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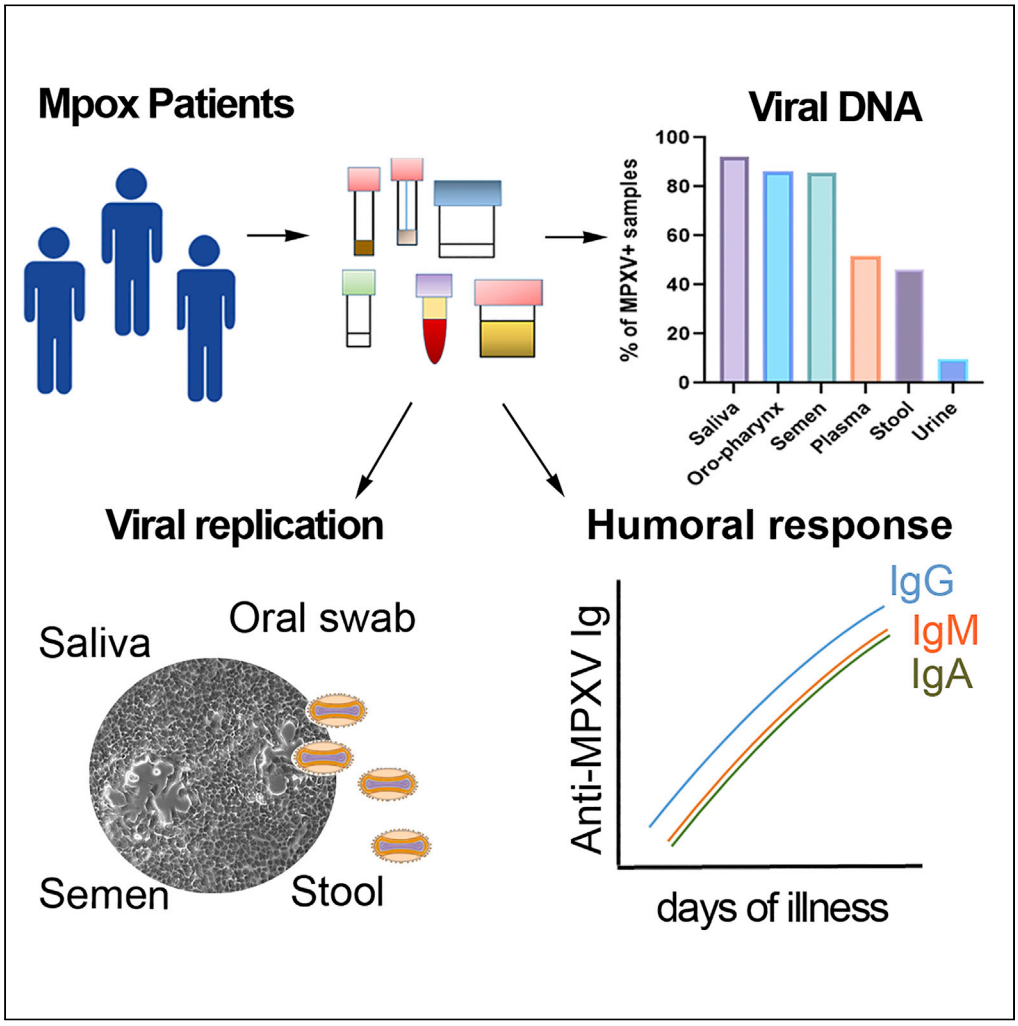

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Highlights

Follow-up study on three patients with MPXV infected during the 2022 global outbreak

Frequent detection of MPXV DNA in saliva, oro-pharynx, and semen

Infectious MPXV cultured from oral swab, saliva, stool, and semen samples

Early seroconversion of specific IgM, IgA, and IgG in MPXV infection

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Kinetics of viral DNA in body fluids and antibody response in patients with acute Monkeypox virus infection

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SUMMARY

We report the follow-up laboratory investigation of three MPXV cases infected in May-June 2022 from diagnosis to disease resolution, monitoring viral shedding in different body fluids and antibody kinetics. Out of 138 non-lesion samples, viral DNA was found in 92.3% saliva, 85.7% semen, 86.2% oropharyngeal swabs, 51.7% plasma, 46.1% stool, and 9.5% urine samples. Viral load quantified by digital PCR widely varied, but tend to be higher in oropharyngeal swabs, saliva, and stool. Replication competent virus was recovered from four out of seventeen samples, including 1 saliva, 1 oropharyngeal swabs, 1 semen, and 1 stool. The analysis of the antibody kinetics revealed that IgM, IgA, and IgG antibodies were detected within two weeks post-symptoms onset for all three patients, with IgG detected early on at day 4-8 and IgM and IgA showing lower titers along the time frame of the study. Antibody levels increased during the second week of illness with IgG reaching high titers.

