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SHORT COMMUNICATION

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Smallpox vaccination-elicited antibodies cross-neutralize 2022-Monkeypox virus Clade II

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Abstract

Since early May 2022, some monkeypox virus (MPXV) infections have been reported from countries where the disease is not endemic. Within 2 months, the number of patients has increased extensively, becoming the most considerable MPXV outbreak described. Smallpox vaccines demonstrated high efficacy against MPXVs in the past and are considered a crucial outbreak control measure. However, viruses isolated during the current outbreak carry distinct genetic variations, and the cross-neutralizing capability of antibodies remains to be assessed. Here we report that serum antibodies elicited by first-generation smallpox vaccines can neutralize the current MPXV more than 40 years after vaccine administration.

KEYWORDS

immunity, monkeypox, neutralizing antibodies, vaccines, vaccinia virus

1 | INTRODUCTION

Beginning in May 2022, a novel cluster of monkeypox virus (MPXV-2022) infections was reported in humans. The virus has spread rapidly to nonendemic countries, sparking global concern. Unlike previous outbreaks, which were localized[1] and resulted in small numbers of infections due to limited human-to-human transmission,[2] the current outbreak has already reached almost 82 353 confirmed cases, of which only 1186 in locations with historically reported MPXV infections.[3] On 23 July 2022, the World Health Organization (WHO) declared the 2022 monkeypox outbreak a global health emergency of international concern.[4]

Vaccines based on the vaccinia virus (VACV) were originally developed against smallpox; today, they are one of the interventions available for preventing and controlling monkeypox infections. Four major types of VACV-based vaccines have been developed. First-generation vaccines include live VACV, which were used for the eradication of smallpox in the last century.[5] Then, the routine vaccination strategy was suspended in all Western countries during the 1970s and 1980s. In fact, in the United States the last case of smallpox occurred in 1949, and vaccination was stopped in 1972. In Italy, vaccination was suspended in 1977. Nowadays, a third-generation vaccine, Bavarian Nordic's modified vaccinia virus Ankara (MVA-BN), is recommended by WHO and the United States Centers for Disease Control and Prevention in high-risk groups for controlling the current monkeypox outbreak.[6] MVA-BN is a highly attenuated VACV-based vaccine incapable of replication in humans.[7] While safe and easier to administer than earlier generation vaccines, MVA-BN is available only in limited quantity to date. Last, a fourth generation of vaccines is developing thanks to the complete genome sequencing of orthopoxvirus species and strains pathogenic for man,[8] targeted mutagenesis,[9] and knowledge of the functions of the genes of these

viruses.[10] This knowledge allowed to implement novel approaches toward generating attenuated VACVs.[11]

Despite differences in replication and safety profiles, several studies and clinical trials reported that VACV-based vaccines induce high neutralizing antibody titers and effective T-cell responses among vaccinated individuals.[12,13] In humans, the first-generation VACV-based vaccines were reported to offer 85% protection against MPXV during monkeypox outbreaks in Africa in the 1980s.[14] However, these efficacy results were obtained using the Clade I of MPXV (or Congo Basin clade, MPXV-CB). To date, a couple of studies focused on the cross-reactive immunity of VACV-based vaccines, in both humans and animals, against viruses belonging to the Clade II of MPXV (or West African clade, MPXV-WA), which appears to be most relevant to the sequences observed in the current outbreak.[15] First, a longitudinal study proved the durability over 40 years of specific and neutralizing anti-vaccinia virus antibodies in nearly all individuals who have been vaccinated with first-generation VACV vaccines.[16] Furthermore, a recent work[17] showed that MPXV-neutralizing antibodies can be detected after MPXV infection and after historic smallpox vaccination. As the role of MPXV-neutralizing antibodies as a correlate of protection against disease and transmissibility is currently unclear, they also conclude that cohort studies following vaccinated individuals are needed.

2 | MATERIAL AND METHODS

2.1 | Clinical samples

Eighty-four serum samples from smallpox vaccinated and 96 from unvaccinated healthcare professionals from San Raffaele Hospital were collected as part of the CE:199/INT/2020 study approved by the San Raffaele Hospital, Milan, Italy, Institutional Ethical Review Boards (Supporting Information: Table S1). Written informed consent was obtained from all participants.

