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Original Article

Detection of *Torque Teno Virus* DNA in Exhaled Breath by Polymerase Chain Reaction

Kumiko Chikasue, Miyuki Kimura, Kazuyuki Ikeda, Takuma Ohnishi,
Satoshi Kawanishi, Tomoe Iio, Mikio Kataoka, and Yujiro Arao*

Department of Medical Technology, Graduate School of Health Sciences, Okayama University, Okayama 700-8558, Japan

To determine whether exhaled breath contains *Torque teno virus* (TTV) or not, we tested exhaled breath condensate (EBC) samples by semi-nested PCR assay. We detected TTV DNA in 35% (7/20) of EBC samples collected from the mouth of one of the authors, demonstrating that TTV DNA is excreted in exhaled breath with moderate frequency. TTV DNA was detected also in oral EBC samples from 4 of 6 other authors, indicating that TTV DNA excretion in exhaled breath is not an exception but rather a common phenomenon. Furthermore, the same assay could amplify TTV DNA from room air condensate (RAC) samples collected at distances of 20 and 40 cm from a human face with 40 (8/20) and 35% (7/20) positive rates, respectively. TTV transmission has been reported to occur during infancy. These distances seem equivalent to that between an infant and its household members while caring for the infant. Taken together, it seems that exhaled breath is one of the possible transmission routes of TTV. We also detected TTV DNA in 25% (10/40) of RAC samples collected at a distance of more than 180 cm from any human face, suggesting the risk of airborne infection with TTV in a room.

Key words: *Torque teno virus*, exhaled breath, airborne infection, polymerase chain reaction

*T*orque teno virus (TTV) is one of human anelloviruses that have small non-enveloped virions containing single-stranded, circular DNA genomes [1]. The more sensitive polymerase chain reaction (PCR) assays that have been recently developed have demonstrated that 70 to 100% of the general population throughout the world is chronically infected with TTV except in the United Kingdom [2].

Although TTV has not yet been etiologically linked to known diseases, TTV infection has been associated with accelerated disease development, poorer clinical outcome, higher morbidity and/or increased mortality in many diseases [1]. For example, subjects with periodontitis had a significantly higher TTV DNA load in the gingival tissue than those without disease [3]. TTV DNA prevalence and loads in both nasal swabs and plasma were significantly higher in patients with bronchopneumonia than in those with milder disease [4]. Serum prevalence of TTV DNA was significantly higher in patients with severe idiopathic inflammatory myopathies than in those with milder disease [5].

Because there is neither an effective antiviral therapy nor a vaccine available for TTV at the present time, the only way to reduce the prevalence of TTV and thereby counteract TTV-associated diseases

is to block TTV transmission from infected to uninfected individuals. Recently, Naganuma *et al.* [6] studied saliva samples from healthy children and detected TTV DNA in 6, 34 and 90% of children aged 1, 4 and 42 months, respectively. From this data they concluded that TTV acquisition during the prenatal or neonatal period seemed infrequent and that TTV prevalence increased with age and apparently reached a plateau at or before 42 months of age. Thus, we must prevent TTV transmission to children less than 42 months old.

TTV DNA has been detected in various body fluids including semen [7, 8], cervical fluid [9, 10], nasal and throat secretions [4, 11], bile juice [12], breast milk [13, 14] and saliva [14–16]. The body fluids of parents and/or other household members have been regarded as possible sources of TTV infection of their children. If TTV is transmitted only via the body fluids, we can block TTV transmission to uninfected children by protecting children from contact with TTV-positive body fluids. However, exhaled breath of the TTV-infected adults may be contaminated with the virus since it passes through their nose, mouth and throat, which are moistened by TTV-positive body fluids. If so, it would only be natural that the children could be infected by TTV in the breath exhaled from TTV-positive adults. In this case, we need to make other plans to cope with TTV-contaminated exhaled breath in addition to protecting children from contact with TTV-positive body fluids. However, there is no information regarding contamination of exhaled breath with TTV.

The aim of this study was to determine whether exhaled breath had potential risk as a vehicle of TTV transmission. In order to check for the existence of TTV in exhaled breath, we collected exhaled breath condensate (EBC) samples from the 7 authors by chilling exhaled air and examined the condensates for the presence of TTV. Laboratory diagnosis of TTV infection has been dependent on PCR detection of viral DNA due to lack of other appropriate assays. Unequivocal culture systems and well-characterized animal models are not available for the virus isolation. There are no easy-to-use immunological methods to detect anti-TTV antibody and TTV antigens. Southern blot hybridization is not sensitive enough to detect TTV DNA in samples such as body fluids and serum. In the present study, TTV presence in EBC samples was tested by semi-nested PCR assay. As a result, we demonstrated that TTV DNA was detectable in numerous EBC samples of 5 of the 7 authors.

Materials and Methods

Sample collection. We collected 20 samples of oral and nasal EBC and saliva from author A and 5 samples of oral EBC and saliva from each of authors B, C, D, E, F and G. These seven authors included 4 males and 3 females. Their mean age ($\pm$ standard deviation) was 33.0 ($\pm$ 13.0) years old and the age range was 21–53 years old. They were not suffering from any illness during the sampling period. Also, we collected 20 samples of room air condensate (RAC) for each collection condition. Informed consent with appropriate documentation was obtained from ourselves. The EBC and saliva samples were collected after gentle tooth brushing and mouth rinsing with water. During the EBC collection, we kept normal tidal breathing by nasal inhalation and oral or nasal exhalation without coughing, sneezing or speaking. Oral and nasal EBC and RAC samples were collected using 3 different hand-made collection devices (Fig. 1).

