



Exploring novel sero-epidemiological tools—Effect of different storage conditions on longitudinal stability of microarray slides comprising influenza A-, measles- and *Streptococcus pneumoniae* antigens



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In this study we evaluated the long-term stability of a microarray-based serological screening platform, containing antigens of influenza A, measles and *Streptococcus pneumoniae*, as part of a preparedness research program aiming to develop assays for syndromic disease detection. Spotted microarray slides were kept at four different storage regimes with varying temperature and humidity conditions. We showed that under the standard storage condition in a temperature-controlled (21 °C) and desiccated environment (0% relative humidity), microarray slides remained stable for at least 22 months without loss of antigen quality, whereas the other three conditions (37 °C, desiccated; Room temperature, non-desiccated; Frozen, desiccated) produced acceptable results for some antigens (influenza A, *S.pneumoniae*), but not for others (measles). We conclude that these arrays for multiplex antibody testing can be prepared and stored for prolonged periods of time, which aids laboratory-preparedness and facilitates sero-epidemiological studies.

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1. Introduction

Infectious diseases can be diagnosed using two types of laboratory tests: [a] pathogen detection, for instance, polymerase chain reaction (PCR), immunoassays or culture, or [b] detection of antibodies against infectious agents, such as, the hemagglutination inhibition assay, microneutralization assay, enzyme-linked immunosorbent assay or Western Blotting. Molecular methods, like PCR, are fast and useful tools, given correct types of samples are collected within an appropriate time from onset of illness. However, with some acute infections e.g. caused by Dengue- (WHO, 2009) or Chikungunya virus (Taubitz et al., 2007), the relatively short period of viremia limits the applicability of virus detection methods as they are usually rapidly cleared in an immunocompetent host.

Serological methods, on the other hand, detect antibody responses triggered by infection, and can thereby provide information on exposure when the agent may no longer be present. Antibody detecting techniques are not only useful to retrospectively confirm infections in individuals when paired sera are available; they also provide important information during outbreak settings. The ability to detect mild or asymptomatic infections allows estimation of attack rates, transmissibility and geographic distribution of a pathogen on a population level, as well as unbiased case fatality rates. In combination with epidemiological and clinical data, these measures are important to guide effective control strategies to contain infectious disease outbreaks (Cauchemez et al., 2012; Kumar and Henrickson 2012; Laurie et al., 2013).

For example, patients with influenza-like illness or acute respiratory infection are identified when referred by physicians. This forms the basis for the global sentinel surveillance systems (Beauté et al., 2012). Whereas such a symptom-dependent system is useful for virological surveillance, it tends to predominantly capture the most severe cases, as patients with mild- or asymptomatic infection are less likely to seek health care (Gibbons et al., 2014). Hence, during infectious disease outbreaks there is a risk that morbidity and

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mortality rates can be biased when only severe cases are included (Ejima et al., 2012; Gibbons et al., 2014).

With the recent influenza pandemic caused by a novel H1N1 subtype in 2009 [A(H1N1)pdm09], the importance of including serological studies into pandemic preparedness planning and the use of standardized serological assays for improved comparability between studies became apparent (Laurie et al., 2013). Serological methods, such as the hemagglutination inhibition- and microneutralization assay were widely used during different stages of the pandemic. However, despite the availability of an international antibody standard, limited awareness thereof precluded its wide use. Another challenge was that laboratory capacity and storage for conducting extensive and high-volume serological studies was insufficient (Laurie et al., 2013).

We previously reported on the development and use of a standardized serological assay termed protein microarray, which is a platform that is able to simultaneously screen for antibodies against multiple influenza hemagglutinin types in humans (Koopmans et al., 2012; Boni et al., 2013; Huijskens et al., 2013; de Bruin et al., 2014), chickens (Freidl et al., 2014) and bats (Freidl et al., 2015). This antibody detection assay was developed as part of an emerging disease preparedness program, and was piloted to monitor the evolution of the A(H1N1)pdm09 in 13 countries (de Bruin et al., 2014). In another study, this technique was used in real-time to assess pre-existing antibody levels to H7 subtypes during the emergence of a novel zoonotic A(H7N9) avian influenza virus subtype in rural and urban locations of Vietnam (Boni et al., 2013). For such large-scale seroepidemiological studies, a high number of spotted microarray slides are required and are ideally stockpiled within the framework of laboratory preparedness for rapid deployment during outbreak situations. The potential use of this technology is conditional on validation of storage conditions. In the current study, we evaluated the long-term stability of viral proteins and bacterial polysaccharides printed onto microarray slides and investigated the influence of four different storage conditions on antigen quality over a period of 22 months.