2.2 | Virus and cells

Vero E6 (Vero C1008, clone E6-CRL-1586; ATCC) cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with nonessential amino acids (NEAA), penicillin/streptomycin (P/S), HEPES buffer, and 10% (v/v) fetal bovine serum (FBS). A clinical isolate of MPXV was obtained and propagated in Vero E6 cells (hCoV-19/Italy/UniSR1/2020; GISAID Accession ID: EPI_ISL_413489).

In detail, 0.8 mL of the transport medium of an oral lesion was mixed 1:1 with DMEM without FBS and supplemented with P/S and Amphotericin B. The mixture was added to an 80% confluent Vero E6 cells monolayer seeded into a 25 cm^2 tissue culture flask. After 1 h adsorption at 37°C, 3 mL of DMEM supplemented with 2% FBS and Amphotericin B were added. One day postinfection (dpi), the monolayer was washed in PBS and 4 mL of DMEM supplemented with 2% FBS and Amphotericin B were added. The cytopathic effect (CPE) was monitored in inverted phase-contrast microscopy (Olympus CKX41) and the supernatant was collected at monolayer complete disruption (3 dpi), and an aliquot was used for sequencing analysis.

2.3 | DNA extraction and sequencing

The sample was heat-inactivated at 56°C for 30 min and the viral genome was extracted using the ELITe InGenius® system (ELITechGroup) following the manufacturers' instructions. Extracted DNA was processed following the Nextera XT DNA library prep protocol (Illumina) and sequenced with MiSeq Reagent Kit v2 (300-cycles) (Illumina) on the MiSeq platform. Genomic reconstruction was performed using the Galaxy tool open-source platform.[18]

2.4 | Virus titration

Virus stocks were titrated using Endpoint Dilutions Assay (EDA, $TCID_{50}$/mL). Vero E6 cells were seeded into 96-well plates and infected at 95% of confluency with serial base 10 dilutions of virus stock. After 1 h of adsorption at 37°C, the cell-free virus was removed, cells were washed with PBS ×1, and the complete medium was added to the cells. After 72 h, cells were observed to evaluate the presence of a CPE. $TCID_{50}$/mL of viral stocks were then determined with the Reed–Muench formula.

2.5 | Plaque reduction assay

Vero E6 cells were seeded into 96-well plates 24 h before the experiment (performed at 95% cell confluency for each well). Serum samples were decomplemented by incubation at 56°C for 30 min, (1:40 dilution) were incubated with MPXV Clade II at 0.01 MOI for 1 h at 37°C. Virus–serum mixtures and positive infection control were applied to Vero E6 monolayers after washing cells with PBS ×1, and virus adsorption was carried out at 37°C for 1 h. Cells were then washed with PBS ×1 to remove cell-free virus particles and virus-containing mixtures and cultured in complete DMEM supplemented with 2% FBS. Plates were incubated at 37°C in the presence of CO_2 for 72 h, then the cells were stained with crystal violet. The experiments were performed in triplicate. 4-Plate ELISPOT Reader with Automated ELISA-Spot Assay Video Analysis Systems A.EL.VIS was used for plates acquisition, and images were examined for cell-lysis (areas and numbers of plaques) using Fiji software (ImageJ2, version 2.3.0/153q).

2.6 | Semi-quantitative measurement of human IgG antibody against MPXV

The MPXV A29L Protein Human IgG ELISA Kit (RayBiotech) is an in vitro indirect ELISA and was used to quantify the IgG antibody fraction against MPXV A29L of 72 sera selected from the vaccinated

subjects' cohort, and 8 sera from the unvaccinated cohort, following manufacturer's instructions.

2.7 | Statistics analysis

Unpaired *t*-test was used to compare number and area of plaques, and for analyze the anti-MPXV IgG content of selected sera. The correlation matrix between numerical variables was evaluated by means of Pearson's correlation method.

