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Retrospective detection of asymptomatic monkeypox virus infections among male sexual health clinic attendees in Belgium

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1 **1. Extended Data**

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Figure #	Figure title	Filename	Figure Legend
	One sentence only	This should be the name the file is saved as when it is uploaded to our system. Please include the file extension. i.e.: *Smith_ED_Fig1.jpg*	If you are citing a reference for the first time in these legends, please include all new references in the main text Methods References section, and carry on the numbering from the main References section of the paper. If your paper does not have a Methods section, include all new references at the end of the main Reference list.
Extended Data Fig. 1	Visualisation of MPXV-PCR template sizes.	Extended_data _Fig_1.jpg	Tapestation 4150 (Agilent, Santa Clara, US) was used and the experiment was only performed once. PC: Positive control; NC: Negative control; C: cases 1 to 4, respectively
Extended Data Fig. 2	Single nucleotide variations in the monkeypox virus genome of asymptomatic case 2	Extended_data _Fig_2.tiff	Single nucleotide variations in the monkeypox virus genome of asymptomatic case 2 (ITM_pt31), as compared to a reference genome from the 2018 – 2019 outbreak in Israel (MN648051.1). Numbers in the top row denote the alignment coordinates of each position. Visualisation by snipit (https://github.com/aineniamh/snipit).
Extended Data Fig. 3	Ful version of Figure 1	Extended_data _Fig_3.tiff	Phylogeny of the monkeypox virus genome of asymptomatic case 2 in the current study (ITM_pt31, in blue), in the context of monkeypox virus genomes collected from seven recent symptomatic cases at the same institution, submitted to Genbank, in green), a range of monkeypox virus genomes from samples collected in non-endemic countries between April 1 and July 1, 2022 (downloaded from GISAID, https://www.gisaid.org, in black), and a reference genome from the 2018 – 2019 outbreak in Israel (MN648051.1, in red). Phylogenetic tree created by parsnp (default parameters).

Figure #	Figure title	Filename	Figure Legend
	One sentence only	This should be the name the file is saved as when it is uploaded to our system. Please include the file extension. i.e.: *Smith_ED_Fig1.jpg*	If you are citing a reference for the first time in these legends, please include all new references in the main text Methods References section, and carry on the numbering from the main References section of the paper. If your paper does not have a Methods section, include all new references at the end of the main Reference list.
Extended Data Table 1	Clinical evaluation, sampling and laboratory analyses	Extended_data _Table_1.jpg	No legend (added to the Table)
Extended Data Table 2	Single nucleotide variants (SNVs) in the monkeypox virus genome recovered from asymptomatic case 2	Extended_data _Table_2.jpg	No legend (added to the Table)

3   ## 2. Supplementary Information:
4

5   ## A. Flat Files
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Item	Present?	Filename	A brief, numerical description of file contents.
		This should be the name the file is saved as when it is uploaded to our system, and should include the file extension. The extension must be .pdf	i.e.: *Supplementary Figures 1-4, Supplementary Discussion, and Supplementary Tables 1-4.*
Supplementary Information	No		

| Reporting Summary | Yes | nr-reporting-summary_final_20220802.pdf |
| Peer Review Information | No | *OFFICE USE ONLY* |

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Title

Retrospective detection of asymptomatic monkeypox virus infections among male sexual health clinic attendees in Belgium

Keywords

Monkeypox, asymptomatic, subclinical, transmission, infectiousness, epidemic

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34   Abstract

35   The magnitude of the 2022 multi-country monkeypox virus outbreak has surpassed any preceding

36   outbreak. It is unclear whether asymptomatic or otherwise undiagnosed infections are fuelling this

37   epidemic. We aimed to assess whether undiagnosed infections occurred among men attending a Belgian

38   sexual health clinic in May 2022. We retrospectively screened 224 samples collected for gonorrhoea and

39   chlamydia testing using a monkeypox virus (MPXV) PCR assay, and identified MPXV DNA-positive samples

40   from four men. At the time of sampling, one man had a painful rash, and three men had reported no

41   symptoms. Upon clinical examination 21 to 37 days later, these three men were free of clinical signs and

42   they reported not having experienced any symptoms. Serology confirmed MPXV exposure in all three

43   men, and MPXV was cultured from two cases. These findings show that certain cases of monkeypox

44   remain undiagnosed, and suggest that testing and quarantining of individuals reporting symptoms may

45   not suffice to contain the outbreak.

<u>Main text</u>

47 **Introduction**

48 Monkeypox is a viral disease that is endemic in several African countries.[1] While rodents are thought to

49 act as the main reservoir, monkeys and humans are accidental hosts. Animal-to-human transmission

50 probably occurs through direct or indirect contact with live or dead infected animals,[1] and human-to-

51 human transmission of monkeypox virus (MPXV) is thought to occur mainly through close contact with

52 symptomatic cases.[2] All those infected with MPXV are assumed to develop symptoms[3] and the secondary

53 attack rate (SAR) is rather low: a systematic review estimated a SAR of 10% for unvaccinated household

54 contacts of cases infected with the Congo Basin monkeypox clade and the SAR of the Western African

55 clade, which is involved in the 2022 multi-country outbreak, is assumed to be even lower.[4] These features

56 imply that, in the absence of repeated animal-to-human transmission, an outbreak in the general

57 population tends towards extinction with relatively minor hygienic interventions, as observed in several

58 outbreaks in endemic regions.[1,5] Similarly, several instances have occurred in recent decades where cases

59 imported by travel from endemic countries have caused small outbreaks in non-endemic countries that

60 could be quickly contained.[2]

61 The current monkeypox epidemic in non-endemic countries differs from previous outbreaks, with respect

62 to the affected population and the clinical presentation.[6] Indeed, the current outbreak appears to

63 primarily affect men who have sex with men (MSM)[6], and many present with symptoms that are largely

64 limited to the anogenital region; some have only minimal signs or symptoms.[3,6] Viral DNA has been found

65 in saliva, semen and anogenital samples, and many infections are linked with sexual contact.[6,7]

