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Rapid Communication

Using next generation sequencing to identify yellow fever virus in Uganda

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ABSTRACT

In October and November 2010, hospitals in northern Uganda reported patients with suspected hemorrhagic fevers. Initial tests for Ebola viruses, Marburg virus, Rift Valley fever virus, and Crimean Congo hemorrhagic fever virus were negative. Unbiased PCR amplification of total RNA extracted directly from patient sera and next generation sequencing resulted in detection of yellow fever virus and generation of 98% of the virus genome sequence. This finding demonstrated the utility of next generation sequencing and a metagenomic approach to identify an etiological agent and direct the response to a disease outbreak.

The study

Uganda is endemic for multiple hemorrhagic fever viruses, as well as other zoonotic and vector-borne diseases. In October 2010, Virus Special Pathogens Branch (VSPB) of the Centers for Disease Control and Prevention (CDC; Atlanta, USA) received reports of an unusual outbreak occurring in the Abim, Agago, and Kitgum districts in northern Uganda. While clinical information was limited, viral hemorrhagic fever was suggested as a possible etiology due to reported bleeding and patient deaths. In response, patient samples were collected and sent to the CDC viral hemorrhagic fever laboratory at Uganda Virus Research Institute in Entebbe, Uganda.

Thirty-six clinical specimens were tested from individuals with evidence of illness. Tests designed to diagnose the acute phase of Ebola and Marburg hemorrhagic fevers were performed by PCR and/or antigen detection ELISA (Towner et al., 2008) (Ksiazek et al., 1999a, 1999b). All 36 samples were negative, indicating that neither Ebola nor Marburg virus was the etiologic agent of the outbreaks. A subset of 8 samples was subsequently sent to VSPB to test for additional hemorrhagic fever viruses. All were negative for Rift Valley fever virus by PCR and IgM ELISA, and for Crimean Congo hemorrhagic fever virus by PCR (Bird et al., 2007; Madani et al., 2003).

Since the initial diagnostic tests for hemorrhagic fever viruses were negative, we applied a metagenomic approach that uses next generation sequencing (NGS) to detect pathogen genomic material amplified directly from patient sera in an unbiased fashion. Such methods have proven useful in identifying infectious agents that are difficult or impossible to isolate in cell culture or laboratory animals (Su et al., 2011). No assumptions regarding the identity of the pathogen are made; therefore, new strains of known organisms, novel pathogens, or agents not under suspicion as the cause of the illness may be identified.

To increase the chance of detecting an infectious agent, samples from patients believed to be in the acute stage of illness were chosen for study. Samples from 4 patients who were one day post onset of illness were further analyzed using NGS. Total RNA was extracted from patient serum, purified using RNeasy mini columns (Qiagen Corp., Valencia, USA), and treated with DNase I (Ambion Inc., Austin, USA) for 40 min. cDNA was generated using a random octomer primer with a 17-mer anchor sequence and SuperScript III (Invitrogen, Carlsbad, USA) reverse transcriptase by incubating for 10 min at 25 °C, 50 min at 42 °C, and 15 min at 70 °C. Residual RNA was removed by RNase H (Sigma-Aldrich Co., St. Louis, USA). The cDNA library was amplified with the random octomer primer and a primer with the 17-mer anchor sequence with an additional

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Table 1
Single nucleotide variations detected by NGS. Variants that contribute to amino acid changes are indicated. The frequency of variants and number of reads for the window are listed.

