

Supplementary Appendix

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Antimicrobials

Before hospitalization, the patient was treated with doxycycline (day of illness, [DOI] # 6–10, DOI # 15–19); levofloxacin DOI # 15–19); cefepime DOI # 13–15; ceftriaxone DOI # 8–10; azithromycin DOI # 8–11; cefuroxime DOI # 10–13; oral sulfamethoxazole-trimethoprim (DOI # 34–50, DOI # 52–62); intravenous vancomycin (DOI # 50–52); prophylactic acyclovir (duration of illness and hospitalization)

During his hospital stay, the patient was treated with daptomycin (DOI # 74–80); linezolid (DOI # 81–86); levofloxacin (DOI # 80–86); metronidazole (DOI # 80–86); ceftriaxone (DOI # 86–90); intravenous tecovirimat twice per day (DOI # 87–107); intravenous vaccinia immunoglobulin (DOI # 90 and 91); oral brincidofovir (DOI # 92 and 100); cefazolin (DOI # 92–93); intravenous remdesivir (DOI # 107–111); and piperacillin/tazobactam (DOI # 137–138)

Methods

Specimen collection

Clinical specimens for orthopoxvirus diagnostic testing were collected using the U.S. Centers for Disease Control and Prevention (CDC) guidelines.²⁷ Lesion swabs in viral transport media and fresh, unfixed tissue from major organs were submitted for real-time polymerase chain reaction (PCR) diagnostic testing. Formalin-fixed tissues from major organs were used to prepare paraffin embedded tissue blocks for histology. This activity was reviewed by CDC and was conducted consistent with applicable federal law and CDC policy (45 C.F.R. part 461.02(l)(2), 21 C.F.R. part 56; 42 U.S.C. §241(d); 5 U.S.C. §552a; 44 U.S.C. §3501 et seq.).

Diagnostic testing

Plasma microbial cell-free DNA (mcfDNA) sequencing was performed as previously described⁴ in the Karius, Inc. clinical laboratory (Redwood City, California), certified under the Clinical Laboratory Improvement Amendments of 1988 and accredited by the College of American Pathologists. Sequencing data were analyzed using the validated Karius Test[®] bioinformatic pipeline version DC-31.4, which was designed to detect mcfDNA from >1,000 microbes,⁴ including poxviruses, and reports the absolute plasma concentration of mcfDNA in molecules per microliter for each microbe detected.

The orthopoxvirus generic OPX3 real-time PCR assay targeting the orthopoxvirus E9L gene was conducted on DNA extracted from the lesion-swab specimens by CDC.⁵ Serum samples were drawn on DOI # 90, 92, 106, and 111; they were tested by CDC for anti-orthopoxvirus IgG and IgM antibodies by modified enzyme-linked immunosorbent assay (ELISA).⁸

Histopathology

Formalin-fixed, paraffin-embedded biopsy tissues were sectioned at 4 µm and stained with hematoxylin-eosin (H&E) for histopathological evaluation, or by laboratory-developed immunohistochemical (IHC) assay for detection of orthopoxvirus antigens.

An IHC assay for detection of orthopoxvirus antigens was performed using a rabbit polyclonal anti-monkeypox virus (MPXV) antibody [CDC, Atlanta, GA]²⁸ in combination with alkaline phosphatase polymer detection system and Fast Red chromogen for visualization. Deparaffinized and rehydrated tissue sections were placed in an autostainer and digested in 0.1 mg/mL

proteinase K. Tissue sections were incubated with a rabbit polyclonal anti-MPXV antibody at 1:1000 dilution for 30 min followed by sequential incubations with Mach4 Universal AP Polymer detection kit [Biocare Medical, Pacheco, California] with Permanent Red Chromogen [Cell Marque/Millipore Sigma, Rocklin, California] or SIGMAFAST Fast Red TR/Naphtol AS-MX Tablets [Sigma-Aldrich, Saint Louis, Missouri]. Counterstaining was with Mayer's hematoxylin [Polyscientific, Bay Shore, New York]; slides were mounted with aqueous mounting medium. Appropriate positive and negative controls were run in parallel; negative controls used normal rabbit serum in place of the primary antibody. The rabbit polyclonal antibody was generated against MPXV but is broadly reactive with other viruses of the genus Orthopoxvirus, including variola (smallpox) virus, vaccinia virus, and cowpox virus. This laboratory did not allow cross-react with bovine parapoxviruses, orf virus, human herpes simplex viruses -1 and 2, varicella zoster virus, cytomegalovirus, or adenoviruses.

