High-throughput multiplex immunoassay for the detection of mpox and MVA-BN vaccination up to 2 years after exposure in Belgium: a retrospective diagnostic accuracy study

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Summary

Background Mpox, caused by monkeypox virus (MPXV), has gained global attention following several international outbreaks. Accurate and high-throughput detection of MPXV-specific antibodies is essential for surveillance programmes, diagnosis, and vaccine trials. We therefore evaluated the long-term accuracy of an mpox multiplex immunoassay that measures IgG responses to 11 orthopoxvirus antigens.

Methods The assay was validated in a diagnostic accuracy study involving a Belgian cohort of clade IIb MPXV-infected individuals (n=211) and modified vaccinia virus Ankara-Bavarian Nordic (MVA-BN) vaccine recipients (n=202), who were followed up for 2 years after exposure. We used receiver operating characteristic analysis to assess diagnostic performance at multiple timepoints after infection or vaccination compared with negative controls (n=287). To explore the assay's broader utility, we also investigated correlations between antigen-specific IgG responses and neutralising antibody titres.

Findings In individuals without previous smallpox vaccination (n=149), the assay accurately detected mpox infection up to 2 years after infection, achieving 92% sensitivity (95% CI 89–97) at 95% specificity (91–98) when multiple antigens were combined. By contrast, diagnostic performance was low in previously vaccinated individuals (n=62), with sensitivities of most antigens dropping below 60% (38–77) after 6 months. Most antigen-specific IgG responses correlated strongly with neutralising antibody titres.

Interpretation The assay shows high sensitivity and specificity for detecting previous mpox infection in unvaccinated individuals, with reliable performance up to 2 years after infection. Strong concordance with neutralising antibody titres supports the assay's potential for monitoring long-term infection and vaccine-induced immunity. However, previous vaccination status should be considered when interpreting results.

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Introduction

Mpox, formerly known as monkeypox, is a zoonotic disease caused by monkeypox virus (MPXV), a member of the genus *Orthopoxvirus*.[1] Two clades are historically known to cause disease: clade I, originating in central Africa, and clade II, originating in west Africa.[2] In 2022, subclade IIb spread efficiently through worldwide sexual networks, leading to a global outbreak with continued circulation to date.[3] In response to the 2022 outbreak, targeted campaigns using the modified vaccinia virus, Ankara-Bavarian Nordic (MVA-BN) vaccine, were implemented in at-risk communities worldwide.[4,5] Despite vaccination campaigns, mpox continues to circulate globally, with the highest burden reported in central and west Africa.[6,7] Mpox therefore remains a global public health concern for which improved diagnostic and surveillance capabilities are highly needed.[8–11]

Traditional serological assays have several limitations when used for mpox diagnostics.[8] Virus neutralisation tests, although regarded as the gold standard for vaccine studies, are expensive, time-consuming, and require specialised laboratory infrastructure.[8,12] ELISA offers a more affordable and high-throughput option. However, this type of assay assesses only one analyte (whole pathogen or single antigen) at once, making ruling out cross-reactivity with antibodies against other pathogens difficult.[13,14] High accuracy is especially crucial in low-incidence settings where false-positive results can significantly bias surveillance outcomes.[10,11,15] Multiplex immunoassays have emerged as powerful

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Research in context

Evidence before this study

The global emergence of monkeypox virus (MPXV), particularly following the 2022 clade IIb outbreak and the emergence of novel clade I lineages in 2023 and 2024, has underscored the urgent need for accurate serological tools to support diagnostics, surveillance, and vaccine evaluation. In response, several high-throughput multiplex immunoassays have recently been developed to measure IgG responses to various orthopoxvirus antigens. These assays have generally shown good sensitivity and specificity and the potential to distinguish between infection-induced and vaccine-induced immunity. However, most studies were constrained by small sample sizes and short follow-up durations, typically no longer than 1 year. Concurrently, other studies have reported a rapid decline in IgG and neutralising antibody titres following modified vaccinia virus Ankara-Bavarian Nordic (MVA-BN) vaccination, raising concerns about the long-term reliability of immune markers used in serodiagnostic applications. We searched PubMed from database inception without language restrictions for studies evaluating the development of multiplex serological assays for mpox from Jan 5, 2022, to Feb 1, 2025, using the search terms ("mpox" OR "monkeypox") AND ("serology" OR "immunoassay") AND ("multiplex" OR "Luminex"). We identified three studies conducted before or shortly after the onset of the global mpox outbreak in 2022. One study, published before the epidemic, evaluated antibody responses against three MPXV-specific peptides. The two subsequent studies expanded on this work by incorporating the same three peptides along with additional antigens. We also identified five publications, all published between 2023 and 2025, which evaluated broader panels of orthopoxvirus antigens using multiplex approaches. Among these, a 2025 study described the development and validation of MpoxPlex, a multiplex-based assay targeting 11 orthopoxvirus antigens. Our current study builds directly on that work, retaining ten of the original 11 antigens and replacing one MPXV antigen to enhance diagnostic coverage.

Added value of this study

Multiplex serological assays developed for MPXV diagnostics show high sensitivity and specificity in detecting IgG antibodies against multiple orthopoxvirus antigens. However, all these studies had a short validation period—typically not exceeding 1 year after infection or vaccination—and analysed samples from a small number of naturally infected and vaccinated individuals. Using a unique cohort of individuals infected with mpox (n=211) and MVA-BN vaccine recipients (n=202), we were able to follow participants for up to 2 years after exposure and evaluate the diagnostic accuracy for a high-throughput multiplex immunoassay using 11 orthopoxvirus antigens, with findings that differ from previous studies.