66 On July 23, the Director of the World Health Organization (WHO) declared this MPXV outbreak as a public

67 health emergency of international concern as more than 16,000 cases had been confirmed to date from

68 75 countries which vastly exceeds case numbers in previous outbreaks in endemic countries.[8] Researchers

69   have raised a number of questions that might explain this extraordinary surge in cases.[9] One hypothesis

70   is that a proportion of monkeypox infections remains undiagnosed, and that undiagnosed cases continue

71   spreading the disease unknowingly. This could occur if patients did not experience any symptoms

72   (asymptomatic infection), or because their signs and symptoms were not attributed to a possible MPXV

73   infection (unrecognized infection). While the challenge of unrecognized infection can be overcome by

74   increased information about the natural history of monkeypox and improved awareness among

75   populations at risk of infection and healthcare providers, asymptomatic infection is more difficult to

76   contain due to lack of healthcare seeking of infected individuals and inability to diagnose by mere history

77   taking.

78   We therefore aimed to retrospectively assess whether MPXV infections remained undiagnosed among

79   men attending a large sexual health clinic in Belgium, in May 2022. To this end, we assessed the presence

80   of MPXV-DNA in stored samples that had been collected for routine oropharyngeal and anorectal

81   gonorrhoea/chlamydia testing at the Institute of Tropical Medicine in Antwerp (ITM), Belgium from

82   individuals who consented with additional analysis of their samples.

83   **Results**

84   Throughout May 2022, 237 men underwent sampling for anorectal or oropharyngeal

85   gonorrhoea/chlamydia testing at ITM. Indications for sampling were either diagnostic evaluation in case

86   of symptoms compatible with gonorrhoea or chlamydia, or gonorrhoea/chlamydia screening in

87   asymptomatic men at risk of infection due to high-risk sexual behaviour. These men included MSM living

88   with HIV, MSM using HIV pre-exposure prophylaxis and men who were notified by a recent sex partner

89   with gonorrhoea or chlamydia. Men who denied having symptoms were not clinically examined at the

90   time of sampling, which is in line with common clinical practice. Anorectal swabs were self-sampled,

91   whereas oropharyngeal swabs were taken by a clinician. From samples of 224 men, left-over DNA extracts

92   were available for testing by MPXV-PCR. These included two oropharyngeal swabs, 60 anorectal swabs,

93   and 162 pooled samples (the combination of a patient's first-void urine, oropharyngeal swab, and

94   anorectal swab).[10] Extended Data Table 1 provides an overview of the clinical assessment, sampling and

95   analyses that were performed.

96   MPXV-PCR was positive on four DNA extracts: three from anorectal swabs, and one from a pooled sample

97   (Table 1). These MPXV-positive samples were collected from four men. At the time of sampling (further

98   referred to as day 0), one of the four men suffered from a painful vesicular perianal rash, which was

99   misdiagnosed as a flare-up of herpes simplex. The remaining three men did not report any symptoms at

100   day 0. All men were contacted as soon as their retrospective diagnosis was made, and recalled to the clinic

101   for additional case investigation.

102   The three MPXV-positive men that had not reported symptoms on day 0 returned to the clinic within 21

103   to 37 days after sample collection (day 0). They were between 30 and 50 years old, had a well-controlled

104   HIV infection under antiretroviral therapy (viral load < 20/µL, CD4 counts above 350/µL), and had a history

105   of multiple sexually transmitted infections (STIs). None of the three men were previously vaccinated

106   against smallpox. Upon return to the clinic, the men were thoroughly questioned about potential

107   monkeypox-related and other symptoms, and clinically examined for signs of monkeypox with particular

108   attention to the skin, oropharynx and anogenital region (Extended Data Table 1). All three men denied

109   having noticed any symptoms during the two months prior to day 0 and up till their return visit. No signs

110   of monkeypox were observed during clinical examination. All three men had condomless sexual

111   intercourse with at least one male partner within a few days to one month before day 0. Two out of three

112   men had sexual contacts while travelling abroad within two weeks before day 0, and all had sex with at

113   least one partner after day 0. According to the index cases, none of their main partners had reported

114   symptoms of monkeypox, and casual partners could not be traced. Results of basic laboratory

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115   investigations at day 0 including renal and liver function tests as well as C-reactive protein were within

116   normal limits (data not shown).

117   The retrospective diagnosis of monkeypox in the three men with asymptomatic infection was confirmed

118   by multiple techniques. First, we repeated MPXV PCR on new DNA extracts of the stored original patient

119   samples, which were positive for all three samples. PCR template size analysis confirmed specific

120   amplification of the targeted MPXV genomic region (Extended Data Fig. 1). Second, another PCR targeting

121   a wider range of orthopox viruses was positive on all day 0 samples.[11] Third, we performed whole genome

122   sequencing and recovered 98% of the MPXV genome in the anorectal swab of case 2 (Genbank

123   ON950045). Of note, phylogenetic (Fig. 1) and single nucleotide variant (SNV) analyses from the

124   interpretable genome fraction (Extended data Fig. 2, Extended data Table 2) did not reveal apparent

125   divergences between the MPXV genome from case 2 and publicly available MPXV genome sequences

126   from other monkeypox cases generated during the current monkeypox outbreak in non-endemic

127   countries. Fourth, viral isolation confirmed presence of replication-competent MPXV in the anorectal

128   swabs of case 2 and 3 at day 0. Anorectal samples taken at the return visit were MPXV-PCR negative for

129   all three men, indicating that the infection had cleared spontaneously by that time. Lastly, orthopox-

130   directed IgG antibodies were detected in convalescent patient sera (day 21-37) of all three men using an

131   EN ISO 15189 accredited orthopox IgG immunofluorescence assay previously established for MPXV IgG

132   detection (Methods).[12,13] IgG titres ranged from 1:40 to 1:320 (cut-off 1:20) which is similar to titers

133   observed in symptomatic cases two to four weeks after symptom onset.[13] Importantly, all day 0 sera were

134   IgG negative. This seroconversion provided final evidence of recent orthopox virus exposure.

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Discussion

140   To summarise, we found four monkeypox cases that had remained undiagnosed among men consulting

141   for gonorrhoea/chlamydia testing in May 2022. Besides one unrecognised symptomatic case, these

142   included three men who had not noticed any symptoms. Interestingly, case 1 predated the first detected

143   symptomatic case in Belgium by several days,[14] and could not be epidemiologically linked to any other

144   monkeypox case through contact tracing, nor did he report international travel or participation in mass

145   gatherings before day 0, indicating that MPXV had been circulating in Belgium before the first cases were

146   formally detected. While it cannot be excluded that those three asymptomatic men had unnoticed signs

147   of monkeypox at the time of infection, the significance of these cases lies in the fact that they would not

148   have sought medical care if it were not for a scheduled visit for routine HIV follow-up and STI screening.

