Novel Poxvirus Infection in an Immune Suppressed Patient

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Background. Human and animal poxvirus infections are being reported with increasing frequency. We describe a challenging case history and treatment of a previously unknown poxvirus rash illness in a renal transplant patient.

Methods. A combination of classical microbiology techniques, including viral culture and electron microscopy, were used to provide initial clinical diagnosis. Subsequent standard polymerase chain reaction assays available in 2001 were noncontributory. Next generation sequencing was used to provide definitive diagnosis.

Results. Retrospectively, next generation sequencing methods were used to ultimately provide the definitive diagnosis of a novel poxvirus infection initially identified by electron microscopy. The closest relative of this poxvirus, identified in North America, is a poxvirus collected from a mosquito pool from Central Africa in 1972.

Conclusions. This diagnostic quandary was ultimately solved using next generation DNA sequencing. This article describes the use of classical and next generation diagnostic strategies to identify etiologic agents of emerging infectious diseases and once again demonstrates the susceptibility of immunosuppressed patients to novel pathogens. The virus identified is closely related to Yoka virus; these viruses appear to have independently diverged from a common ancestor of all known orthopoxviruses.

Keywords. poxvirus; immunosupression; pathology.

A 45-year old Caucasian male with a stable renal allograft presented to his local hospital with a 3-week history of rash on the right lateral chest that had become progressively more tender, erythematous, and indurated. Three weeks prior, the patient initially noted a blister-like lesion in his axilla. No fever or chills were reported, and he was admitted for intravenous antibiotics with a diagnosis of cellulitis. The erythema and induration worsened, and an ulceration appeared on the right flank. An incision and drainage of the right flank “abscess” was attempted. Little pus was obtained, and bacterial and fungal cultures failed to grow any causative organism. His antibiotics were changed with no improvement noted. On the 5th day he was transferred to the regional tertiary hospital. He remained afebrile with a normal white blood cell count.

The patient’s medical history was significant for end stage renal disease secondary to Alport syndrome and receipt of a living unrelated donor kidney 26 months earlier; other illnesses included hypertension, hyperparathyroidism, hypercholesterolemia, and hearing loss. Past surgical history included parathyroidectomy, hemoaccess procedures, cleft lip repair, and orthopedic surgery of the left forearm. The patient was maintained on mycophenolate mofetil, cyclosporine, prednisone, lansoprazole, terazosin, metoprolol, diltiazem, simvastatin, furosemide, and potassium chloride.

Physical exam at the tertiary care facility revealed an enlarging area of erythema measuring 15.0 × 15.0 cm in greatest dimension. The red and indurated areas had progressed to a lighter pink and spread around the lateral flank anteriorly to involve the inguinal ligament (Figure 1A) accompanied by right axillary lymphadenopathy.
Six days after transfer to the tertiary care facility, new flat vesiculopustular lesions were observed (Figure 1B). Multiple debridements were performed over the course of his hospital stay and sent for pathology review. The major consistent finding in all specimens was a dense inflammatory infiltrate composed of lymphocytes, histiocytes, and focal eosinophils extending into the subcutaneous adipose tissue. There was no evidence of herpes simplex virus or varicella zoster virus (VZV) with immunohistochemical staining. Special stains for acid fast and fungal organisms were negative. Definitive viral inclusions were not initially recognized (see below); the features were deemed consistent with eosinophilic panniculitis [1]. Because a reported case of eosinophilic panniculitis associated with polyarteritis nodosa was treated with cytoxan and corticosteroids [2], the patient was given a trial of pulse corticosteroids initiated on day 11 of hospitalization, and the patient also underwent hyperbaric oxygen therapy post-operatively. Three days after pulse steroids, the patient developed additional pruritic vesicles on his right flank and bilateral extremities. Similar to that depicted in Figure 1B, these vesicles were beige, round, umbilicated, and approximately 2–4 mm in diameter, raised about 1 mm, flat-topped, and filled with a thick cream-colored material. Famciclovir was started to treat a presumptive VZV infection. Over the next several days, the number of vesicles increased considerably and coalesced; the patient became febrile (up to 39.6 °C) and developed leukocytosis for the first time.