Implications of all the available evidence

Together with existing evidence, our findings highlight the potential of multiplex immunoassays to provide accurate, high-throughput tools for long-term serological detection of mpox infection. The ability to detect past MPXV infection up to 2 years after exposure makes this approach suitable for seroepidemiological studies. In addition, the strong correlation between antigen-specific IgG responses and neutralising antibody titres suggests that multiplex assays might serve as practical alternatives to labour-intensive neutralisation tests, especially in resource-limited settings. However, our results also show that several of the original antigens suggested in previous studies do not have the sensitivity at later timepoints for serodiagnosis. Furthermore, the declining diagnostic performance in individuals with previous smallpox vaccination and the limited ability to distinguish natural infection from MVA-BN vaccination at later timepoints underscore the need for assay refinement. Future research should focus on identifying more specific antigens or immune signatures capable of discriminating between these exposures over longer timeframes.

alternatives, enabling improved accuracy by integrating responses across targets. This approach could be particularly valuable when attempting to differentiate infection-induced from vaccine-induced antibody profiles, because it uses subtle variations across multiple immune targets rather than relying on any single antigen.[9–11,16,17]

Jones and colleagues[16] developed MpoxPlex, a Luminex-based serological assay designed for mpox diagnostics. This assay distinguishes between infection and MVA-BN vaccine-induced seropositivity, showing high sensitivity and specificity in detecting IgG antibodies against multiple antigens. Antigen selection was based on previous evidence of immunogenicity and included MPXV-specific and shared orthopoxvirus antigens. The study had a short validation period, analysing samples from a small number of MPXV-infected individuals (up to 81 days) and vaccinated individuals (up to 240 days). Because several studies have shown a substantial decline in IgG and neutralising antibody concentrations as early as 1 year after MVA-BN vaccination,[12,18,19] validating the assay's diagnostic performance across later timepoints is essential to ensure its suitability for long-term serosurveillance.

We hypothesised that serological assay performance of MpoxPlex diminishes over time. To test this hypothesis, we applied the MpoxPlex multiplex immunoassay—retaining ten of the original 11 antigens and adding B16R—to samples from a large long-term Belgian cohort of MVA-BN-vaccinated and MPXV-infected individuals.[20] We also assessed the assay's ability to distinguish infection from vaccination.

Methods

Study design and participants

We conducted a retrospective diagnostic accuracy study using samples from a prospective cohort of mpox patients and MVA-BN vaccine recipients enrolled at the Institute of Tropical Medicine (ITM; Antwerp, Belgium; ClinicalTrials.gov NCT05879965).[20] Belgium introduced the MVA-BN vaccine in August, 2022.[21] Guidelines recommended two vaccination doses with an interval of 4 weeks or one dose in those vaccinated during childhood with a replicating vaccinia virus (VACV)-based vaccine against smallpox. Although smallpox vaccination campaigns in Belgium continued until 1976, many individuals born in or before 1976 still received two doses of MVA-BN.

Samples were obtained from 211 individuals diagnosed with clade IIb MPXV, who were enrolled in a 2-year follow-up study starting on May 13, 2022 (table). Of these, 149 individuals had not received childhood smallpox vaccination, whereas 62 were born in or before 1976 and had presumably been vaccinated during childhood. We also included samples from 202 individuals who received the MVA-BN vaccine in 2022 and were followed up during visits at similar timepoints after their last vaccine dose. Of these, 126 individuals had not received childhood smallpox vaccination, whereas 76 were born in or before 1976 and had presumably been vaccinated during childhood. Vaccine recipients were eligible if they received two doses of MVA-BN at least 28 days apart, except for those with previous childhood smallpox vaccination, for whom one or two doses sufficed. Participants diagnosed with mpox who also received the MVA-BN vaccine were excluded from the analyses.

All participants in the prospective mpox and vaccine cohorts provided written informed consent before any study procedures. Individuals included in the retrospective arm of the mpox cohort were enrolled on the basis of our clinic's standard policy, provided they had not opted out of the use of their data and samples for research purposes. Ethical approvals were granted by the institutional review board of the ITM (references 1628/22, 1641/22, and 34/2023) and the ethics committee of the University Hospital of Antwerp (references 3797 and 4981). The study was conducted in accordance with the Declaration of Helsinki and adhered to the latest Good Clinical Practice guidelines.

Procedures

Baseline data on demographics, sexual orientation, and HIV status were collected prospectively at diagnosis or first vaccination or retrospectively from medical records. Serum was collected in serum separator tubes at diagnosis or vaccination and during follow-up visits at 0–0·5, 0·5–6, 7–10, 15–20, and 23–27 months after diagnosis or vaccination. In addition to the cohort of mpox and MVA-BN vaccinees, we included negative control samples for assay validation. We used a combination of 184 samples from cohort participants (samples remaining from routine care before MPXV infection and MVA-BN vaccination) and 103 samples from non-cohort participants who attended the ITM clinic for unrelated reasons before May, 2022 (ie, before the global mpox outbreak). In total, 114 samples were from individuals born in or before 1976, and 173 samples were from individuals born after 1976 (appendix p 3). The samples were presumed unexposed to orthopoxviruses given the eradication of smallpox in Belgium by 1950, the extreme rarity of cowpox infections, and the absence of reported mpox cases in Belgium before 2022.[22]

See Online for appendix

	Exposure status	Time post-immunising event (months)					Total participants (n)
		0–0·5	0·5–6	7–10	15–20	23–27	
Born in or before 1976	Infected	33	62	27	43	17	62
	Vaccinated	2	2	73	52	54	76
Born after 1976	Infected	103	150	74	99	45	149
	Vaccinated	9	6	125	93	86	126

Data are n. Low sample sizes in the early post-vaccination phase reflect the retrospective recruitment of vaccinees after the initial mass vaccination campaign had concluded. A small subset of individuals with MPXV contributed more than one sample within the same interval (collected on different dates). MPXV=monkeypox virus. MVA-BN=modified vaccinia virus Ankara-Bavarian Nordic.

Table: **Overview of tested samples in cohorts of individuals with MPXV infection and participants with MVA-BN-vaccination, assessed at various months post-infection or vaccination**

Luminex multianalyte IgG immunoassay

Building on the methodology described by Jones and colleagues,[16] we used commercially available recombinant proteins derived from MPXV and VACV, including VACV A33R and B5R and MPXV A35R, H3L, B2R, A5L, A27L, B6R, E8L, B22R.64/65, and B16R (details and suppliers listed in appendix p 4) for the Luminex multianalyte IgG immunoassay development. Unlike the original MpoxPlex assay, we replaced the M1R antigen with B16R. M1R yielded weak reactivity in samples from infected patients with high anti-mpox antibody concentrations, despite being sourced twice (similar issues were noted previously[10]), whereas B16R showed strong performance in a previous study[23] and proved more robust in our assay. Antigen coupling was done on paramagnetic MAGPLEX COOH-microspheres (Luminex Corporation, Austin, TX, USA) following established protocols.[11,24] Each antigen was coupled at a concentration of 4 µg per 1×10^6 beads. Bovine serum albumin (BSA; Sigma-Aldrich, St Louis, MO, USA; 3 µg) and tetanus toxoid (EMD Millipore #582231-25UG, Burlington, MA, USA) were coupled separately to additional bead sets to serve as background controls.