Oral EBC samples were collected in the device shown in the panel A of Fig. 1. The device was composed of a mouthpiece, a 10-ml pipette, a one-way bulb and 2 bellows-tubes. The 2 bellows-tubes were connected by a one-way bulb. The mouthpiece was attached to one end of the connected bellows-tubes and the pipette was linked to another end. The bellows-tube adjacent to the pipette was rotated to prevent samples from contamination with saliva droplets. All components of the device were made of plastic and the device was used only one time. Exhaled air was sent into the device via the mouthpiece, passed through the 2 bellows-tubes and one-way bulb and was chilled in the pipette by 2 refrigerants. The refrigerants were pre-cooled at $-80\,℃$. The fluid that was accumulated in the device for approximately 30 min was poured into a 20-ml plastic container and assayed as an oral EBC sample.

Nasal EBC samples were collected by the same method as oral EBC samples except for the collection device (panel B of Fig. 1). The nasal EBC collection device had a nosepiece and a straight bellows-tube instead of the mouthpiece and rotated bellows-tube of
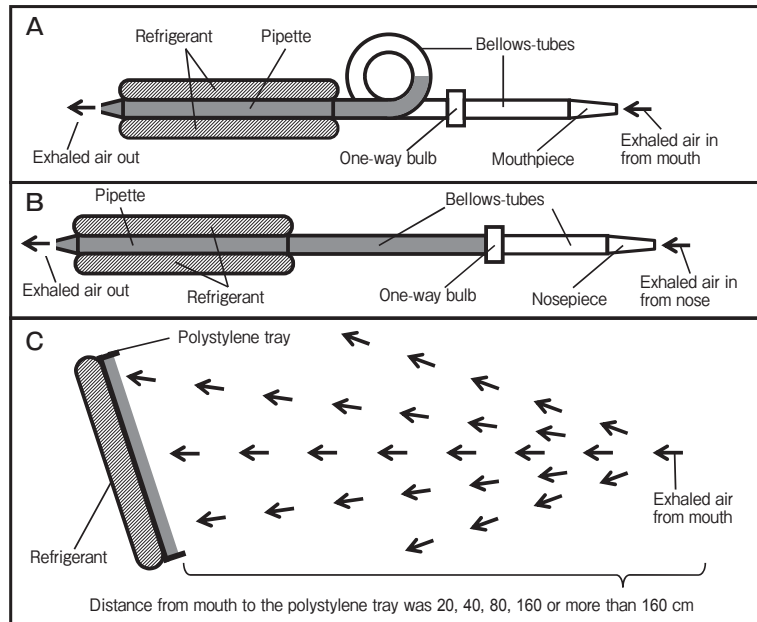
Fig. 1   Schematic illustration of EBC and RAC collection devices. (**A**) Oral EBC collection device. Nasally inhaled air was exhaled into the device through a mouthpiece held in a mouth, passed through a straight bellows-tube, a one-way bulb and a rotated bellows-tube that prevented passage of saliva droplets. The breathed air that passed through the rotated bellows-tube was chilled in a pipette by pre-cooled refrigerants at $-80\,^{\circ}\mathrm{C}$. The condensate that accumulated within both the pipette and the latter half of the rotated bellows-tube (the gray area) was poured into a 20-ml plastic container and assayed as described in Materials and Method. (**B**) Nasal EBC collection device. Nasally inhaled air was exhaled into the device trough a nosepiece inserted into nostrils, flowed through 2 straight bellows-tubes and a one-way bulb between the tubes. Then, nasal EBC was trapped in the gray area, collected and assayed in the same way as the oral EBC. (**C**) RAC collection device. Room air was cooled on the surface of a brand-new sheet of polyvinylidene chloride film covering a polystyrene tray by the use of refrigerant on the opposite side of the tray. The refrigerant was pre-cooled at $-80\,^{\circ}\mathrm{C}$. RAC accumulated on the film (the gray area) was collected by a micropipette with a filtered tip and assayed by the same methods as oral and nasal EBCs. RAC was collected at distances of 20, 40, 80, 160 or more than 160 cm from a human face.

the oral EBC collection device. We inhaled nasally through a hole in the top side of the nosepiece and sent exhaled air into the device by closing the hole with a gloved finger.

RAC samples were collected by an open system using the device shown in panel C of Fig. 1. This device was made of a plastic tray $(16 \times 24 \times 2\,\mathrm{cm})$, the inner surface of which was covered with a brand-new polyvinylidene chloride film. We breathed out through the mouth and at a distance of 20, 40, 80, 160 or more than 160 cm from the RAC collection device. An RAC sample was accumulated on the surface of the film for approximately 30 min by chilling the opposite side of the tray with refrigerant and was collected into a 20-ml plastic container by a micropipette with a filtered tip. The refrigerants were pre-cooled at $-80\,^{\circ}\mathrm{C}$. The device was set at the same height as a human face and the collection side of the device fronted on the face when we collected RAC samples at sites that were a specific distance (20 to 160 cm) from a human face. Alternatively, when collecting at sites far from any face, the device was placed at a height of 180 cm from the room floor, and the collection side faced the room wall.