2. Materials and methods

2.1. Antigen selection and production of microarray slides

We evaluated the stability of recombinant proteins of the HA1 of different influenza virus hemagglutinins, whole inactivated measles virus and capsular polysaccharides of *Streptococcus pneumoniae* spotted onto microarray slides (Table 1). The stability of antigens was evaluated at four different temperature- and humidity conditions as further specified below. Recombinant HA1 proteins were produced in HEK293 cells and purified using HIS-tag purification as described by the manufacturers (Table 1). Antigen H1.09 was validated extensively in serosurveillance studies in humans during the H1N1 influenza virus pandemic of 2009 (Koopmans et al., 2012; Huijskens et al., 2013; de Bruin et al., 2014). Antigens H5.05 and H5.07 were validated for antibody screening of chicken serum samples (Freidl et al., 2014). Measles virus and *Streptococcus pneumoniae* (used as a surrogate for *Streptococcus suis*) antigens are currently being validated for the use in diagnostics and were included to evaluate antigen stability in the microarray platform for future purposes.

Optimal concentration per antigen was determined by checkerboard titration using antisera as shown in Table 1. Influenza and measles viral antigens were diluted in working strength protein arraying buffer (Maine Manufacturing, ME, USA) containing proteinase inhibitor cocktail (BioVision, Mountain View, CA, USA). Bacterial polysaccharides were printed in working strength protein arraying buffer only. All antigens (Table 1) were spotted onto

nitrocellulose-coated glass slides of the same lot number (16-pad, Oncyte Avid, Grace Biolabs, Bend, OR, USA) using a non-contact spotter (Piezotray, Perkin Elmer, Mass., USA). Two microarray batches were produced on the same day using the same reagents and antigens. Each batch consisted of 25 slides, which constituted the maximum capacity per spot run. Immediately after spotting, slides were transferred to a dark plastic box and were stored in a drying chamber to allow optimal protein linkage to the nitrocellulose. All slides were kept in the drying chamber with an average temperature of 21 °C under dark conditions until further use (~3 weeks later). Based on previous experience with short-term storage of spotted slides it is known that antigen quality does not change within three weeks. For quality control prior to the study, we tested one slide per batch at the onset of the study and demonstrated that results with slides from different batches were comparable as overall antibody titers did not differ significantly at baseline (Time point 0, Fig. 2; Wilcoxon rank sum test, p-value = 0.69). Similarly, batches 1 and 2 did not differ over the entire study period (Wilcoxon rank sum test, p-value = 0.92). Calculation of geometric coefficients of variation (GCV) showed comparable variations in titers for both batches (GCV batch 1: 126%, GCV batch 2: 130%).

2.2. Microarray protocol

Microarray slides were essentially tested as described before (Koopmans et al., 2012). Briefly, we first incubated microarray slides with Blotto blocking buffer containing 0.1% Surfact-Amps (both Thermo Fisher Scientific, Rockford, MA, USA), followed by incubation of serum pools (see explanation hereafter) and finally used specific conjugates to visualize bound antibodies. All incubation steps were one hour in duration. Conjugates used were AlexaFluor647 AffiniPure labeled goat-anti-rabbit IgG, and Alexa647 AffiniPure labeled goat-anti-human IgG (both Fc-fragment specific and polyclonal, Jackson Immuno Research, West Grove, USA), at dilutions 1:1300 as determined using checkerboard titration. Following the manufacturer's instructions, we updated both conjugates once during the study period. Before replacing the conjugates (same product from same manufacturer), we tested and verified that old- and new conjugates yielded comparable fluorescence signals at the same dilution (data not shown). After slide analysis, fluorescent signals were quantified using a ScanArray Gx Plus microarray scanner (Perkin Elmer) and sigmoidal fluorescence curves were converted into titers as described previously (Koopmans et al., 2012).