Beads and serum samples were diluted in phosphate-buffered saline (PBS) containing 1% BSA and 0·05% sodium azide (final serum dilution 1:100) and added to each well, resulting in a total volume of 50 µL per well. Plates were incubated at 37°C on a shaker for 30 min, then washed three times with 200 µL per well PBS containing 0·05% Tween 20 and 1% BSA (PBS-TBN). Subsequently, 50 µL of secondary antibody (1:500, R-phycoerythrin-conjugated AffiniPure F[ab′]2 fragment of goat anti-human IgG, Jackson ImmunoResearch Laboratories, West Grove, PA,

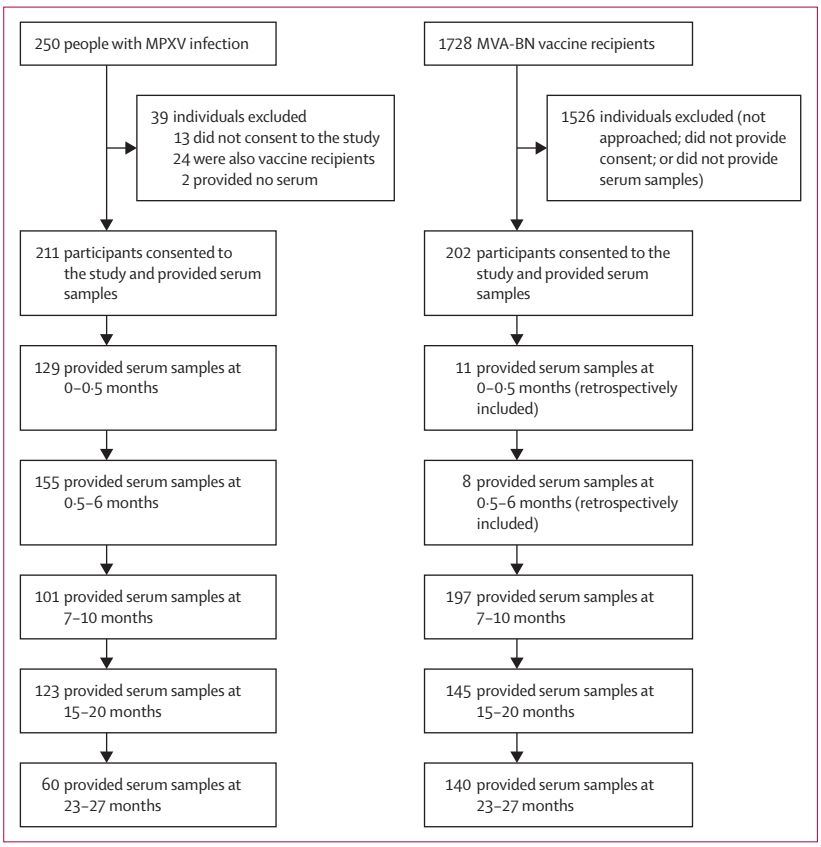

Figure 1: **Participant flowchart of infected or vaccinated individuals included in the study**
MPXV=monkeypox virus. MVA-BN=modified vaccinia Ankara-Bavarian Nordic.

USA) in PBS-TBN was added. The plates were incubated for an additional 30 min at 37°C on a shaker set to 600 rpm.

Data acquisition was done on a Luminex 200 analyser (Austin, TX, USA), measuring an acquisition volume of 50 µL per well, with double discriminator gate settings of 5000–25 000 and the high photomultiplier tube option enabled. Samples with abnormal high background against BSA-coupled beads (defined as >500 MFI) or abnormal low signals against tetanus toxoid-coated beads (defined as <500 MFI) were excluded from further analysis (n=12).

In addition to the Luminex analyses, all serum samples were tested for VACV-specific IgG binding antibodies with an in-house ELISA based on VACV-Elstree-infected cell lysate, as previously described.[12] 30% endpoint titres were calculated by transforming the net optical density at 450 nm responses per sample dilution series to the S-curve of a positive reference serum pool. If no binding was detected, a 30% endpoint titre of 10 was assigned (lower limit of detection). Additionally, a random subset of samples from mpox-infected patients (n=105, stratified over the predefined timepoints after infection) was further assessed using an in-house MPXV clade IIb-based plaque reduction neutralisation test (PRNT) following established protocols.[12]

Outcomes

The primary outcome was diagnostic accuracy of mpox antigens over time among individuals with varying orthopoxvirus vaccination histories. Secondary outcomes were correlations between antigen-specific IgG responses measured by the Luminex assay, IgG reactivity to whole viral lysate by ELISA, and neutralising antibody titres. Participants were divided into subgroups according to exposure type (infection *vs* vaccination) and age. We stratified by age to account for previous childhood vaccination. Participants were divided into a group born in or before 1976 and a group born after 1976, the year when routine vaccination with replication-competent VACV was discontinued in Belgium. No formal power analysis was done for this study, because we included the maximum number of participants that were recruited at the institute. Missing data resulting from participant attrition were not included, because analyses were based on the number of participants with available data at each follow-up timepoint.

Statistical analysis

We first assessed the predictive value of each antigen individually by generating receiver operating characteristic (ROC) curves and calculating the corresponding area under the curve (AUC) values. For each subgroup, the results obtained from either MPXV-infected individuals or MVA-BN vaccinated individuals were compared with each other or with negative controls from the corresponding age cohort. As a result, for each antigen, five comparisons were done: (1) MPXV-infected individuals versus negative controls born after 1976; (2) MPXV-infected individuals versus negative controls born in or before 1976; (3) MVA-BN-vaccinated individuals versus negative controls born after 1976; (4) MVA-BN-vaccinated individuals versus negative controls born in or before 1976; and (5) MPXV-infected individuals versus MVA-BN-vaccinated individuals in both age cohorts. Because individuals born in or before 1976 are presumed to be vaccinated against smallpox during childhood, comparing MPXV-infected individuals with negative controls born in or before 1976 allowed us to also assess the assay's ability to distinguish natural infection from smallpox vaccine-induced immunity.

Subsequently, we assessed whether combining antigens could enhance diagnostic accuracy. We used a supervised machine learning approach (random forest; randomForest in R version 4.4.2; details in appendix pp 1–2). The classifier maps the multidimensional antigen median fluorescence intensity (MFI) profile to a probability of belonging to the positive class, which we convert to a binary outcome. Models were trained on 70% of the data and validated on the remaining 30% using 5000 iterations, with classification performance evaluated using both accuracy metrics (mean decrease in accuracy and Gini) and ROC curve analysis.

