THE LANCET
Infectious Diseases

## Supplementary appendix

This appendix formed part of the original submission. We post it as supplied by the authors.

Supplement to: Raccagni AR, Canetti D, Mileto D, et al. Two individuals with potential monkeypox virus reinfection. *Lancet Infect Dis* 2023; published online April 6. https://doi.org/10.1016/S1473-3099(23)00185-8.

**Supplementary Appendix**

**Table of contents**

## Methods

### Molecular diagnostics

A real-time polymerases chain reaction (RT-PCR) (RealStar® Orthopoxvirus PCR Kit 1.0 – Altona Diagnostics) targeting variola virus and non-variola Orthopoxvirus species (cowpox, mpox, raccoonpox, camelpox, vaccinia virus) was used to detect the presence of non-variola DNA on rectal, oropharyngeal and lesions swabs, urines, plasma, and seminal fluids. Cycle thresholds (Ct) for positive samples at Orthopoxvirus PCR test are presented. A specific RT-PCR targeting mpox DNA (Jiangsu Bioperfectus Technologies Co., Ltd.) was subsequently used to confirm the presence of mpox on the specimen with the lowest Ct. Rectal, oropharyngeal and lesions swabs were collected with Universal Transport Medium swabs (UTM-RT; COPAN Diagnostics, Italy). Mpox testing on rectal biopsies was performed with PCR following 60 seconds of samples vortexing. A sexually transmitted infection (STI) screening was performed at time of mpox diagnoses, including *Chlamydia trachomatis* and *Neisseria gonorrhoeae* on rectal, pharyngeal swabs and urines and syphilis.

### Serological diagnostics

Plaque reduction neutralization test (PRNT) was used to assess the presence of neutralizing anti-mpox antibodies in the serum at different timepoints. Briefly, 50µL of each serum, starting from a 1:10 dilution followed by serial two-fold series, were transferred in two wells of 96-weel microtiter plates (COSTAR, Corning Incorporated, NY14831, USA) and mixed with 50µL of tissue culture infecting dose 50 (TCID50) of mpox virus (EPI_ISL_13302316). All dilutions were made in DMEM with 1% penicillin and streptomycin. After one-hour incubation at 37°C and 5%CO2, 50µL of 2x104 VeroE6 (VERO-C1008-ATCC®-CRL-1586™) cells were added to each well. After 6 days of incubation at 37°C and 5%CO2, wells were stained with 0.1% crystal violet solution (Merck KGaA, 64271 Damstadt, Germany) plus 5% formaldehyde 40%m/v (Carlo ErbaSpA, Arese, Italy) for 30 minutes; microtiter plates were washed in running water. Wells were scored to evaluate the degree of cytopathic effect (CPE) compared to the virus control; blue staining of wells indicated the presence of neutralizing antibodies. Neutralizing titer was the maximum dilution with the reduction of 90% of CPE, a positive titer was defined as >=1:10. Positive and negative controls were included in all test runs: every test included serum control (1:10 dilution), cells control (VeroE6 cells alone) and viral control (three-fold series dilution). Elecsys® Anti-SARS-CoV-2 (Roche Diagnostics Rotkreuz, Switzerland) was used to measure antibodies against SARS-CoV-2 Nucleoprotein on serum samples.

### Virus isolation

Viral isolation was performed for mpox-positive samples. An aliquot of 200µL of each transport medium was plated in duplicate in 24-well plates containing 80–90% confluent VeroE6 cells, adding 800µL of Dulbecco's Modified Eagle Medium with L-glutamine (Gibco ThermoFisher Scientific) supplemented with 2% of heat-inactivated fetal bovine

serum (Gibco ThermoFisher Scientific) and 1% penicillin-streptomycin (5,000 U/mL; Pen-Strep, Gibco ThermoFisher Scientific). Plates were incubated at 37 °C and 5%CO2 atmospheric pressure and evaluated daily. CPE was observed in VeroE6 cells showing typical monolayer separation and cell rounding; viral isolation was confirmed by Orthopoxvirus screening RT-PCR.

**Virus sequencing**

The viral whole genome sequencing was performed with a metagenomics approach: 500ng of the extracted DNA from the virus isolates (QIAamp DSP virus Spin Kit - Qiagen) were used to generate paired-end libraries via Illumina DNA Prep kit (Illumina) and sequenced on an iSeq100 instrument with a read length of 150 nucleotides. Raw reads were analyzed developing a specific Galaxy Project workflow to obtain the consensus sequences.