INTRODUCTION

Monkeypox virus (MPXV) is a zoonotic virus taxonomically located within the Orthopoxvirus genus of the Poxviridae family. It has a linear, enveloped, double-stranded DNA genome of about 200 kilobases and contains at least 190 non-overlapping ORFs more than 180 nucleotides long.[1,2] Genetically, MPXV sequences cluster into two distinct clades, the West African clade, and the Central African (Congo basin) clade, with the latter having historically caused more severe diseases and being more transmissible.[3–5] Until recently, the MPXV has been classified as a neglected zoonotic pathogen with a limited inter-human transmission, largely confined to Africa, where outbreaks mainly occurred in household and healthcare settings.[6,7] Sporadic cases were reported outside Africa, mainly linked to travels from endemic countries.[8–10] In May 2022, the MPX outbreak was identified in several non-endemic countries, and as of 25 August 2022, a total of 45,355 cases were reported from 124 countries worldwide that have not historically reported monkeypox, most cases in the EU/EEA countries.[11–18]

Much still needs to be learned about this infection and research is underway worldwide to gain more knowledge about the pathobiology of the virus, the clinical features of the disease, and its peculiarities, such as the routes of this unprecedented inter-human transmission or the distinct features of clinical presentation.[19–21] Data on the kinetics of viral shedding and antibody response could help to define MPXV transmission and pathogenesis, to support diagnostic algorithms, surveillance, and clinical management in this new current global outbreak.

Here, we reported both the dynamic of viral release in different body fluids and the serum antibody profile over the disease course in three MPX-confirmed cases diagnosed and followed up at the National Institute for Infectious Diseases "Lazzaro Spallanzani" (INMI) in Rome, Italy. These three cases were previously described clinically either because they were observed early during the current outbreak,[15,22] or because of the unusual clinical presentation.[23] The current report describes original unreported data on virological dynamics (the quantitative measure of MPXV viral load by using the ultra-sensitive method of digital PCR

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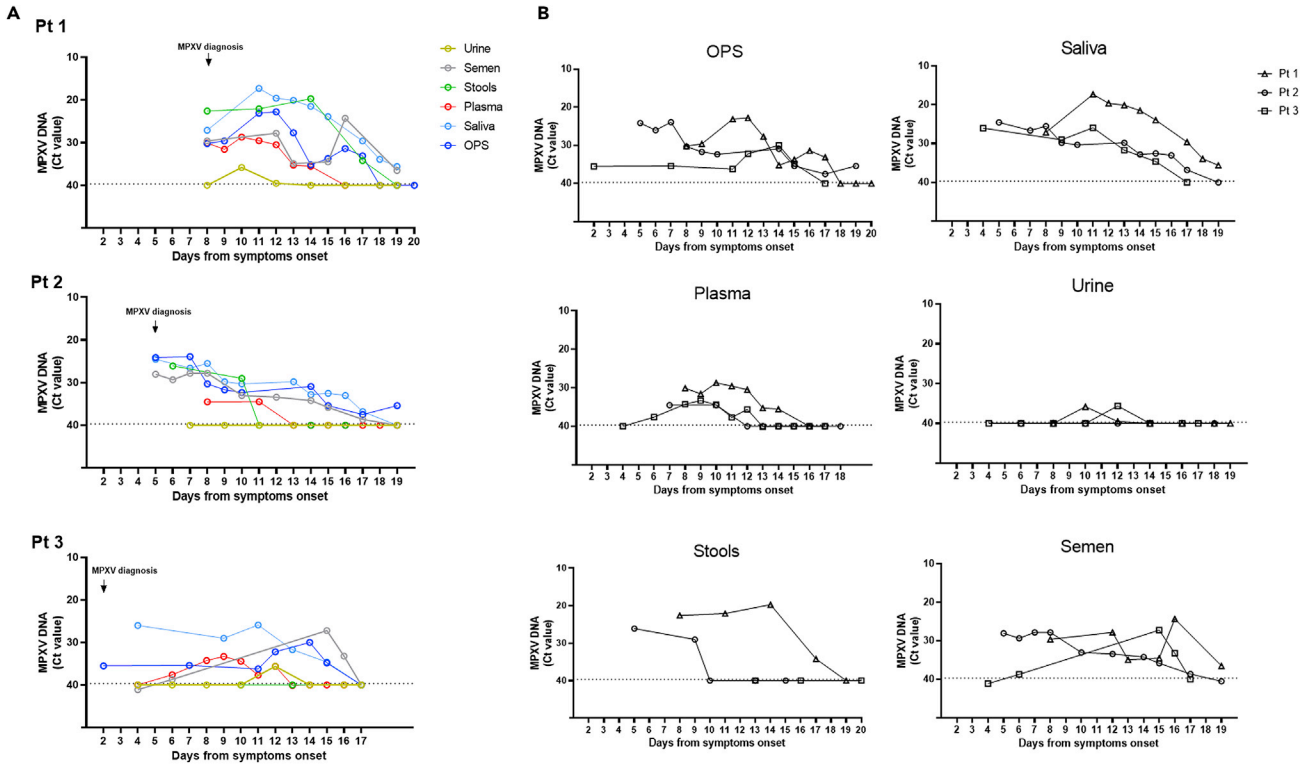