149   Indeed, as they were not aware of their MPXV infection, none of the men had self-isolated and all of them

150   had sexual contacts around the time of detectable MPXV DNA. The presence of replication-competent

151   virus in two out of three asymptomatic cases indicates that they may have been able to transmit the virus,

152   but the possibility of onward transmission could not be verified by the retrospective nature of our study.

153   While other retrospective studies have found serological evidence of MPXV infection in asymptomatic

154   MPXV-exposed individuals,[15–21] our study adds the finding of replication-competent virus particles in

155   asymptomatic individuals. Prospective serological, molecular and epidemiological studies involving

156   monkeypox cases and their contacts will need to establish the proportion of MPXV infections that present

157   without symptoms, or without recognized symptoms, whether they present any clinical signs of infection

158   at any point in time and how likely it is that they transmit the virus.

159   Asymptomatic carriership was thought to play a negligible role in the spread of orthopox viruses.[22,23]

160   Despite the fact that smallpox virus could be detected in the upper respiratory tract of asymptomatic

161   contacts of smallpox cases,[22] smallpox eradication was primarily, and successfully, based on the

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162   identification and quarantining symptomatic cases, and tracing their contacts.[23] Monkeypox outbreaks in

163   endemic settings have successfully been contained by similar measures.[1] However, undiagnosed

164   infections may play a much more significant role in terms of overall disease transmission in the current

165   outbreak among MSM compared to previous orthopox epidemics because of the dense sexual network

166   including anonymous contacts of some MSM, which hampers efficient contact tracing. Moreover, viral

167   transmission in the absence of noticeable symptoms could explain why self-isolation at symptom onset

168   has been insufficient to halt the epidemic thus far.

169   In conclusion, the finding of several monkeypox cases that remained undiagnosed at the beginning of the

170   epidemic implies case finding should be intensified. First, healthcare workers and individuals at risk of

171   infection should be aware that monkeypox symptoms may overlap with those of other diseases, in

172   particular STIs. Second, not all individuals with monkeypox infection notice symptoms, and so may not

173   seek medical attention. Increased awareness of the sometimes subtle signs of disease, as well as

174   intensified testing and contact tracing, may be helpful to diagnose additional cases. Populations at risk of

175   infection should be encouraged to keep record of their close contacts and, until there is more clarity about

176   the extent to which asymptomatic individuals are contagious, high-risk contacts of infected cases should

177   be aware that they might transmit the virus even if asymptomatic. Beyond the general recommendation

178   for close contacts to self-monitor for symptoms and the advice by the European Centre for Disease Control

179   to abstain from sexual activities for a period of 21 days,[24] our data suggest that in the absence of

180   symptoms, monkeypox testing may need to be considered to confirm the end of this period. Further

181   research is needed to determine the duration of the infectious period in symptomatic as well as

182   asymptomatic monkeypox cases in order to guide clinical recommendations. Third, high-risk populations

183   should have access to low-threshold monkeypox testing and health care providers may consider screening

184   for monkeypox in high-risk populations. The availability of performant rapid diagnostic (self-)tests could

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185   further lift testing barriers. Finally, undiagnosed monkeypox cases will need to be taken into account when

186   determining the usefulness of pre- or post-exposure vaccination of individuals at highest risk of infection.

198   Authors Contributions statement

199   IDB, MVE, KV, FV, DVDB conceptualized the study. IDB, MVE, KV, FV, DVDB, KKA, PS supervised and

200   coordinated the laboratory analyses at the Institute of Tropical Medicine. PG, SZ, and JJB supervised and

201   coordinated the laboratory analyses at the Bundeswehr Institute of Microbiology. HS, JM, TDB and SC

202   performed the testing. IDB, AMR & CVD analysed the data. CK managed the cases clinically. IDB & CVD

203   wrote the first draft of the manuscript with revision by KV & MVE. IDB and CVD contributed equally; KKA

204   and AMR contributed equally; KV and MVE contributed equally. All authors reviewed and approved the

205   final version of the manuscript.

206   Competing Interests Statement

207   The authors declare no competing interests.

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209 <u>Tables</u>

210 **Table 1: Patient and sample characteristics**

211

Case	Time point	Sample type	Symptoms	MPXV-PCR on left-over DNA extract (Ct value)	MPXV-PCR on original sample (Ct value)	Orthopox virus-PCR on left-over DNA extract (Ct value)	Whole genome sequencing	Viral viability study	Orthopox virus IgG antibodies (titer)
1	Day 0	Pooled sample*	None reported	Positive (27.63)	Anorectal swab: positive (26.69); oropharyngeal swab: negative	Positive (30.35)	ND	Negative	Negative (<1:20)
	Day 37	Anorectal swab	None reported	NA	Negative	NA	NA	NA	Positive (1:320)
2	Day 0	Anorectal swab	None reported	Positive (22.25)	Positive (20.05)	Positive (23.50)	Yes	Positive	Negative (<1:20)
	Day 21	Anorectal swab	None reported	NA	Negative	NA	NA	NA	Positive (1:40)
3	Day 0	Anorectal swab	None reported	Positive (19.19)	Positive (17.16)	Positive (21.43)	ND	Positive	Negative (<1:20)
	Day 24	Anorectal swab	None reported	NA	Negative	NA	NA	NA	Positive (1:80)
4	Day 0	Anorectal swab	Painful vesicular perianal rash	Positive (29.06)	Positive (27.38)	Positive (28.3)	ND	Negative	NA