Reference position	Reference	Variants	Allele variations	Frequencies	Counts	Coverage	Amino acid change
1484	C	2	C/T	93.4/6.6	57/4	61	
2078	T	2	T/C	97.9/2.1	190/4	194	
2087	T	2	T/G	97.3/2.7	178/5	183	Phe656Leu
2089	G	2	G/T	97.2/2.8	176/5	181	Gly657Val
2108	T	2	T/C	87.8/7.3	72/6	82	
2145	A	2	A/G	93.5/6.5	58/4	62	Lys676Glu
2347	T	2	T/G	87.1/12.9	115/17	132	Val743Gly
2398	T	2	T/C	94.0/6.0	157/10	167	Met760Thr
2641	T	2	T/C	97.8/2.2	182/4	186	Leu841Pro
2760	CCT	2	CCT/ACC	97.9/2.1	191/4	195	Pro881Pro,Thr
2780	T	2	T/C	98.7/1.3	304/4	308	
2824	T	2	T/C	93.5/6.5	257/18	275	Phe902Ser
2844	G	2	G/A	93.6/6.4	175/12	187	Gly909Ser
2956	T	2	T/A	92.5/7.5	161/13	174	Phe946Tyr
2983	T	2	T/C	89.5/10.5	162/19	181	Val955Ala
4225	G	2	G/A	61.4/38.6	43/27	70	Gly1369Glu
5143	G	2	G/A	93.3/6.7	56/4	60	Gly1675Glu
5411	T	2	T/C	96.7/3.3	384/13	397	
5422	A	2	A/G	98.5/1.5	390/6	396	Glu1768Gly
5499	A	2	A/G	98.8/1.2	342/4	346	Arg1794Gly
5584	A	2	A/G	98.8/1.2	330/4	334	Glu1822Gly
5650	A	2	A/G	98.5/1.5	320/5	325	Lys1844Arg
5669	T	2	T/C	98.2/1.8	322/6	328	
7127	T	2	T/C	97.7/2.3	211/5	216	
7141	T	2	T/C	97.3/2.2	219/5	225	Ile2341Thr
7367	A	2	A/G	98.5/1.5	333/5	338	
7382	T	2	T/G	98.8/1.2	318/4	322	
7630	C	2	C/T	93.8/6.2	150/10	160	Thr2504Ile
7855	A	2	A/G	84.5/15.5	49/9	58	Lys2579Arg
9176	A	2	A/T	97.7/1.9	208/4	213	
9190	G	2	G/A	98.8/1.2	325/4	329	Gly3024Asp
9292	A	2	A/G	98.8/1.2	335/4	339	Glu3058Gly
9428	T	2	T/A	97.6/2.4	162/4	166	Ser3103Arg
10236	C	2	C/A	75.9/24.1	22/7	29	His3373Asn

CGCC 5′ extension in a 1:9 ratio; the samples underwent 8 cycles at 25 °C and 49 cycles at 55 °C (Palacios et al., 2007). Unincorporated nucleotides and fragments shorter than 70 nucleotides were removed using a Qiagen MinElute column. The quality and complexity of the library fragment size was analyzed on a BioAnalyzer 7500 labchip (Agilent Technologies, Santa Clara, USA). Library fragments were directionally ligated to adapters for 454 NGS (Roche Diagnostics, Indianapolis, USA). Emulsion-based PCR and pyrosequencing were carried out using GS FLX Titanium chemistry and procedure as described by the manufacturer (Roche). NGS resulted in an average of 564,204 sequence reads with an average length of 235 nucleotides.

The sequence reads were analyzed using a series of bioinformatic tools. Random library amplification and 454 sequencing primers were removed using the Trimseq script by Mothur (Schloss et al., 2009). After trimming, overlapping sequence reads were assembled into contiguous segments (contigs) using the GS De Novo Assembler Newbler (Roche) and CLC bio Genomics De Novo assembler (CLC bio, Aarhus, Denmark). To determine the genomic origin of each sequence read, contiguous and unassembled sequences were translated and compared to the non-redundant protein database from the National Center for Biotechnology Information using the basic local alignment search tool (BLASTx). The sequence reads linked to a BLASTx result were parsed into groups of taxa using the program MEtaGenome Analyzer (MEGAN) (Huson et al., 2007).

Yellow fever virus (YFV) sequences were identified in 1 of the 4 patient serum samples tested. MEGAN analysis categorized 5 sequence contigs as similar to the YFV genome sequence isolated from a 1961 outbreak in Ethiopia. The remaining contigs were of human origin (500 contigs), bacteria (16 contigs), or unassigned taxa (19 contigs). The 5 contigs aligning to YFV ranged from 828 to 3587 nucleotides in length and created a contiguous segment spanning 98% of the YFV RNA genome (10,441 of the 10,823 nucleotides). Additional sequence reads were identified as YFV when mapped to the Ethiopian 1961 YFV genome using a mapping reference assembler program (Genomics CLC bio). In total, 0.5% (3229 out of 599,158 reads) of the sequenced patient library was identified as YFV. The reads averaged 309 nucleotides in length with an average coverage of 92 reads. A gap of 167 nucleotides in the YFV sequence was completed using primers specific for Uganda 2010 YFV and sequenced by the traditional Sanger method. The 5′ end of the genome was sequenced from the NGS library. We determined the sequence of the final ~101 nucleotides at the 3′ end of the genome using 3′ RACE and Sanger sequencing.