Figure 1. Kinetics of MPXV DNA shedding in different biological samples (other than skin lesions samples) from the onset of the symptoms throughout infection

(A and B) Viral DNA levels detected in different longitudinal samples are shown for the three patients with MPXV followed up during infection (A) and the single type of sample (B). Viral DNA levels are expressed as cycle threshold values (Ct) of CrmB gene amplification. The monkeypox diagnosis indicates the day of the confirmatory diagnosis of skin lesion samples. Dashed lines represent the limit of detection of the real-time PCR (Ct: 40).

(ddPCR) and isolation of viable MPXV) in different biological samples other than skin lesions and on the antibody response during the acute phase of the diseases.

RESULTS

Kinetics of the viral DNA in different body fluids

Clinical diagnosis of MPXV infection was confirmed for all three patients by positivity to MPXV-specific rtPCR on skin lesion samples collected at the diagnosis, days 8, 5, and 2 for Pt1, Pt2, and Pt3, respectively, after the onset of the symptoms. Additional samples of different body fluids (i.e., OPS, saliva, plasma, urine, stool, and semen) were collected at multiple time points from the diagnosis along with the follow-up of the disease and tested for MPXV DNA.

The dynamics of viral DNA levels in these specimens, different from the skin lesions, are shown in Figure 1. MPXV DNA was detected in OPS, saliva, plasma, semen, and stool samples collected from Pt1 on day 8 after the onset of the symptoms. Pt2 revealed MPXV DNA presence in OPS, saliva, and semen samples collected on day 5 after the onset of the symptoms, and in the first available stool sample the day after. OPS was the only MPXV-positive sample collected from Pt3 two days after the onset of symptoms, followed by saliva positive at four days, and plasma and semen between days 5 and 6 from the onset of the symptoms (Figure 1A). In all three cases, MPXV DNA was detected in several follow-up samples collected between 2- and 19-day post-symptoms onset (Figure 1B and Table 1). None of the urine and stool samples from Pt2 and Pt3, respectively, resulted positive for viral DNA. Overall, out of 138 biological samples collected from the three patients between 2 and 20 days after the onset of the symptoms, MPXV DNA was detected in 24/26 (92.3%) saliva, 25/29 (86.2%) OPS, 18/21 (85.7%) semen, 15/29 (51.7%) plasma, 6/13 (46.1%) stool, and 2/21 (9.5%) urine. Prolonged detection for up to 15-19 days after the onset of the symptoms was observed in saliva, OPS, and semen samples collected from all patients, and in the stool samples from Pt1 (up to 17 days) (Table 1).

Table 1. Summary of the MPXV DNA results in different body fluids collected from the three patients with MPXV

	Saliva		OPS		Semen		Stool		Plasma		Urine	
Pt	N. pos/tot (Ct)[a]	Last day/ f-up	N pos/ tot (Ct)	Last day/ f-up	N pos/ tot (Ct)	Last day/ f-up	N pos/ tot (Ct)	Last day/ f-up	N pos/ tot (Ct)	Last day/ f-up	N pos/ tot (Ct)	Last day/ f-up
1	9/9 (25.4 ± 6.5)	19/19	9/12 (29.6 ± 4.4)	17/20	6/6 (31.3 ± 4.8)	19/19	4/5 (24.7 ± 6.5)	17/19	7/8 (31.7 ± 2.6)	14/16	1/7 (37.7)	10/19
2	10/11 (30.2 ± 3.8)	17/19	10/10 (30.8 ± 4.8)	19/19	9/10 (31.2 ± 4.4)	19/19	2/5 (27.6 ± 2.1)	9/16	2/9 (34.5 ± 0.02)	11/19	0/6	-/19
3	5/6 (29.4 ± 3.8)	15/17	6/7 (33.7 ± 2.6)	15/17	3/5 (33.0 ± 5.8)	16/17	0/3	-/17	6/12 (35.5 ± 1.8)	12/17	1/8 (35.6)	12/17

Abbreviation: OPS, oro-pharyngeal swab; N pos/tot, number positive samples over the total tested; Last day/f-up, last day from the onset of the symptoms with the detection of MPXV DNA over the total days of follow-up per type of specimen.

[a]Mean Ct ± SD value measured in the MPXV DNA positive samples. Samples found negative using rtPCR were excluded from this analysis.

