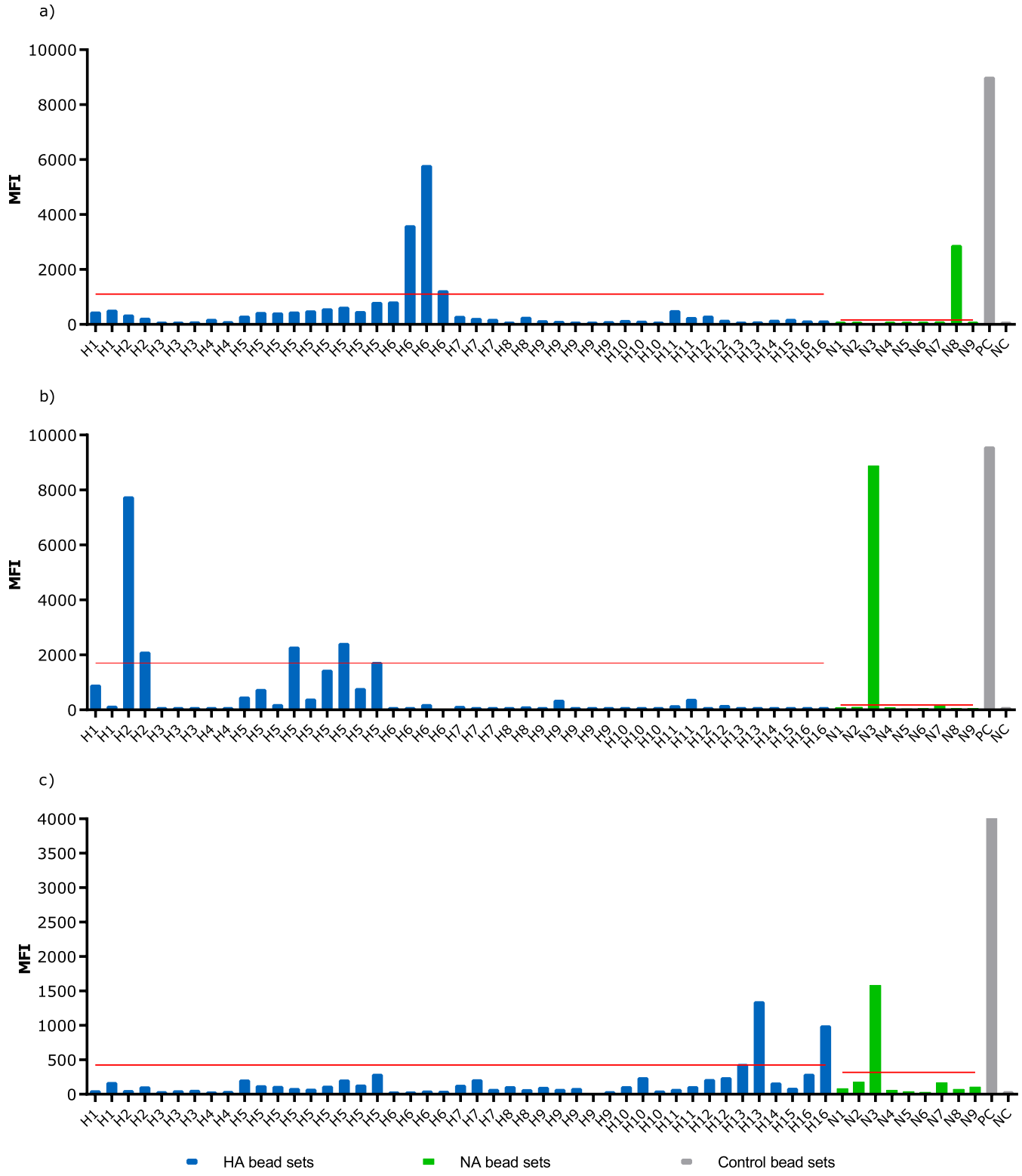

Fig. 1. Three examples of the multiplex serological assay output for three different reference sera: a) correct subtyping of a H6N8 reference serum, b) subtyping of a H2N3 serum, for which a cross-reaction with H5 antigens was observed with a final subtyping of H2N3, c) a subtyping of a H16N3 serum for which cross-reactivity with the H13 antigens was observed and because the H13 bead set gave the highest MFI signal this resulted in an incorrect subtyping as H13N3. The red lines are the calculated sample specific cut-offs for the HA and NA beads. PC: positive control bead set; NC: negative control bead set. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Inhibition was also often observed when the serum and antigen had a homologous neuraminidase subtype. Cross-reactivity was seen between genetically closely related HA antigens, i.e.: H1 and H5 or H6; H2 and H5; H3, H4 and H14; H5 and H6; H7 and H15; H8, H9 and H12; and H13 and H16. Cross-reactions were not frequently observed for NA, although some cross-reactivity was seen occasionally.