Antisera used for checkerboard titration were used to prepare specific antiserum pools, one containing rabbit antisera (i.e. anti-influenza and anti-*S. pneumoniae*) and another one consisting of human antisera (i.e. anti-measles) (Table 1). After pooling, we prepared twelve aliquots per serum pool which were subsequently stored at -80 °C to keep them stable until further use. At every 2-months interval, we used one aliquot per the serum pool to test the stability slides. From each aliquot we prepared two-fold dilution series in Blotto blocking buffer containing 0.1% Surfact-Amps, starting at a dilution of 1:80 for the rabbit- (anti-influenza A and anti-*S.pneumoniae*), and 1:320 for the human serum pool (anti-measles). For periodic testing, four slides – one per storage condition – were tested simultaneously.

2.3. Storage conditions

All slides were stored under dark conditions. The stability of spotted microarray slides was evaluated under the following four storage conditions:

Table 1

Selection of antigens and antisera used for spotting and testing of microarray slides. Optimal antigen concentrations were determined by checkerboard titration using antisera shown below the respective antigen. Two anti-serum pools (rabbit and human) were prepared for periodic testing of spotted microarray slides. Serum pools were filled out in twelve aliquots and were stored at -80°C until further use.

	Code	Nature of antigen/antiserum	Strain/Serotype	Concentration	Source
Influenza A virus	H1.09	Recombinant protein (HA1 ^a)	A/California/6/2009 (subtype H1N1)	~0.125 mg/ml	Immune Technology Corp., USA
	Anti-H1.09	Polyclonal rabbit serum	A/California/06/2009	~0.250 mg/ml	Immune Technology Corp., USA GenScript USA Inc., USA
	H5.05	Recombinant protein (HA1 ^a)	A/Indonesia/5/2005 (subtype H5N1)		
	Anti-H5.05	Polyclonal rabbit serum	A/Indonesia/5/05	~0.5 mg/ml	Immune Technology Corp., USA Sino Biological Inc., China
H5.07	Recombinant protein (HA1 ^a)	A/duck/Hokkaido/167/2007 (subtype H5N1)			
Measles virus	Measles	Inactivated whole virus	Edmonston strain	Baseline concentration unknown; 2x dilution used	In-house vaccine formulation, RIVM, the Netherlands
	Anti-measles	Pool of measles-seropositive humans			RIVM, surplus of anonymized diagnostic samples
<i>Streptococcus pneumoniae</i>	<i>S. pneumoniae</i>	Purified capsular polysaccharides	Serotype 14	0.5 mg/ml	Statens Serum Institute, Denmark
	Anti- <i>S. pneumoniae</i>	Polyclonal rabbit antiserum (Serotype 14)			Statens Serum Institute, Denmark

^a Head domain of the hemagglutinin receptor.

- Temperature-controlled, desiccated (hereafter abbreviated 'TC.D'): Slides were stored in a desiccation chamber placed in a temperature-controlled room (average temperature 21°C).
- Frozen at -20°C , desiccated (abbreviated 'Frozen'): Slides were stored in individual plastic boxes containing silica sachets and were sealed in plastic bags prior to freezing.
- 37°C , desiccated (abbreviated '37C'): Slides were stored in a dark plastic box containing silica sachets and were stored in an incubator set to 37°C .
- Room temperature, non-desiccated (abbreviated RT.ND). As this was the only storage condition for which temperature and humidity were not controlled, we recorded daily temperature in degrees Celsius (C) and relative humidity (RH) expressed in percentages (Lascar Electronics, Easy Log USB, version 7.2.0.0) over a period of ~16 months (data was logged between October 24th, 2012 and January 16th, 2013, as well as between March 19th, 2013 and February 21st, 2014, respectively).

The above conditions were chosen to evaluate the suitability of the currently employed standard storage condition 'TC.D' and to compare it to potentially alternative long-term storage options ('Frozen'). We furthermore examined the influence of a temperate climate on spotted slides kept at room-temperature without regulation of temperature and humidity (RT.ND), and aimed to study the effect of warm temperature in combination with controlled humidity (37°C) to mimic conditions in countries with warmer climates in which collaborations are ongoing.

2.4. Study set up

As microarray slides were printed in two separate spot runs (two batches) we checked for equal quality of batches by simultaneously testing one slide from each batch. This initial test served as a baseline for subsequent measurements established during twelve time points spaced at two-month intervals, covering a total study period of 22 months (Fig. 1). At each testing time point four slides – one slide per storage condition – were analyzed simultaneously. Spotted microarray slides were randomly allocated to the four storage conditions, in a way that slides from the two batches alternated at every test point.

