Case Report
Pathogenic characterization of a cervical lymph node derived from a patient with Kawasaki disease

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Abstract: Kawasaki disease (KD) is the most common cause of multisystem vasculitis in childhood. Although cervical lymphadenitis is one of the major symptoms in KD, lymph node biopsy is rarely performed, because KD is usually diagnosed by clinical symptoms. A cervical lymph node biopsy was taken from a girl aged 1 year and 8 months who had suspected lymphoma, but she was diagnosed with KD after the biopsy. The cervical lymph node specimen was analyzed with multivirus real-time PCR that can detect >160 viruses, and unbiased direct sequencing with a next-generation DNA sequencer to detect potential pathogens in the lymph node. Histologically, focal necrosis with inflammatory cell infiltration, including neutrophils and macrophages, was observed in the marginal zone of the cervical lymph node, which was compatible with the acute phase of KD. Multivirus real-time PCR detected a low copy number of torque teno virus in the sample. Comprehensive direct sequencing of the cervical lymph node biopsy sample sequenced more than 8 million and 3 million reads from DNA and RNA samples, respectively. Bacterial genomes were detected in 0.03% and 1.79% of all reads in DNA and RNA samples, respectively. Although many reads corresponded to genomes of bacterial environmental microorganisms, Streptococcus spp. genome was detected in both DNA (77 reads) and RNA (2,925 reads) samples. Further studies are required to reveal any association of microbial or viral infection with the pathogenesis of KD.

Keywords: Kawasaki disease, lymph node, next-generation sequencer, multivirus real-time PCR, torque teno virus, streptococcus

Introduction
Kawasaki disease (KD) is an acute systemic vasculitis of infancy and early childhood that is characterized by prolonged fever, conjunctivitis, inflammation of the oropharynx, rash, erythematous induration of the distal extremities, and cervical lymphadenopathy [1]. Its etiology remains to be elucidated, and epidemiological studies have shown that it is more frequently observed in winter [2]. The disease occurs worldwide, but its incidence is 10 times higher in Japan than in western countries [3], suggesting that race-specific genotypes might be associated with the disease.

The resemblance of KD to toxic shock syndrome (TSS) suggests a causative role for as-yet-uncharacterized infectious agents. TSS is caused by the superantigen (SAg) TSS toxin-1 [4]. SAgS are toxins of microbial or viral origin that cross-link antigen-presenting cells and T cells by simultaneously binding to class II major histocompatibility complex molecules [5-7] and the variable b-chain of T-cell receptors (TCR Vb) [8-10]. Although the etiology of KD is unknown, Lactobacillus cell wall [11], immunization with Bacillus Calmette–Guerin (BCG) [12], or Candida albicans fractions [13, 14] induce vasculitis and coronary arteritis in animal models, whereas tumor necrosis factor (TNF)-α has been suggested as necessary for this induction [15]. These observations suggest that, in addition to microbial SAg, infectious agents could be potential candidates for the onset of KD. To
date, a number of SAgs-producing bacteria [16], including *Streptococcus pyogenes*, *Staphylococcus aureus* and *Yersinia pseudotuberculosis* [17, 18], as well as viruses, such as Epstein–Barr virus [19], have been speculated to be the causative agents of KD; however, there is a lack of consistency among reports. These disparate findings suggest that the inflammation observed in KD is not the result of a single agent, but rather from numerous infectious agents in genetically susceptible individuals [20-22].

Cervical lymphadenitis is one of the major symptoms in KD [1, 23]. A pathological study demonstrated that lymph node biopsy from KD patients showed focal necrosis with inflammatory cell infiltration [24]. This observation implied that lymphadenopathy is a symptom of the acute phase of KD, and suggested the association of bacterial or virus infection with the pathogenesis of KD. However, lymph node biopsy is rarely performed in KD patients, because KD is usually diagnosed by clinical symptoms [23].