Bioinformatics

CDC sequencing and analysis

Extracted DNA (15 µL) from the patient's lesion swab sample was sequenced directly using the Illumina DNA Prep (Illumina® DNA Prep (M) Tagmentation (96 Samples, IPB) 20060059) method according to the manufacturer's protocol with ½ reagent volumes throughout and sequenced on a NextSeq 1000 using 300 cycle P2 Reagents (NextSeq 1000/2000 P2 Reagents (300 Cycles) v3 20046813) and Illumina indexes (Illumina® DNA/RNA UD Indexes, Tagmentation (96 Indexes, 96 Samples)) according to manufacturer's recommendations. Reads were trimmed using FaQCs v 1.34 (<https://github.com/LANL-Bioinformatics/FaQCs>) with minimum length of 50 bp, minimum quality 20, and removal of 3 nt from each end. Reads were then mapped to 2015 BRPV reference genome (Genbank accession MN240300.1) using bwa mem v 0.7.17 (<https://github.com/lh3/bwa>). Mapped reads were extracted using samtools v 1.9 (<https://github.com/samtools/samtools>) and *de novo* assembled using SPAdes v 3.13.0 (<https://github.com/ablab/spades>) using the --careful flag and --cov-cutoff off. Contigs with coverage >10 were manually assembled in Geneious Prime 2023.0.4 (Dotmatics, Boston, Massachusetts, USA) after mapping to 2015 BRPV reference genome. The draft genome was manually edited in the inverted terminal repeats and repeat regions at approximate positions 150, 163, 174, and 200 kb to match repeat lengths from the 2015 BRPV genome. Trimmed reads were then mapped back to the draft genome to generate a polished genome using ivar v 0.1 and a minimum read depth of 10. Annotations were transferred from the 2015 BRPV reference genome using LiftOff (<https://github.com/agshumate/LiftOff>). No novel open reading frames were identified using ORF finder in Geneious Prime 2023.0.4.

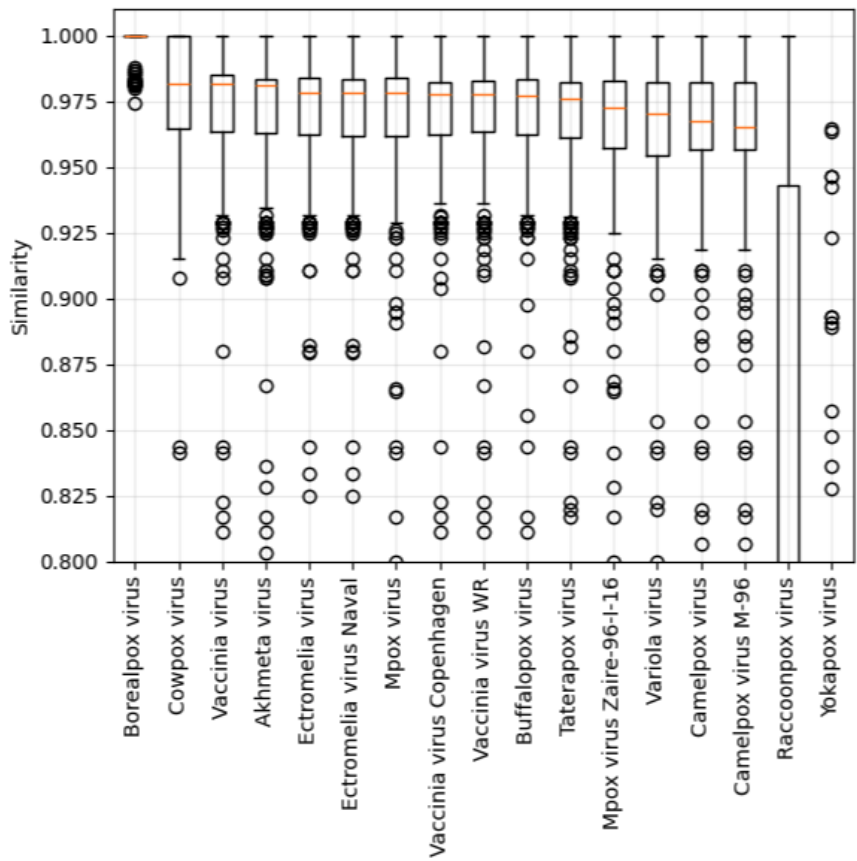
Clinical Diagnostic Results

Karius plasma microbial cell-free DNA (mcfDNA)