NGS enables the detection of infectious pathogens directly from clinical material by sequencing extremely large amounts of genetic material without a bias of origin. NGS also provides extraordinary depth of sequence coverage, which allows detection of variants in a genome population. YFV is a positive-stranded RNA virus that generates frequent mutations upon replication. Mapping almost 3230 sequence reads to YFV gave an average coverage of 92 reads with a range of 2 to 397 sequence reads over the entire genome. Sequence variations in the library were detected using single nucleotide polymorphism (SNP) detection function in the CLC bio Genomics package, with a window of 25 nucleotides and minimum average and center base quality score of 35. With a minimum count of 4 variations per site and at least 10 sequence reads of coverage, 34 variations in the YFV genome were detected; 10 variants were silent nucleotide changes yet 24 would contribute to a change in the encoded amino acid (Table 1).

Genetic comparison of YFV isolates has previously established 2 South American genotypes and 5 genotypes from Africa (Lepiniec et al., 1994). A full-length sequence for the Uganda 2010 isolate was compiled from the consensus of NGS library reads and Sanger sequencing. YFV sequences available from the NCBI database comprising the full-length genome (approximately 10,823 nucleotides) and sequence fragments with at least 10% of the genome were imported into the SeaView program and aligned using Muscle (Table 2).

Table 2
Sequences used in the phylogenetic analysis. GenBank accession number and length of sequence included in the analysis are listed.

Name	Accession	Length
Angola1971	AY968064	10,823
Brazil1979	U23570	1479
BurkinaFaso1986	AY960139	1813
Cameroon1991	GU073131	1521
CAR1974	GU073130	1521
CAR1977A	U52392	1320
CAR1977B	U52395	1320
CAR1977C	U23571	1479
CAR1985	U23573	1479
Colombia1979	U23580	1479
Ecuador1981	U23566	1479
Ethiopia1961Couma	DQ235229	10823
Gambia2001	AY572535	10,862
Ghana1927Asibi	AY640589	10,862
Guinea2000	AY502949	1479
IvoryCoast1973	GU073134	1431
IvoryCoast1982	U54798	10,862
IvoryCoast1995	GU073133	1485
IvoryCoast1996	GU073132	1473
IvoryCoast1999	AY603338	10,862
Kenya1993A	U23569	1479
Mali1987	AY960138	1813
Peru1977	U23565	1479
Senegal1927Mayali	U21056	10,862
Senegal1953	AH005112	2953
Senegal1965	U23574	1479
Senegal1976	GU073155	1521
Senegal1977	GU073156	1521
Senegal1978	GU073141	1521
Senegal1990	AY839635	1479
Senegal1993	GU073157	1487
Senegal1995A	GU073161	1521
Senegal1995B	GU073164	1476
Senegal1995C	GU073162	1480
Senegal1995D	GU073166	1521
Senegal1996A	GU073142	1483
Senegal1996B	GU073140	1484
Senegal2000A	GU073148	1521
Senegal2000B	GU073149	1485
Senegal2000C	GU073146	1476
Senegal2000D	GU073143	1521
Senegal2001A	GU073154	1521
Senegal2001B	GU073152	1521
Senegal2001C	GU073151	1419
Senegal2001D	GU073158	1521
Sudan2003	DQ068260	1479
Trinidad1979	AF094612	10,760
Trinidad2009	HM582851	10,236
Uganda1948a	AY968065	10,823
Uganda1948B	U52422	1320
Uganda1964	U23578	1479
Uganda2010	JN620362	10,820
Nigeria1986	U23572	1479
Nigeria1991	U23567	1479
Gambia1979	GU073135	1521
Mali1986	AY960138	1813
Concatenated sequences		
DRC1958	AY541445	578
DRC1958	AF369697	670
Ethiopia1961Serie	AY541407	574
Ethiopia1961Serie	AF369674	670
Kenya1993	AY541408	516
Kenya1993	AF369676	670
Uganda1972	AY541440	571
Uganda1972	AF369696	670