Saliva samples were collected by accumulating saliva quietly in the oral cavity for approximately 10 min and by drooling it into a 20 ml plastic container.

Sample pretreatment.   There has been no report about materials which are constantly contained in EBC and can be used as internal standards. For example, the β-actin gene which has been used as an internal control in many PCR experiments could not be detected in 42% of EBC samples [17]. Also, other materials such as a total DNA, which have been used to standardize the amount of template DNA in PCR assays, do not seem to be contained in EBC at the

same concentration. In order to make it possible to detect TTV DNA under the same condition without using internal standards, we did not extract nucleic acids from the samples and amplified TTV DNA from exactly the same volume of sample solution. This assay concept avoided the laborious use of an external standard to determine the extraction efficiency and also was expected to reduce the risk of contamination of the samples with exogenous target DNA.

To put the assay concept into practice, we pretreated the samples as follows: We froze each sample at $-80℃$ immediately after collection and concentrated it by a Freeze dryer ALPHA 1-2 (Martin Christ Gefriertrocknungsanlagen Gm bH, Osterode am Harz, Germany). After being thawed, the concentrated sample was supplemented with 10 mM Tris•Cl (pH 8.0), 1 mM EDTA•2NA (pH 8.0), 0.5% SDS and 0.2 mg/ml proteinase K and its volume adjusted to one-tenth of that before the sample was concentrated with DNase, RNase-free distilled water. Subsequently, the sample was incubated at 53℃ for 3 h to digest proteins, further incubated at 95℃ for 10 min to inactivate the enzyme and then stored at $-80℃$ until analysis by semi-nested PCR. For saliva, each sample was supplemented with 10 mM Tris•Cl (pH 8.0), 1 mM EDTA•2NA (pH 8.0), 0.5% SDS and 0.2 mg/ml proteinase K right after collection and treated in the same way as EBC and RAC samples.

In addition to the samples for the semi-nested PCR assay, EBC, RAC and saliva samples were stored at $-80℃$ for α-amylase assay without any pretreatment.

Detection of TTV DNA using semi-nested PCR. We detected TTV DNA in EBC, RAC and saliva samples by the *in-house* semi-nested PCR assay. The primers for detection of TTV DNA were designed based on already-reported TTV genome sequences. A conserved region of the TTV genome was selected by aligning the genome sequences of 20 TTV strains (GenBank accession number AB008394, AB025946, AB028668, AB028669, AB038619, AB038620, AB064604, AB064605, AB064606, AB064607, AF351132, AF435014, AJ620233, AJ620234, AJ620235, AM712032, AM712033, AM712034, AY666122 and NC002076), and 3 primers corresponding to the region were synthesized. The forward and reverse primers of the outer pair were 5'-attttgctacgtcactaaccacg-3' (TF5m) and 5'-cccgaatt-

gccccttgac-3' (TR6), respectively. The forward and reverse primers of the inner pair were TF5m and 5'-taaactcacctccggcac-3' (TR1c), respectively. TF5m, TR6 and TR1c are located at nucleotide positions 1-23, 204-221 and 177-194 in the TTV genome (AB008394), respectively. The predicted length of the amplicons produced by the inner primers is 194 bp.

The first PCR was performed in a 20-μl reaction mixture containing $1 \times$ Taq DNA polymerase reaction buffer (Roche Diagnostics K.K., Tokyo, Japan), 200 μM of each deoxynucleotide triphosphate (Roche Diagnostics K.K.), 0.5 U of Taq DNA polymerase (Roche Diagnostics K.K.), 0.1 μM of forward and reverse primers, 10% Tween 20 and 10 μl of each pretreated sample. In the cases of EBC and RAC, 10 μl of the pretreated sample contained template DNA quantitatively equivalent to that in 100 μl of each sample before pretreatment. In the case of saliva, the amount of template DNA in a 10-μl pretreated sample was equal to that in 9.18 μl of each untreated sample. The following amplification program was used for the first PCR mixture: 7 min at 94℃; 35 cycles of 30 sec at 94℃, 45 sec at 59℃ and 30 sec at 72℃; and a final extension of 5 min at 72℃. Conditions for the second PCR mixture were the same as those for the first one except that forward and reverse primers were contained at a final concentration of 1 μM in place of 0.1 μM; also, 1 μl of the first PCR product was added instead of 10 μl of pretreated sample and Tween 20 was not included in the mixture. Cycling conditions for the second PCR were the same as the first one except for the annealing at 63℃ instead of 59℃ and the cycling number of 25 times. After amplification was completed, aliquots of the semi-nested PCR products were separated by electrophoresis (3% agarose gels) and photographed under UV light after ethidium bromide staining. The minimum detection limit of our semi-nested PCR was determined using 10-fold serial dilutions of the cloned TTV DNA fragment. It was estimated that 10 copies of the fragment or more were detectable per reaction.