**Mpox confirmation tests**

Mpox confirmation tests, following positive pan-Orthopoxvirus PCR testing, were performed on the following samples:

**Case 1.**

*First mpox episode.* Mpox confirmation test was conducted on the oropharyngeal swab (24th May, cycle threshold=20).

*Second mpox episode.* Mpox confirmation test was conducted on the oropharyngeal swab (27th September, cycle threshold=36).

**Case 2.**

*First mpox episode.* Mpox confirmation test was conducted on the oropharyngeal swab (20th July, cycle threshold=24).

*Second mpox episode.* Mpox confirmation test was conducted on the anal swab (22nd August, cycle threshold=31).

**Evidence of SARS-CoV-2 re-infection**

**Case 2.**

Antibodies against SARS-CoV-2 Nucleoprotein supported evidence of SARS-CoV-2 re-infection during the second mpox infection in Case 2. Titers were 33.4, 36.8 and 51.9 cutoff index for serum, on 7th July, 23rd August and 9th November, respectively. Mpox testing performed on the 22nd of August highlighted presence of mpox DNA on the rectal (cycle threshold=29; negative mpox isolation) and oropharyngeal (cycle threshold=32; negative mpox isolation) swabs, with negative plasma. Positive SARS-CoV-2 isolation was obtained on the oropharyngeal swab.

**Mpox neutralizing antibodies titers**

**Case 1.**

Following the first episode of mpox (3rd June) neutralizing antibodies were barely detectable (titer=1:10). Later, we witnessed the presence of detectable neutralizing antibodies (23nd August, titer=1:40), which were stable during the second episode (28th September, titer=1:40).

**Case 2.**

At time of first mpox episode neutralizing antibodies were not detectable (7th July, titer<1:10). Later, at the complete resolution of symptoms, neutralizing antibodies were detectable (2nd August, titer=1:20), with increase during the second episode (26th August, titer=1:40; 7th September, titer=1:160).

**Virus sequencing interpretation**

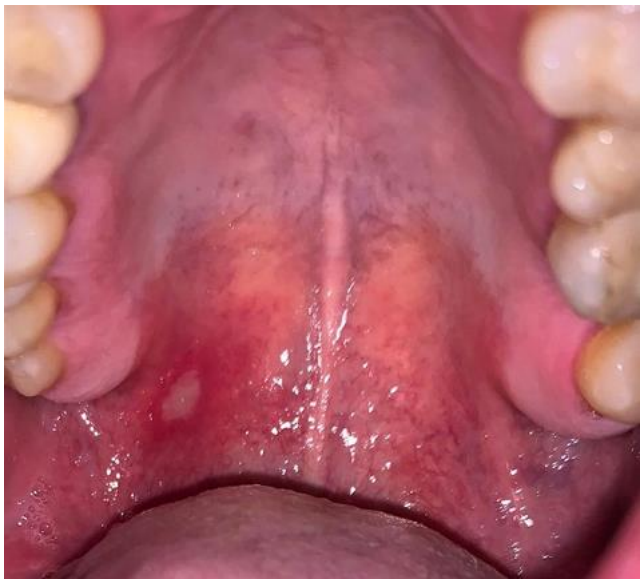
In both Case 1 and Case 2 during the first episode of mpox, the virus was isolated and sequencing was successfully performed. During both potential mpox re-infections it was not possible to isolate the virus and therefore perform sequencing. In Case 1, this could be linked with the observed high cycle threshold values, that made difficult to perform successful viral cultures. In Case 2, SARS-CoV-2 was isolated in the viral culture and with PCR on the oropharyngeal swab, which had a lower mpox cycle threshold, possibly allowing successful mpox isolation. The presence of SARS-CoV-2 on the oropharyngeal swabs might have negatively influenced mpox isolation. The rapid cytopathic effect of SARS-CoV-2 (48-72 hours), could have significantly reduced the cellular substrate for appreciable mpox replication (4-5 days). As viral culture could not be obtained in the second episodes, and according to recommendations on mpox sequencing, we underline that, given the observed cycle threshold values, with also the possible negative influence of SARS-CoV-2 infection, sequencing data are available for the first episodes of mpox and not during potential re-infection.

**Table 1.** Clinical history, virologic and serologic data of two episodes of potential mpox re-infection, occurring in a 36-year-old man who have sex with men living with HIV and a 33-year-old man who have sex with men PrEP user.