On hospital day 35 a 4th debridement was performed, and human epithelial type 2 (HEp-2) cells were inoculated with tissue extract. The Hep-2 cells manifested viral cytopathic effect (CPE) and reacted with a pan-enterovirus antibody but not with species-specific antibodies. Thin section electron microscopy (EM) showed intracytoplasmic inclusions containing many mature and developing forms that were classic for poxvirus A-type inclusions (Figure 2). The Centers for Disease Control and Prevention (CDC) was contacted for identification of the virus and therapeutic options. Samples sent to the CDC produced CPE in BSC40 cells, but at that time (year 2000) it could not be identified using standard polymerase chain reaction (PCR) primer sets targeting hemagglutinin [3] or A-type inclusion body protein genes [4] used to identify North American and Eurasian orthopoxviruses. Review of biopsy histopathology revealed dense pink cytoplasmic inclusions, typical for Guarneri bodies (Figure 2). As the virus could not be identified as an orthopoxvirus, vaccinia immune globulin was not recommended, and the renal transplant team was uncomfortable with the use of cidofovir given its known renal toxicity; at that time, cidofovir was the only antiviral with known anti-poxvirus activity.

Additional history revealed the patient had no travel outside of his community in upper New York state and had no contact with animals other than an adopted (feral) cat, which had recently been treated for an abscess on its back. Material from the cat’s lesion was not available for analysis.

By the 61st day of illness, the patient was no longer febrile, the right flank wound had ceased to progress, no new vesicular lesions developed, and the existing lesions had healed. A final debridement was done on hospital day 59, and he was discharged a few days later. Six weeks following discharge, a successful skin graft was performed. Long-term follow-up showed the patient to be well and complication-free as of 2011.

Figure 1. A, Photograph of patient, showing large ulcerative lesion involving right flank, after debridements. B, Image of a lesion that appeared 12 days after the case patient first sought medical care at outside hospital, 6 days after hospitalization at the tertiary hospital, and 33 days after first notation of a blister under the arm.
Subsequent sequencing analyses, using next generation technologies, in addition to tissue pathologic and virologic studies, are presented to characterize this novel poxvirus. Phylogenetic analyses were done to provide additional taxonomic information about this virus.

METHODS

Viral DNA Cloning and PCR Amplicon Sequencing
DNA was purified from viral infected BSC40 cell and extracted using the protocols described previously [5]. The purified viral DNA was cloned into pGEM-3Z Vector (Promega, Wisconsin) following manufacturer’s protocols, the cloned DNA fragments were sequenced and sequence similarity searches were performed using BLAST [6].

Genomic Sequencing Using 454 and Illumina Platforms
The purified viral DNA was initially sequenced with high-throughput pyrosequencing using the Roche GS20 platform (standard protocols; 454 Life Sciences, Branford, Connecticut), sequences were generated using de novo assembly with an average coverage of 25. The same extracted viral DNA was sequenced using the newer Illumina platform (Illumina, Inc, San Diego, California). The de novo assembling of the viral genome was performed using Charcot–Leyden crystal (CLC) genomic workbench software (CLC bio, Aarhus, Denmark) with an average coverage of 500. The sequence data sets were compared to resolve the differences between two assembled genomes, particularly the homopolymeric stretches. Gaps, due to large repetitive sequences, were filled using PCR amplification of predicted regions followed by Sanger sequencing of the PCR amplicons.

Phylogenetic Analysis
Nine coding sequences (A7L, A10L, A24R, D1R, D5R, H4L, E6R, E9L, and J6R (vaccinia virus strain Copenhagen (VACV-Cop) nomenclature [7]), were selected from the central region of the genome [8, 9]; these 9 genes are among the 49 genes conserved across poxviruses [10]. Sequences were concatenated and aligned

Figure 2. Photomicrographs H&E stain (A–D) and Transmission electron micrographs (E–G). A, Low magnification showing skin with dense dermal inflammatory infiltrate, edema and sub- and intra-epidermal vesicles. B, Epidermis showing multinucleated cells and numerous intracytoplasmic eosinophilic inclusions (Guarnieri bodies) (arrows). C, Extensive multinucleated giant cells in epidermis. D, Prominent eosinophils in subcutaneous adipose tissue. E, Cultured HEP2 cell from primary inoculation, showing intracytoplasmic A-type inclusion (arrow) containing many mature and developing viral particles (scale bar = 2.0 micrometers). F, Numerous intracytoplasmic brick-shaped virions (arrow) in cultured cell (scale bar = 250 nm). G, Biopsy tissue (recovered from paraffin block) showing classic intracytoplasmic virions (arrows) (scale bar = 250 nm).
by using the ClustalW alignment option in Geneious v6.0.5 (www.geneious.com/). Phylogenetic analysis was performed using the Bayesian analysis software package (v1.7.5, http://beast.bio.ed.ac.uk), BEAST, BEAUti, and Tracer [11].