Quantitative measure of monkeypox virus viral load in different body fluids

The MPXV DNA loads showed differences among patients and types of biological samples. OPS, saliva, and stool samples were more frequently the samples with DNA loads equal to or below Ct 25 (Figure 1). Because Ct value can be only considered as a surrogate marker for the viral load, a total of 39 biological samples (9 OPS, 9 saliva, 8 semen, 7 plasma, and 6 stool samples) were also tested by an *in-house* ddPCR for measuring the exact number of MPXV DNA copies. The results are shown in Table 2, where the samples analyzed are grouped based on the observation period of days 2-8, 9-14, and 15-18 after the onset of the symptoms. Importantly, a significant inverse correlation ($r = -0.893$; $p < 0.0001$; Spearman correlation test) was observed between the Ct values and the absolute count of target DNA copies (data not shown). Most of the samples collected in the 2-14-day periods, tested MPXV positive at copy numbers that varied widely among patients and specimen types, but tend to be relatively high (Table 2). Interestingly, two samples from Pt1 (one stool and one saliva) at the 9-14 days sampling point showed the highest MPXV loads (6.9 and 6.4 log copies/μg extracted DNA, respectively), and 7 specimens (2 OPS, 2 saliva, and 3 stool samples) from Pt1 and Pt2 harbored viral DNA at levels greater than 5.0 log copies/μg extracted DNA. Subsequently, MPXV DNA levels underwent a decline that was similar in kinetic among almost all patients and samples but variable in extent, as suggested by the high reduction of the mean load detected at 15-18 days in stool and saliva samples (approximately 4.0 and 3.0 log copies/μg DNA reduction, respectively).

Infectivity of monkeypox virus in different body fluids

To investigate the infectivity of MPXV in different types of body fluids, virus isolation was attempted on the available residual samples collected on different days from the onset of symptoms (fso) (Table 3). Apart from urine and plasma samples (mostly negative or with very low viral DNA levels), the replication-competent virus, as measured by the observation of cytopathic effect and increase of viral DNA during culture, was recovered from all types of samples. Specifically, over 17 samples tested, replication-competent virus was isolated from 4 samples, including a saliva (collected on day 13 fso), an OPS (day 12 fso), a stool (day 11 fso), and a semen sample (day 6 fso), the latter had previously been reported for Pt2[22] (Table 3). Figure 2 shows the increase in viral DNA levels in cell culture supernatants collected at selected time points post-inoculum removal.

Kinetics of the antibody response during the early phase of monkeypox virus infection

The levels of specific anti-MPXV IgG, IgM, and IgA were evaluated on serial serum samples collected in a time frame between 4 and 15 days from the onset of the symptoms (Figure 3). IgM, IgA, and IgG were detected in serum samples from all three patients. Regardless of the previous smallpox vaccination and HIV status, IgG were detected early (day 4 in Pt2 and Pt3, day 8 in Pt1) and with titers significantly higher than those observed for IgM and IgA at each time-point tested (overall median titer: IgM = 1:40 vs IgA = 1:80 vs IgG = 1:320, 95%CI < 1:20-1:160 vs < 1:20-1:160 vs 1:80-1:1280, respectively; Friedman matched-pairs rank test, $p = 0.0003$) (Figure 3A). For two patients (Pt1 and Pt3), both with no history of previous vaccination and HIV infection, IgM and IgA were detected after the first week from the onset of the symptom (IgM, days 9 and 11; IgA, days 11 and 14), while the third case (Pt2) showed all three isotypes positive within the first week (Figure 3B). Overall, the antibody levels steadily increased during the second week of illness with IgG reaching the highest titers.

Table 2. MPXV DNA quantitation by ddPCR on different types of specimens collected from the three patients with MPXV

Sample	Pt	2-8 days[a]	9-14 days	15-18 days
OPS	1	3.8[b]	5.6	3.1
	2	5.7	3.1	<2.4
	3	2.9	3.4	2.6
	Total mean ± SD	*4.1 ± 1.4*	*4.0 ± 1.3*	*2.7 ± 0.3*
Saliva	1	4.4	6.4	2.6
	2	5.2	3.5	<2.4
	3	4.6	3.3	2.5
	Total mean ± SD	*4.7 ± 0.4*	*4.4 ± 1.7*	*2.5 ± 0.1*
Plasma	1	3.9	3.9	2.7
	2	3.3	ND	<2.4
	3	<2.4	3.1	<2.4
	Total mean ± SD	*3.4 ± 0.8*	*3.5 ± 0.6*	*2.5 ± 0.2*
Stool	1	5.4	6.9	<2.4
	2	5.2	3.2	<2.4
	3	ND	ND	ND
	Total mean ± SD	*5.3 ± 0.1*	*5.0 ± 2.6*	*2.4 ± 0*
Semen	1	4.2	2.7	<2.4
	2	3.0	<2.4	<2.4
	3	<2.4	ND	4.1
	Total mean ± SD	*3.2 ± 0.9*	*2.5 ± 0.2*	*2.9 ± 0.9*

Abbreviation: SD, standard deviation; ND, not done.

