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A New Arenavirus in a Cluster of Fatal Transplant-Associated Diseases

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ABSTRACT

BACKGROUND

Three patients who received visceral-organ transplants from a single donor on the same day died of a febrile illness 4 to 6 weeks after transplantation. Culture, polymerase-chain-reaction (PCR) and serologic assays, and oligonucleotide microarray analysis for a wide range of infectious agents were not informative.

METHODS

We evaluated RNA obtained from the liver and kidney transplants in two recipients. Unbiased high-throughput sequencing was used to identify microbial sequences not found by means of other methods. The specificity of sequences for a new candidate pathogen was confirmed by means of culture and by means of PCR, immunohistochemical, and serologic analyses.

RESULTS

High-throughput sequencing yielded 103,632 sequences, of which 14 represented an Old World arenavirus. Additional sequence analysis showed that this new arenavirus was related to lymphocytic choriomeningitis viruses. Specific PCR assays based on a unique sequence confirmed the presence of the virus in the kidneys, liver, blood, and cerebrospinal fluid of the recipients. Immunohistochemical analysis revealed arenavirus antigen in the liver and kidney transplants in the recipients. IgM and IgG antiviral antibodies were detected in the serum of the donor. Seroconversion was evident in serum specimens obtained from one recipient at two time points.

CONCLUSIONS

Unbiased high-throughput sequencing is a powerful tool for the discovery of pathogens. The use of this method during an outbreak of disease facilitated the identification of a new arenavirus transmitted through solid-organ transplantation.

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METHODS OF CLONING NUCLEIC ACIDS of microbial agents directly from clinical specimens offer new opportunities for the surveillance and discovery of pathogens. Molecular techniques have been used successfully in the identification of infectious agents such as the Borna disease virus, hepatitis C virus, Sin Nombre virus, human herpesviruses 6 and 8, *Bartonella henselae*, *Tropheryma whipplei*, West Nile virus, and the coronavirus associated with severe acute respiratory syndrome.¹

The arenaviruses are enveloped, negative-strand RNA viruses in rodents; these viruses are most frequently transmitted to humans through exposure to infected urine. Infection with the prototype virus, lymphocytic choriomeningitis virus (LCMV), is typically asymptomatic or associated with mild, transient illness; however, LCMV has also been implicated in aseptic meningitis.² Human-to-human transmission of LCMV during pregnancy has been reported, and infection during the gestational period can result in fetal death, neurologic sequelae, and chorioretinopathy.³ Fatal outbreaks of disease associated with human-to-human transmission of LCMV in recipients of solid-organ transplants have also been described.⁴ We report the use of unbiased DNA sequencing in the discovery of a new LCMV-related arenavirus that caused fatal disease in three recipients of organs from a single donor.

METHODS

PATIENTS AND CLINICAL COURSE

Three women in Australia who were 63 years of age (Recipient 1), 64 years of age (Recipient 2), and 44 years of age (Recipient 3) received a liver transplant (Recipient 2) or kidney transplants (Recipients 1 and 3) from one male donor who was 57 years of age. The donor died of cerebral hemorrhage 10 days after returning to Australia from a 3-month visit to the former Yugoslavia, where he had traveled in rural areas. The immediate post-transplantation course in the three transplant recipients was unremarkable; however, febrile illnesses with varying degrees of encephalopathy developed in all three, and they died 4 to 6 weeks after transplantation (Table 1). Bacterial and viral cultures; polymerase-chain-reaction (PCR) assays for herpesviruses 1 through 8, lyssavirus, influenza A and B viruses, respiratory syncytial virus,

picornavirus, adenovirus, human parainfluenza virus, flavivirus, alphavirus, hantavirus, polyomavirus, Crimean–Congo hemorrhagic fever virus, Rift Valley fever virus, toxoplasma, *Mycobacterium tuberculosis*, and *Mycoplasma pneumoniae*; and viral and panmicrobial oligonucleotide microarray analysis⁴ revealed no candidate pathogens.