3.3. Performance of the multiplex serological assay with reference sera

The performance of the multiplex serological assay was first assessed using the 43 reference sera. Three typical examples of the multiplex assay output are presented in Fig. 1. The first example (Fig. 1A) shows the subtyping of a H6N8 reference serum. Three out of four H6 bead sets gave positive MFI signals, with MFIs ranging from 1244 to 5805. The N8 signal was 2893 MFI, well above the determined NA cut-off of 163 MFI. The positive control bead set gave a signal of 9022 MFI, which verified the addition of test serum and secondary antibody, and the low MFI signal (44 MFI) of the negative control bead set indicates a low background signal. The second example (Fig. 1B) shows subtyping of the H2N3 serum, for which in addition low positive MFI signals of the H5 bead sets, resulting from cross-reactivity between these genetically closely related subtypes, were observed. The third example (Fig. 1C) shows subtyping of a known H16N3 serum. In this case, the highest MFI signals were observed for the H13 bead sets, what resulted in an incorrect subtyping as H13N3. This is an example of cross-reactivity between antibodies against H16 in the serum and the genetically closely related H13 antigens on the bead sets. Both cross-reactions presented in the figure were similar to the cross-reactivity which was observed for this sera in the HI assay (Table A3).

To study the reproducibility of subtyping, we tested the sera of the standard control serum panel multiple times (between 26 and 54 repeats). The results are summarized in Table 2. All results were valid, because the signals of the positive control bead set were ≥2000 MFI and the signals of the negative control bead set were ≤200 MFI for all samples. The results show that the multiplex assay correctly subtyped 97.8% of the reference sera, which were generated by infection of chickens with known subtypes of AI virus. The estimated Cohen's kappa was 0.782 (95% CI: 0.66–0.91), which indicates a significant good agreement. The few sera that were not correctly subtyped are due to cross-reactions, which resulted in higher MFI signal for genetically closely related HA subtypes than for the correct subtypes. The cross-reactivity for these sera was also seen in the HI assay (Table A3). Most important cross-reactions were of H2 serum with H5 antigens, reaction with the H8 and H9 antigens of H12 sera and cross-reactions of H16 sera with H13 antigens. Similar results were obtained using the same batches of coupled bead sets stored for over 12 months, demonstrating that the bead sets have a long shelf life (not shown). We also tested sera obtained after experimental infection of chickens with avian leukosis virus, adenovirus (EDS), reticuloendotheliosis virus, avian encephalomyelitis virus, Marek disease virus, gallid herpesvirus 1 (ILT), infectious bursal disease virus, infectious bronchitis virus, reovirus, avian metapneumovirus (TRT), avian nephritis virus and avian avulavirus 1, 2, 3 and 7. These sera all tested negative, showing that no cross-reactions occur in the multiplex serology assay with antibodies against these avian (respiratory) viruses.

Table 2
The performance of the multiplex serological assay testing the standard control serum panel. The number of times the serum was subtyped correctly is shown relative to the total number of tests. The observed cross-reactions resulted in the highest MFI signal in that assay, but the signal for the correct subtype was also detected and above the cut-off. Symbols: - : no cross-reaction.

Serum	Correct/Total tested	Cross-reactions
H1N1	29/29	–
H2N3	48/54	5× H5; 1× H11
H3N9	27/28	1× H14
H4N6	28/28	–
H5N2	50/50	–
H6N5	43/43	–
H7N7	29/29	–
H8N4	29/29	–
H9N5	28/28	–
H10N4	27/27	–
H11N6	27/27	–
H12N8	24/26	2× H8H9
H13N2	28/28	–
H14N7	26/26	–
H15N9	27/27	–
H16N3	25/27	2× H13
Total	**495/506 (=97.8%)**	

Table 3
Comparison of the detection limit between the HI assay and multiplex serological assay. The multiplex serological assay was more sensitive for all three sera. Symbols: + : positive result (HI assay ≥ 2^1; multiplex serological assay ≥ 200 MFI for the HA and NA bead sets), − : negative result.