In the present study, we analyzed a cervical lymph node biopsy from a girl aged 1 year and 8 months who had suspected lymphoma, but she was diagnosed with KD after biopsy. The recently developed next-generation sequencer is a powerful tool for detecting pathogen genomes in clinical samples. Comprehensive direct sequencing without any filtering preparation steps by a next-generation sequencer enables one to detect pathogens in representative and unbiased conditions in a small number of clinical samples [25-28]. The multivirus real-time PCR system developed recently by our group is another powerful tool to detect virus genomes in pathological samples. This system can detect >160 viruses in frozen or formalin-fixed paraffin-embedded (FFPE) tissues based on the techniques of the Taqman real-time PCR system [29]. To identify the potential pathogens in KD patients, the lymph node sample was analyzed with multivirus real-time PCR and comprehensive direct sequencing using a next-generation sequencer.

**Materials and methods**

**Ethics statement**

The study protocol was approved by the Institutional Medical Ethics Committee, National Institute of Infectious Diseases, Japan (Approval No. 295), and Niigata City General Hospital. The study was conducted according to the principles of the Declaration of Helsinki.

**KD patient**

A girl aged 1 year and 8 months had prolonged fever and cervical lymphadenopathy. She was subjected to a lymph node biopsy for suspicion of malignant lymphoma; however, histological features were compatible with KD and suggested no malignancy (Figure 1) [24]. After the biopsy, conjunctivitis, skin rash, and “strawberry tongue” were observed in the patient. These symptoms met the diagnostic criteria for KD.
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established by the Japanese Kawasaki Diseases Research Committee [1, 23]. The patient was positive for 5 markers (fever, conjunctiva, exanthema, strawberry tongue, and lymphadenopathy) of the 6 KD symptom criteria at day 10 from the appearance of their earliest symptom. Administration of intravenous immunoglobulin and aspirin resulted in rapid decline of fever, and all symptoms disappeared at day 20 from the appearance of the earliest symptom.

Histopathology

Hematoxylin–eosin (HE), periodic acid–Schiff (PAS), Gram, and Giemsa staining was performed on the paraffin sections. In immunohistochemistry, monoclonal or polyclonal antibodies to herpes simplex virus (HSV)-1 and -2 [30], varicella–zoster virus (VZV) [31], human cytomegalovirus (CMV) [32], human herpesvirus 6 (HHV-6) (P101; Millipore, Bedford, MA, USA), Kaposi’s sarcoma-associated herpesvirus (KSHV) [33], and human papillomavirus (HPV) [34] were used as the primary antibodies. The labeled avidin–biotin method was used to detect virus antigens. In situ hybridization was performed to detect EBV-encoded small RNA (EBER) as described previously [35].

Total nucleic acid preparation from specimens

Total DNA and RNA were prepared from a FFPE 7-mm cube of the autopsy lymph node tissue using QIAamp DNA extraction kit for FFPE (Qiagen, Hilden, Germany) and PureLink FFPE RNA isolation kit (Invitrogen, Carlsbad, CA, USA), respectively. Total RNA was treated with DNase (TurboDNase, Ambion, Austin, TX, USA).

Multivirus real-time PCR system

The multivirus real-time PCR system that detects >160 viruses was used as reported previously [29]. Total DNA and RNA (each 1 µg) were applied to the system.

Short-read DNA sequencing

The first-strand cDNA was prepared from ~0.5 µg total RNA using the random priming method with the RT2 First Strand kit (Supper Array Bioscience, Frederick, MD, USA), and double-strand cDNA (ds-cDNA) was synthesized with DNA polymerase I (Escherichia coli, 10 U/µl, Invitrogen) with RNase H (2 U/µl; Invitrogen). The ds-cDNA was purified using a QIAquick PCR Purification Kit (Qiagen). A DNA library was prepared using a Genomic DNA Sample Prep Kit (Illumina, San Diego, CA, USA), and DNA clusters were generated on a slide using a Cluster Generation Kit (version 2) on an Illumina cluster station, according to the manufacturer’s instructions. To obtain ~1.0 × 10⁷ clusters for 1 lane, the general procedure, as described in the standard protocol (Illumina), was performed as follows: template hybridization, isothermal amplification, linearization, blocking, denaturation, and hybridization of the sequencing primer. All sequencing runs for 75–80 mers were performed with the Illumina Genome Analyzer IIx sequencing kit. Fluorescent images were analyzed using the Illumina base-calling pipeline 1.4.0 to obtain FASTQ-formatted sequence data. The detailed sequencing parameters are summarized in Table 2. The short read archives have been deposited in the DNA Data Bank of Japan (DDBJ; accession numbers: DRA000437).