212 Ct = cycle threshold; MPXV = monkeypox virus; NA = not applicable/not available
213 * combination of a patient's first-void urine, oropharyngeal swab, and anorectal swab

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214 <u>Figure Legends/Captions (for main text figures)</u>

215 <u>Fig. 1:</u> Phylogeny of the monkeypox virus genome of case 2 (ITM_pt31, in blue), in the context of

216 monkeypox virus genomes collected from seven recent cases with monkeypox symptoms at the same

217 institution (submitted to GenBank: in green), a range of monkeypox virus genomes from samples

218 collected in non-endemic countries between April 1 and July 1, 2022 (downloaded from GISAID,

219 https://www.gisaid.org, in black), and a reference genome from the 2018 – 2019 outbreak in Israel

220 (MN648051.1, in red). Phylogenetic tree created by *parsnp* (default parameters). Branches containing no

221 samples from our institute were collapsed for simplicity. The full version of the tree can be found in

222 Extended Data Fig. 3.

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282 <u>Methods</u>

283 **Ethical considerations**

284 All subjects included in this study were informed that their pseudonymized samples and data could be

285 used for additional research purposes and that they could be notified of findings relevant for their health.

286 Those who preferred not to participate in additional research were given the opportunity to opt out, and

287 their samples and data were not used in the current study. In addition, retrospective written informed

288 consent was obtained from all MPXV-positive asymptomatic cases for publication of their data upon

289 return to the clinic. The study protocol was approved by the Institutional Review Board of the Institute of

290 Tropical Medicine (1600/22).

291 **Samples**

292 To increase the potential yield of the study, only samples from individuals who self-identified as men

293 attending our sexual health clinic were included as the majority of reported cases to date in the current

294 epidemic were men. Included sample types were anorectal swabs (Eswab, Copan Diagnostics, Brescia,

295 Italy), oropharyngeal swabs (Eswab, Copan Diagnostics, Brescia, Italy), or a combination of a patient's

296 first-void urine, oropharyngeal swab, and anorectal swab (pooled sample, as described[10]). All samples

297 were collected for routine diagnostic testing or screening of gonorrhoea/chlamydia in men with or

298 without symptoms compatible with a gonorrhoea/chlamydia infection. Samples were processed with the

299 Abbott Real Time CT/NG assay which includes the Abbott m2000sp for DNA extraction (Abbott Molecular

300 Des Plaines, Illinois, USA). The original swab samples and their left-over DNA extracts were frozen (-20°C)

301 or refrigerated (2-8°C), respectively, until processing in the current study. Repeat DNA extraction of the

302 original samples was done with Maxwell® Promega, using 300 µL sample input and 75 µL elution volume.

303 **Molecular detection of monkeypox- and orthopox-virus**

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304   The monkeypox virus (MPXV) PCR in this study made use of previously described primer sets targeting the

305   MPXV-TNF receptor gene.[25] The Applied Biosystems Quantstudio PCR system was used for PCR

306   amplification. To verify specificity of the MPXV-PCR, PCR template sizes were analysed by TapeStation

307   4150 (Agilent, Santa Clara, US) with the HS D1000 kit.

308   The orthopox virus PCR was performed as previously described.[11]

309   **Whole genome sequencing analyses**

310   Whole genome sequencing was performed on the anorectal sample of case 2, as follows. Extracted DNA

311   was amplified using sequence-independent, single-primer amplification (adjusted from[26]) and used as

312   input into Oxford Nanopore ligation sequencing kit SQK-LSK109 before sequencing on a MinION flowcell

313   (R9.4.1, Oxford Nanopore Technologies, Oxford, UK). Reads longer than 200 bp were filtered from the

314   sequencing data before mapping against the human genome T2T[27] using *minimap2*[28]. Unmapped reads

315   were used as input for the MetaMaps classification tool[29] using the set of complete genomes from the

316   RefSeq database (https://www.ncbi.nlm.nih.gov/refseq). The classified reads belonging to the

317   Monkeypox genome were aligned against reference genome MN648051.1, present at GenBank database

318   using *minimap2* and the *Medaka* tool (https://github.com/nanoporetech/medaka). The mapping result

319   was then used to produce a consensus sequence applying the *iVar* tool *consensus* module

320   (https://github.com/andersen-lab/ivar). The sequencing depth was calculated by *samtools depth* [30] and

321   the BAM file was generated by *minimap2* and *Medaka*. The reference genome was covered in 98% of its

322   extension at an average sequencing depth of 161.4x. The MPXV consensus sequence of asymptomatic

323   case 2 in the current study was used for sequence alignment using MAFFT,[31] along with MPXV consensus

324   sequences from seven recent symptomatic cases that were diagnosed at our institution (Genbank

325   submission numbers: 2608876, 2608883, 2608888, 2608899, 2608905, 2608908, 2608909), as well as

326   other   complete   MPXV   genomes   recovered   from   GISAID   (all   genomes   available   from

20

327   https://www.gisaid.org from samples collected between April 1 and July 1, 2022). The alignment was

328   cleaned with GBlocks[32] (default parameters) to keep only the informative sites. The original alignment was

329   composed of 329 sequences and 206,797 sites, whereas the cleaned data contained 188.882 sites. After

330   manual verification of the alignment (for alignment site consistency and artificial divergence), 324

331   sequences were retained. The cleaned alignment was used as input for *parsnp*

332   (https://github.com/marbl/parsnp) to produce a phylogenetic tree. We applied the *SNP-sites* tool[33] to

333   identify single nucleotide variations (SNVs) based on the MAFFT-alignment of the consensus sequence of

334   case 2 and the reference genome mentioned above. SNVs were checked for sequencing depth and

335   agreement on the sequencing data for the alternative allele using the tool *bam-readcount*.[34]

336   **Viral viability studies**

337   Anal swab samples were passed through a 0.45µm filter, and spinoculated (at 2500 g / 37°C for 2 hours)

338   on confluently grown VERO cells (obtained from ATCC, ref. CCL-81) in a 96-well cell culture plate before

339   incubation at 37°C and 7% CO2. Cultures were microscopically checked for cytopathic effect (CPE) typical

340   for MPXV infection. On day seven of the primary culture, supernatant of CPE-positive wells was harvested

341   and used as inoculum for secondary culture on confluently grown VERO cells in a 24-well culture plate.