Phylogenetic analysis using the maximum likelihood method shows that the Uganda 2010 YFV belongs to the East/Central Africa genotype and is most closely related to yellow fever viruses isolated in the Central African Republic (CAR) in 1977 and 1985 (Fig. 1). Among the African genotypes, the greatest diversity is between isolates from East Africa and West Africa, which differ by 21% at the nucleotide level and 9% at the amino acid level using a pair-wise comparison. East Africa and East/Central Africa genotypes differ by 15% at the nucleotide level. The Uganda 2010 full-length sequence differs by 8% from those of viruses in the East Africa genotype, and by 5% from those of other members of the East/Central Africa lineage. This sequence divergence is within the range (1 to 5%) generally seen among the East/Central Africa viruses. The polyproteins of YFV are very conserved with East Africa and East/Central Africa genotypes sharing 99% amino acid identity.

In order to gain further insight into the evolutionary history of the YF lineages found in East Africa, a Bayesian analysis was carried out similar to that performed in earlier YFV investigations (Sall et al., 2010). The trees generated by Bayesian methods were identical (with minor exceptions) to those described above generated by likelihood (Fig. 1). Bayesian analysis of virus E genes estimated that the most recent common ancestor shared by all YFVs was approximately 2187 years ago (Fig. 1, nodes indicated in bold). This is within the estimated range obtained in earlier analyses (Sall et al., 2010). The Angola genotype shared a common ancestor with the East Africa and East/Central Africa genotypes approximately 867 years ago whereas the YFVs of the East Africa and East/Central Africa genotypes shared an ancestor 441 years ago. These analyses indicate that the virus genetic diversity seen in the East African region is not recently generated, but has accumulated over several hundred years. The pattern of diversity would also suggest that there have been multiple, independent introductions of YFV into the human population from an endemic source as opposed to transmission from another region of Africa.

The genome of Uganda 2010 shares particular molecular elements with viruses from the East Africa and East/Central Africa genotypes. A previously reported threonine is present at amino acid position 21 of NS4B in all East and East/Central Africa lineages, yet is absent in YFV from the West Africa genotype (von Lindern et al., 2006). The NS4B gene of Uganda 2010 also encodes a threonine at position 21 similar to genotypes of East Africa viruses. Another distinguishing characteristic of yellow fever viruses of different genotypes is the number of copies of a 40 nucleotide repeat element in the 3′ non-coding region (3′ NCR), called RYF. Viruses of the West Africa genotype contain 3 copies of RYF separated by four to six nucleotides. YFV from the East and East/Central Africa have only two RYF copies present in the first and third positions and YFV from South America has only one RYF in the third position (Mutebi and Barrett, 2002) (Wang et al., 1996). Similar to the East and East/Central Africa genotypes, the Uganda 2010 sequence contains only two copies of RYF. There are additional 5′ and 3′ NCR nucleotide variations in the Uganda 2010 that could potentially influence RNA structural elements, however the complementary sequences thought to be important for cyclization of the 5′ and 3′ NCR, as well as a predicted stem loop RNA structure at the 3′ terminal core are present as expected (Villordo and Gamarnik, 2009).

Conclusions

YFV is a mosquito-borne flavivirus. The majority of human infections are asymptomatic, and clinical disease can vary from an undifferentiated type fever to severe disease with hemorrhage (Monath and Barrett, 2003). Yellow fever is often difficult to diagnose because its symptoms are similar to those of other diseases such as malaria, leptospirosis, or infection with other endemic flavivirus infections such as Zika virus, West Nile virus, or dengue virus. YFV infections often occur in children who have yet to acquire immunity from exposure or vaccination (Monath, 2008). The patient from whom we identified YFV in this study was a 16-year-old schoolboy from the village of Golgotha, Aremo parish, Morulem county, located in northeastern Uganda. His symptoms were reported as headache, fever, diarrhea, vomiting blood, and cloudy urine, but with the remarkable absence of jaundice. He arrived at the regional hospital on November 9, 2010, and died 2 days later. The serum sample