Species identification

After quality trimming of reads, human-derived sequencing reads were identified and removed by alignment to a curated human reference database. Subsequent pipeline stages removed control molecules and common external synthetic oligonucleotides; the remaining reads are highly enriched for microbial-derived sequences. The resultant read set was aligned to a diverse panel of representative orthopoxvirus reference genomes using BLASTn. As summarized in Figure S1, borealpox was the highest identity match to the clinical isolate.

Figure S1: Similarity of cell-free DNA fragments to orthopoxvirus genomes.



Plasma mcfDNA sequences were aligned to the published borealpox virus genome²⁹ using bowtie2. Variants were summarized using samtools mpileup and consensus. This resulted in 560 SNPs, 128 deletions (221 deleted bases) and 130 insertions (226 inserted bases), for a frequency of 0.27 SNPs, 0.06 deletions and 0.06 insertions per 100 bases. This indicates that the isolate in this sample is highly similar to the 2019 strain, and still genetically distinct from it (Table S1).

Table S1: Summary of alignment statistics of mcfDNA fragments to various orthopoxviruses

accession	species	number reads aligned	% identity to genome
GCA_031192875.1	Borealpox virus	259556	99.63 ± .90
GCA_023535675.1	Cowpox virus	89874	95.95 ± 2.51
GCA_000841905.1	Ectromelia virus	84969	96.02 ± 2.58
GCA_000869985.1	Taterapox virus	78296	95.87 ± 2.54
GCA_020809625.1	Camelpox virus	77086	95.84 ± 2.55
GCA_023536395.1	Vaccinia virus	76024	95.93 ± 2.52
GCA_021870145.1	Buffalopox virus	74846	95.90 ± 2.52
GCA_006465225.1	Mpox virus	74164	95.81 ± 2.51
GCA_023536475.1	Variola virus	71288	95.77 ± 2.54
GCA_001029045.1	Raccoonpox virus	26318	95.38 ± 2.83
GCA_000892975.1	Yokapox virus	1722	95.02 ± 3.41

De-novo genome assembly

Given the significant coverage obtained for this isolate, we were able to de-novo assemble the genome from the clinical borealpox isolate directly from plasma mcfDNA. The assembly was performed independently with two different methodologies and then manually merged to obtain the final draft genome. Specifically, the assembly was performed using paired-end sequencing using SPADes, using kmer sizes 15,17, and 19 as well as single-end (just the primary read) under default SPADes settings. The former better resolved the repetitive termini while the latter was found to be more contiguous in the bulk. In cases where one genome bridged two contigs of the other, if there were raw reads found to support the bridge, the contigs were manually spliced together (alignment of plasma mcfDNA sequences to the genome sequence are published).

Table S2: Patient's past medical history and prescribed medications upon initial presentation.

Medical History	PTA Home Medications	Comments
Rheumatoid arthritis	Meloxicam Aspirin	In hands
Aortic stenosis (AS) s/p transcatheter aortic valve replacement (TAVR)	..	Severe, resolved with TAVR
Chronic lymphocytic leukemia (CLL)	Rituximab q28 days 5/2023-9/2023 Venetoclax 3/2016-7/2016, 5/2023-12/2023 IVIG infusions 5/2023-12/2023 Allopurinol Acyclovir Ibrutinib 7/2020-5/2023 Obinutuzumab 3/2016-7/2016	In remission; organizing pneumonia secondary to lung parenchymal leukemic infiltrates 1/2022
Chronic back pain attributable to motor vehicle accident, 2016	Oxycodone Ibuprofen Acetaminophen	..
Coronary artery disease (CAD)	Atorvastatin Nitroglycerin	..
<i>Helicobacter pylori</i> infection	Lansoprazole	Noted in PAMC admission history
Hyperlipidemia	Atorvastatin	..
Hypertension (HTN)	Furosemide Lisinopril	..
Obstructive sleep apnea (OSA)	..	CPAP at night