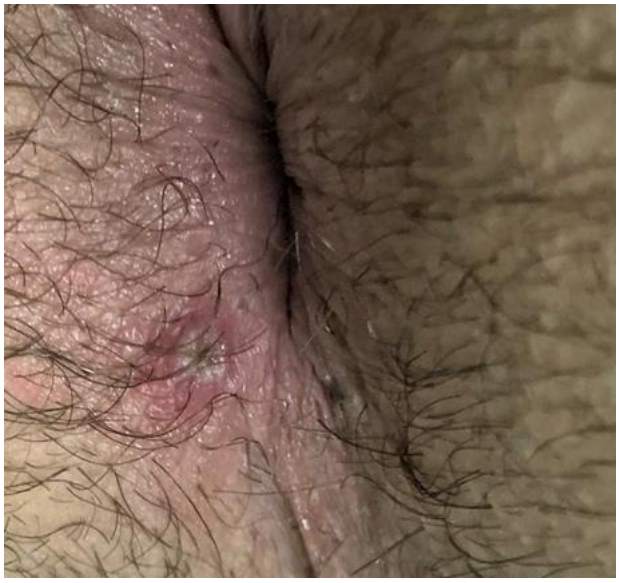
| Date | Events | Rectal swab | Oropharyngeal swab | Lesion swab | Plasma | Urines | Semen | PNRT |
|---|---|---|---|---|---|---|---|---|
| **Case 1. First episode** | | | | | | | | |
| ***18th May*** | Onset of symptoms | | | | | | | |
| ***24th May*** | Chlamydia proctitis | Positive (Ct=23)<br>VI positive | Positive (Ct=18)<br>VI positive | Positive (Ct=32) (pharyngeal)<br>VI negative | NA | NA | NA | NA |
| ***1st June*** | Resolution of symptoms | | | | | | | |
| ***3rd June*** | | Negative | Negative | NA | Negative | NA | NA | 1:10 |
| ***23rd August*** | Erythematous lesions and chlamydia urethritis | Negative | Negative | Negative | Negative | Negative | NA | 1:40 |
| **Case 1. Second episode** | | | | | | | | |
| ***27th September*** | Onset of genital lesion | | | | | | | |
| ***28th September*** | Chlamydia proctitis | Negative | Positive (Ct=34)<br>VI negative | Positive (Ct=37) (genital)<br>VI negative | Negative | Negative | Negative | 1:40 |
| ***1st October*** | Resolution of symptoms | | | | | | | |
| ***3rd October*** | | Negative | Negative | NA | Negative | Negative | NA | NA |
| ***10th October*** | | Negative | Negative | NA | Negative | Negative | NA | NA |
| **Case 2. First episode** | | | | | | | | |
| ***4th July*** | Onset of proctitis symptoms | | | | | | | |
| ***6th July*** | Onset of lower lip lesion | | | | | | | |
| ***7th July*** | Chlamydia proctitis | Positive (Ct=37)<br>VI negative | Negative | Negative (Lip) | Negative | Negative | Negative | <1:10 |
| ***14th July*** | Resolution of proctitis symptoms and lower lip lesions; onset of pharyngodynia | Negative | Positive (Ct=25)<br>VI positive | NA | Negative | NA | NA | NA |
| ***15th July*** | Onset of shoulder lesion | | | | | | | |
| ***20th July*** | | Negative | Positive (Ct=22)<br>VI positive | Positive (Ct=37) (shoulder)<br>VI negative | Positive (Ct=34) | Negative | Negative | NA |
| ***26th July*** | Resolution of pharyngodynia and shoulder lesion | NA | Positive (Ct=26)<br>VI positive | NA | Positive (Ct=36) | NA | NA | NA |
| ***2nd August*** | | NA | Negative | NA | Negative | NA | NA | 1:20 |
| **Case 2. Second episode** | | | | | | | | |
| ***22nd August*** | Onset of proctitis symptoms; gonorrhoea proctitis | Positive (Ct=29)<br>VI negative | Positive (Ct=32). MPox VI negative, SARS-CoV-2 positive | NA | Negative | NA | NA | NA |
| ***23rd August*** | | NA | NA | Negative (Biopsies) | Negative | Negative | Negative | NA |
| ***26th August*** | Resolution of proctitis symptoms | Negative | Positive (Ct=31)<br>VI negative | | Negative | Negative | Negative | 1:40 |
| ***31st August*** | | Negative | Negative | Negative | Negative | Negative | Negative | NA |
| ***7th September*** | | Negative | Negative | Negative | Negative | Negative | Negative | 1:160 |

**Legend.** PCR: polymerases chain reaction. CT: cycle threshold. VI: mpox virus isolation. NA: not available. PNRT: plaque reduction neutralization test.