342   After two hours, the supernatant was replaced by fresh cell culture medium. The plate was further

343   incubated until maximum CPE was reached (i.e. total destruction of the cell layer). For both CPE-positive

344   patient samples (case 2 and 3), MPXV-PCR on culture supernatant showed increased viral DNA titers at

345   the end of both the primary and secondary cultures, compared to their respective inocula, confirming that

346   the observed CPE was MPXV-induced. These viral viability studies were not standardized but based on a

347   previously published method.[35]

348   **Orthopox virus serology**

21

349   Orthopox virus IgG antibodies in paired serum or plasma samples were screened using an EN ISO 15189

350   accredited in-house assay at the Bundeswehr Institute of Microbiology, Germany, following a method

351   previously used to confirm monkeypox virus infection in humans and animals.[12,13] Briefly, chamber slides

352   with African green monkey kidney epithelial cells (MA104; ATCC CRL-2378.1) were infected with 10 to 15

353   plaque forming units of Vaccinia Virus Elstree in MEM with 5% FBS. Plaques were fixed in

354   methanol/acetone 40 hours post infection. Chambers were blocked with dilution buffer (PBS - 10% goat

355   serum) for 1 hour at 37°C. Then twofold dilutions of human sera (samples and controls) in dilution buffer

356   were prepared and incubated for 1 hour at 37°C. Samples were washed with PBS-0.25 Tween20. FITC-

357   labelled anti-human IgG (1/20) was added with Evans Blue (1/50) as counterstain and incubated for 30

358   minutes at 37°C. After a washing step with sterile water the chamber slides were air-dried, followed by

359   immunofluorescence microscopy using a Nikon Eclipse 50i instrument with a 40x objective. Human

360   Vaccinia immunoglobulin was used as positive controls (reference >1:20) and dilution buffer as negative

361   control.

362   **Data Availability**

363   The data supporting the findings of this publication can be found in Table 1.

364   The assembled consensus sequence for the MPXV genome of asymptomatic case 2 was deposited in the

365   National Center for Biotechnology Information (NCBI) under the GenBank accession number ON950045.

366   **Statistics & Reproducibility**

367   A descriptive analysis was performed. No other statistical analyses were done. No statistical method was

368   used to predetermine sample size. One individual returned twice to the clinic for gonorrhoea/chlamydia

369   screening during May 2022. We only included one data point as both samples were MPXV negative. The

370   experiments were not randomized and the investigators were not blinded to allocation during

371   experiments and outcome assessment.

372 We cross-validated the MPXV positivity of the asymptomatic cases with multiple techniques: 1. Repetition

373 of the MPXV PCR on new DNA extracts of the stored original patient samples. 2. Orthopox virus PCR on

374 day 0 samples. 3. Whole genome sequencing of MPXV of case 2. 4. Viral isolation. 5. Orthopox-directed

375 IgG antibody detection on paired sera (day 0 and follow-up visit) of all three men.

376

377 <u>Methods-only References</u>

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401

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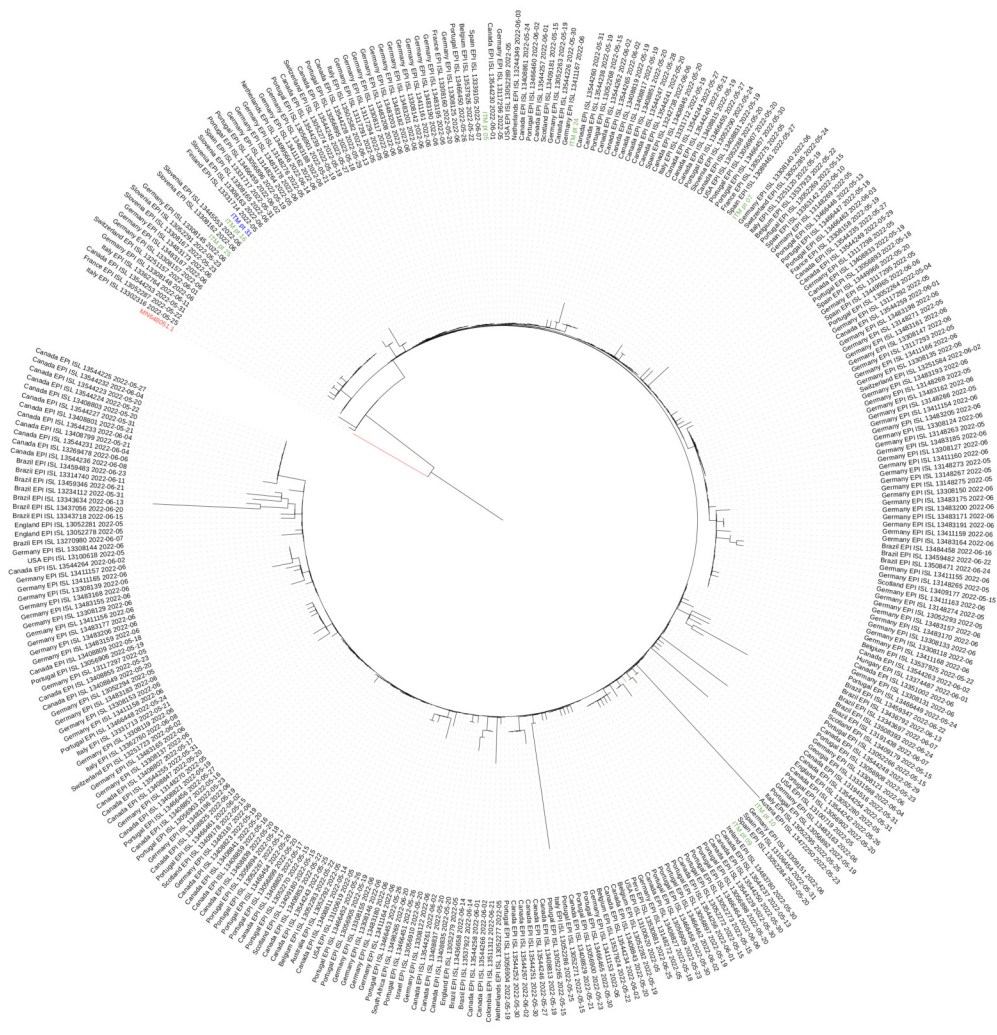
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MN648051.1