Measure	Reference range	One day after initial punch biopsy Day of illness #28	On admission to local hospital Day of illness #65, Hospital Day #1	On admission to referral hospital Day of illness #80, Hospital Day #16	At time of transfer to long-term care hospital Day of illness #108 and #111, Hospital Day #44 and #47	Day before death Day of illness #137, Hospital Day #71
White-cell count (K/ μ l)	4–11	5.43	7.06	6.98	8.5	31.76 (H)
Red-cell count (M/ μ l)	4.3–6	4.05	3.81 (L)	2.54 (L)	3.32 (L)	3.40 (L)
Platelet count (K/ μ l)	140–440	174	243	246	231	146
Hemoglobin (g/dL)	13.5–17.5	11	11.1 (L)	7.6	9.8 (L)	11 (L)
Hematocrit (%)	40–51	33.5 (L)	33.3 (L)	22.8 (L)	29.9 (L)	33.2 (L)
Sodium (mmol/L)	136–144	134 (L)	133 (L)	132 (L)	139	145 (H)
Potassium (mmol/L)	3.5–5.1	4.1	4.1	4.2	4.6	4.5
Chloride (mmol/L)	98–107	98	99	96	107	110 (H)
Carbon dioxide (mmol/L)	21–32	27	21	29	27	27
Anion gap (mmol/L)	4–12	9	13	7	5	8
Glucose (mg/dL)	74–106	107 (H)	98	138 (H)	108 (H)	140 (H)
Blood urea nitrogen (mg/dL)	7–18	15	15	21	20 (H)	77 (H)
Creatinine (mg/dL)	0.70–1.30	0.64 (L)	0.84	1.03	0.95	1.67 (H)
Total protein (g/dL)	6.4–8.3	6.4	6.3 (L)	4.2	4.5 (L)	4.6
Albumin (g/dL)	3.5–5.2	4.4	3.6	2.3 (L)	2 (L)	3 (L)
Total bilirubin (mg/dL)	<1.2	0.5 (L)	0.4 (L)	0.2 (L)	0.4 (L)	1 (L)
Procalcitonin (ng/mL)	<0.5	..	0.49 (L)	..	..	..
Alanine aminotransferase (U/liter)	7–55	9	20	8	10	8

Aspartate aminotransferase (U/L)	8–48	19	31	21	14	16
Alkaline phosphatase (U/liter)	40–115	85	173 (H)	96	..	109
Lactate (mmol/L)	0·5–2	..	0·9	..	..	..
Calcium (mg/dL)	8·5–10·1	9	8·7	7·5 (L)	8·9	11·2 (H)
C-reactive Protein (mg/L)	<=0·80	..	111 (H)	93	..	42·6 (H)
Estimated glomerular filtration rate (mL/min/1·73m ²)	>=60	>60	>60	>60	>60	44 (L)
Neutrophils (K/ µl)	1·50–7	3·20	3·73	4·77	5·7	28·33
Neutrophils %	39–73	59	52·8	68·4	67	90·8 (H)
Lymphocytes (K/ µl)	1–4·80	1·74	0·98 (L)	0·71 (L)	0·87	0·78
Lymphocytes %	19–45	32	13·9 (L)	10·2 (L)	10·2 (L)	2·5 (L)
Monocytes (K/ µl)	0·00–0·80	0·27	0·95 (H)	0·60	0·71	0·53
Monocytes %	3–11	5	13·5 (H)	8·6	8·4	1·7 (L)
Eosinophils (K/ µl)	0·00–0·50	0	1·36 (H)	0·84 (H)	1·01 (H)	0
Eosinophils %	0·0–4	0	19·3 (H)	12 (H)	11·9 (H)	0
Lactate Dehydrogenase (U/L)	122–222	258 (H)	..	..	172	..

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Figure S2: Major clinical event and symptoms timeline.