[a]Days since symptoms onset.

[b]MPXV DNA log copies/μg of extracted DNA; for values under 2.4 log copies/μg, a value of 2.4 log was considered to calculate mean and SD.

DISCUSSION

The present study describes both virological and serological features of the MPXV infection during the follow-up of three symptomatic patients infected during the current global outbreak. These cases were previously described clinically either because they were observed early during the current outbreak,[15,22] or because of unusual clinical presentation.[23]

Although the small number of cases is limited to draw definitive inferences about transmission mode or clearance time, the evaluation of the *in vivo* kinetics of MPXV DNA in these patients extends our and others' findings,[14–16,22–27] confirming that MPXV can be detected in several biological specimens during acute infection. Samples found more frequently positive for MPXV DNA in our patients included saliva (92.3%), OPS (86.2%), and semen (85.7%), while viral DNA was detected in about half of the plasma (51.7%) and stool (46.1%) samples. Only two urine samples contained the virus, confirming the very low MPXV DNA detection in this specimen.[11,25] In addition, saliva, OPS, and stools, followed by semen, can contain high viral loads as confirmed by ddPCR, which provides a sensitive and reproducible measuring of the viral DNA copies number.[28,29] These findings, coupled with the evidence of viable virus isolated in cell culture from OPS, saliva, stool, and semen samples, might indicate that MPXV can spread in humans through several alternative modes, although probably not all as primary drivers of infection.[24,26,30,31] Duration of viral shedding has implications for clinical case management and public health policies. Persistent MPXV DNA detection in non-lesions samples was reported for up to 54 and 67 days from symptoms onset in semen and saliva samples respectively[31]; however, data on infectivity are limited and difficult to obtain, but crucial to define the real implications for transmissibility. In our patients, the follow-up of viral DNA in the different non-lesion specimens showed that they were likely to be most infectious in the first two weeks of illness with a drastic decline of the viral loads in almost all biological materials during the third week of observation. Accordingly, of the samples other than skin lesions, the live virus was isolated from samples collected up to 13 days of symptoms.

Table 3. MPXV isolation from different body fluids

Type of specimen	Pt	Days from symptoms onset	MPXV rtPCR Ct value[a]	Viral culture
Saliva	1	13	20.2	Pos
	1	15	23.9	Neg
	1	17	29.7	Neg
OPS	**1**	**12**	**23.1**	**Pos**
	1	13	22.8	Neg
	1	16	31.4	Neg
Semen	**2**	**6**	**29.3**	**Pos**
	1	8	29.7	Neg
	2	8	27.9	Neg
	1	12	27.8	Neg
	1	16	24.3	Neg
Stool	2	5	26.2	Neg
	1	**11**	**22.1**	**Pos**
	1	14	19.8	Neg
Plasma	1	8	28.7	Neg
	1	10	29.5	Neg
	1	11	30.5	Neg

[a]The Ct value is referred to the clinical sample tested for MPXV DNA. Positive results are shown in bold.

The kinetics of antibody response is one of the gaps in our present comprehension of the early phase of the MPXV infection and data on the antibody response in infected patients are still scarce. IgM and IgG detection may support the diagnosis of the infection as the development of specific IgM or evidence of seroconversion in paired samples indicates recent exposure to orthopoxviruses.[32] However, validated diagnostic tests are limited and cross-reactivity with other orthopoxvirus or residual immunity due to the smallpox vaccination complicate the serological evaluation of poxviruses.[33,34] Reports from the 2003 US outbreak showed the detection of IgM and IgG after 5 and 8 days from the symptoms onset.[35] Our results showed

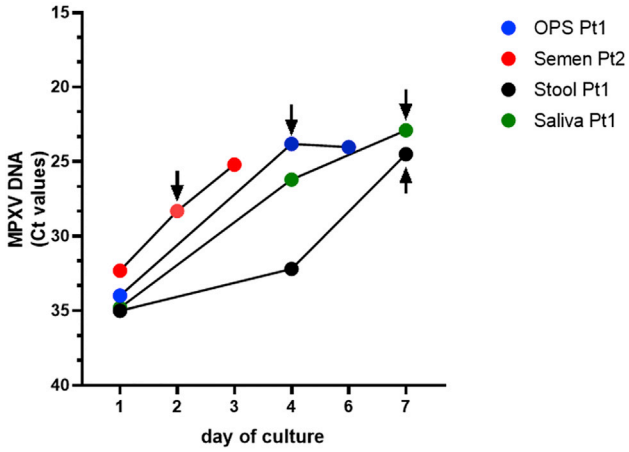

Figure 2. Replication of MPXV from different body fluids

The viral culture was performed by inoculating on Vero E6 cells clinical samples pre-diluted and pre-incubated in MEM containing antibiotics and antimycotic. Viral inoculum was removed after 1 h and replaced with fresh medium. Viral DNA levels expressed as cycle threshold values (Ct) were measured in cell supernatant collected at selected time points post-inoculum to evaluate the viral replication. The patient and type of clinical specimen tested are indicated in figure legend. Arrows indicate the time of first observation of the Cytopathic effect (CPE). OPS Oro-pharyngeal swab; Pt1 patient 1; Pt2 patient 2.