Furthermore, to identify which antigens correlated most strongly with neutralising antibody concentrations, we correlated the MFI values of the different Luminex antigens with 50% reduction of plaques in PRNT ($PRNT_{50}$) values

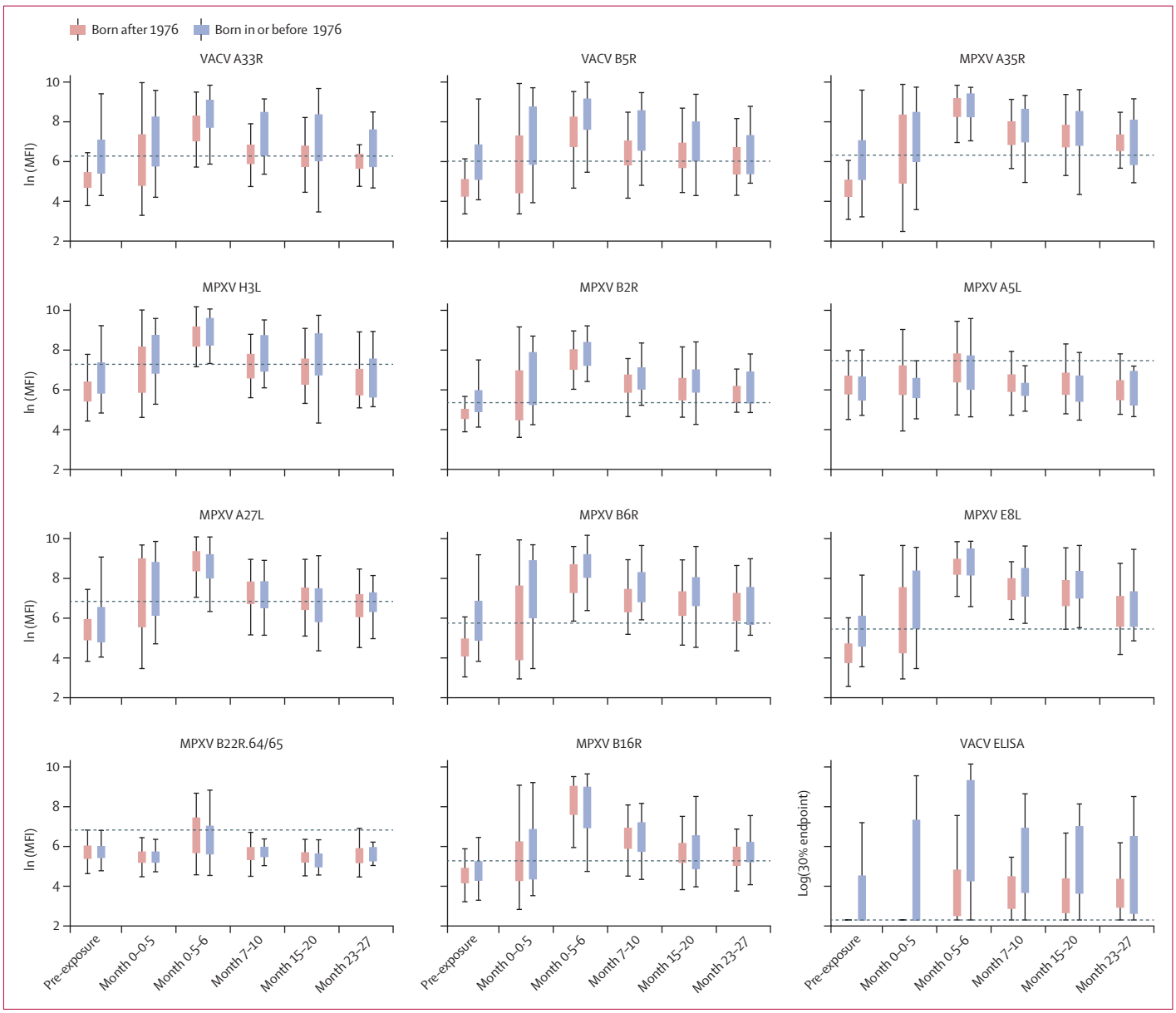

Figure 2: **Distribution of IgG titres in individuals infected with MPXV over time since symptom onset, as measured by the Luminex assay (natural log MFI values) and VACV ELISA (natural log 30% endpoint titres)**

The blue dotted horizontal line represents the 95% specificity cutoff, established using 173 pre-2022 samples from naive individuals born after 1976. Colours reflect participants' birth year, inferring previous smallpox vaccination status (blue=born in or before 1976; red=born after 1976). Boxes show the IQR (Q1–Q3). Whiskers extend to the most extreme observations within 1·5 × IQR below Q1 and above Q3 (Tukey whiskers). E8L and VACV ELISA results are also presented in Van Dijck and colleagues.[20] MFI=median fluorescence intensity. MPXV=monkeypox virus. VACV=vaccinia virus.

using Spearman's correlation with the corrplot package in R version 4.4.2. For this analysis, we included all samples from MPXV-infected participants across all time-points to capture both high and low titres, and we excluded MVA-BN-vaccinated individuals.

Role of the funding source

The funders of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report.

Results

By Jan 1, 2025, 211 people with MPXV infection and 202 MVA-BN vaccine recipients were assessed at least once for the presence of anti-mpox IgG antibodies (table, figure 1). We refer to the appendix (p 3) for detailed participant characteristics of this cohort (age, sex, and comorbidities).

IgG concentrations against all antigens decreased over the 2-year study period, both in individuals with MPXV infection (figure 2) and MVA-BN vaccine recipients (figure 3). For all antigens, baseline (pre-exposure) antibody