Homology search analysis

All of the obtained DNA sequence reads were aligned to a reference sequence of human genomic DNA, followed by quality trimming to remove low-quality reads, and further excluding reads with similarity to ambiguous human sequences by a BLASTN search with a cutoff value of e⁻⁸. The remaining sequence reads were investigated using a BLASTN search against a nucleotide database. The results of the BLASTN search were analyzed and visualized using MEGAN v4.2.6 [36] with the following parameters: minimum support, 1 hit; minimum score 50 for the genus level.

Results

Histopathological findings

HE stain showed focal and severe necrosis mainly in the lymphoid follicle in the marginal zone of the lymph node (Figure 1). Lymph node structure was destroyed by necrosis, and no intact germinal center was found. The necrotic area contained many necrotic ghost cells with hemorrhage and infiltration of neutrophils, eosinophils and macrophages. Apoptotic cells were also frequently found around the necrotic area. The lymph node capsule was thickened with inflammatory cell infiltration. These obser-
vations were compatible with the acute phase of KD [24]. No bacteria or fungi were detected by PAS, Gram and Giemsa staining. HSV-1, CMV, HHV-6, VZV, KSHV and HPV antigens were not detected by immunohistochemistry, and EBER was negative by in situ hybridization.

**Multivirus real-time PCR**

To detect viruses in the lymph node sample, we used the multivirus real-time PCR system, which can detect >160 DNA and RNA viruses (Table 1). RNA extracted from the lymph nodes was negative for all RNA viruses, while internal control RNA (hGAPDH mRNA) was detected. The multivirus real-time PCR system revealed that 30 ng DNA extracted from the lymph node contained 14 copies of torque teno virus (TTV).