Negative controls for the PCR procedure were made by adding water instead of sample solution, and no TTV DNA amplification occurred after the second PCRs. Positive controls for the PCR procedure were prepared by adding water including 100 copies of the cloned TTV DNA fragment in place of the sample solution, and we achieved the target DNA amplifica-

tion after the second PCRs in all cases. The fragment was cloned from the amplicon of the first PCR. The sequence identities between the fragment and each of the above-described 20 TTV strains ranged from 76.8 to 94.1%. In addition to controls for the PCR procedure, we collected 20 samples of outdoor air condensate as negative controls for sample collection and pretreatment. The outdoor air condensate samples were collected on an emergency stairway of 5th floor of a building in the device used for collection of oral EBC except that air was brought into the device by an electric suction pomp. The samples were pretreated and examined in the same way as described above, and all of the samples turned out to be negative for TTV DNA. The data of these controls are not shown in the figures of this report.

Assay of α-amylase activity. Activities of α-amylase in EBC, RAC and saliva samples were measured enzymatically with Salivary α-Amylase Assay Kit (Salmetrics LLC, PA, USA). Frozen samples that were not pretreated were thawed, and $8\mu l$ of each sample was assayed according to the manufacturer's instruction.

Statistical analysis. The statistical significance between sets of data was evaluated by the Pearson's chi square test with $p < 0.05$ being significant. The relationship between sets of data was evaluated by calculating Pearson's product-moment correlation coefficient (r).

Results

Activity of α-amylase in oral and nasal EBC samples. From one of the present authors (author A), we gathered saliva samples by drooling and collected oral and nasal EBC samples in the devices shown in panels A and B of Fig. 1. Then, we examined the samples for activity of α-amylase, a well-known enzyme that is secreted in saliva (Panels A, B and C of Fig. 2). Detection of α-amylase activity in a sample would serve as evidence that the sample contained saliva. While there were α-amylase activities ranging from 107 to 505U/ml in saliva samples, the activity of the enzyme was lower than the lowest detection limit (0.0164U/ml) in all of the oral and nasal EBC samples. Thus, the oral and nasal EBC samples seem not to have been contaminated with saliva.
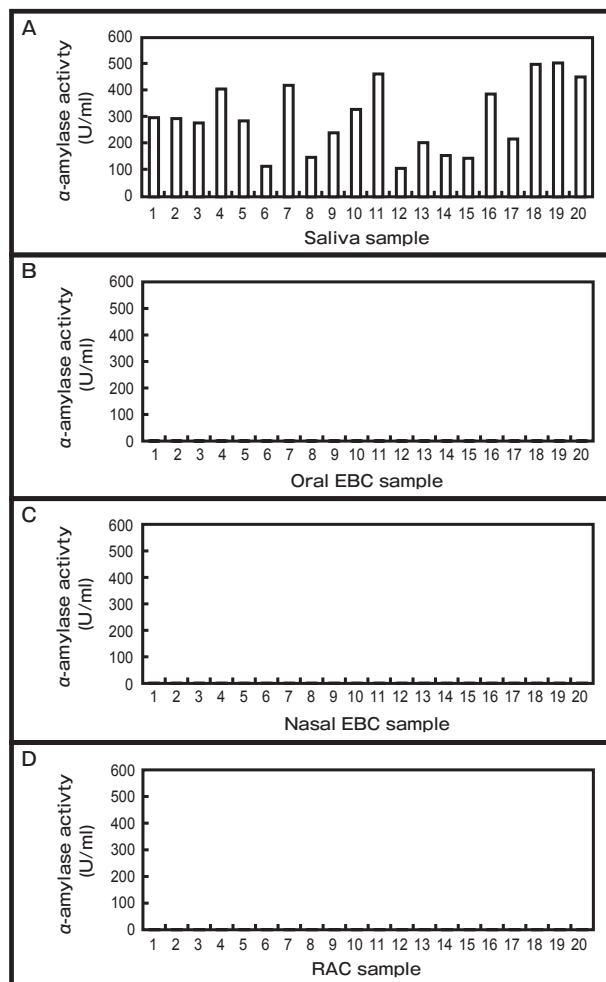

Fig. 2   Activities of α-amylase in saliva (**A**), oral EBC (**B**), nasal EBC (**C**) and RAC (**D**) samples. All saliva and EBC samples were collected from author A as described in the Materials and Methods. RAC samples were collected at a distance of 20cm from the face of author A. The lowest detection limit of the activity was 0.0164U/ml.