UNBIASED HIGH-THROUGHPUT SEQUENCING

RNA was extracted from the brain, cerebrospinal fluid, serum, kidney, and liver of Recipient 1, who had received a kidney transplant, and from the cerebrospinal fluid and serum of Recipient 2, who had received a liver transplant. As shown in Figure 1, after digestion with DNase I to eliminate human chromosomal DNA, RNA preparations were amplified by means of reverse-transcriptase PCR (RT-PCR) with the use of random primers.^{5,6} Amplification products were pooled and sequenced with the use of the GSL FLX platform (454 Life Sciences), but DNA fragmentation was omitted.⁷ After trimming to remove sequences derived from the amplification primer and after filtration to eliminate highly repetitive sequences, the data set was analyzed by subtracting fragments that matched human sequences, clustering non-redundant sequences,⁸ and assembling them into contiguous sequences⁹ for direct comparison with the GenBank databases of nucleic acids and proteins with the use of BLASTN and BLASTX software.¹⁰ We analyzed the resulting alignments and assigned them to nodes in the National Center for Biotechnology Information taxonomy database, using a custom software application written in Perl (BioPerl version 5.8.5).

PCR QUANTITATION OF THE ARENAVIRUS BURDEN

RNA obtained from tissues, plasma, serum, and cerebrospinal fluid was reverse transcribed with the use of random hexamers. PCR was performed with the use of a SYBR Green assay (Applied Biosystems). The following cycling conditions were used: 50°C for 2 minutes and 95°C for 10 minutes, followed by 45 cycles at 95°C for 15 seconds and 60°C for 1 minute. Real-time PCR assays were performed with the following primer set: 5'AGTGCYTGACACAACATCGTTT3' (forward) and 5'CAATGCCAGCYTGACAAT3' (reverse). Thermal cycling was performed with the use of an ABI 7500 real-time PCR system (Applied Biosystems).

Table 1. Characteristics of the Organ-Transplant Recipients.

Recipient No.	Age yr	Diagnosis	Organ Transplanted	Clinical Course	Interval between Transplantation and Death days
1	63	End-stage renal failure due to polycystic kidney disease	Kidney	Fever, sepsis, encephalopathy, acute tubular necrosis, graft rejection, radiographic evidence of chest infiltrates	36
2	64	Decompensated cirrhosis and hepatocellular cancer due to hepatitis C infection	Liver	Fever, confusion, encephalopathy with myoclonus, chest infiltrates	30
3	44	End-stage renal failure due to polycystic kidney disease	Kidney	Fever, graft rejection, intraabdominal hematomas and effusion, transplant nephrectomy, encephalopathic illness	29

VIRAL ISOLATION AND ANALYSES

Kidney tissue from Recipient 1 was homogenized in phosphate-buffered saline, centrifuged to pellet cellular debris, filtered, and used to inoculate Vero E6 cells. The cells were monitored daily by means of light microscopy for cytopathic effect and by means of RT-PCR for the presence of arenavirus nucleic acid in supernatant. Monolayers of cells showing cytopathic effects that were also positive for arenavirus nucleic acid were fixed with buffered 4% paraformaldehyde for indirect immunofluorescence and immunohistochemical microscopy and with buffered 2.5% glutaraldehyde for thin-section electron microscopy. Rabbit polyclonal antiserum against Old World arenaviruses, including LCMV, was used as the source of primary antibodies for immunohistochemical analysis. Secondary antibodies were alkaline phosphatase-conjugated goat antibodies against rabbit IgG.⁴ Immunohistochemical assays were also performed with the use of formalin-fixed, paraffin-embedded tissue sections obtained from the liver and kidney of Recipient 1.

Virus-infected and noninfected (control) Vero E6 cells were fixed with methanol. Serum specimens from the donor, from the recipients, and from 100 randomly chosen control recipients of solid-organ transplants were applied to the fixed cells followed by fluorescein-labeled antihuman IgG or IgM secondary antibodies.