3 | RESULTS

In the present study 180 sera were collected from both VACV-vaccinated and unvaccinated healthcare professionals ($n = 84$ and $n = 96$, respectively), with no clinical history of MPXV infection. MPXV used in the present study (hMpxV/Italy/LOM-UniSR-1/2022) was isolated on May 27, 2022 from an oral pustule swab of a subject infected with MPXV on permissive Vero E6 cells. Whole genome sequencing was also performed (GISAID accession ID: EPI_ISL_13157812). According to the current classification, the clinical virus isolate belongs to clade IIb lineage B1 (MPXV-WA). After complement heat inactivation, all sera were tested for their ability to inhibit MPXV (0.01 multiplicity of infection, MOI) infection and prevent the lytic damage of the cell monolayer (Figure 1A). As MPXV infection results in lysis plaques formation, the neutralizing activity was assessed by measuring the area and number of plaques (Figure 1B). The comparison of the overall count of the number of plaques between the two groups corroborated this observation ($p < 0.01$). Also, measuring the area of the plaques and comparing the overall activity of the two cohorts showed that the inhibitory activity of sera was different ($p < 0.01$, Figure 1C). When we performed a deeper analysis on the characteristics of the selected subjects, we obtained

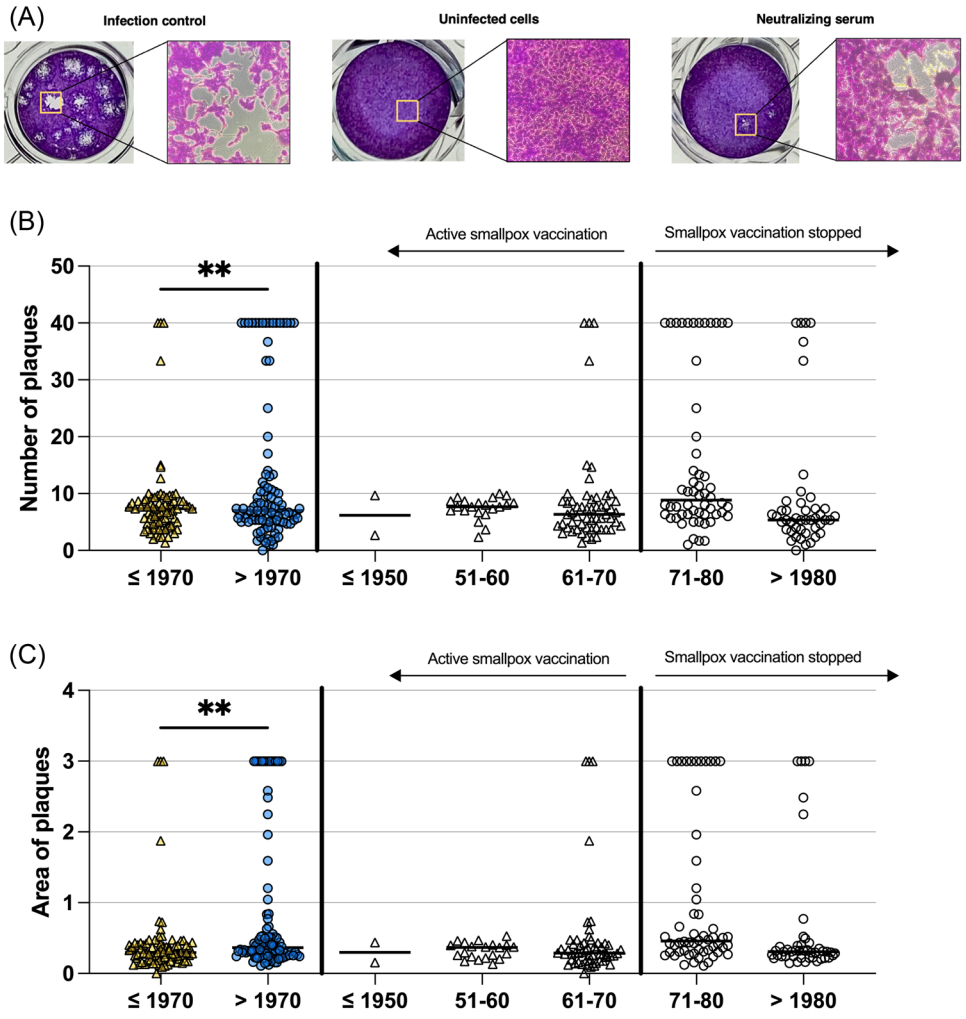

FIGURE 1 Neutralizing activity of sera collected from smallpox vaccinated subjects. (A) Representative image of the CPE resulting after MPXV infection in combination with a neutralizing serum (dilution 1:40), compared to uninfected cells and infection control. (B, C) Neutralizing activity assessed in an age-panel of $n = 180$ biologically independent sera against 