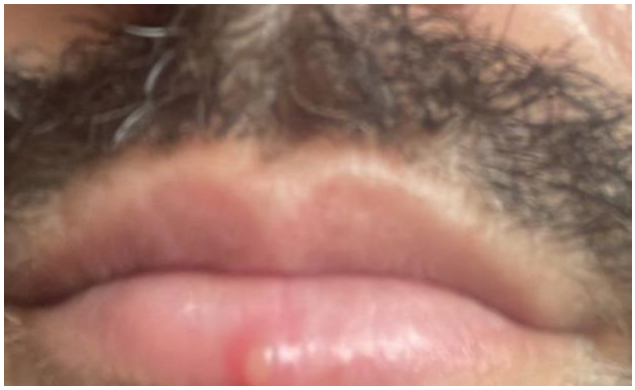
**Supplementary Figure 1.** ***Case 1. Single vesicular pharyngeal lesion in a 36-year-old man who have sex with men living with HIV, which tested positive for mpox virus.***



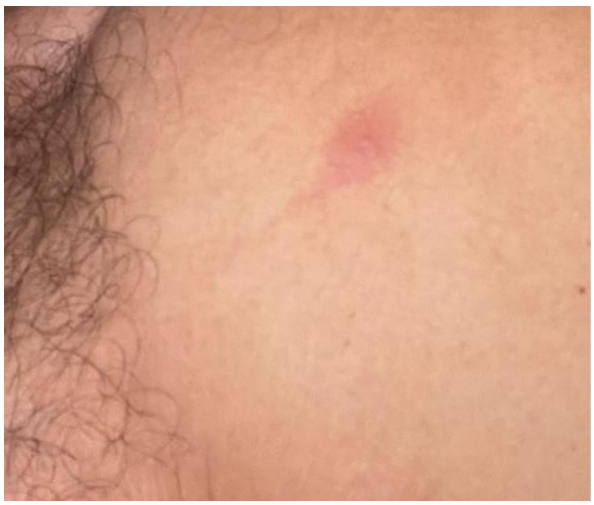
**Supplementary Figure 2.** ***Case 1. Single ulcerated perianal vesicular lesion in a 36-year-old man who have sex with men living with HIV, which tested positive for mpox virus.***



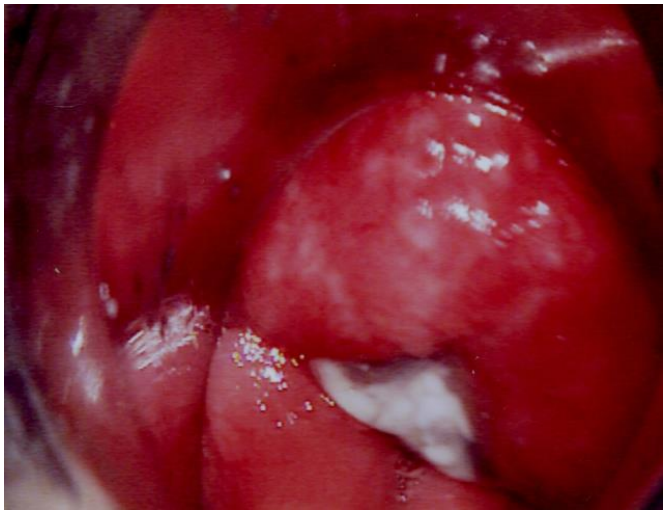
**Supplementary Figure 3.** ***Case 2.*** **Single vesicular, non-umbilicated, lesion on the lower lip in a 33-year-old man who have sex with men PrEP user, which tested negative for mpox virus.**



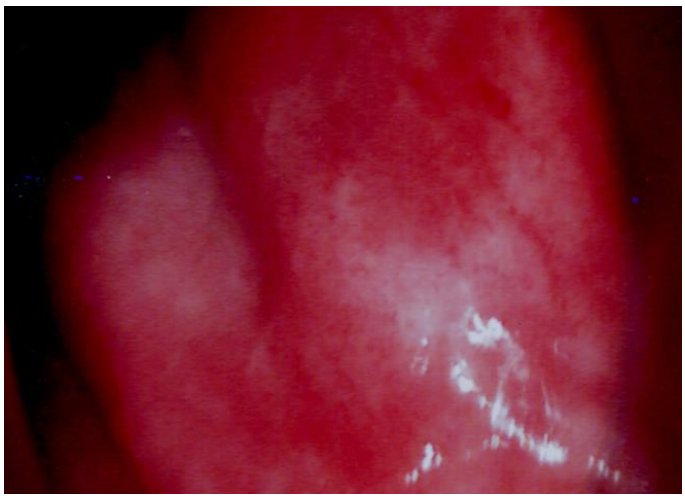
**Supplementary Figure 4.** ***Case 2.*** **Single non-tender papular lesion on the right shoulder in a 33-year-old man who have sex with men PrEP user, which tested positive for mpox virus.**