2.5. Statistical analysis

All statistical analyses were performed in R (language for statistical computing, version 3.1.0, Vienna, Austria). Descriptive analyses were performed using the 'psych' package (Revelle 2015). Line graphs were created using package 'ggplot2' (Wickam, 2009). To compare the variation between the two microarray slide batches we calculated *geometric* coefficients of variation (GVC, expressed as percentages) on natural log-transformed data, due to the underlying log-normal distribution of antibody titers (Limpert et al., 2001). We furthermore examined comparability of the two microarray batches by means of a non-parametric Wilcoxon rank sum test. To account for day-to-day variation and determine whether titer measurements remain within an acceptable range over the duration of the study, we calculated mean titers on log₂ transformed data (equivalent to geometric mean titer, GMT) per antigen and storage condition, and calculated how many measurements transgressed the range of plus/minus one dilution step around the mean. Variation between antibody titer measurements is considered acceptable if it does not exceed a range of plus/minus one dilution step from a central tendency measure, e.g. GMT or median (Ferngren and Granström 1986; Noah et al., 2009; Messmer et al., 2001).

To quantify the effect of long term storage under different storage conditions on titers per respective antigen over time, we used a linear mixed effects model using package 'lme4' (Bates et al., 2015; p.4). We chose this approach as this type of analysis is frequently applied in longitudinal studies that accounts for dependence (repeated measures of the same entity) and potentially unbalanced nature of the data (different number of measurements per storage condition, Fig. 1) (Cheng et al., 2010; Laird and Ware 1982; van Montfort et al., 2010). The package 'multcomp' was used to retrieve 95% confidence intervals (95% CI) for the estimates (Hothorn et al., 2015). To meet the assumption of a normal distribution of residuals, titers were similarly log₂-transformed for this analysis. We entered the predictors 'storage condition' and 'testing time point' as fixed effects combined in an interaction term into the linear mixed effects model to allow for different slopes per storage condition. To account for an observed – possibly seasonal – trend (Fig. 2), we added trigonometric (sine & cosine) functions. To account for the data possibly being clustered by batch and anti-

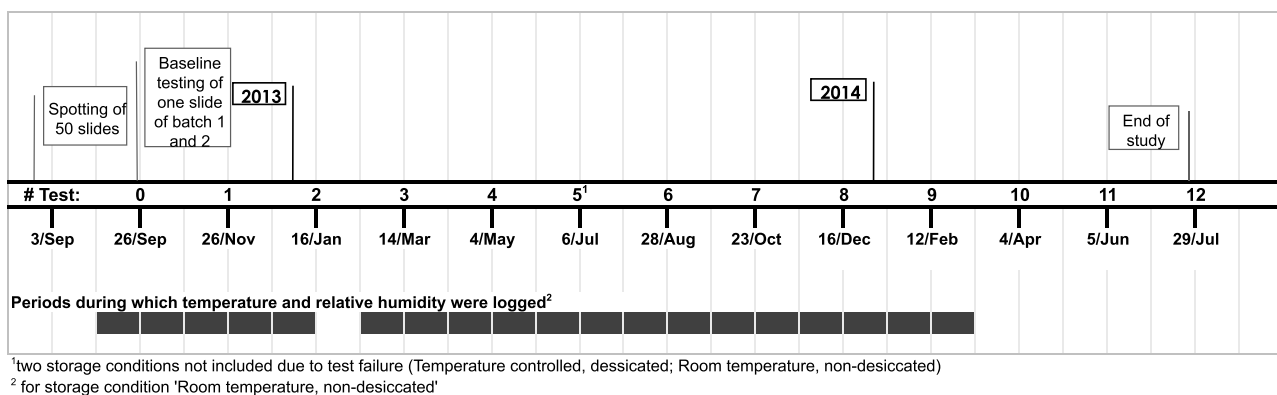


Fig. 1. Experiment timeline. The study period spanned a total of 22 months. Periodic testing of spotted microarray slides was performed at a 2-month interval. Per testing time point, four slides – one slide per storage condition – were tested simultaneously. Temperature and relative humidity were logged (indicated by grey blocks) during part of the study for condition 'Room temperature, non-dessicated'.