COMPLETE GENOME SEQUENCING AND PHYLOGENETIC ANALYSES

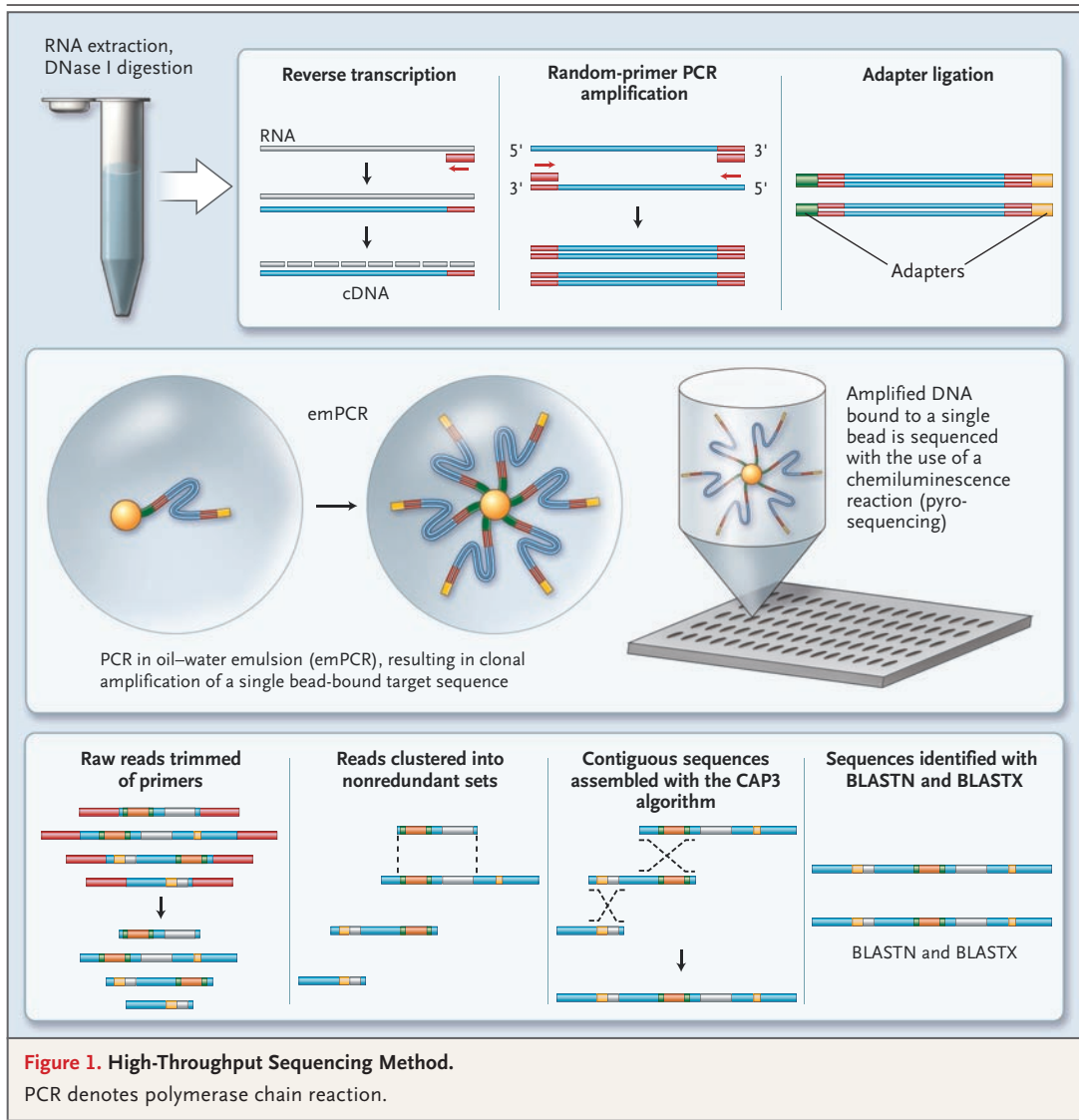
RNA extracted from the liver in Recipient 1 was used as a template to clone and sequence the L and

S segments of the virus. The gene fragments obtained by means of pyrosequencing were used to design specific PCR primers; thereafter, consensus primers were designed on the basis of alignments of other arenavirus sequences with the use of the SCPrimer program.¹¹

The L and S segments were assembled and sequenced as a series of overlapping genetic fragments. Evolutionary distances between the assembled segments were computed with the use of the Poisson correction method and expressed in units of amino acid substitutions per site in relationship to arenavirus L, glycoprotein precursor, and nucleoprotein amino acid segments in the GenBank database with the use of the MEGA program.¹² The percentage of replicate trees in which taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. All positions containing alignment gaps and missing data were eliminated only in pairwise sequence comparisons (see Fig. 1a, 1b, and 1c of the Supplementary Appendix, available with the full text of this article at www.nejm.org). The nucleotide and amino acid homologies of each of the arenavirus genes (Z, L, GPC, and NP) to LCMV (the closest completely sequenced relative) are shown in Table 2. The sequences are deposited in GenBank (accession numbers EU136038 and EU136039).