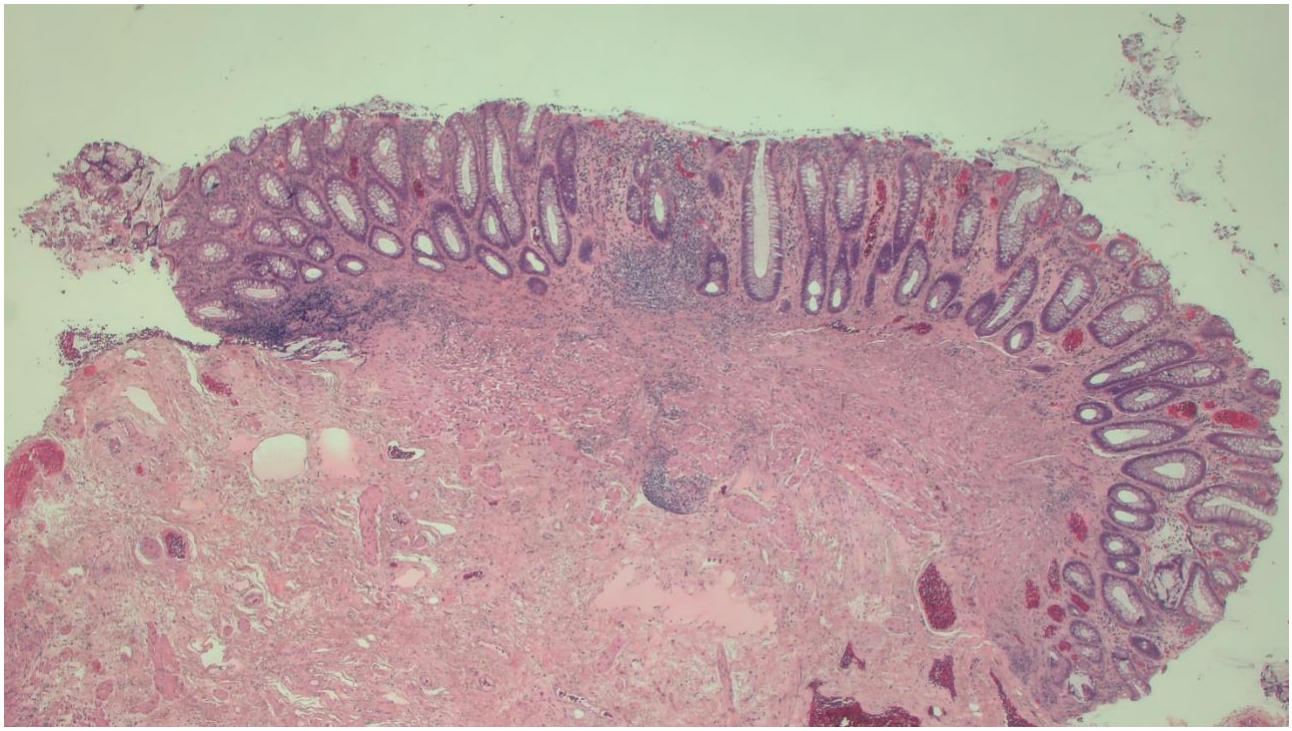
**Supplementary Figure 5.** ***Case 2.*** **High-resolution anoscopy with Zeiss OPMI Pico colposcope showing presence of diffuse mucosal edema, superficial erosions, telangiectasias and vesicular-scarred lesions during the second episode of mpox (7x).**



**Supplementary Figure 6.** ***Case 2.*** **High-resolution anoscopy with Zeiss OPMI Pico colposcope showing presence of mucosal diffuse edema, superficial erosions, telangiectasias and vesicular-scarred lesions during the second episode of mpox (10x).**



**Supplementary Figure 7.** ***Case 2.*** **Histology of the rectal biopsies performed during the second episode of mpox proctitis. The histological examination of the biopsies showed minimal architectural distortion, with moderate fibrosis of the connective tissues of the mucosa and submucosa (2,5x).**



**Supplementary Figure 7.** ***Case 2.*** **Histology of the rectal biopsies performed during the second episode of mpox proctitis, magnification of the previous picture.**