Serum	Assay	Dilutions										
		2^1	2^2	2^3	2^4	2^5	2^6	2^7	2^8	2^9	2^{10}	2^{11}
H2N3	HI assay	+	+	+	+	+	+	−	−	−	−	−
	Multiplex serological assay	+	+	+	+	+	+	+	+	+	−	−
H5N2	HI assay	+	+	+	+	+	−	−	−	−	−	−
	Multiplex serological assay	+	+	+	+	+	+	+	+	−	−	−
H6N5	HI assay	+	+	+	+	+	+	+	−	−	−	−
	Multiplex serological assay	+	+	+	+	+	+	+	+	−	−	−

The detection limit of the multiplex serological assay was compared to that of the HI assay by testing three sera from the standard control serum panel (H2N3, H5N3 and H6N5) (Table 3). These reference sera were diluted 2-fold in negative chicken serum, to generate dilution series of the anti-AI antibodies present. The detection limit for the assay was defined as a signal of ≥200 MFI in the multiplex serological assay for HA and NA bead sets and the HI assay should show at least a titre against the homologous antigens. The multiplex assay is able to correctly subtype the sera in a 2–8 times lower concentration compared to the HI assay. These results demonstrate that the multiplex serological assay has a lower detection limit, and thus is more sensitive than the HI assay. The sensitivity compared to the NA ELISA was not determined.

3.4. Performance of the multiplex serological assay with field sera

Finally, the performance of the multiplex serological assay with field sera was evaluated by testing 87 selected individual field samples from 13 poultry flocks. To allow subtyping using the traditional assays, chicken sera from the same flock and submission date were pooled to create enough volume of serum. The results of the 16 distinct HI assays (against H1–H16 antigens) and nine (N1–N9) different NA ELISAs were subsequently compared to the results of the multiplex assay. Similar subtypes were detected using the multiplex assay and traditional assays for 79/87 of the samples tested (90.8%) (Table 4). Most likely, 4.6% of the different results in the multiplex serological assay and the HI assay were caused by cross-reactions between genetically related HA antigens. In these cases, the signal(s) of the bead set(s) of genetically related HA antigens was higher than the subtype that was defined by the traditional assays. The cross-reactions observed in the multiplex assay were similar to those seen in the HI assay (Table A3). For 3.4% of the samples, the differences in subtyping in the multiplex assay could not be explained by cross-reactions between genetically related HA antigens. Also, one sample tested negative in the multiplex assay. These results are possibly due to the fact that individual sera were tested in

Table 4
The performance of the multiplex serological assay with field sera. Field sera were tested individually in the multiplex serological assay and most results were similar with the results of the 16 distinct HI assays (against H1–H16 antigens) and nine (N1–N9) different NA ELISAs. The large volume of serum required for the HI assays and the NA ELISAs was obtained by pooling multiple sera from the same flock. [a] individual samples, [b] pooled samples, [c] cross-reactions, when a genetically related HA subtype gives a positive higher signal than the subtype detected by the traditional assays but the subtype defined by the HI assay and NA ELISA was still above the cut-off, [d] subtype defined by the HI assay and the NA ELISA.

Flock	Results multiplex serological assay[a]	Results HI assay[b]	Results NA ELISA[b]	Subtype field sera[d]
1	7× H2N5	H2	N5	H2N5
2	4× H2N7, 1× H5N7[c], 1× H5N7	H2	N7	H2N7
3	6× H5N2, 1× H2N2[c]	H5	N2	H5N2
4	6× H6N1	H6	N1	H6N1
5	7× H6N2	H6	N2	H6N2
6	6× H6N5	H6	N5	H6N5
7	7× H6N8	H6	N8	H6N8
8	6× H7N7, 1× H15N7[c]	H7	N7	H7N7
9	5× H7N9, 1× H15N7[c]	H7	N9	H7N9
10	7× H8N4	H8	N4	H8N4
11	7× H9N2	H9	N2	H9N2
12	5× H9N7, 1× H5N7; 1× H14N7	H9	N7	H9N7
13	6× H10N7, 1× negative	H10	N7	H10N7

the multiplex assay, whereas pooled sera were tested in the traditional assays. Individual sera from a tested poultry flock may be negative for the infection, or may be infected with a different subtype of AI virus.

4. Discussion

In this study we report the development of a multiplex serological assay that allows the detection of antibodies against all AIV subtypes in poultry sera using Luminex xMAP technology. For this assay, HA and NA proteins were selected and expressed in vitro that represent all 16 HA and 9 NA subtypes. The recombinant HA and NA proteins were purified and coupled to multiple bead sets. The binding of the AI antibodies to specific beads was measured using the Luminex MAGPIX device. The sera were serotyped based on the bead sets with the highest positive MFI for both HA and NA.