RESULTS

RNA from tissue from Recipient 2, who had received a liver, and Recipient 1, who had received a kidney, was pooled and amplified for unbiased high-throughput sequencing,⁷ yielding 103,632 se-



quence fragments. The sequences recovered ranged in size from 45 to 337 nucleotides, with a mean length of 162. Sequences derived from the amplification primer and highly repetitive sequences were eliminated, yielding a net of 94,043 sequences. These sequences were processed with the use of algorithms that subtract vertebrate sequences, assemble contiguous sequences, and compare the residual nucleotide and deduced amino acid sequences in all six potential open reading frames with motifs represented in databases of microbes.

At the nucleotide level, sequence data were uninformative; however, BLASTX analysis of the deduced protein sequence revealed 14 fragments that were consistent with Old World arenaviruses

(12 S-segment and 2 L-segment fragments) sharing the closest relationship to LCMV.

Primers were designed for RT-PCR experiments to detect viral RNA in clinical specimens, assess the similarity of viral sequences among individual organs and recipients, and extend the viral sequence needed to facilitate characterization. Viral RNA was present in a total of 22 of 30 specimens of tissue, blood, or cerebrospinal fluid from all three transplant recipients (Table 3). The sequence was identical in all specimens, a finding that was consistent with the introduction of a single virus into all the recipients.

Fresh-frozen kidney tissue from Recipient 1 was homogenized and used to inoculate cultures of

Vero E6 cells. A cytopathic effect was observed only in the first passages; thereafter, morphologic characteristics did not differ between infected and control cells. Indirect immunofluorescence assays with the use of polyclonal antibodies against arenaviruses and LCMV showed cytoplasmic distribution of viral antigen. Immunostaining of viral antigens was also seen in infected cells by means of an indirect immunoalkaline phosphatase technique (Fig. 2A). Quantitative RT-PCR assays showed increasing concentrations of viral nucleic acid with serial passage. Examination of infected Vero E6 cells by means of thin-section electron microscopy revealed extracellular particles with morphologic features that are characteristic of arenaviruses (Fig. 2B).

Immunofluorescence assays for serum antibodies that are reactive with infected Vero E6 cells revealed virus-specific IgM and IgG antibodies in the donor that were consistent with acute infection. Plasma and serum specimens from Recipient 2 that had been collected at two time points 19 days apart (11 days and 30 days after transplantation) were available for analysis. Virus-specific IgG and IgM antibodies were detectable only at the second time point, consistent with seroconversion.

Immunohistochemical analysis of specimens of the liver (Fig. 3A) and kidney (Fig. 3B) obtained from Recipient 1 showed focal immunostaining of arenavirus antigens. PCR surveys of 100 archived serum or plasma specimens from solid-organ transplant recipients who were not linked to the cluster and who had undergone transplantation in the same city and during the same time period revealed no evidence of infection with this pathogen.

The 3301-nucleotide S-segment and 7215-nucleotide L-segment sequences were cloned from the kidney of Recipient 1 by means of PCR and sequenced. Phylogenetic characterization was limited by the paucity of available sequences deposited in public databases; nonetheless, L- and S-segment analyses were consistent with the presence of a new arenavirus. Whereas sequences in the nucleoprotein and glycoprotein genes on the S segment were closest to the LCMV strain LE¹³ and M1 and M2 isolates¹⁴ (Table 2, and Fig. 1b and 1c of the Supplementary Appendix), the L-segment sequence indicated a closer relationship to Kodoko virus. Strain LE was isolated in France from an infected fetus. M1 and M2 were isolated in Japan

Table 2. Nucleotide and Amino Acid Homologies of the New Arenavirus to Other Arenaviruses.*