0.01 MOI of MPXV. CPE was detected by counting (B) and measuring the area (pixels, C) of cell-lysis plaques. Sera obtained from individuals born in or before 1970 (triangles) or after 1970 (circles) are merged on the left side of the graphs and shown per decade on the right side of the graphs. Mean values ± SD are reported, and each condition was tested in triplicate, $^{**}p < 0.01$. CPE, cytopathic effect; MOI, multiplicity of infection; MPXV, monkeypox virus; SD, standard deviation.

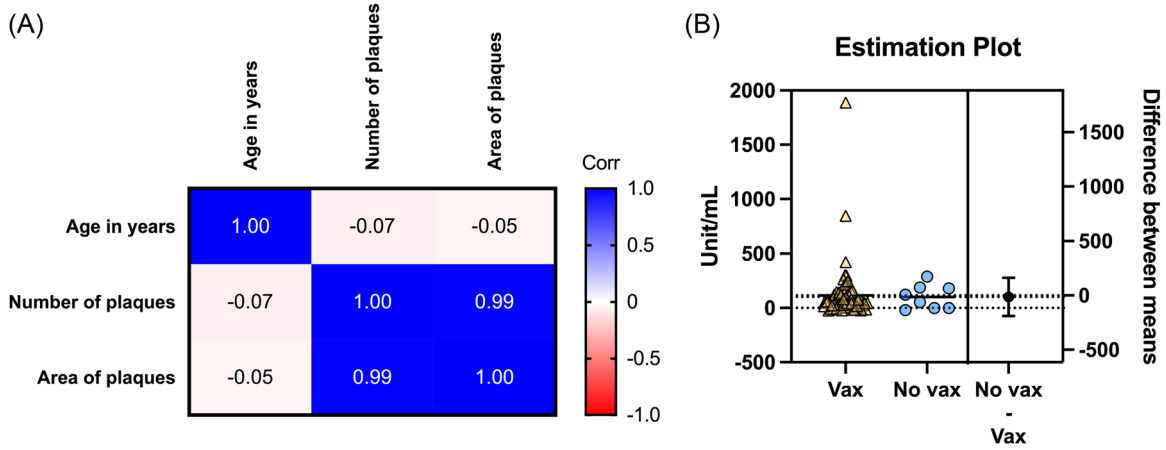

FIGURE 2 Multivariate analyses on the selected cohort. (A) Correlation matrix in R. Matrix heatmap plot corresponding to ρ correlation values between age of the selected subjects, and the biological activity of sera expressed as number of plaques and their area. (B) Semi-quantitative measurement of human IgG antibody against MPXV. The analysis showed that there is no statistical difference between the selected sera from vaccinated ($n = 72$) and unvaccinated subjects ($n = 8$) in their ability to bind viral A29L surface protein. MPXV, monkeypox virus.

that the biological activity of sera is related to vaccination status, but not to the gender. Also, their age was not directly correlated neither to the number of plaques, nor the area ($\rho = -0.07$ and -0.05, respectively), as the two variables are among themselves ($\rho = 0.99$) (Figure 2A).

Last, we tested the binding activity of sera against a viral envelope glycoprotein, A29L. It was selected as it highly conserved in poxviruses, and it is the target of neutralizing antibodies.[19] However, no correlation was found between the titer of anti-A29L antibodies in the selected sera and their ability to neutralize MPXV infection (Figure 2B).