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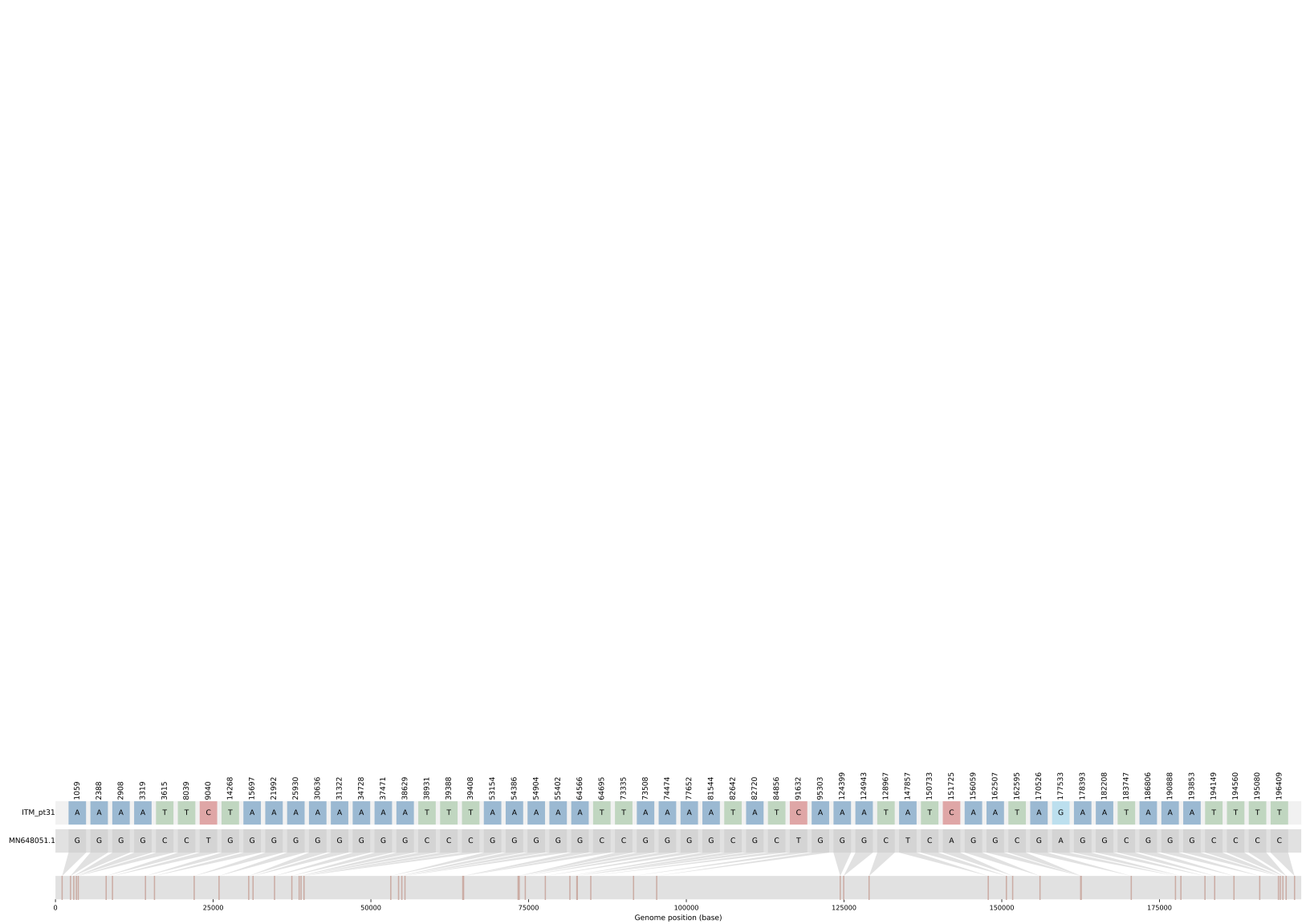
Tree scale: 0.001

| Time point | History | Clinical examination | Sample type | Analyses done | | | | | |
| | | | | On left-over DNA extract | | On DNA-extract of the original, un-pooled swab material | | On original sample | |
				MPXV-PCR[a]	Ortho-pox virus-PCR	MPXV-PCR	WGS	Viral viability assay	Orthopox virus IgG antibodies
Initial sampling visit (day 0)	Patients were asked whether they had noticed any new or persisting symptoms, past and recent illnesses or other health concerns in general, and related to the anogenital / oropharyngeal region and skin in particular.	Symptom-oriented (*i.e.* clinical examination in the presence of symptoms)	Pooled sample[b]	Yes[c]	Yes[c]	NA	NA	No	
			Anorectal swab	Yes[c]	Yes[c]	Yes	Yes	Yes	
			Oropharyn-geal swab	Yes[c]	Yes[c]	NA	NA	NA	
			Serum or plasma						Yes
Follow-up visit (day of return to clinic)[d]	Patients were asked whether they noticed any new symptoms in the two months prior to day 0 and up till the follow-up visit. They were specifically asked if there were/had been any rash or lesions of the skin or mucosa of the oral cavity or anus, anal irritation or symptoms of proctitis, fever, headache, general feeling of illness, fatigue, myalgia, or lymphadenopathies	Full clinical examination including thorough examination of the oropharynx, the anogenital area and skin	Anorectal swab	NA	NA	Yes	NA	NA	
			Serum						Yes

MPXV = monkeypox virus; WGS: Whole genome sequencing; NA = not applicable/not available

[a] MPXV-PCR on left-over DNA extracts was used as the first screening test. All other analyses were only performed if the first screening test was positive.