Gene	Accession No.	LCMV Strain	Homology	
			Amino Acid	Nucleotide
			<i>percent</i>	
GPC	AB261990	M2	94	86
NP	AB261990	M2	97	87
L	DQ286932	Marseille 12	82	79
Z	DQ286932	Marseille 12	79	72

* LCMV denotes lymphocytic choriomeningitis virus.

from wild mice. Kodoko virus was recently isolated in Africa from wild mice (Fig. 1a of the Supplementary Appendix).¹⁵ Reassortment is well described in arenaviruses and could account for differences in phylogenetic relationships based on L- and S-segment sequences. However, reassortment cannot be implicated without a complete genomic sequence for the viruses used in these phylogenetic analyses.

DISCUSSION

Two clusters of transmission of arenavirus through solid-organ transplantation have been reported.⁴ In each cluster, recipients linked to a single donor died of an unexplained infectious disease 9 to 76 days after transplantation. In neither cluster did the donor have a history of acute infectious disease or evidence of infection by PCR or serologic analysis; however, in one cluster, a pet hamster that had recently been introduced into the donor's household was found to be infected with the same virus that was detected in the recipients. LCMV was implicated after the results of viral culture and electron microscopy triggered specific immunohistochemical and molecular tests for arenaviruses.

In our cluster, a new arenavirus was first detected through unbiased high-throughput sequencing. Thereafter, the infection was confirmed by means of culture, electron microscopy, and specific immunohistochemical and serologic tests. As in the other two reported clusters of transplant-associated transmission, we detected no viral nucleic acids in the donor and found no history of acute infectious disease; however, the presence of IgG and IgM antibodies confirmed recent infection. We were also unable to obtain any infor-

Table 3. Viral RNA and Antibody Titers in the Donor and Recipients.*

Specimen	Interval between Transplantation and Collection of Specimens	Viral RNA	Antibody Titer
	days		
Donor			
Serum	0	ND	1:80 IgG, 1:20 IgM
Spleen	0	ND	NA
Pancreas	0	ND	NA
Recipient 1 (kidney transplant)			
Plasma	0	ND	<1:10 IgG, <1:10 IgM
Plasma	27	889,200	NP
Plasma	33	614,900	NP
Cerebrospinal fluid	33	5,500	NP
Plasma	35†	1,000,000	NP
Urine	35†	88,000,000	NA
Heart	35†	33,200	NA
Spleen	35†	52,600	NA
Liver	35†	2,362,800	NA
Lung	35†	498,600	NA
Cerebrospinal fluid	35†	63,700	NP
Serum	35†	1,440,400	<1:10 IgG, <1:10 IgM
Brain	35†	16,600	NA
Rectal swab	35†	623,200	NA
Nasal swab	35†	55,400	NA
Axillary swab	35†	ND	NA
Kidney	35†	85,900	NA
Recipient 2 (liver transplant)			
Plasma	12	121,900	<1:10 IgG, <1:10 IgM
Mouth swab	24	457,000	NA
Bronchoalveolar lavage	19	1,163,400	NA
Cerebrospinal fluid	24	ND	NP
Plasma	24	346,200	NP
Serum	31†	347,600	1:40 IgG, 1:20 IgM
Recipient 3 (kidney transplant)			
Serum	-235	ND	<1:10 IgG, <1:10 IgM
Serum	0	ND	NP
Serum	24	415,500	NP
Serum	28	565,100	<1:10 IgG, <1:10 IgM

* NA denotes not applicable, ND not detected, and NP not performed.

† Specimens were obtained after death.

mation indicating that the donor had been exposed to rodents; however, his history of recent travel suggests that he may have been infected before returning to Australia from southern Europe,

where such exposure may have occurred in a rural area.