This is the first assay that is able to subtype all AI antibodies in one single assay using Luminex xMAP technology. Based on this technology, assays have been developed for the detection of antibodies against AI and other avian respiratory viruses [25–27], but without the possibility of discrimination between subtypes. The performance of the multiplex serological assay was compared to the HI assay and NA ELISA, which are used to determine the HA and NA subtype, respectively. Similar results were obtained for 97.8% of the reference sera, and for 90.8% of the field sera. Most results that differed between the multiplex and the serological assay may be explained by cross-reactions with genetically related HA subtypes [22]. Similar cross-reactions were observed in the HI assay. However, for the field sera, we tested samples from individual chickens in the multiplex assay, whereas pooled sera had to be used for testing in the traditional assays. Variation in the infection status of individual chickens may have caused some of these differences.

Possibly, the calculation of the results of the assay can be further optimized to distinguish between reaction and cross-reaction. As earlier described, the cut-off is now specific calculated per sample and defined for HA and NA separately. In assay setups like this, where multiple analysis result in one final result (e.g. subtype), it is difficult to calculate cut-offs, since defined positive and negative samples for each antigen must be available in large quantities. This makes using straightforward rules like 'the average of negatives +3× standard deviation' [28] impractical. However, since usually just one subtype or two subtypes of AIV infect an animal, bead sets with antigens of other subtypes can be used as background of that serum sample. So, this issue was overcome by calculating the cut-off per sample for the two types of antigen (HA and NA) separately, based on the assumption that 40 out of the 45 HA bead sets and 7 out of the 9 NA bead sets will return low signals that match with the absence of antibodies against the corresponding antigens. This is the reason a specific cut-off is calculated for each sample. We are currently investigating the possibility to define a specific cut-off for every bead in the multiplex assay.

This study shows that there are multiple advantages of this novel multiplex serological assay. The detection limit of the multiplex assay proved to be lower than that of the traditional HI assay. The multiplex assay is fast and efficient. The traditionally used ELISA and HI assay are labour-intensive and time consuming, and therefore costly. These assays have to be performed for all HA and NA subtypes separately which requires a large amount of serum, and therefore complete subtyping of the serum is for most samples not feasible. For comparison, subtyping for each antigen with the HI assay and the NA ELISA requires a serum volume of at least 490 µl, whereas only 2 µl of serum is required for complete serotyping with the multiplex serological assay. Also, because a serum can be tested against all antigens simultaneously, it is possible to detect double infections of two different AI viruses in poultry. The subtype is called according to the highest MFI signal. However, careful interpretation of the raw data will allow the detection of other significant MFI signals related to other subtypes. Subsequently, the results can be verified with traditional HI assays. Multiple AI infections in the same poultry flock do not occur very often, but the detection of a double infection is an advantage of the multiplex assay over the HI assay that is normally performed only for H5 and H7 antibodies. This study was performed using sera derived from chickens, however similar results were obtained using sera from other poultry types, such as turkeys, pheasants, guinea fowl, ducks and swans (results not shown). The only disadvantage of the multiplex assay is that the assay requires sophisticated equipment to analyse data, so the start-up costs will be high compared to the HI assay. However, this high start-up costs will be compensated by the multiplex assay being less labour-intensive.

The multiplex assay may be further optimized by coupling all representative HA proteins for one subtype to a single bead set. The multiplex assay now contains 9 bead sets coupled to H5 proteins, coupling all H5 proteins to one single bead set in equimolar concentrations will limit the number of beads required in this assay. A major advantage of the multiplex assay is its flexibility to add novel or remove obsolete HA and NA antigens. The genetic evolution of AI viruses is very rapid in wild birds, which cause changes in antigenicity. In the Netherlands, several outbreaks of HPAI H5 viruses occurred in poultry in recent years. In 2014 [29] and 2016 H5N8 [30] subtypes were introduced, whereas H5N6 was detected in 2017 [31]. Transmission of new variants of the existing AIV subtypes or new subtypes to poultry is unpredictable, but when novel strains are detected, new HA and NA proteins can be included in the assay to screen poultry farms for the presence of this novel virus.

In conclusion, we developed a multiplex serological assay for AI subtyping in poultry sera. This assay has a higher sensitivity than the traditionally used HI assay, and requires a smaller sample volume. Therefore, the assay will allow complete serotyping of different poultry species sera samples, which will improve the monitoring of AI subtypes circulating in poultry in the Netherlands significantly.

5. Declarations of interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ymeth.2019.01.012.

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