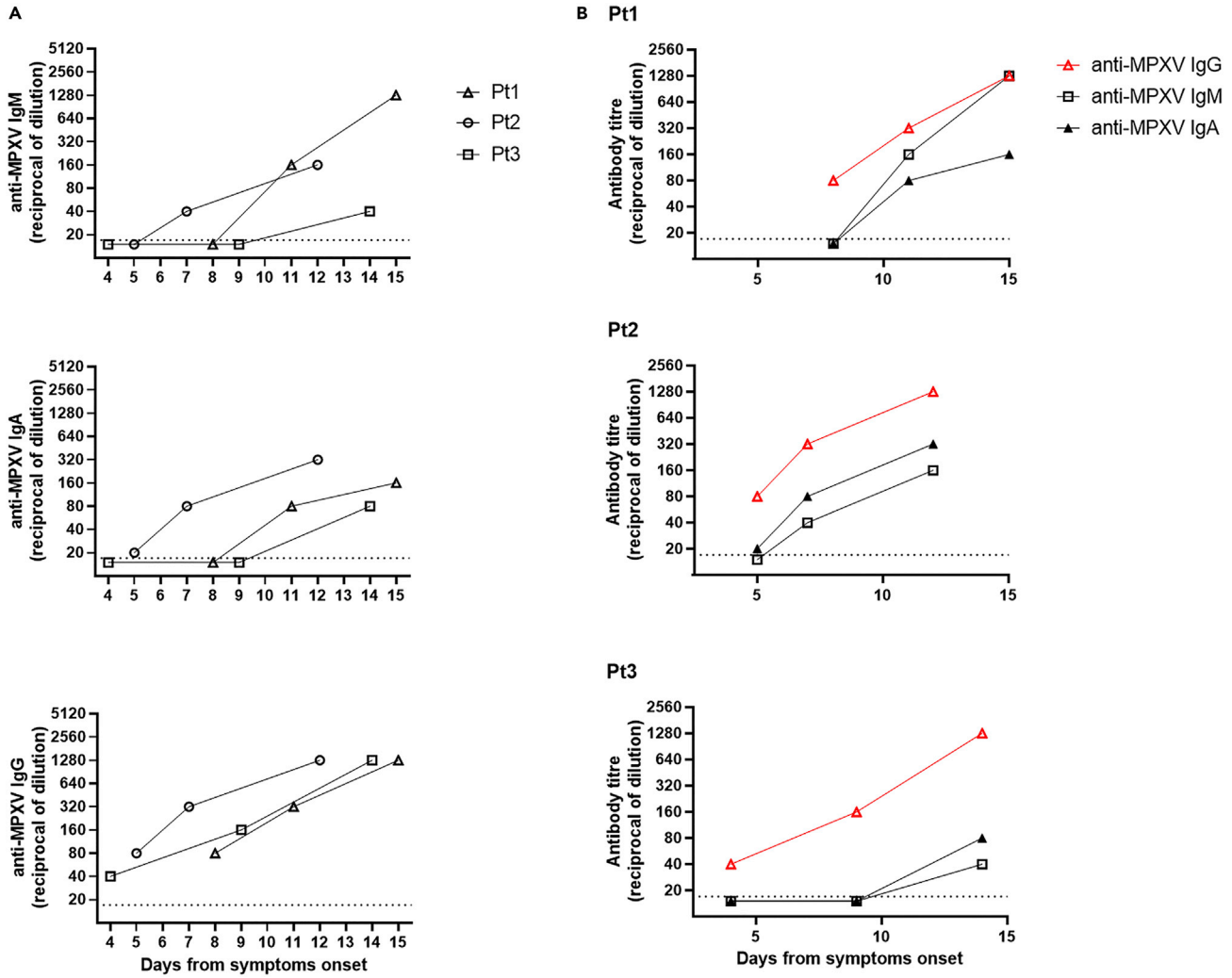

Figure 3. Kinetics of anti-MPXV antibody response throughout the infection

(A and B) The trend of the anti-MPXV IgM (up-panel), IgA (central), and IgG (down) titers detected in the three patients with MPXV is shown in (A). Seroconversion observed in each patient during the infection is shown in (B). Antibody titers are expressed as the reciprocal of serum dilution. Dashed lines represent the detection limits of IFA (1:20).