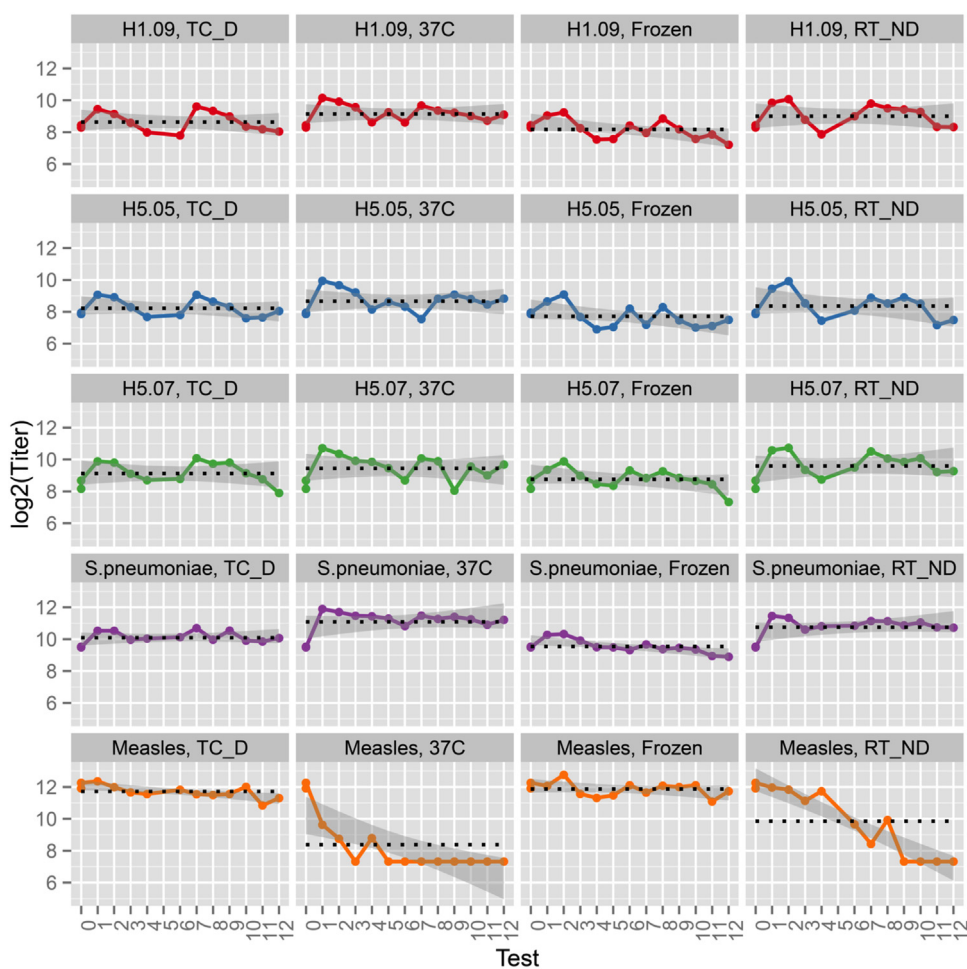


Fig. 2. Stability of viral (influenza A and measles virus)- and bacterial antigens (*S. pneumoniae*) printed onto microarray slides stored at four different temperature- and humidity conditions. Antigen stability was assessed by periodic testing using specific serum pool aliquots at two-month-intervals (x-axis) covering a total study period of 22 months. Log₂-titers are shown on the y-axis. Horizontal panels represent different storage conditions per antigen. Colored lines show titer measurements per antigen and storage condition. Horizontal, dotted black lines correspond to respective geometric mean titers. Measurements were acceptable if they remained within a range of plus/minus one dilution step from the mean log₂-titer. Grey ribbons show a 95% confidence interval around an invisible linear regression line to indicate trends in antigen stability over time. Baseline measurements (Test 0) are combined under one tick.

TC.D: temperature-controlled and dessicated; 37C: 37 °C and dessicated; Frozen: frozen and dessicated; RT.ND: room temperature and non-dessicated.

gen, we included random intercepts for 'batch' and 'antigen', and moreover, random effects for the interaction of time and storing condition per antigen. This allows that for antigen, the slopes

of time can differ for each storing condition. We checked model assumptions by inspecting residual plots, which did not reveal overt deviations from homoscedasticity and normality. P-values

were obtained by means of the likelihood ratio test. A p-value of less than 0.05 was considered statistically significant.