**Table 1. Results of multivirus real-time PCR**

<table>
<thead>
<tr>
<th>Virus Type</th>
<th>Results</th>
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<tbody>
<tr>
<td>DNA viruses</td>
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<tr>
<td>Polyomavirus: JC virus, BK virus, Simian virus 40</td>
<td>–</td>
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<tr>
<td>Papillomaviruses: HPV 6, 11, 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68, 73</td>
<td>–</td>
</tr>
<tr>
<td>Parvoviruses: adeno-associated virus 1, 2, 3 and 5; parvovirus B19; human bocavirus; adenovirus A–F</td>
<td>–</td>
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<tr>
<td>Herpes viruses: HHV 1–8, B virus</td>
<td>–</td>
</tr>
<tr>
<td>Poxviruses: variola virus, monkey pox virus, molluscum contagiosum virus</td>
<td>–</td>
</tr>
<tr>
<td>Anellovirus: TTV</td>
<td>+</td>
</tr>
<tr>
<td>Hepadnavirus: Hepatitis B virus</td>
<td>–</td>
</tr>
<tr>
<td>Others: mimivirus</td>
<td>–</td>
</tr>
<tr>
<td>RNA viruses</td>
<td></td>
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<tr>
<td>Filoviruses: Ebola virus, Marburg virus</td>
<td>–</td>
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<tr>
<td>Bunyaviruses: Crimean–Congo hemorrhagic fever virus, hemorrhagic fever with renal syndrome virus (Hantaan, Dovrava, Puumala, and Seoul), Rift valley fever virus, sin nombre virus</td>
<td>–</td>
</tr>
<tr>
<td>Arenaviruses: Lassa virus, Junin, Guanarito, Machupo and Sabia viruses</td>
<td>–</td>
</tr>
<tr>
<td>Togaviruses: equine encephalitis virus (Venezuelan, Eastern, and Western), Sindbis virus, Mayaro virus, Getah virus, Chikungunya virus, rubella virus</td>
<td>–</td>
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<tr>
<td>Enteroviruses: enterovirus 68 and 71; poliovirus 1–3; coxsackievirus A2–A6, A8–A10, A16, A21, A24, B1–B6; echovirus 5, 6, 7, 9, 11, 13–18, 25, 30; parechovirus 1 and 3; rhinovirus A and B; rotavirus; reovirus 1–4; Melaka virus; Colorado tick borne fever virus</td>
<td>–</td>
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<tr>
<td>Flaviviruses: dengue virus 1 and 2; Japanese encephalitis virus; Murray Valley encephalitis virus; St. Louis encephalitis virus; West Nile virus; Tick-borne encephalitis virus; yellow fever virus</td>
<td>–</td>
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<tr>
<td>Orthomyxoviruses: influenza virus A-C and H5N1</td>
<td>–</td>
</tr>
<tr>
<td>Paramyxoviruses: parainfluenza virus 1–3; Hendra virus; mumps virus; measles virus; Sendai virus; RS virus A and B; metapneumovirus; Nipah virus</td>
<td>–</td>
</tr>
<tr>
<td>Rabdoviruses: rabies virus, lyssavirus 5 and 6, Chandipura virus, Duvenhage virus</td>
<td>–</td>
</tr>
<tr>
<td>Coronavirus: coronavirus OC43, 229E and NL63; SARS virus</td>
<td>–</td>
</tr>
<tr>
<td>Caliciviruses: sapovirus, Norwalk-like virus 1 and 2</td>
<td>–</td>
</tr>
<tr>
<td>Hepatitis viruses: hepatitis A virus, hepatitis C virus, hepatitis D virus, hepatitis E virus, GB virus</td>
<td>–</td>
</tr>
<tr>
<td>Retroviruses: human immunodeficiency virus 1, human T cell leukemia virus 1 and 2</td>
<td>–</td>
</tr>
<tr>
<td>Others: astrovirus, Borna disease virus</td>
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All other DNA viruses examined in the system were negative. **Sequencing reads by next-generation sequencer**

To determine the potential pathogens in the patient, direct sequencing was performed on the DNA or ds-cDNA from total RNA extracted from an FFPE sample of the lymph node biopsy. The Illumina Genome Analyzer Iix sequencing system produced several million 75-mer reads from the DNA or ds-cDNA libraries (Table 2). 99.59% and 77.43% of the sequences from the DNA and RNA samples were originated from a possible human source, respectively (Table 2 and Figure 2). The remaining reads were further analyzed using a BLASTN search against Table 1.
non-redundant databases, revealing potential bacterial and viral sequences (Figure 2). Regarding the bacterial hits, genus classification was determined from the results of a BLASTN search (Figure 3). The most abundant bacteria in both DNA and RNA samples were from the genera *Methylotenera*, *Cytophaga*, *Flavobacterium* and *Polynucleobacter*, which are well-known environmental microorganisms, but our other sequencing trials with clinical specimens suggested that these bacteria could be the result of contamination through the handling of FFPE samples, because these species were always detected (data not shown). In addition, *Escherichia* and *Propionibacterium* spp. were frequently detected as possible contaminants. Sequencing of the lymph node showed that *Streptococcus* spp. was present, but it could not be confirmed as the specific pathogen related to the onset of KD (Figure 3; panel Lymph-RNA). No virus genome was detected by the direct sequencing.