Detection of TTV DNA in exhaled breath. Next, we tested the samples for the presence of TTV DNA by semi-nested PCR assay. The assay amplified TTV DNA from 85% (17/20) of saliva samples (Panels A and D of Fig. 3), suggesting that author A was surely positive for the virus and the EBC samples from the author were worth being assayed for the virus. Then, we assessed the EBC samples for the presence of TTV DNA. We detected TTV DNA in 35% (7/20) of oral EBC samples and in 10% (2/20) of nasal EBC samples (Panels B, C and D of Fig. 3).
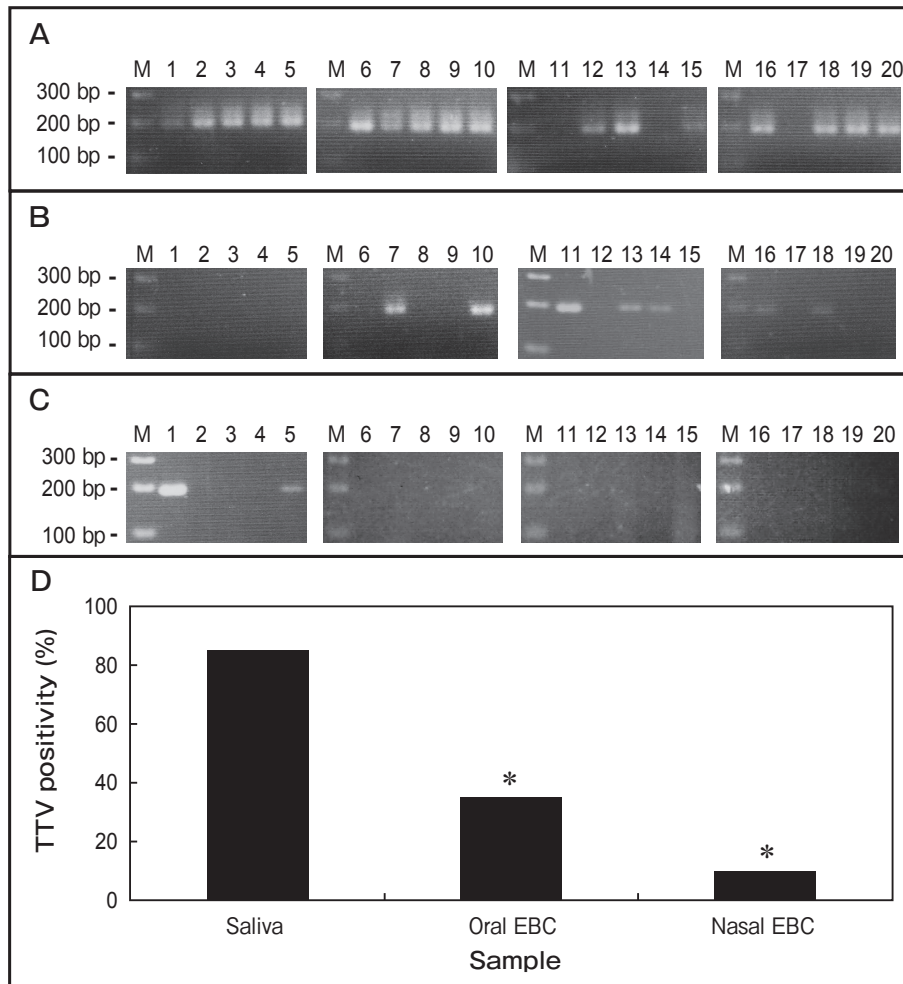
Fig. 3   Detection of TTV DNA in exhaled air. Electrophoretic profiles of the semi-nested PCR products amplified from saliva, oral EBC and nasal EBC samples are shown in panels **A**, **B** and **C**, respectively. We collected all samples from author A and assayed them as described in Materials and Methods. Signals observed at a position of approximately 200 bp were positive signs for TTV DNA. The abbreviation M means a lane of molecular markers. (**D**) TTV DNA-positive rates in saliva, oral EBC and nasal EBC samples. Columns represent the TTV DNA-positive rates calculated from the results in the panels **A**, **B** and **C**. A single asterisk means that the value is significantly lower than that in saliva samples ($p < 0.05$).

The results indicate that exhaled breath from the author A undoubtedly contained TTV DNA, although the TTV DNA-positive rates in the oral and nasal EBC samples were significantly lower than that of the saliva samples.

Generality of TTV excretion in exhaled breath.   To determine whether or not author A's exhalation of TTV DNA was an exception, we examined six other authors (authors B, C, D, E, F and G) using semi-nested PCR. With regard to saliva, we detected TTV DNA in 90% (27/30) of the samples and found that all of the 6 authors were positive for

TTV (Panels A and C of Fig. 4). As the result of examining oral EBC samples from these TTV-positive authors, we could detect TTV DNA in 20% (6/30) of the samples and in 4 of the 6 authors (Panels B and C of Fig. 4). Therefore, it seems that excretion of TTV in exhaled breath is not an exceptional case but is common.