3. Results

3.1. Effect of storage conditions on different antigen classes and longitudinal trends

Antibody titer measurements per storage condition and antigen for the entire study period are presented in Fig. 2. Spotted microarray slides stored at the current standard storage condition 'TC.D' proved the most stable for all antigen classes and showed the least variation over time, compared to other conditions (Figs. 2 and 3). Freezing spotted microarray slides at -20°C yielded the second best results (Figs. 2 and 3).

In contrast, slides stored under conditions 'RT.ND' and ' 37°C ' resulted in higher variation in titers for different antigens (Figs. 2 and 3). While results were reasonable for influenza and *Streptococcus* antigens, the measles antigen was no longer detectable after test time point 5 for 'RT.ND', and test time point 9 for ' 37°C ', respectively. A further observation was that storage conditions affected the titer estimates, again in an antigen dependent manner: microarray slides stored under condition ' 37°C ' generally showed higher mean/median log₂-titers to influenza A- and *S.pneumoniae* antigens, compared to other conditions (Figs. 2 and 3). Conversely, mean/median log₂ titers against these antigens were lowest for frozen slides, whereas this was not observed for the measles antigen (Figs. 2 and 3).

To quantify the titer trends across different storage conditions, we fitted a linear mixed effects model on log₂-titers in which we accounted for the observed seasonal fluctuations (Fig. 2) and used predictors as described in the methods section. To rule out more subtle changes between batches over time we included 'batch' in the model which confirmed results of our initial comparison that no variation between the two microarray batches was observed over time (data not shown). Given the different nature of the antigens, as expected, the model output showed some variation between the different antigen classes (variance: 1.65). Overall, we found a slight decreasing trend on antigen stability for all storage conditions with estimates ranging from -0.012 to -0.065 . The smallest signal decrease over time for all antigens combined was observed for condition 'TC.D' (estimate: -0.012 , 95%CI [-0.049 , 0.024]). Back-transformed to titers, the estimate can be interpreted as follows: every two months (interval between testing time points) for condition 'TC.D', the overall titer for all antigens combined decreased by 0.86%. This further corroborates our observation that spotted microarray slides stored at condition 'TC.D' remain the most stable, irrespective of antigen type. Titer decreases for other conditions were slightly higher but still relatively low, with overall decreases across different antigens of 2.73% for 'RT.ND' [estimate: -0.040 ; 95% CI (-0.253 , 0.173)], 3.18% for ' 37°C ' [estimate: -0.047 ; 95% CI: (-0.338 , 0.245)] and 4.37% for 'Frozen' [estimate: -0.065 ; 95% CI (-0.134 , 0.005)], respectively. When comparing a model including 'storage condition' versus a model without this predictor, 'storage condition' did significantly improve the model (Likelihood Ratio χ^2 -test: 10.4, p-value = 0.0158), confirming that storage conditions have a significant influence on antigen stability. Titer changes over time for individual antigens, expressed as percentage increase or decrease, differed markedly between the different storage conditions (Table 2).

3.2. Titer fluctuations due to seasonal and day-to-day variation

Temperature and relative humidity measured at condition 'RT.ND' showed fluctuations concordant with changing seasons and

ranged from 16 to 26°C (median 21.5°C) and 36–68% RH (median 47% RH), respectively. Overall, temperature and humidity did not seem to have a large influence on antigen stability, as relative to each other, titers against different antigens were largely clustered across the different storage conditions and showed a comparable trend over the entire study period (Fig. 2). When applying the conservative quality

score of less than one log₂-dilution step deviation from the mean for acceptance of the results, likewise, storage condition 'TC.D' yielded the best result for all antigens. For this condition, only one measurement of one antigen, influenza H5.07, was rejected (time point 12; Table 3). For condition 'Frozen', all measurements against *S. pneumoniae*- and measles virus antigens remained within the acceptable titer range throughout the study period, but some ($n=4$) of the influenza antibody titer estimates fluctuated more than the accepted norm. For storage conditions ' 37°C ' and 'RT.ND', the number of measurements deviating one dilution step from the mean was higher for all antigen classes (Table 3). For these two conditions, means for the measles antigen and deviations of one dilution step from it were calculated using only titer measurements of testing time points 0–4 and 0–8, respectively. Time points thereafter were excluded due to antigen degradation (37°C : test 5–12, RT.D: test 9–12, respectively).