**Discussion**

Using a next-generation DNA sequencer and multivirus real-time PCR system, we detected...
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Figure 3. Dot plot of the short read numbers for archaea and all bacterial genera from the lymph node biopsy. The sequencing reads from lymph node DNA or RNA samples were analyzed by BLASTN homology search with a threshold score of 50.0, and the similarity results were classified into 11 and 653 genera of archaea and bacteria, respectively. The phylum taxonomy of the bacteria is shown below the dot plot. Notable genera and the corresponding number of sequencing reads are shown beside each dot.
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possible pathogens from a cervical lymph node biopsy specimen, which is a tissue that is more likely to be associated with pathogens responsible for the pathogenesis of KD. Direct sequencing from DNA or RNA revealed that abundant environmental bacteria were unexpectedly identified, but variable bacterial genera were identified in the lymph node. Although we could not definitively identify the responsible pathogen for the lymphadenopathy, the presence of *Streptococcus* spp. in the lymph node suggests the association of streptococcal infection with the lymph node lesion in a KD patient.

Pathological findings in the lymph node from this patient, such as focal necrosis with abundant apoptotic cells, are compatible with previous findings in the lymph nodes of KD patients [24]. Focal necrosis with abundant apoptotic cells and severe inflammatory cell infiltration are frequently observed in any bacterial or viral infection, non-specifically. However, any specific pathogen has not been identified in the lymph node of KD patients so far. These previous findings suggest that etiological pathogens are already excluded by inflammation in the necrotic lesion, and some bacterial toxins are associated with formation of necrotic lesions in the lymph node. In such necrotic lesions, it is not easy to detect the pathogens. Another reason why the pathogen has not been identified in the lymph node of KD patients is that lymph node biopsy is not usually performed in such patients because the disease can be diagnosed from clinical symptoms. Thus, the lymph node sample in the present study is important for investigation of KD pathogenesis.

The next-generation sequencer identified *Streptococcus* spp. in the lymph node as one of the probable suspected pathogens for the pathogenesis of KD. Although detailed species classification could not be performed in our study, some oral *Streptococcus* spp. such as *Streptococcus salivarius*, *Streptococcus thermophilus*, *Streptococcus pneumoniae*, *Streptococcus mitis*, *Streptococcus sanguinis* and *Streptococcus dysgalactiae* are known to induce platelet aggregation [37]. In fact, increased platelet count is one of the significant parameters detected at the onset of KD [38, 39]. Streptococcal platelet activation is dependent on the species and strain. Some *S. sanguinis* strains produce platelet-aggregation-associated protein [40], the adhesion of *S. pneumoniae* to platelets occurs as a consequence of platelet activation by thrombin [41], and although still controversial, *S. mitis* stimulates platelet aggregation in a strain-dependent manner [42].

In this study, we were unable to identify S. pyogenes using bacterial culture and comprehensive detection; however, it has been reported that the SAg genes of group A streptococci might be transferred among themselves [43]. It has been reported that the *Streptococcus* spp. isolated from KD patients induce expansion of T-cell populations expressing Vβ2 [44]. Thus, it is possible that an uncharacterized SAg gene could be transferred to the identified *Streptococcus* spp. Indeed, multiple SAg gene clusters have been identified in stool samples from KD patients [45]; however, this finding remains to be confirmed.

The multivirus real-time PCR revealed the absence of viruses, except TTV, in the lymph node from our patient. TTV was originally identified as an etiological agent of hepatitis [46, 47]. However, TTV has been detected frequently in various tissues and even from healthy individuals [48, 49]. Thus, TTV is thought to be a ubiquitous virus that is not associated with any specific diseases. TTV has been detected frequently even in samples from other children by the multivirus real-time PCR system (data not shown), therefore, it should not be thought of as an etiological agent of KD. However, increased copy numbers of TTV have been found in some disease tissues, suggesting that TTV is activated under certain conditions in some diseases [50-52]. Further studies are required to clarify the association of TTV infection and KD pathogenesis in the lymph node.

Finally, this study used multivirus real-time PCR and unbiased direct sequencing to identify possible causative agents of KD, and demonstrated the presence of TTV and *Streptococcus* spp. in the lymph node of a KD patient. It remains to be elucidated which species of streptococci are closely related to the etiology of KD. We are currently trying to identify additional specific isolates from KD patients, and whole genome sequencing of these isolates may identify a crucial virulence factor for KD pathogenesis, such as unique SAgS and platelet-activating factors.
Acknowledgment

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