both IgM and IgG detected within the first two weeks post-symptoms onset in all three patients, regardless of the pre-existing immunity. Patient 2, who received smallpox vaccination during childhood, showed a more robust and early response compared to the other two patients with a concomitant faster decline of the viral DNA levels in the different body fluids evaluated along the follow-up (Table 2). Given the evidence of MPXV detection in mucosal and respiratory tract samples, we also evaluated the IgA levels in serum samples. In fact, IgA are produced and predominantly present in mucosal tissues, providing the first line of defense in mucosal immunity. Our data showed that the dynamics observed for IgA mirror those of IgM during the acute phase of the disease. Further studies could be of interest to extend these findings and assess the mucosal immunity against MPXV. The early detection and higher titers of specific IgG compared to IgM and IgA needs to be carefully considered and interpreted. These findings could be related to unconventional antibody kinetics as reported for other viral infections including other orthopoxviruses,[36–38] or to a cross-reactive response to past exposure to other orthopoxviruses.

In conclusion, we believe that our results contribute to providing insight into MPXV infection and the natural antibody response with a picture of the virological and serological short-term kinetics after the onset of symptoms in MPXV-infected patients. These data extend and improve the understanding of the disease

and support decision-making regarding risk assessment, protective measures, and guidelines for patients with monkeypox. Finally, information about the antibody response during the acute phase of the infection is important to help the design of integrated diagnostic and surveillance algorithms for MPX case management.

Limitation of the study

Limitation of the study is the small cases series, as the number of patients here described narrows the conclusion about clearance time, transmission mode, and antibody kinetics, for which larger cohort studies are necessary. In addition, the serological results could be influenced by the single method used, therefore further studies are crucial to better define the serological response in MPXV-infected patients.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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AUTHOR CONTRIBUTIONS

F.Co., G.R., G.M., I.A., S.M., F.Ca., D.L., C.M., E.S., and A.B.: were directly involved in the laboratory activities and performed the virological and serological assays; V.M., C.P., A.M., S.V., C.A., and R.G.: were directly involved in the patient care; F.Co. and F.M.: wrote the original draft; F.Co., F.M., G.M., I.A., G.R., A.A., V.M., E.N., and E.G.: reviewed and edited the article; F.M., E.N., and A.A: supervised the activities; F.V. and E.G.: funding acquisition. All authors have read and agreed to the published version of the article. All members of the "INMI Monkeypox group" were involved in patient care and microbiological experiments.

DECLARATION OF INTERESTS

The authors declare that no conflicting financial interests or other competing relationships exist for the present study.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
FITC-labelled anti-human IgA	EUROIMMUN	AF 101-0160
FITC-labelled anti-human IgG	EUROIMMUN	AF 102-0160
FITC-labelled anti-human IgM	EUROIMMUN	AF 103-0160
Bacterial and virus strains		
Monkeypox virus isolate (from clinical sample)	INMI L. Spallanzani	GenBank: ON745215.1 (clinical sample)
Biological samples		
Clinical samples from patients (i.e., plasma, OPS, Urine, Stool, Semen, Saliva)	Recruited at INMI L.Spallanzani (Ethical approvement: 40z/2022)	N/A
Chemicals, peptides, and recombinant proteins		
Minimum Essential Medium Eagle	Sigma-Aldrich	M2279-500ml
Fetal Bovine Serum	Corning	35-079-CV
Trypsin, 1X, 0.05% Trypsin/0.53 mM EDTA in HBSS	Corning	25-051-CI
L-glutamine	Corning	25-005-CI
Penicillin (5,000 IU)/Streptomycin (5,000 µg/mL)	Corning	30-001-CI
Amphotericin B	Sigma-Aldrich	A2942-20 ML
Evans blue	EUROIMMUN	Z200903BF
EUROSORB	EUROIMMUN	ZF 1270 0145
Critical commercial assays		
QIAsymphony® DSP Virus/Pathogen Midi Kit	Qiagen	937055
Qubit dsDNA BR Assay Kit	Thermo Fisher Scientific	Q32850
Droplet Generation Oil for Probes	Bio-Rad	1863005
SuperScript III Platinum One-Step qRT-PCR	Invitrogen	11732–088
ddPCR Supermix for Probes (No dUTP)	Bio-Rad	1863024
Experimental models: Cell lines		
Cercopithecus aethiops Vero E6 Cells	ATCC	CRL-1586
Oligonucleotides		
MPXV real time PCR (primers, probes)	Li Y, et al. 2010	https://doi.org/10.1016/j.jviromet.2010.07.012
Software and algorithms		
GraphPad Prism 8	GraphPad	https://www.graphpad.com/
QuantaSoft software Version 1.7.4	Bio-Rad	https://www.bio-rad.com/it-it/life-science/digital-pcr/qx200-droplet-digital-pcr-system/quantasoft-software-regulatory-edition