4. Discussion

In this study, we assessed the stability of spotted microarray slides containing different antigen classes under four storage conditions with varying temperature- and humidity settings over a total period of 22 months. A broad range of antigens, varying from crude lysates of infected cells or microbial lysates to highly purified microbial components (e.g. expressed proteins and bacterial polysaccharides) are routinely used for serological assays, e.g. ELISA, hemagglutination inhibition assay, Western Blot, as well as multiplex techniques (protein microarrays and bead-based platforms) (Tang and Stratton, 2013; Balmer et al., 2007). Antigen stability can be influenced by the composition of the antigen, and by various chemical and physical factors, such as temperature, pH, oxidation, salt concentration, freeze-thaw cycles, proteases, mechanical destruction as well as contact with solvents or contamination with bacteria.

In our study, slides kept in a temperature-controlled and desiccated environment showed less overall variation than slides stored at 37°C or slides stored at room temperature and uncontrolled humidity. This is in contrast to the recommendations by the manufacturer to store slides at room temperature before and after spotting. However, the use of a desiccant during the storage period was not recommended as it supposedly could negatively influence results (GraceBioLabs, 2012). Humidity is known to negatively affect protein properties (Hageman 1988), however, this effect seemed to have a larger impact on measles and *Streptococcus* compared to influenza virus antigens.

On the other hand, influenza virus antigens showed slightly more variation when kept frozen and desiccated compared to the other storage conditions and antigens. Previous reports showed that proteins linked to nitrocellulose slides remained stable for at least 3 years when kept under dry conditions at -20°C (Espina et al., 2011). It is conceivable that freeze-thawing might negatively affect the stability of influenza antigens to a larger – but still moderate – extent compared to other antigens (Cao et al., 2003). However, as these effects cannot be directly compared given the different nature of antigens (influenza: hemagglutinin part 1; measles: whole inactivated virus; *Streptococcus*: capsular polysaccharides) and the differences in methods used for antigen preparation and purification (influenza: 6xHIS-tag; measles: discontinuous sucrose

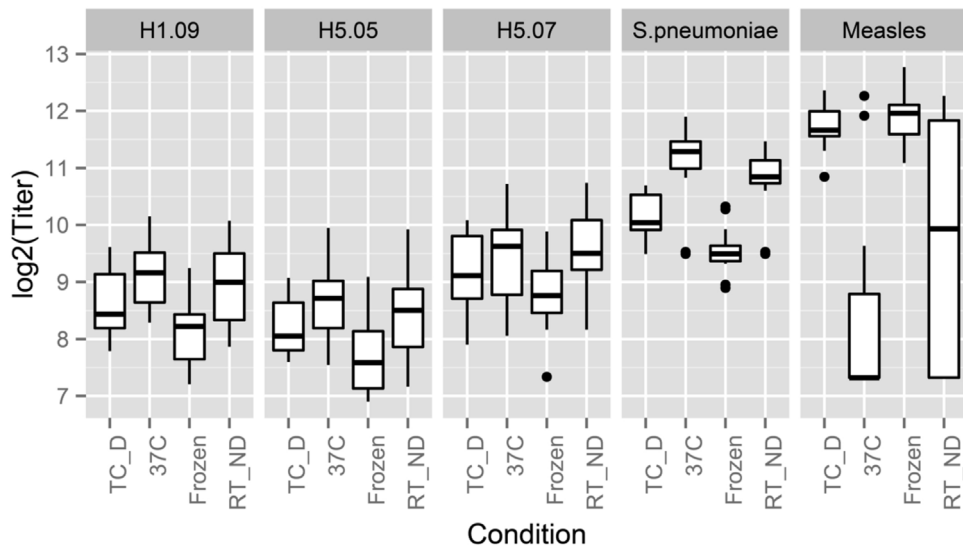


Fig. 3. Boxplots showing distributions of log₂-titers (y-axis) against different storage conditions (x-axis) presented per antigen (panel) over the entire study period. Titers to *S. pneumoniae* antigens showed the least variation for all storage conditions (log₂-titer ranges: 1.2–2.41). Within the three influenza A antigens (H1.09, H5.05, H5.07) we found comparable titer distributions across the four storage conditions, with the lowest variation observed for condition 'TC.D' (ranges: 1.47–2.18) and the highest for 'RT.ND' (log₂-titer ranges for H1.09: 2.2; H5.05: 2.75) and 37 °C (H5.07: 2.66), respectively. With exception of conditions '37C' and 'RT.ND' which had a detrimental effect on antigen stability, variation in log₂-titers against the measles antigen was low.