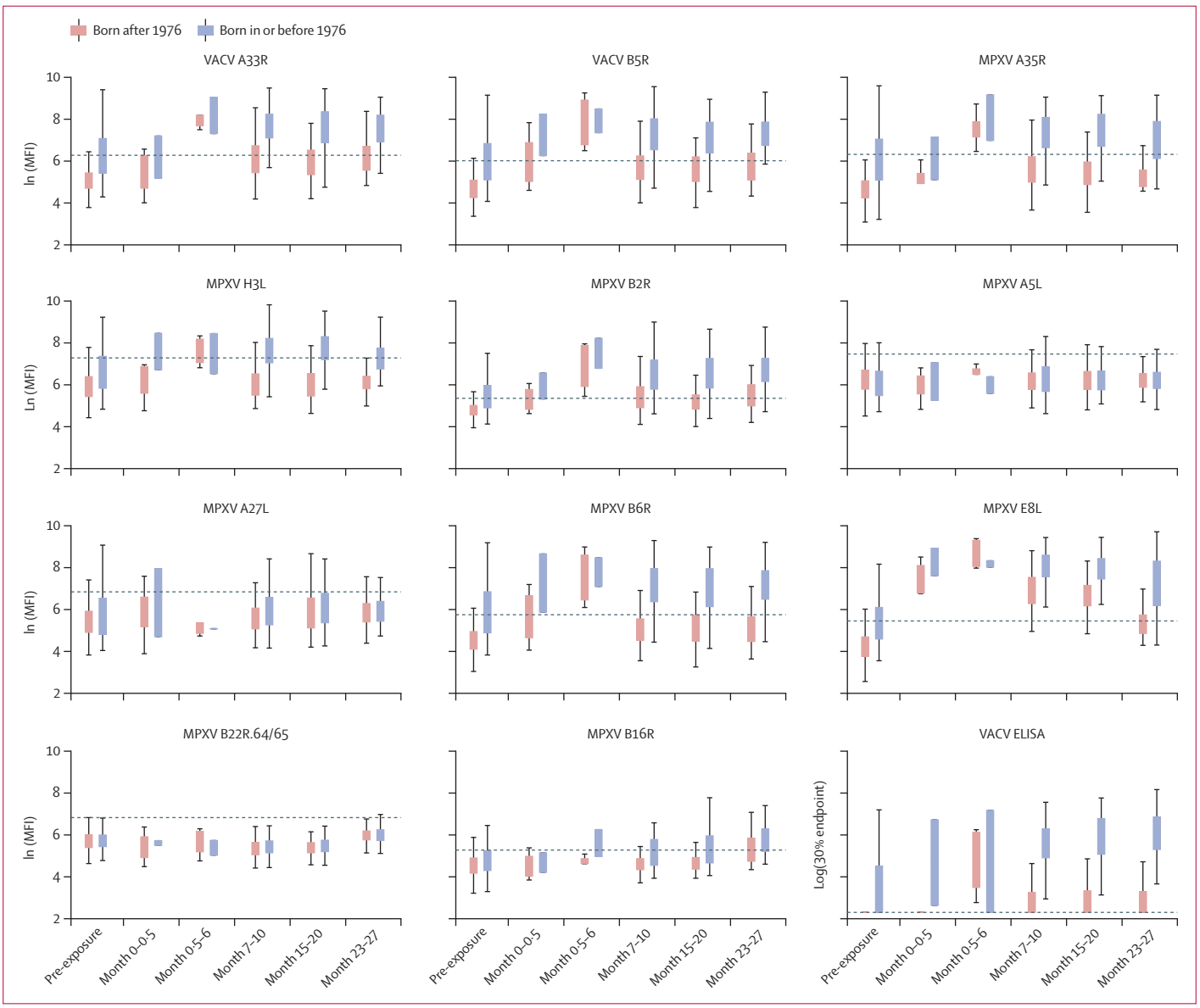

***Figure 3*: Distribution of IgG titres in individuals vaccinated with MVA-BN over time since last vaccine dose, as measured by the Luminex assay (natural log MFI values) and VACV ELISA (natural log 30% endpoint titres)**

The blue dotted horizontal line represents the 95% specificity cutoff, established using 173 pre-2022 samples from naive individuals born after 1976. Colours reflect participants' birth year, inferring previous smallpox vaccination status (blue=born in or before 1976; red=born after 1976). Boxes show the IQR (Q1–Q3). Whiskers extend to the most extreme observations within 1·5 × IQR below Q1 and above Q3 (Tukey whiskers). E8L and VACV ELISA results are also presented in Van Dijck and colleagues.[20] MFI=median fluorescence intensity. MPXV=monkeypox virus. MVA-BN=modified vaccinia Ankara-Bavarian Nordic. VACV=vaccinia virus.

titres were higher among participants born in or before 1976 than in participants born after 1976. Different patterns emerged in the five subgroups. In individuals with MPXV infection who did not receive childhood vaccination (born after 1976), six antigens—VACV B5R, MPXV A35R, B2R, B6R, E8L, and B16R—reliably detected IgG antibodies from 2 weeks up to 20 months after infection. When compared with negative controls from the same age cohort, these antigens yielded AUC values exceeding 0·85 and showed sensitivities of more than 60% (95% CI 49–69) at 95% specificity (91–98; figure 4; appendix p 5). MPXV antigens A35R, B2R, B6R, and E8L also maintained excellent diagnostic accuracy, with AUC values consistently above 0·94 and sensitivities exceeding 80% (95% CI 64–90) even 2 years after infection, underscoring their potential for reliable long-term serological surveillance. By contrast, antigens such as A27L, H3L, and VACV A33R showed a more rapid decline in performance over time, whereas B22R.64/65 and A5L showed overall low diagnostic performance.