Detection of TTV DNA in room air near a human face.   Although the above-described TTV DNA-positive data demonstrated TTV DNA excretion in exhaled breath, the data were obtained by closed systems using devices in which the exhaled breath was
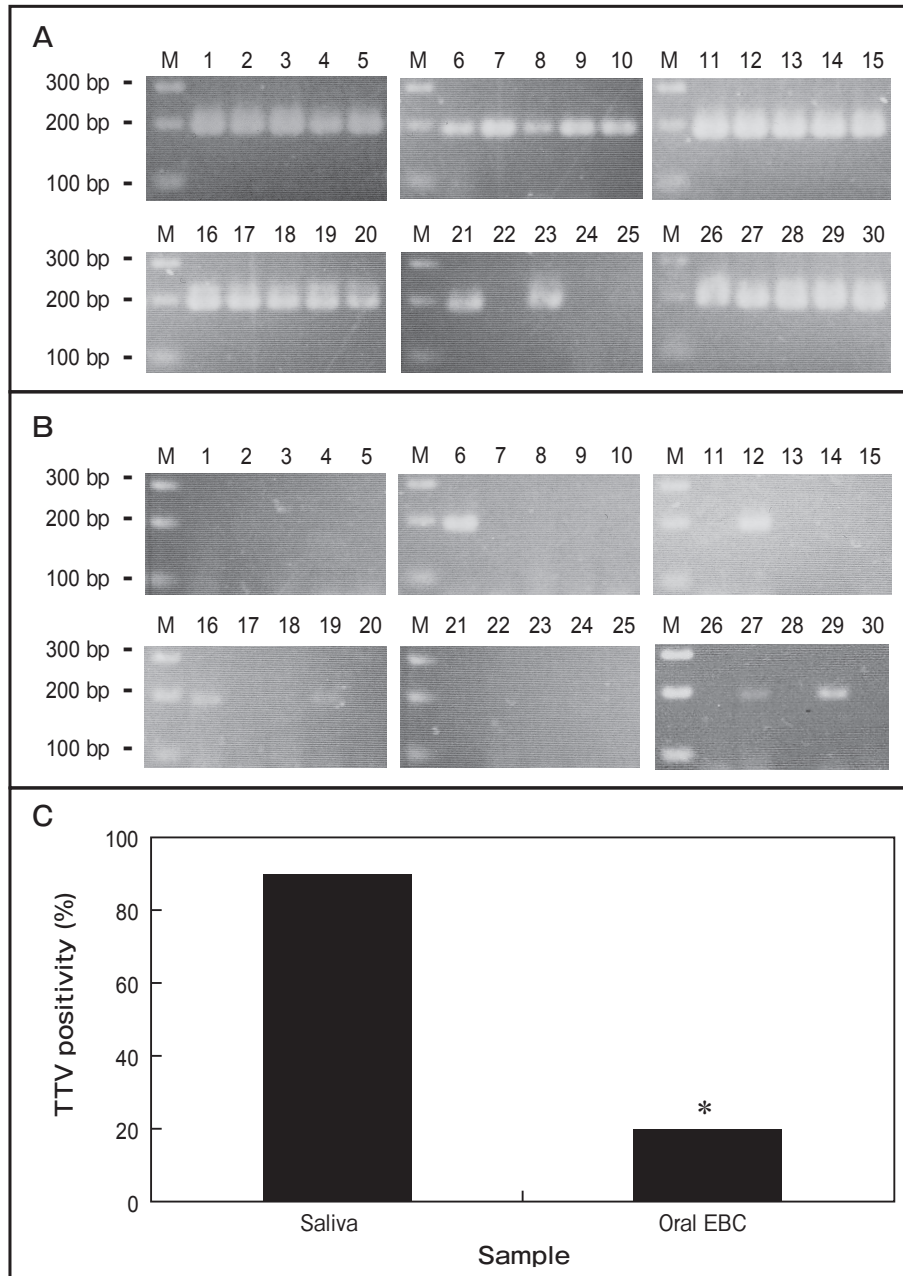
Fig. 4   Generality of TTV DNA detection in exhaled breath. Electrophoretic profiles of the semi-nested PCR products amplified from saliva and oral EBC samples are shown in panels **A** and **B**, respectively. We collected and assayed saliva and oral EBC samples in the same way as in Fig. 3 except for the authors from whom the samples were collected. Samples 1 to 5, 6 to 10, 11 to 15, 16 to 20, 21 to 25 and 26 to 30 were collected from authors B, C, D, E, F and G, respectively. Signals observed at a position of approximately 200 bp are positive signs for TTV DNA. The abbreviation M means a lane of molecular markers. (**C**) TTV DNA-positive rates in saliva and oral EBC samples. Columns represent the TTV DNA-positive rates calculated from the results in panels **A** and **B**. A single asterisk means that the value is significantly lower than that in the saliva samples ($p < 0.05$).

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screened the RAC samples for presence of TTV DNA by the same methods as described above (Fig. 5).

As a result, we found that TTV DNA was detectable in RAC samples collected at distances of 20 and 40 cm from a human face with positive rates of 40 (8/20) and 35% (7/20), respectively. These TTV DNA-positive rates were comparable to that in oral EBC samples from the same author. Thus, it seems that room air near a human face contains an equivalent amount of TTV to orally exhaled breath, and that exhaled breath may be one of the possible transmission routes of TTV in an actual situation. We detected TTV DNA also in RAC samples collected at distances of 80 and 160 cm from the face. There was

a negative correlation between the TTV DNA-positive rates and the distances from the face (r = −0.90). The correlation seems to indicate the natural diffusion of TTV in room air.