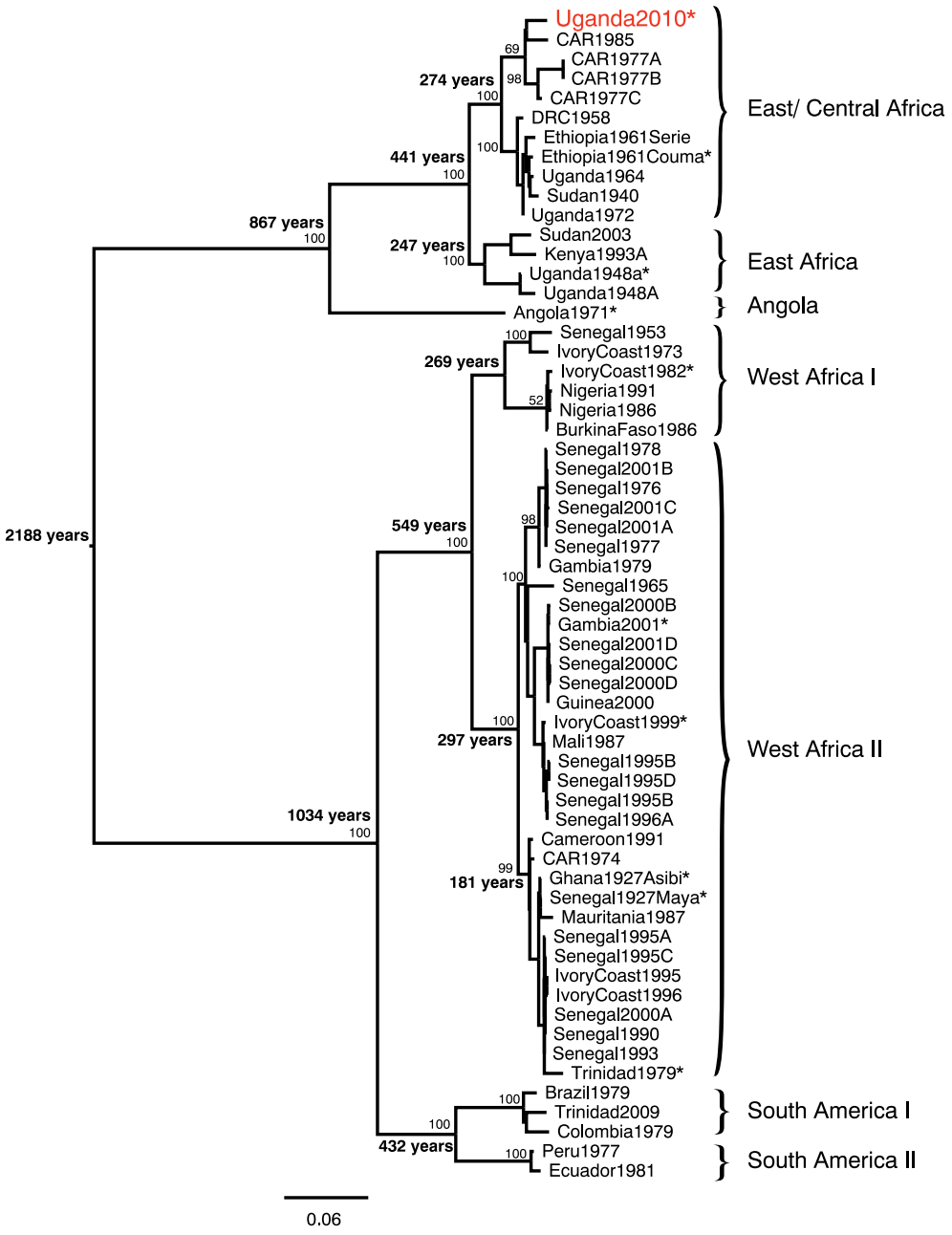

Fig. 1. Phylogenetic analysis of yellow fever virus. The tree topology depicted was generated by maximum likelihood analysis utilizing the Genetic Algorithm for Rapid Likelihood Inference (GARLI) using a general time reversible (GTR) substitution matrix with 6 relative rates and γ-distributed rate variation determined empirically (Zwickl, 2006). Full length virus sequences are indicated with an "*". There are seven YFV genotypes. Depicted at the nodes are the bootstrap values of 500 replicates. In addition, time to most recent common ancestor (TMRCA) estimates were generated by Bayesian analysis and are indicated in bold at nodes of particular interest. Rates of nucleotide substitution and the TMRCA were inferred using the YFV envelope gene data and the BEAST, BEAUTi, and Tracer software packages (Drummond and Rambaut, 2007). A relaxed uncorrected exponential molecular clock was selected by comparing marginal likelihoods generated in an initial analysis of 10,000,000 generations with the GTR matrix and gamma rate variation with a constant population ($\log_{10}$ Bayes factors = 14.33). The Markov chain Monte Carlo (MCMC) was allowed to run for 100,000,000 generations to achieve convergence as assessed with Tracer.

was collected on the day of hospitalization. Additional patients were subsequently identified as being infected with YFV, and these findings will be reported elsewhere.