4 | DISCUSSION

The current study aimed to investigate how the MPXV-2022 outbreak virus might impact the possible cross-neutralizing activity of antibodies elicited by first-generation VACV-based vaccines decades after vaccine administration. Such a hypothesis is even more intriguing as the effectiveness of first-generation VACV-based vaccines in humans was already reported for MPXV Clade I.[10] There is scientific evidence describing antibody-neutralizing epitopes shared between the two MPXV clades *in silico*,[20] and recently MPXV-neutralizing antibodies were detected after historic smallpox vaccination, by testing their activity using a virus stock obtained from Calu-3 cells.[17] Moreover, the study described that no apparent waning in total binding antibody levels as a function of age was detectable in individuals born before 1974, supporting previous assertions about the longevity of antibodies induced by vaccinia-based smallpox vaccination.[16,21]

The present study assessed the neutralizing activity of 180 sera collected from first-generation VACV-vaccinated and unvaccinated healthcare professionals and confirmed our hypothesis. We divided our cohort of subjects on their birth date, selecting 1970 to be sure that the vaccination cycle had been completed, according to Italian health policy. The sera were used at 1:40 dilution, the same defined reliable to assess both the protection against orthopoxvirus challenges in vivo[22] and the detection of asymptomatic MPXV infections in retrospective studies.[23] In addition, the $PRNT_{50}$ titer obtained for 10 sera from unvaccinated and vaccinated subjects with a diverse degree of NT activity (Supporting Information: Figure S1) showed that the selected dilution reflects the capability of sera to hamper MPXV entry into the target cells in vitro. All the neutralizing sera resulted in a $PRNT_{50}$ titer below 1:40, while non-neutralizing sera never reached a $PRNT_{50}$ titer at this dilution. We also demonstrated that the age or the gender of the participants did not impact the biological activity of sera, which instead depended only on the vaccination status. However, no correlation existed between the ability of sera to neutralize MPXV Clade II infection and to bind the viral envelope glycoprotein A29L. This protein was selected as it is required for the fusion of the virus membrane with the host cell plasma membrane and is known to elicit a neutralizing antibody response in vaccinated animals. In fact, the vaccination with a Bovine Herpesvirus 4 (BoHV-4)-based vector expressing this protein proved protective in vivo.[24] Given the complexity of the structure of the orthopoxviruses' virion, it might be that other proteins present on the viral surface can serve as targets for a neutralizing antibody response. Therefore, identifying immunogenic antigens as correlates of protection is fundamental in setting up novel tests to check for neutralizing antibodies in a patient's serum.

Based on studies inferring the genetic composition of known targets of VACV-elicited neutralizing antibodies or T cells, either at the epitope level, when available, or at a protein level, limited genetic variability among the MPXV-2022 was observed.[20] Most MPXV-2022 variations compared to VACV were described in MPXV Clade I. As VACV-based vaccines elicit comparable antibody responses, the present study suggests that the currently available VACV-based vaccines (MVA-BN and ACAM2000) may elicit neutralizing responses

against MPXV-2022.[7,12,25] Our results show that antibodies evoked by first-generation smallpox vaccines can neutralize MPXV Clade II infection more than 40 years after their administration.

AUTHOR CONTRIBUTIONS

Elena Criscuolo, Nicasio Mancini, and Nicola Clementi designed the study. Davide Ferrari and Massimo Locatelli contributed to the collection and processing of clinical specimens. Elena Criscuolo, Benedetta Giuliani and Roberto Ferrarese performed wet lab sequencing procedures. Elena Criscuolo, Nicasio Mancini, and Nicola Clementi were the main contributors for manuscript writing. All authors critically reviewed the manuscript for intellectual content, approved the final version of the manuscript for submission and agreed to be accountable for all aspects of the work.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request. MPXV sequence of the propagated isolate was deposited in the GISAID repository (Accession ID: EPI_ISL_413489). Data are available after reasonable request to the corresponding author.

ETHICS STATEMENT

Clinical samples have been collected within the study CE:199/INT/2020, approved by the San Raffaele Hospital, Milan, Italy, Institutional Ethical Review Board. Informed consent was obtained from all human research participants.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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