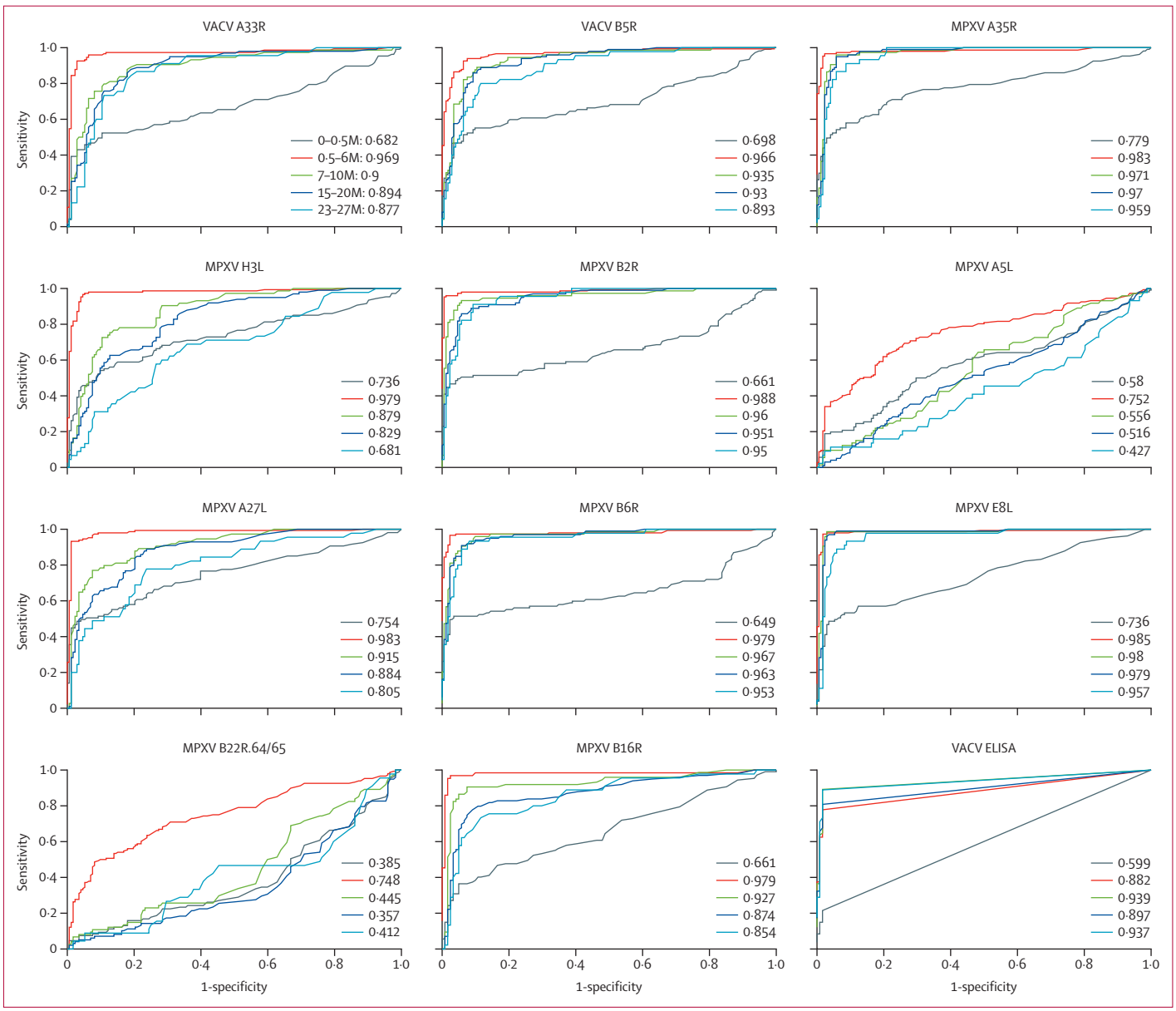

Figure 4: **Receiver operating characteristic curves for different antigens of the IgG Luminex assay and VACV ELISA, comparing samples from individuals infected with MPXV with negative control samples from individuals born after 1976 (smallpox vaccine-naive)**

Different colours represent different time periods after symptom onset (black=0–0·5, red=0·5–6, green=7–10, dark blue=15–20, and light blue=23–27 months). Area under the curve values are presented in the legend. MPXV=monkeypox virus. VACV=vaccinia virus.

In individuals with MPXV infection and previous smallpox vaccination during childhood (born in or before 1976), the antigens showed reduced discriminatory power for detecting past mpox infection when compared with negative controls from the same age group (figure 5; appendix p 5). Although most antigens performed reasonably well up to 6 months after infection, their AUC values dropped, with sensitivity of less than 60% (95% CI 38–77) at later timepoints. The E8L antigen was an exception, with an AUC value of 0·89 and sensitivity of more than 60% (95% CI 42–72) up to 20 months after infection at 90% specificity (95% CI 82–94).

In MVA-BN-vaccinated individuals without previous smallpox vaccination (born after 1976), IgG titres for nearly all antigens declined below the assay's 95% specificity cutoff by 7–10 months after vaccination (figure 3). This decline was accompanied by a drop in AUC values below 0·85 and corresponding sensitivities falling below 60% (95% CI 51–68; appendix p 5; appendix p 6). Again, the E8L antigen stood out, maintaining AUC values of 0·97 at