Although we have not fulfilled Koch's postulates, evidence implicating this new virus in the

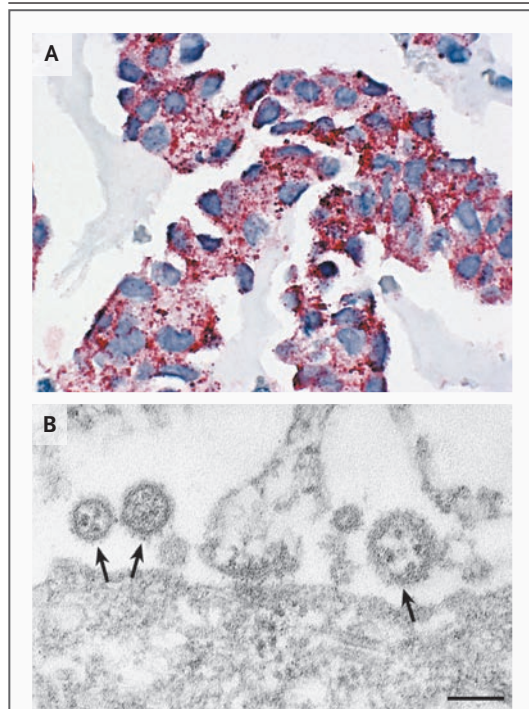


Figure 2. Propagation of the New Arenavirus in Tissue Culture.

Panel A shows immunostaining of viral antigens in infected cells by means of an indirect immunalkaline phosphatase technique. Panel B shows an electron micrograph of extracellular arenavirus-like virions. Particles (arrows) are round, vary in size, and have surface projections on the perimeter. Cellular ribosomes are visible within the virions. The length of the bar corresponds to 100 nm.

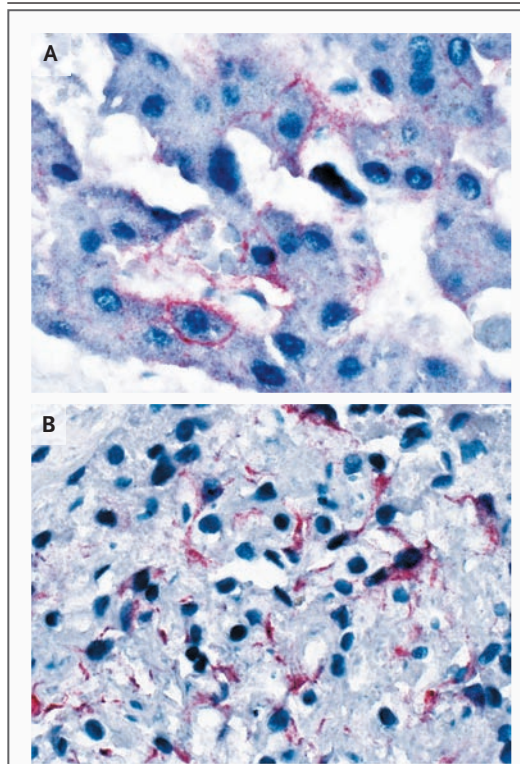


Figure 3. Predominantly Membranous Distribution of Arenavirus Antigen.

The distribution of the arenavirus antigen is shown in the liver (Panel A) and kidney (Panel B) of Recipient 1. Formalin-fixed, paraffin-embedded tissue sections were incubated with polyclonal rabbit antiserum against lymphocytic choriomeningitis virus followed by alkaline phosphatase-conjugated secondary antibodies against rabbit IgG.

outbreak of infection among patients who received transplants is compelling. All three recipients received organs from the same donor and died within days of one another after febrile illness. Identical viral sequences were obtained from all the recipients. The virus is new and was not detected in 100 organ recipients who were not linked to this cluster. The results of serologic analysis of specimens obtained from the donor were consistent with recent infection, and seroconversion was observed in one recipient.

Unbiased high-throughput sequencing has been used to characterize complex mixtures of microflora in environmental contexts¹⁶; we have shown that this strategy can be used to address a suspected outbreak of infectious disease. Its use in the context of investigating a cluster of cases of

acute disease associated with organ transplantation facilitated the rapid implication of a new arenavirus not detected by other methods. This technique may prove useful as a new tool in the identification and surveillance of pathogens in chronic as well as acute disease.

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Drs. Du, Simons, and Egholm report being employees of 454 Life Sciences. Dr. Lipkin reports being a member of the scientific advisory board of 454 Life Sciences during a portion of the time the work reported here was pursued. Drs. Du, Simons, Egholm, and Lipkin report holding stock options in 454 Life Sciences before it was purchased by Roche Diagnostics in May 2007. No other potential conflict of interest relevant to this article was reported.

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