RESULTS

Histopathology and Electron Microscopy

Many histologic findings within debrided tissue were consistent throughout the disease course: a dense inflammatory infiltrate composed of lymphocytes, histiocytes, and eosinophils; eosinophils were concentrated in the dermis with lobules of necrotic subcutaneous adipose tissue and eosinophilic abscesses. Blood vessels with fibrinoid change were also seen within the areas of necrosis. Specimens taken after the vesicles developed showed intra-epidermal vesicles filled with neutrophils and lymphocytes; some of these vesicles had ruptured and were infiltrated with eosinophils. In one of the specimens multinucleated squamous epithelial cells were seen as well as striking eosinophilic cytoplasmic inclusions (Figure 2). The poxvirus isolated from this case, designated NY_v014, showed classic poxvirus particles within A-type inclusions seen within the

Figure 3. NY_v014 (denoted as V014) is closely related to poxvirus Yoka_DakA. The phylogenetic analyses ran MCMC chain lengths of 5 000 000 with a GTR and an invariant sites nucleotide rate substitution models, strict molecular settings and sampling of every 1000 states. The consensus tree was derived from 5000 of the tree samples. Poxviruses used in these analyses are listed below with their GenBank accession numbers in parentheses: genus Orthopoxviruses form 2 subclades, the Old World orthopoxviruses CMLV_M96 (NC_003391), TATV_DAH6 (NC_008291), VARV_BGD7 (DQ437581), VARV_Gar (Y16780), HPXV_MNR7 (DQ792504), VACV_Cop (M35027), MPXV_LBR (DQ011156), MPXV_COG (DQ011154), CPXV_BR (NC_003663), ECTV_Mos (NC_004105), and New World orthopoxviruses RACV_MD19 (FJ807746-54), SKPV_USA1 (FJ807755_63), VPXV_USA1 (FJ807737-45). Poxvirus genus capripoxviruses GTPV_G20L (AY077836), LSVV_LW19 (AF409138), SPPV_TU (NC_004002); Deerpox DPV_W848 (NC_006968); Suipoxvirus SWPV_Neb (NC_003389); Yatapoxviruses YLDV_Davi (NC_002642) and TANV_KEN (NC_009888); and Leporipoxviruses RFV_Kas (NC_001266) and MYXV_Lau (NC_001266) are other members of chordpoxvirinae subfamily. To root the dendrogram, 2 Avipoxviruses CPNV_VR111 and FWPV_Iowa (NC_005309 and NC_002188) are used as the outgroup. The scale bar denotes the genetic distance in substitutions per site. Abbreviations: GTR, general time reversible; MCMC, Markov chain Monte Carlo.
cultured cells. Although tissue recovered from the formalin fixed, paraffin embedded block was less well-preserved, EM showed several classic poxvirus particles (Figure 2).

**Virologic Studies**

Cross neutralization experiments demonstrated that this virus is not efficiently neutralized by pooled, anti-orthopoxvirus serum derived from subjects vaccinated, and revaccinated with smallpox (vaccinia) vaccine. Whereas vaccinia virus strain WR (VACV-WR), a Eurasian orthopoxvirus, was efficiently neutralized (50% reduction in plaque number) with dilutions of pooled serum to 1:640, this virus was only neutralized at dilutions to 1:40 dilution. The North American orthopoxvirus, raccoonpox virus, was more efficiently neutralized by this same pooled serum at dilutions of up to 1:160. To further biologically characterize this novel poxvirus, its mycophenolate sensitivity was compared to VACV-WR [12]. Whereas the growth of VACV-WR is completely inhibited by 10 µM mycophenolate, the growth of NY_014 is inhibited no more than 50%. The IC₅₀ is approximately 2 µM for VACV, and greater than 10 µM for NY_014.

**Sequencing**

Initial PCR testing was nondefinitive in identifying the poxvirus observed by EM. Using random cloning and sequencing methods, the BLAST analyses found the various 200–500 nt sequences from the ends of the cloned viral DNA fragments to be 70%–85% identical to orthopoxviruses and capripoxviruses.

Subsequent genome sequencing has revealed a virus genome of 200,058 nt with 70.5% adenosine and thymidine (A + T), encoding approximately 200 genes. At each end of the genome, there were inverted terminal repeats of 1657 nucleotides. No hemagglutinin (HA) gene was identified, consistent with the negative diagnostic PCR that targets this sequence. Phylogenetic analysis, using 9 essential poxvirus genes, indicates that this virus is most closely related to the Yoka poxvirus [13], which was isolated from mosquitos in the Central African Republic via a 1972 ecologic survey (Figure 3); these viruses form a clade basal to the North American and Eurasian orthopoxviruses.