Detection of TTV DNA in room air far from a human face.   We could detect TTV DNA in RAC samples that were collected not only at sites near a human face (20 and 40 cm) but also at a site far from the face (160 cm). This result may suggest that TTV can drift in room air for a long distance. To better understand the distribution of TTV in room air, we collected RAC samples at 2 different room sites located at a height more than 180 cm from a room floor and at a distance more than 160 cm from any human
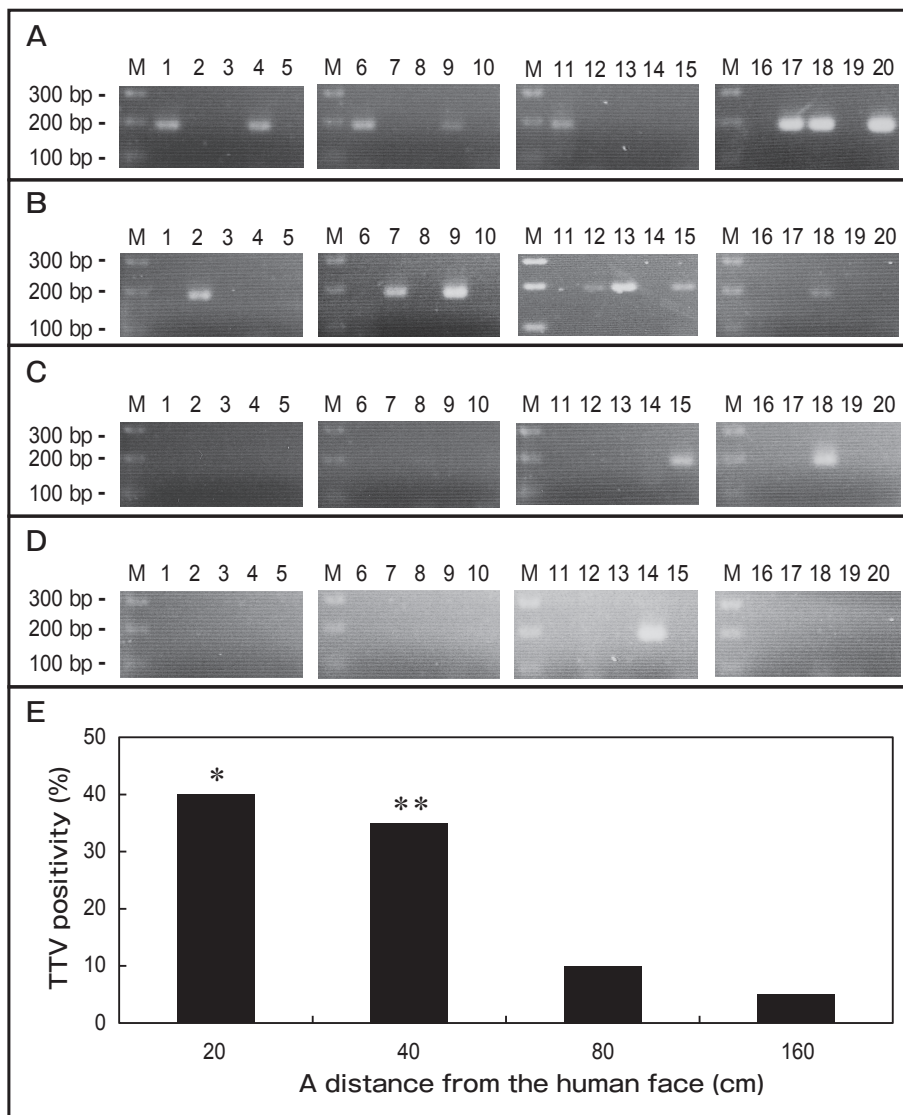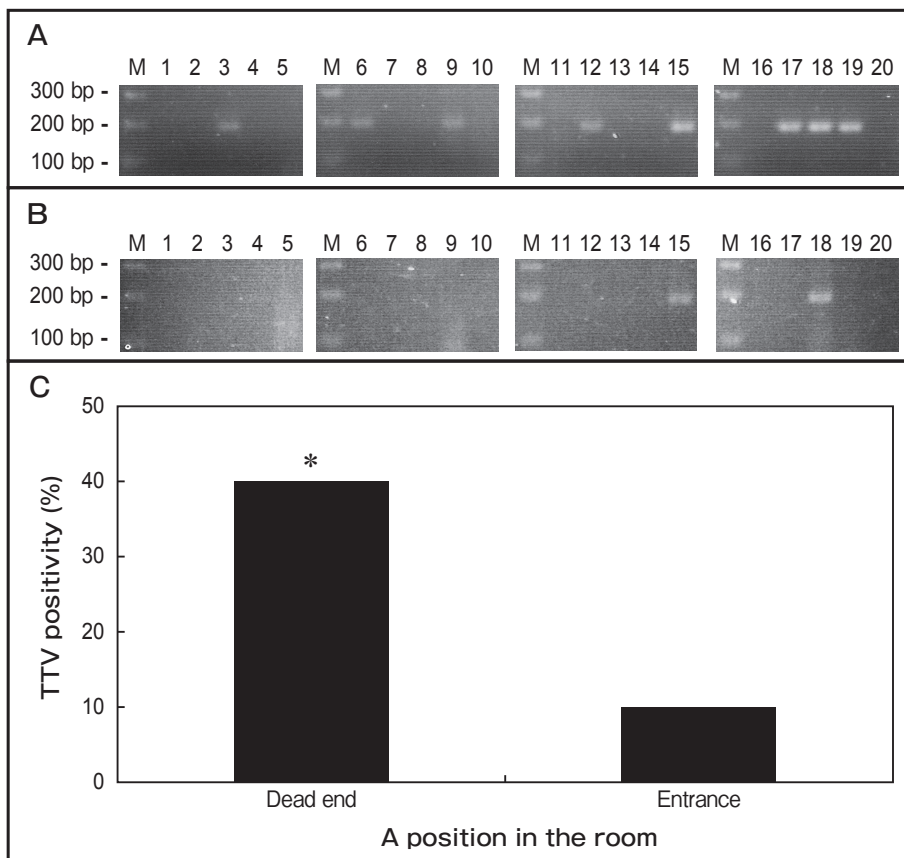