TC.D: temperature-controlled and desiccated; 37C: 37 °C and desiccated; Frozen: frozen and desiccated; RT.ND: room temperature and non-desiccated.

Table 2
Percent titer change (increase or decrease) per antigen and storage condition for one unit change in testing time points, i.e. between two testing time points (2 months apart).

Antigen	Storage conditions			
	TC_D	37°C	Frozen	RT_ND
H1.09	-1.3	3.6	-6.4	1.7
H5.05	-1.7	2.2	-7.3	0.2
H5.07	-0.9	3.0	-5.3	1.8
<i>S. pneumoniae</i>	0.02	9.2	-3.4	7.4
Measles	-0.4	-28.6	0.6	-21.8

TC.D, temperature-controlled and desiccated; 37 °C, 37 ° Celsius and desiccated; Frozen, frozen and desiccated; RT.ND, room-temperature and non-desiccated; Color legend indicating magnitude in titer changes: green: ≤5%, orange: >5 and <10%, red: ≥10%.

Table 3
Number of data points not passing the quality score of deviating less than one dilution step above (>1) or below (<1) the geometric mean titer per storage condition and antigen over time.

Storage condition	Antigen	<1	>1	Total n
Temperature-controlled, desiccated	H1.09	0	0	1
	H5.05	0	0	
	H5.07	1	0	
	<i>S.pneumoniae</i>	0	0	
	Measles	0	0	
Room temperature, non-desiccated	H1.09	1	1	13
	H5.05	1	2	
	H5.07	1	1	
	<i>S.pneumoniae</i>	2	0	
	Measles	3	1	
Frozen	H1.09	0	1	4
	H5.05	0	1	
	H5.07	1	1	
	<i>S.pneumoniae</i>	0	0	
	Measles	0	0	
37 °C	H1.09	0	1	13
	H5.05	1	2	
	H5.07	2	1	
	<i>S.pneumoniae</i>	2	0	
	Measles	2	2	

gradient 20/40/70%; *Streptococcus*: highly purified but exact purification technique not stated by manufacturer), these differences need to be investigated in future research. Similarly, the extent to which the different storage conditions affect the nitrocellulose itself, i.e. the assay background, should be studied further.

Our data also shows that it is important to validate these findings for different antigens types. In this study, the most detrimental effects on antigen quality were observed for the measles antigen, which consisted of crude cell lysate, whereas the recombinant proteins used gave more consistent results throughout the study period. The instability of the measles antigen under the conditions '37°C' and 'RT_ND' showed that this particular antigen would be less suitable for studies in warmer climates, unless stored appropriately. These findings highlight that it is paramount to evaluate the stability and performance of a diagnostic assay under extreme temperature and humidity conditions, if the test is to be deployed in such settings; for instance in tropical countries (Banoo et al., 2008).

We took care to keep variation due to external factors, e.g. operator-induced, different lots of reagents etc., to a minimum. To avoid batch effects, ideally the same batches of assays are used throughout the entire study period. However, particularly in large-scale studies, that is not always feasible and batch effects can have a significant impact on the outcome (Leek et al., 2010) and therefore we compared results for different batches prior to their use. We performed this work as part of a project for improved avian influenza surveillance. There is a strong push for enhanced standardization of serological testing after comparing reproducibility of serological assays for diagnosis of the pandemic A(H1N1)pdm09 which – without using a standard – revealed high inter-laboratory variation; median GCVs ranged from 95 to 345% for hemagglutination inhibition-, and 204–383% for virus neutralization assay, i.e. a 80- and 109-fold difference, respectively (Wood et al., 2012). The microarray described here constitutes an assay with a long shelf life that also allows standardization.

5. Conclusion

In conclusion, this study showed that spotted microarray slides containing influenza, measles and streptococcus antigens can be stored up to 22 months without quality loss when kept in a dark, desiccated and temperature-controlled environment. We also found good results at conditions compatible with situation in developing countries, although this needs to be assessed for each antigen separately. Our findings are assuring for large-scale studies, as this property allows production and stockpiling of multiple batches within a short period of time using the same reagents, thereby keeping variation between batches to a minimum.

Conflict of interest

None declared.

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