The NY_v014 virus contains an A-type inclusion (ATI) gene, about the same size as that expressed by ectromelia (the causative agent of mousepox) and consistent with the A-type inclusion bodies observed by EM. NY_v014 also contains ORFs not previously annotated in any poxvirus genome. Interestingly, the new virus encodes 3 major histocompatibility complex (MHC) class I antigen homologs, which generate best similarity scores with rodent MHC class I antigens. A further analysis of these genes’ functions may help us understand the interactions between the new virus and its host.

**DISCUSSION**

A combination of classical and novel diagnostic approaches was required to ultimately characterize this pathogen. The histopathology of the infected tissues revealed rarely recognized poxvirus type B inclusions (Guarnieri bodies) as reported in smallpox [14], fowlpox, and tanapox [15, 16], and the EM was remarkable for A type inclusions, typical of only the Eurasian orthopoxviruses cowpox and ectromelia and North American orthopoxviruses. Although next generation sequencing technologies were not available at the time this case was initially identified, this report demonstrates their utility in identification of novel human pathogens. It also demonstrates the limitations of PCR diagnostics that target small regions, typically <0.5%, of a poxvirus genome to determine the poxvirus species. Neither the classical standard hemagglutinin nor ATI PCR diagnostics were informative due to the absence of hemagglutinin in NY_014, and 5 nucleotide mismatches within the annealing sites of the ATI primer sets.

The poxvirus family encompasses a range of virus species that infect vertebrates and insects; the viruses are complex double-stranded DNA viruses that replicate in the cytoplasm of the host cell. Prior to this report, 4 species of viruses, parapoxviruses, molluscipoxviruses, yatapoxviruses, and orthopoxviruses, were known to infect humans. Of the orthopoxviruses, most are zoonotic infections of humans, except for variola virus, the causative agent of smallpox. The antigenic similarity among members of a species led to the recognition that infection with a less virulent member of the species could protect against a more virulent member. Within the orthopoxvirus genus, at least cowpox and vaccinia viruses were used as live virus vaccines to protect against smallpox. Worldwide routine vaccination against smallpox ceased in the early 1980s, after the eradication of smallpox. In the intervening time, an increasing number of human infections with other orthopoxviruses have been noted—vaccinia, cowpox, and monkeypox have all been noted as reemerging or emerging orthopoxvirus pathogens [17].

This poxvirus is unique and is a previously unrecognized human pathogen. Its phylogenetic characterization indicates it is most closely related to the recently described Yoka poxvirus. Differences between these 2 viruses include presence or absence of A-type inclusions. NY_v014 forms A-type inclusion bodies, whereas Yoka poxvirus has a large truncation at the 3’ end of the ATI gene; no ATI structures were observed during growth of Yoka poxivirus. This virus, with the related Yoka virus, should probably be designated as belonging to a novel poxvirus genus that shares a common ancestor of orthopoxviruses.

The divergence of the NY_014 genome sequence explains the initial unsuccessful use of orthopoxvirus diagnostics that target the ATI and hemagglutinin genes and other standard assays. Recently a new pan pox low guanine-cytosine (GC) PCR assay was developed [18] that permits NY_v014 and several other highly
diverged chordopoxviruses to be amplified and subsequently sequenced. The antigenic properties of this virus assessed by cross-neutralization experiments with anti-vaccinia sera, when compared to Eurasian and North American orthopoxviruses indicate the remote resemblance of NY_v014 to members of orthopoxvirus genus. This individual is of the age to have been vaccinated against smallpox as a child; the cross-neutralization data provided here suggest that smallpox vaccine may provide little protection against this new poxvirus. Alport syndrome is not known to make individuals more susceptible to poxvirus infections, but immunosuppression has been shown to be associated with more severe poxvirus infections.

Interestingly, Yoka poxvirus was identified in a historic collection of mosquito pools from ecologic surveys being performed in sub-Saharan Africa, and the collection that revealed Yoka virus was obtained in the Central African Republic in 1972. The relatively closely related NY_v014 virus was identified in North America. Additional surveillance to understand the ecology of these viruses will be important for public health considerations. This case serves as a reminder that patients on immunosuppressive medications are prone to novel, pre-existing poxvirus infections.

Notes

Acknowledgments. We thank Nadine Bartholoma who did the initial viral isolations, Maureen Barcza took the transmission electron microscopy images, the Wadsworth laboratory for confirmatory electron microscopy and enterovirus testing, and Miriam Laker who performed some of the initial cloning and blast analyses. Additionally we thank the Biotechnology Core Facility at Centers for Disease Control and Prevention (CDC) for sequencing assistance, and Dhwani Batra for assistance in initial assembly and analyses.

Disclaimer. The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the CDC.

Financial support. Funding was provided by author’s home institutions.

Potential conflicts of interest. All authors: No potential conflicts of interest.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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