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Fabrizio Maggi (fabrizio.maggi@inmi.it).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- All data reported in this paper will be shared by the lead contact upon reasonable request.
- This paper does not report original code.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Patients and specimens

All three patients described here were young adult men who have sex with men (MSM; age ranged from 26 to 39 years). Pt1 and Pt2 reported sexual relationship with multiple partners in the previous two weeks; Pt3 was a contact of a known case of MPXV. They all presented to INMI in May 2022 for the appearance of papular lesions in the genital and anal (Pt1 and Pt2) regions, with systemic symptoms and inguinal painful lymphadenopathy. Pt2 reported one dose of smallpox vaccination during childhood, 30 years earlier. He was HIV-positive on antiretroviral therapy with undetectable viremia and $CD4^+$ T-cell lymphocyte count 884 (49%) cell/mm^3 at last observation; he was concomitantly diagnosed with penile gonorrhea. The other two patients (Pt1 and Pt3) were unvaccinated for smallpox and negative for HIV. Pt1 was taking pre-exposure prophylaxis (PrEP). Overall, the three patients were in good condition: only Pt3 experienced an ocular complication that required antiviral treatment with cidofovir. All were fully recovered.

METHOD DETAILS

MPXV DNA detection and quantification

Viral DNA was extracted by QIAsymphony (QIAgen, Hilden, Germany), and amplified using the real-time PCR (rtPCR) published by Li et al. (Li et al. 2010), targeting the gene encoding the CrmB secreted TNF-alpha-receptor-like protein of the MPXV genome. Samples with values > 40 were considered negative. As a parameter correlated with MPXV DNA concentration in the sample fluids, we used threshold cycles (Ct) values obtained by MPXV-specific rtPCR to study the kinetics of MPXV DNA shedding in different biological samples.

Thereafter, to obtain a direct measure of MPXV DNA concentration in the clinical samples, the molecular design used in the rtPCR was adapted to run in a digital droplet PCR (ddPCR) assay using the Bio-Rad QX200 AutoDG Digital Droplet PCR system (Bio-Rad, Hercules, CA, USA). A concentration of 900 nM of primers and 250 nM of probe (FAM-labeled) was added to ddPCR Supermix for probes (no dUTPs) (Bio-Rad, United States). To ensure consistent quantification, DNA extracted from each clinical sample and time point was run in triplicate wells, which then were merged during the analysis. After the PCR reaction, the droplets were subsequently read by a QX100 droplet reader, and data were analyzed using QuantaSoft software (Bio-Rad, United States). For each sample, DNA concentration was determined by fluorometry (Qubit dsDNA BR Assay Kit, Thermo Fisher Scientific), and the results were expressed as Log of copies for micrograms (μg) of extracted DNA. A detection limit of 5 copies of the CrmB MPX gene for a sample was considered. Since the DNA quantity in the different sample types and patients was variable, to uniform the values obtained from normalization, only values above Log 2.4 (250) copies/μg were reported.

MPXV isolation from biological samples

The viral culture was performed in the BSL-3 laboratory on Vero E6 cells as described elsewhere.[23] Briefly, samples were diluted in MEM (Corning, Glendale, USA) containing a solution of antibiotics and antimycotic. The mixtures were kept at room temperature for 30 min and inoculated on Vero E6 cells. After 1 h at 37°C in 5% CO2 of incubation, the inoculum was discarded and replaced with MEM containing 2% FBS plus the solution of antibiotics and antimycotic. Cytopathic effect (CPE) appearance was observed

by light microscope and aliquots of cell supernatant (70 μL) were collected at selected time points post-infection to evaluate the viral replication.

Anti-MPXV antibodies detection

Indirect immunofluorescence assay (IFA) was used to detect specific IgM, IgA and IgG in serum on slides prepared *in-house* with Vero E6 cells infected using an MPXV isolate from the 2022 outbreak, as described elsewhere (Colavita et al. 2020). Serum samples were tested using 1:20 as screening dilution and 2-fold titrated by limiting dilution up to 1:2,560. To evaluate IgM and IgA, serum was depleted of IgG using Euro-sorb reagent (Euroimmun, Lubeck, Germany). FITC-conjugated anti-human IgM, IgA and IgG (Euroimmun, Lubeck, Germany) were used as secondary antibody and Evans Blue as cell counterstain.

QUANTIFICATION AND STATISTICAL ANALYSIS

Viral load data in the different time ranges or type of samples were presented as mean ± SD. Friedman matched-pairs rank test was used to compare titers of the different antibody isotypes, while Spearman test was performed to evaluate the correlation between the Ct values and the absolute count of target DNA copies. Analyses were performed using GraphPad Prism version 9 (GraphPad Software, La Jolla, California, USA) for Windows statistical software; $p < 0.05$ was considered statistically significant.