[b] combination of a patient's first-void urine, oropharyngeal swab, and anorectal swab

[c] depending on which sample type was available as a stored left-over DNA-extract

[d] only patients with a positive MPXV-PCR on the initial sample were called back to the clinic for a follow-up visit

	SNV coordinate		Nucleotide variant		Reads containing nucleotide variant ITM_pt31
Alignment	MN648051.1	ITM_pt31	MN648051.1	ITM_pt31	n/N reads covering the position (%)
1059	1059	1055	G	A	42/47 (89.4)
2388	2388	2384	G	A	73/78 (93.6)
2908	2908	2904	G	A	121/128 (94.5)
3319	3319	3315	G	A	122/135 (90.4)
3615	3615	3611	C	T	154/176 (87.5)
8039	7790	7987	C	T	24/25 (96.0)
9040	8991	8986	T	C	52/60 (86.7)
14268	14219	14211	G	T	53/58 (91.4)
15697	15648	15638	G	A	166/173 (96.0)
21992	21943	21931	G	A	125/128 (97.7)
25930	25881	25869	G	A	159/177 (89.8)
30636	30587	30573	G	A	219/230 (95.2)
31322	31273	31259	G	A	90/106 (84.9)
34728	34679	34664	G	A	82/95 (86.3)
37471	37422	37406	G	A	142/155 (91.6)
38629	38580	38563	G	A	241/248 (97.2)
38931	38882	38865	C	T	198/228 (86.8)
39388	39339	39322	C	T	124/141 (87.9)
39408	39359	39342	C	T	132/147 (89.8)
53154	53105	53084	G	A	598/620 (96.5)
54386	54337	54316	G	A	193/221 (87.3)
54904	54855	54834	G	A	129/149 (86.6)
55402	55353	55331	G	A	99/108 (91.7)
64566	64517	64492	G	A	265/280 (94.6)
64695	64646	64621	C	T	247/257 (96.1)
73335	73286	73259	C	T	200/218 (91.7)
73508	73459	73431	G	A	39/45 (86.7)
74474	74425	74397	G	A	383/418 (91.6)
77652	77603	77571	G	A	126/138 (91.3)
81544	81495	81459	G	A	168/180 (93.3)
82642	82593	82557	C	T	161/173 (93.1)
82720	82671	82635	G	A	285/291 (97.9)
84856	84807	84771	C	T	151/169 (89.3)
91632	91583	91544	T	C	180/196 (91.8)
95303	95254	95213	G	A	263/295 (89.2)
124399	124350	124301	G	A	281/303 (92.7))
124943	124894	124845	G	A	175/189 (92.6)
128967	128918	128867	C	T	57/58 (98.3)
147857	147807	147677	T	A	31/33 (93.9)
150733	150683	150550	C	T	188/194 (96.9)
151725	151675	151524	A	C	115/125 (92.0)
156059	156009	155854	G	A	72/79 (91.1)
162507	162457	162301	G	A	212/226 (93.8)
162595	162545	162389	C	T	203/216 (94.0)
170526	170476	170316	G	A	137/143 (95.8)
177533	177483	177317	A	G	43/44 (97.7)
178393	178343	178176	G	A	40/41 (97.6)
182208	182158	181990	G	A	90/93 (96.8)
183747	183697	183529	C	T	528/614 (86.0)
186806	186756	186588	G	A	164/181 (90.6)
190888	190838	190669	G	A	47/49 (95.9)
193853	193803	193575	G	A	197/210 (93.8)
194149	194099	193871	C	T	114/128 (89.1)
194560	194510	194282	C	T	115/124 (92.7)
195080	195030	194802	C	T	63/76 (82.9)
196409	196359	196131	C	T	34/36 (94.4)

Single nucleotide variants (SNVs) in the monkeypox virus genome recovered from asymptomatic case 2 (ITM_pt31) as compared to reference sequence MN648051.1 from the 2018 – 2019 outbreak in Israel. Of note, 49 SNVs were consistent with those previously reported from a monkeypox virus genome from the 2022 multi-country outbreak in Portugal (Isidro, J. et al. Nat. Med. 1–1 (2022), indicated in black), and seven others were shared by most (98,8-100%) of the MPXV genome sequences from samples collected in a range of non-endemic countries between April 1 and July 1, 2022 (downloaded from GISAID, https://www.gisaid.org, indicated in red, and visualised in Extended Data Fig 2).

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Last updated by author(s): 2022-AUG-03

Reporting Summary

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For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

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☐	☒	A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
☒	☐	The statistical test(s) used AND whether they are one- or two-sided *Only common tests should be described solely by name; describe more complex techniques in the Methods section.*
☒	☐	A description of all covariates tested
☒	☐	A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
☒	☐	A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
☒	☐	For null hypothesis testing, the test statistic (e.g. F, t, r) with confidence intervals, effect sizes, degrees of freedom and P value noted *Give P values as exact values whenever suitable.*
☒	☐	For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
☒	☐	For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
☒	☐	Estimates of effect sizes (e.g. Cohen's d, Pearson's r), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection	Whole genome sequencing included alignment of the Monkeypox genome of case 2 to reference genome MN648051.1, present at GenBank database using minimap2 and the Medaka tool (https://github.com/nanoporetech/medaka) and MPXV consensus sequences from seven recent symptomatic cases that were diagnosed at our institution (submitted to GenBank), as well as other complete MPXV genomes recovered from GISAID (all genomes available from https://www.gisaid.org from samples collected between April 1 and July 1, 2022).
Data analysis	The mapping result was then used to produce a consensus sequence applying the iVar tool consensus module (https://github.com/andersen-lab/ivar). The sequencing depth was calculated by samtools depth and the BAM file was generated by minimap2 and Medaka. The MPXV consensus sequence of asymptomatic case 2 in the current study was used for sequence alignment using MAFFT, along with MPXV consensus sequences from seven recent symptomatic cases that were diagnosed at our institution (submitted to GenBank), as well as other complete MPXV genomes recovered from GISAID (all genomes available from https://www.gisaid.org from samples collected between April 1 and July 1, 2022). The alignment was cleaned with GBlocks34 (default parameters) to keep only the informative sites. The original alignment was composed of 329 sequences and 206,797 sites, whereas the cleaned data contained 188.882 sites. After manual verification of the alignment (for alignment site consistency and artificial divergence), 324 sequences were retained. The cleaned alignment was used as input for parsnp (https://github.com/marbl/parsnp) to produce a phylogenetic tree. We applied the SNP-sites tool to identify single nucleotide variations (SNVs) based on the MAFFT-alignment of the consensus sequence of case 2 and the reference genome mentioned above. SNVs were checked for sequencing depth and agreement on the sequencing data for the alternative allele using the tool bam-readcount.