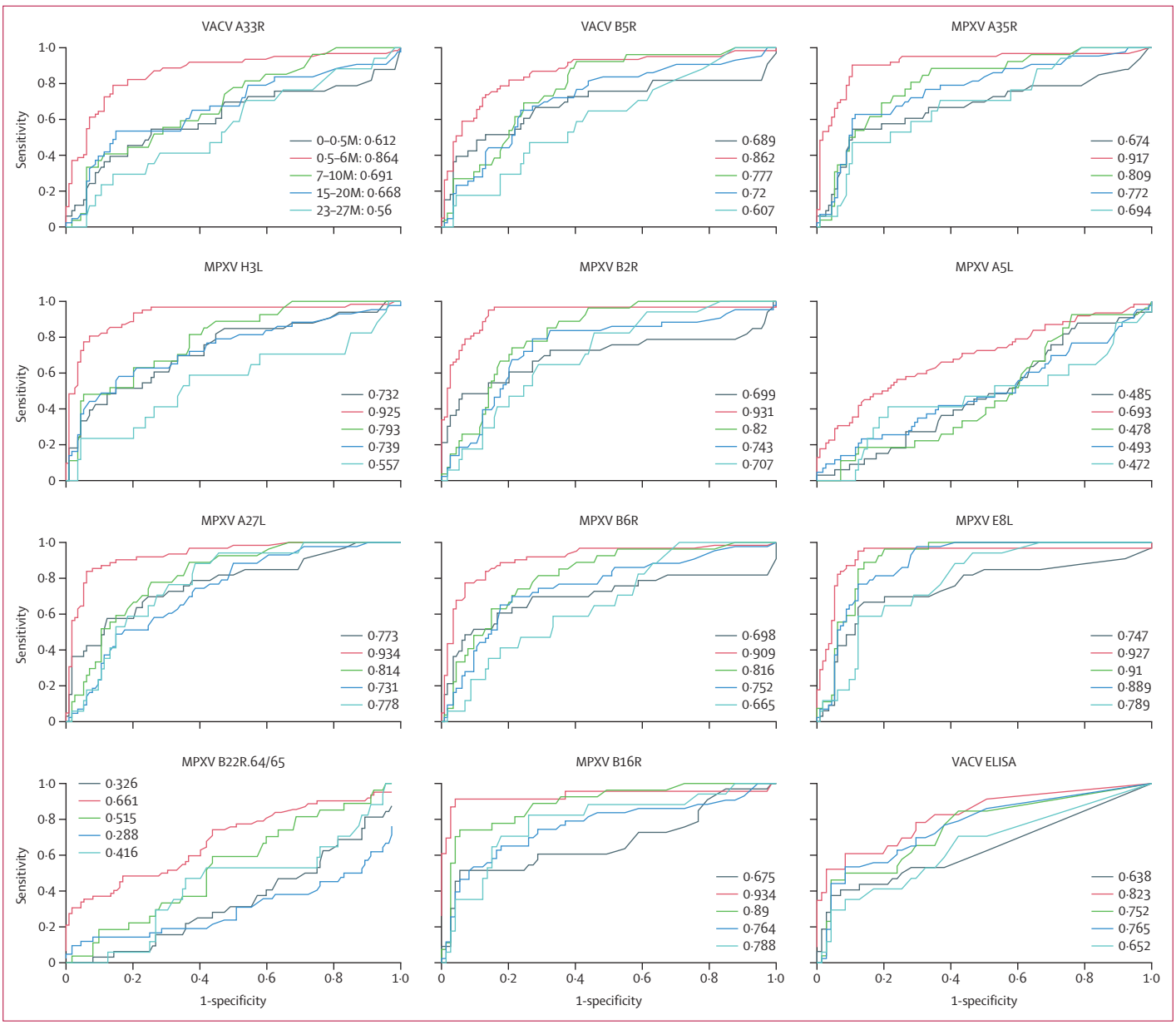

Figure 5: **Receiver operating characteristic curves for different antigens of the IgG Luminex assay and VACV ELISA, comparing samples from individuals infected with MPXV with negative control samples from individuals born in or before 1976 (smallpox vaccinees)**

Different colours represent different time periods after symptom onset (black=0–0·5, red=0·5–6, green=7–10, dark blue=15–20, and light blue=23–27 months). Area under the curve values are presented in the legend. MPXV=monkeypox virus. VACV=vaccinia virus.

15–20 months and 0·87 at 23–27 months after vaccination. Similarly, in MVA-BN-vaccinated individuals born in or before 1976, the assay showed low accuracy in distinguishing vaccinated individuals from negative controls for the same age cohort. Except for E8L, AUC values dropped below 0·90 at months 7–10, with sensitivities of less than 50% (95% CI 38–62) for all other antigens (appendix p 5; appendix p 7). Finally, when we analysed the assay's ability to distinguish between infection and vaccination by comparing individuals with MPXV infection and MVA-BN vaccine recipients in the different birth cohorts, we found AUC values consistently at or below 0·90, with sensitivity falling below 50% (95% CI 38–61; appendix pp 8–9).

To explore whether combinations of antigens could improve the accuracy of our assay, we applied a random forest algorithm to evaluate the relative importance of each antigen at different timepoints, using the mean decrease in accuracy as a measure of contribution (appendix p 10). By combining the top five antigens, the predictive performance of the assay to distinguish previous mpox infection

from negative controls improved at later timepoints among individuals born after 1976 (appendix p 11). For example, at 23–27 months after infection, we observed an increase in AUC of 0·07 when comparing one versus four antigens. At this timepoint, a panel comprising B6R, A35R, B2R, and E8L could effectively predict infection in individuals born after 1976 (AUC 0·98, sensitivity 92% [95% CI 89–97]; appendix p 11). For the same time period, combining antigens only slightly improved prediction of infection in individuals born in or before 1976, with overall remaining low sensitivity at 23–27 months after infection (AUC 0·76, sensitivity 40% [95% CI 15–65]; appendix p 12).

We observed moderate-to-strong positive correlations (pairwise Spearman's ρ>0·60) between most individual antigens, vaccinia whole-virus ELISA, and neutralising antibody titres in individuals with MPXV infection (figure 6). Separately, we observed moderate-to-strong correlations between antibody titres against the different antigens included in the assay (figure 6), with pairwise Spearman's ρ values typically ranging from 0·60 to 0·80. Notably, MPXV A5L and B22R.64/65 were exceptions, showing weak or no correlation with neutralisation titres or other antigens in the assay (pairwise Spearman's ρ<0·6).

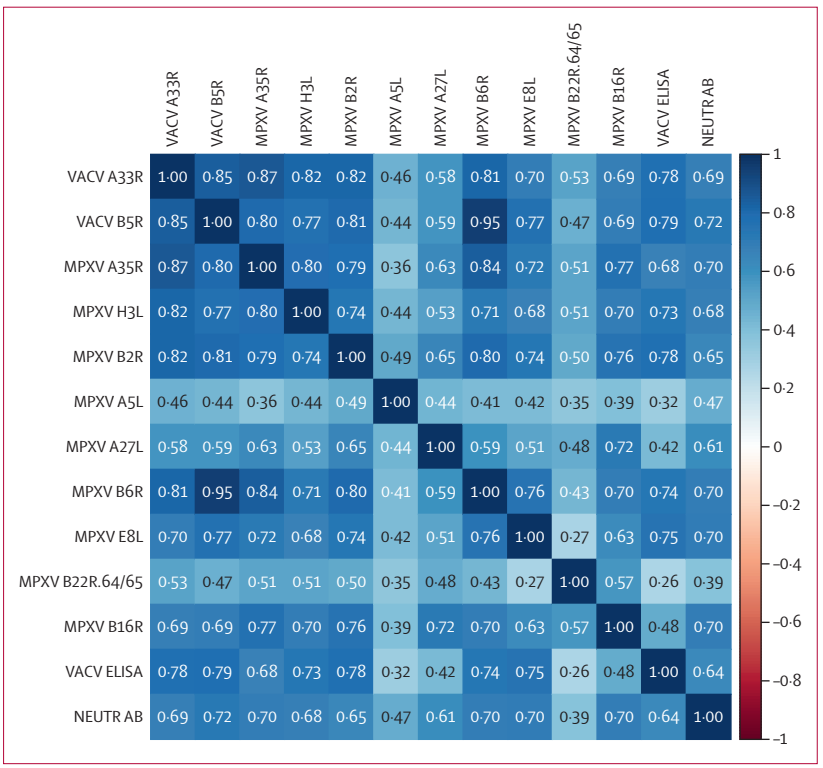

Figure 6: **Spearman's correlation matrix between the different antigens of the Luminex assay, the VACV ELISA, and the neutralisation assay ($PRNT_{50}$), using median fluorescence intensity values (Luminex) and titres (ELISA and neutralisation) from individuals infected with MPXV only**

Blue indicates high positive correlations between variables, white represents no correlations, and red indicates negative correlations. MPXV=monkeypox virus. PRNT=plaque reduction neutralisation test. VACV=vaccinia virus.