Fig. 6   Detection of TTV DNA in room air far from a human face. Electrophoretic profiles of the semi-nested PCR products amplified from RAC samples obtained at the dead end and entrance of the room are shown in panels **A** and **B**, respectively. We collected RAC samples in the same way as in Fig. 5 except that the samples were collected at a height of 180 cm from the room floor and at distances of more than 160 cm from any human face and assayed in the same way as Fig. 3. Signals observed at a position of approximately 200 bp are positive signs for TTV DNA. The abbreviation M means a lane of molecular markers. (**C**) TTV DNA-positive rates in RAC samples obtained at the dead end and entrance of the room. Columns represent the TTV DNA-positive rates calculated from the results in panels **A** and **B**. The single asterisk means that the value is significantly higher than that in RAC samples collected at the entrance of the room ($p < 0.05$).

face. The collection side of the device used to accumulate RAC did not face any human in the room but faced a room wall. We examined the RAC samples for the presence of TTV DNA by the semi-nested PCR assay, resulting in amplification of TTV DNA from the samples with a much higher frequency than we had anticipated (Fig. 6). TTV DNA was detected in 40% (8/20) of the samples that were collected at the dead end of the room and in 10% (2/20) of the samples that were collected at the entrance of the room. The result suggests a wide distribution of TTV in room air. The difference in the TTV DNA-positive rate between the dead end and entrance of the room was statistically significant, suggesting that the virus may accumulate in the inner part of the room.

Discussion

First, we demonstrated TTV DNA in both oral and nasal EBC samples from author A. The mean TTV DNA-positive rate in oral EBC samples reached 35%, indicating that the presence of TTV DNA is not rare in exhaled air. Second, we detected TTV DNA in oral EBC samples not only from author A but also from 4 of 6 other authors examined in the following investigation, suggesting that excretion of TTV in orally exhaled air is not unique to author A but is a common phenomenon. Third, we detected TTV DNA also in RAC samples collected at distances of 20 and 40 cm from a human face with positive rates of 40 and 35%, respectively. These TTV DNA-positive rates are comparable to that of oral EBC samples. Thus, it seems that exhaled air contains TTV in actual situations. TTV transmission occurs during infancy [6] and it has been assumed that saliva droplets from household members of an infant are the sources of TTV transmission to the infant. The above-described results suggest that exhaled air from household members may be a possible source of TTV transmission to the infant in addition to saliva droplets.

Although α-amylase activity was more than 107 U/ml in saliva, the activity of the enzyme in exhaled air was below the lowest detection limit (0.0164 U/ml). Thus, it seems that our oral EBC samples were not contaminated with saliva, and the TTV DNA-positive results obtained in the oral EBC samples were not directly due to the contamination of samples with the TTV DNA-positive saliva. Nevertheless, the TTV

DNA-positive rate in saliva samples from author A was significantly higher than those in oral and nasal EBC samples collected from him. Hence, we can not completely deny the possibility that TTV in EBC samples is associated with the virus secreted in saliva; the origin of TTV DNA in the EBC samples is still unknown.

In addition to directly exhaled breath and room air near a human face, we could detect TTV DNA also in room air far from human faces, suggesting that TTV drifts for long distances and is widely distributed in room air. During RAC sample collection at sites far from a human face, the ventilation of the room was not so good because the air in the room was ventilated only through the entrance door and the door was opened for a short time, when someone entered or exited the room. Since TTV is a non-enveloped virus, TTV is considered highly resistant to environmental stressors [18]. For example, TTV infectivity has been reported not to be lost even after 95 h of dry heat treatment [19]. Together with this remarkable stability of TTV particles, our result may suggest the risk of airborne infection with TTV in rooms without good ventilation.

Breath is exhaled constantly by TTV-infected persons, and so the generation frequency of exhaled breath is much higher than those of other vehicles of TTV spread. TTV acquisition seems to occur during infancy in most cases [6]. It is assumed that members of a household in which there is an infant may attempt to protect the infant from contact with their body fluids but not usually from their exhaled air. As for the risk of TTV transmission, therefore, it seems possible that airborne infection via exhaled air may be comparable to infection though other routes. However, at this moment in time, no one can evaluate the risk of airborne infection with TTV quantitatively as we cannot estimate TTV infection efficiencies in humans via other vehicles of spread, largely because there is a paucity of experimental systems to determine TTV infection efficiency. Infection experiments in humans cannot be performed for ethical reasons. Also, practical animal models for the quantitative assay of TTV infection are not available. In addition, studies of the infectivity of TTV are very limited due to lack of cell lines that support sufficient TTV replication. These difficulties should be resolved in order to enable risk assessment of TTV transmission routes including

airborne infection.

Even in oral EBC from the same author, there were both TTV DNA-positive and -negative samples. This suggests the possibility that differences in the physical and mental conditions of a host in ordinary life may influence TTV replication and/or TTV excretion from the host. Information on the conditions affecting TTV replication and excretion would seem useful for preventing transmission of the virus and related disease deterioration.

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