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors and reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.

Data

The data supporting the findings of this publication can be found in Table 1. The assembled consensus sequence for the MPXV genome of asymptomatic case 2 was deposited in the National Center for Biotechnology Information (NCBI) under the GenBank accession number ON950045 and in the GISAID database.

Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

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For a reference copy of the document with all sections, see nature.com/documents/nr-reporting-summary-flat.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size	As this was a retrospective study, all anorectal, pharyngeal or pooled samples that were collected from men for CT/NG screening, were tested for the presence of MPXV.
Data exclusions	One patient returned to the clinic twice for CT/NG screening. Both samples were negative for MPXV. To avoid misunderstandings we decided to report this patient only once in this descriptive analysis (the oldest sample was included)
Replication	We cross-validated the MPXV positivity of the asymptomatic cases with multiple techniques: . 1. We repeated MPXV PCR on new DNA extracts of the stored original patient samples, which came back positive for all three samples. PCR template size analysis confirmed specific amplification of the targeted MPXV genomic region. 2. Another PCR targeting a wider range of orthopox viruses was positive on all day 0 samples. 3. We performed whole genome sequencing and recovered 98% of the MPXV genome in the anorectal swab of case 2. 4. Viral isolation confirmed presence of replication-competent MPXV in the anorectal swabs of case 2 and 3 at day 0. 5. Orthopox-directed IgG antibodies were demonstrated on convalescent patient sera (day 21-37) of all three men using an EN ISO 15189 accredited orthopox IgG immunofluorescence assay previously established for MPXV IgG detection (Methods). Importantly, all day- 0 sera were IgG negative. This seroconversion provided final evidence of recent orthopox virus exposure.
Randomization	Due to the retropsective design of the study, randomization was not applicable
Blinding	Due to the retrospective design of the study, blinding was not applicable

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

n/a	Involved in the study
☐	☒ Antibodies
☐	☒ Eukaryotic cell lines
☒	☐ Palaeontology and archaeology
☒	☐ Animals and other organisms
☐	☒ Human research participants
☒	☐ Clinical data
☒	☐ Dual use research of concern

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☒	☐ Flow cytometry
☒	☐ MRI-based neuroimaging

Antibodies

Antibodies used	FITC-labelled anti-human IgG antibodies Rabbit F(ab')2 Anti-Human IgG(H+L), Mouse ads-FITC (Cat. No.: 6005-02) from Southern Biotech were used for orthopox virus serology.
Validation	These react with the heavy and light chains of human IgG and light chains of human IgM and IgA. Relevant citations:

Validation	1. Baselmans PJ, Pöllabauer E, van Reijsen FC, Heystek HC, Hren A, Stumptner P, et al. IgE production after antigen-specific and cognate activation of HLA-DPw4-restricted T-cell clones, by 78% of randomly selected B-cell donors. Hum Immunol. 2000;61:789-98. (ELISA) 2. Piesche M, Ho VT, Kim H, Nakazaki Y, Nehil M, Yaghi NK, et al. Angiogenic cytokines are antibody targets during graft-versus-leukemia reactions. Clin Cancer Res. 2015;21:1010-8. (ELISA)

Eukaryotic cell lines

Policy information about cell lines

Cell line source(s)	For the viral viability studies, VERO cells were obtained from ATCC - ref CCL-81. For orthopox serology, MA 104 (ATCC: CRL-2378.1 was used.
Authentication	Cells from ATCC were used in both experiments. ATCC provides authenticated cell lines.
Mycoplasma contamination	Cell lines were not tested for mycoplasma contamination.
Commonly misidentified lines (See ICLAC register)	Not applicable

Human research participants

Policy information about studies involving human research participants

Population characteristics	This was a retrospective study. We only included men that underwent anorectal and/or oropharyngeal gonorrhoea/chlamydia screening at the Institute of Tropical Medicine, Antwerp, Belgium during the month of May 2022 (n=237). To increase the potential yield of the study, only samples from individuals who self-identified as men attending our sexual health clinic were included as the majority of reported cases in the current epidemic were men. Indications for sampling were either diagnostic evaluation in case of symptoms compatible with gonorrhoea or chlamydia, or gonorrhoea/chlamydia screening in asymptomatic men at risk of infection due to high-risk sexual behaviour. These men included MSM living with HIV, MSM using HIV pre-exposure prophylaxis and men who were notified by a recent sex partner with gonorrhoea or chlamydia. Only left-over material of 224 men was available. The three MPXV-positive men that had not reported symptoms on day 0 were between 30 to 50 years old. None of the men received financial compensation.
Recruitment	Not applicable
Ethics oversight	The study protocol was approved by the Institutional Review Board of the Institute of Tropical Medicine (1600/22). In our clinic, all subjects included in this study were informed that their pseudonymized samples and data could be used for additional research purposes and that they could be notified of findings relevant for their health. Those who preferred not to participate in additional research were given the opportunity to opt out, and their samples and data were not used in the current study. In addition, retrospective written informed consent was obtained from all MPXV positive asymptomatic cases for publication of their data.

Note that full information on the approval of the study protocol must also be provided in the manuscript.