In 1936, a serological survey of arboviruses reported yellow fever immunity distributed throughout Uganda; however, there have been only 4 human cases reported since 1941 with the most recent case prior to this study detected in 1964 (Ellis and Barrett, 2008; Henderson et al., 1970). Although YFV infections are frequently reported in West Africa, notably in Senegal and most recently in Ivory Coast (January 2011) and Sierra Leone (February 2011), infections in East Africa are curiously intermittent (World Health Organization, 2011). The scarcity of yellow fever cases in East Africa could be due to subclinical disease, infrequent exposure to carrier mosquitoes, or underreporting. Phylogenetic analysis relates the Uganda 2010 genome to the East/Central Africa lineage and it is most closely related to YFV isolated in CAR in 1977 and 1985. Most yellow fever virus genotypes also share common geographical locations and have been referred to as topotypes, but there are some exceptions (Deubel et al., 1986). YFV sequences from both East Africa and East/Central Africa genotypes circulate in Uganda. CAR is endemic to YFV classified as both East and West African genotypes. It is interesting that neither Uganda nor CAR has yet to experience a sizeable YFV outbreak or report more than just a few human infections despite the observation of such endemic YFV genetic diversity.

The first verified East African YFV epidemic of was in 1940 in the Nuba Mountains of Sudan with an estimated 15,000 cases (Kirk, 1941). Subsequently, only infrequent cases of 1 to 8 were reported for all of eastern Africa until 1958 (Ellis and Barrett, 2008). A large outbreak of 1800 cases occurred in the Upper Nile region of Sudan in 1959 and spread into neighboring Ethiopia, leading to the largest yellow fever epidemic with 200,000 cases from 1960 to 1962. In contrast, Uganda reported only a single case in 1941, 1952, and 1959. Additional serological studies performed in 1967–69 demonstrated Uganda to be endemic for YFV, yet only 1 human case was reported in 1964. This case remained the most recent report of a YFV human infection in Uganda until this report.

Phylogenetic analysis infers that YFV from the 1940 Sudan and 1961 Ethiopian outbreaks belong to the East/Central Africa genotype (Fig. 1). Prior to this report, the two most recent YFV reported from Uganda: a human case identified in 1964 and an isolate from a mosquito in 1972, also share homology with YFV from these outbreaks. The Uganda 2010 genome sequence is more closely related to those available from virus isolates from CAR than of past outbreaks in East Africa as well as more recent outbreaks in Sudan 2003. The genetic variation observed among YFV isolated from Uganda argues that YFV is endemic to the region, and that circulating virus maintained in sylvatic cycles with nonhuman hosts is being introduced into a human host at independent events.

YFV was the first hemorrhagic fever virus to be isolated in 1927 and was sequenced in its entirety in 1985, yet there is surprisingly little yellow fever genomic information on isolates from previous outbreaks and strains from different geographical locations (Lepiniec et al., 1994; Rice et al., 1985). Indeed, there are only 25 full-length YFV sequences in the NCBI database, including 7 sequences of YFV vaccine and 9 sequences from human cases with an adverse event to vaccination.

Rapid pathogen detection and disease-specific intervention are crucial in minimizing morbidity and mortality associated with viral hemorrhagic fever outbreaks. Here, we demonstrated the ability to rule out commonly suspected viral hemorrhagic fevers in a timely manner, and implemented an NGS approach when other diagnostic testing failed to yield the etiologic agent of this outbreak. After the identification of YFV, the outbreak team amended the clinical patient case definition, distributed samples for yellow fever specific diagnostic testing, and began a vaccination campaign with the highly effective YFV vaccine 17D (Monath, 2005). The fact that YFV was not the suspected etiological agent in this outbreak demonstrates the power of unbiased NGS for pathogen surveillance and discovery.

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