Discussion

In this study, we assessed the performance of an 11-antigen multiplex immunoassay for detecting IgG antibodies up to 2 years after natural mpox infection or vaccination. The assay showed strong long-term discriminatory capacity for diagnosing previous mpox infections, particularly in individuals without childhood vaccination. By contrast, the assay's performance in detecting previous vaccination with MVA-BN decreased substantially from 6 months after vaccination onwards.

Our findings are consistent with recent results from our group and others, which show that MVA-BN-induced antibody responses decline substantially over time.[12,18,20] By 1 year after vaccination, most individuals had neutralisation titres approaching or falling below the detection threshold.[18] Our results confirm these observations, with IgG concentrations for most Luminex antigens falling below the assay cutoff as early as 7 months after vaccination. Notably, Jones and colleagues[16] reported more persistent IgG titres at 7 months after vaccination for several antigens, including B6R, A33R, A35R, and E8L. However, their study was based on a small sample size (n=8). In our cohort, the E8L antigen, associated with surface attachment and viral entry,[25] stood out as an exception, with antibody titres remaining above the assay cutoff for up to 20 months after vaccination. Given its sustained performance, E8L could likely serve as a reliable singleplex marker for long-term detection.

Among individuals infected with MPXV who were born after 1976, several MPXV antigens (A35R, B2R, B6R, and E8L) showed strong predictive power (AUC >0·94) in singleplex format, up to 2 years after infection. By contrast, the assay performed considerably less well in differentiating infection from vaccination in individuals who received smallpox or MVA-BN vaccines. In these groups, multiplexing antigens improved performance during early timepoints, with sensitivity increasing from 50% (95% CI 37–62) to above 80% (67–88). These results are consistent with findings from other studies.[11,17] However, at later timepoints, the discriminatory power diminished substantially, even when combining antigens (AUC <0·75 up to 2 years). So far, no serological test has been able to reliably distinguish mpox infection from vaccinia-based vaccination at these later timepoints, which poses a challenge for population-based surveillance, especially in age groups that have received smallpox vaccination during childhood.

We further tested whether specific antigens on the Luminex assay correlated with $PRNT_{50}$ neutralising titres in participants infected with MPXV.[12,26] Neutralising antibodies are widely recognised as a key correlate of protection against many viral infections, potentially including mpox.[18,27] We observed strong positive correlations between most antigens tested and neutralising antibody levels, as observed in other viral diseases.[24] This finding suggests that high-throughput Luminex-based assays might serve as practical surrogates for neutralisation in seroepidemiological studies. However, given the absence of an

established mpox correlate of protection,[18,27] we interpret these findings as evidence of a signal of a correlate of neutralisation rather than a surrogate for protection.

Because responses correlate strongly with neutralising titres and show a sustained response, some antigens of our assay might be attractive targets for mRNA vaccine development. In addition to A35R and B6R, which are already being evaluated as mRNA candidates,[28,29] our data suggest that E8L and B2R also merit consideration as immunogens. By contrast, H3L (although included in some current programmes) appeared to wane more rapidly and might be less suitable for durable protection. These results align only partially with those by Byrne and colleagues,[9] who reported the strongest responses for B6R, followed by A27L and weaker responses for E8L and A35R. The discrepancy likely reflects our explicit adjustment for time since infection, as A27L also performed well early in our cohort but declined more quickly at later timepoints.

One limitation of this study is that all infected participants were exposed to clade IIb MPXV, the lineage responsible for the 2022 global outbreak. Antigenic differences across clades might influence assay performance, and the current public health emergency in central Africa is driven primarily by clade I lineages. Validation in these settings will therefore be essential to assess generalisability. Notably, the six best-performing antigens in our panel were derived from a clade Ia background, suggesting that the assay is likely to perform equally well—or potentially even better—against clade I viruses (appendix p 4). A second limitation is that cross-reactivity with other orthopoxviruses remains a concern. This limitation is evident from the reduced discriminatory power in individuals previously vaccinated against smallpox and might also apply to individuals from regions where immune responses induced by other orthopoxviruses (eg, cowpox, camelpox, or vaccinia-derived vaccines) are present. Although multiplexing improved specificity in our analysis, future work should include cohorts from endemic clade I settings and additional controls exposed to orthopoxviruses to better establish specificity and cross-reactivity profiles. Finally, we intentionally do not fix a single sensitivity and specificity cutoff per antigen. Main figures present the full ROC trade-offs so readers can select context-appropriate cutoffs. In low-prevalence serosurveys or highly vaccinated cohorts, we recommend prioritising high specificity to limit false-positive results. For outbreak reconstruction or vaccine evaluation, higher sensitivity might be preferable despite some loss of specificity. To guide these choices, we report positive and negative predictive values across plausible prevalences (appendix pp 13–16).

In conclusion, our study showed that a subset of antigens from the MpoxPlex assay are robust and reliable for long-term serological detection of mpox infection, maintaining strong diagnostic performance for up to 2 years after infection. These antigens reliably distinguished individuals infected with MPXV from uninfected individuals, but only among unvaccinated individuals. A strong correlation was observed with neutralising antibody concentrations, and multiplexing antigens further improved classification accuracy. Taken together, these results highlight the assay's potential for use in serosurveillance in both endemic and non-endemic settings, although previous vaccination status should be carefully considered.

Contributors

JM, CVD, NB-R, and LL: conceptualisation and protocol writing. SB, JV, FV, EB, NDC, BS, LV, and AvH: data collection. BS and OG: human sampling design. EW, LMZ, SJ, BH, AO, JLY, DTH, and WTL: sample processing and analysis. CVD, NBR, JM, and LMZ: data analysis. CVD, NBR, JM, and LMZ: data management. LL, KKA, JM, RDdV, WA, KV, MVE, AM, and PS: supervision of study proceedings. JM, KKA, WA, RDdV, MVE, and KV: supervision of laboratory procedures. JM: writing of the initial manuscript. JM, LL, KKA, LMZ, and RDdV: funding acquisition. All authors reviewed the manuscript. All authors contributed to the review and approved the final version of the manuscript. JM, CVD, and LL verified the data and had full access to all the data in the study. JM had the final responsibility for the decision to submit for publication.

Declaration of interests

LL has received institutional consultancy fees from BioNTech and institutional research funding from GSK; both are not relevant for this work. The other authors declare no competing interests.

Data sharing

De-identified participant data collected for the study will be made available upon reasonable request to the corresponding author (ie, when ethically viable without violating the protection of participants or other valid ethical, privacy, or security concerns).

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