Since December 2019, the number of cases of the coronavirus disease (COVID-19) has increased in Wuhan, Hubei Province, China (1). As of January 2020, new cases of COVID-19 have also been identified and confirmed in Japan. The new coronavirus has been named severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (2), and the complete genome of the original SARS-CoV-2 isolate has been sequenced. In Japan, individuals infected with SARS-CoV-2 have been identified as returnees from Wuhan, China and their close contacts (3).

The Tokyo Metropolitan Institute of Public Health (TMIPH) has initiated testing of individuals and close contacts suspected of harboring the SARS-CoV-2 by using nucleic-acid based methods including real-time RT-PCR (qRT-PCR) (4) as described in the manual distributed by the National Institute of Infectious Diseases (NIID) (5).

SARS-CoV-2 specific sequences were detected from pharyngeal swabs of three individuals who were travelers or returnees from China, using qRT-PCR QuantStudio™ 12K (Thermo Fisher Scientific, Waltham, MA, USA). We tried to sequence the complete genomes of these new isolates directly using next-generation sequencing (NGS) of RNAs from the three clinical specimens that were evaluated using qRT-PCR, including 30.0–34.1 (CT values) for the nucleocapsid protein set no. 1 (N1) and 26.0–29.2 for the nucleocapsid protein set no. 2 (N2) (4,5). Next, virus culture was performed using Vero and VeroE6 cells. The virus growth in the culture was detected using RNA extracted from the supernatants of infected Vero or VeroE6 cells using QIAamp viral RNA mini kit (Qiagen, Hilden, Germany). RNA libraries were prepared using the NEB Next Ultra RNA Library Prep Kit for Illumina (New England Biolabs, MA, USA) according to the manufacturer’s protocols. NGS was performed with MiSeq (Illumina, San Diego, CA, USA), and genome data were analyzed using a web-based integrated NGS analysis tool, Virus Tap, available at the NIID (6).

This study was approved by the Institutional Review Board of TMIPH, according to the Declaration of Helsinki 2013 (acceptance number: 31KenKenKen-2007).

Using NGS, 37 contigs (100–700 bp fragments) were identified in one clinical specimen (asymptomatic returnee from China) that matched the original Wuhan seafood market SARS-CoV-2 isolate, Wuhan-Hu-1, (GenBank Accession No. NC045512). NGS provided genome sequences of SARS-CoV-2 in only one of the three specimens; qRT-PCR findings including Ct values of 30.0 and 26.0 were determined with the aforementioned qRT-PCR sets N1 and N2, respectively. Our results suggest that the direct use of NGS examination is somewhat limited when the goal is a comprehensive search for pathogens associated with poorly characterized illnesses.

We inoculated the monolayers of Vero and VeroE6 cells (distributed by the Osaka Institute of Public Health) with three clinical specimens to isolate SARS-CoV-2. The cells were grown in Eagle’s minimum essential medium (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) with 2% FCS in 25 cm² flasks at 36°C in 5% CO₂ in a biosafety level 3 laboratory. Three days after inoculation, cytopathic effect (CPE) of one specimen (asymptomatic returnee from China) was observed in the VeroE6 culture, but not in Vero cells. Total RNA was extracted from culture supernatants using QIAamp viral RNA mini kit (Qiagen, Hilden, Germany). RNA libraries were prepared using the NEB Next Ultra RNA Library Prep Kit for Illumina (New England Biolabs, MA, USA) according to the manufacturer’s protocols. NGS was performed with MiSeq (Illumina, San Diego, CA, USA), and genome data were analyzed using a web-based integrated NGS analysis tool, Virus Tap, available at the NIID (6).
Fig. 1. SARS-CoV-2 (TKYE6182) levels in cell supernatant by qRT-PCR. SARS-CoV-2 amplification plots (Cp values) of supernatants in both Vero and VeroE6 cells inoculated with the specimens were measured by qRT-PCR using primer-probe set, open reading frame 1a/b. (A) supernatant at day 4 after inoculation of Vero cells, (B) Vero cell supernatant on day 3, (C) VeroE6 supernatant on day 4, (D) VeroE6 supernatant on day 3, (E) supernatant from VeroE6 cells alone (no virus) on day 3 and (F) day 4.

Fig. 2. Comparison of whole genome sequences. Included in this analysis are the original SARS-CoV-2 (NC045512), our new isolate TKYE6182_2020, and additional 5 isolates from Japan. The sequence of TKYE6182_2020 differs from the original SARS-CoV-2 by only 3 out of 29,903 bases, but differed at 1–8 bases from each of the additional isolates included. There are two subtypes (S and L) in Japan; TKYE6182_2020 is a subtype L.

5′-CCCACCTCGGTCTCCATT-3′, N-28356-Probe: VIC-5′-TCAACTGGCAGTAACC-3′-MGB). Three days after the inoculation, C slaughtered well values (amplification curves) determined via qRT-PCR in the culture supernatants of Vero and VeroE6 cells were found to have increased. On day 4, Ct values in the supernatants of both cells increased even further as compared to those on day 3 (Fig. 1).

From the viewpoint of usefulness, by preferentially analyzing the supernatant of VeroE6 cells showing CPE, the whole genome sequence of the new SARS-CoV-2 isolate, TKYE6182_2020, of VeroE6 cells was determined using NGS. The genome of TKYE6182_2020 included a total of 29,903 bases (Genbank Accession No. LC529905). We compared this sequence to SARS-CoV-2 isolates already registered in the GenBank (NC045512) and GISAID (EPI_ISL_408665-408667, 408669, 407084). The genome of TKYE6182 differed by only three bases from that of the original reference sequence (NC045512), and also differed by 1–8 bases from each of the earlier isolates included in Japan (Fig. 2). The SARS-CoV-2 genome has been classified into two major subtypes (designated L and S), and can be distinguished by the 8,782nd and 28,114th sequence of the Wuhan-Hu-1 (NC045512) (7); TKYE6182_2020 was L type.

We added 50 µL of 8% paraformaldehyde to 300 µL of culture supernatant from infected VeroE6 cells.
and visualized the virion structure under a Tecnai™ 12 electron microscope (FEI, Tokyo, Japan). Our results revealed a virion size of about 100 nm (Fig. 3), and confirmed the characteristic envelope structure of coronaviruses as reported by NIID (8).

Our new virus isolate (TKYE6182_2020) can be used for evaluating new genetic testing and for the development of antiviral drugs and vaccines.

Conflict of interest None to declare.

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Fig. 3. Electron microscope image of SARS-CoV-2 (TKYE6182_2020) isolated in Tokyo. The virion is ~100 nm in diameter with unique envelope structure that are typical for coronaviruses as indicated.