

Hemagglutination mediated by the spike protein of cell-adapted bovine torovirus

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Abstract Bovine torovirus (BToV)-Aichi, recently isolated in cultured cells, showed hemagglutination (HA) activity, although the virus has a truncated hemagglutinin-esterase (HE) protein, judging from its gene structure, indicating the existence of another viral protein with HA activity. We examined whether the spike (S) protein possesses HA activity. A BToV antiserum used in this study, reactive to S but not to HE, inhibited HA activity. Furthermore, cells infected with BToV and those expressing S showed hemadsorption (HAD) activity, which was inhibited by the anti-BToV serum; however, HAD activity by expressed HE was not blocked. These data indicate that the S protein of BToV-Aichi is responsible for its HA activity.

Members of several different species of torovirus (ToV) have been found in various types of animals and usually cause diarrhea in those animals [17, 22]. Also, animals of a

variety of different species display antibodies against ToV, indicating that ToV infects animals of a wide range of species [2]. However, only two ToVs, equine and bovine ToVs (BToVs), have been reported to have been isolated in cultured cells [12, 21], and other ToVs have only been detected in the specimens by RT-PCR [8, 17].

Toroviruses belong to the subfamily *Torovirinae*, family *Coronaviridae*, which implies that the genome structure and replication strategy of ToVs in cells are similar to those of coronaviruses [17]. Although coronavirus replication in cultured cells has been studied extensively, that of ToV is not sufficiently understood because of the lack of cultured cells that are permissive for ToV infection. There are some differences between ToVs and CoVs; CoVs have a leader sequence at the 5' end of the genomic RNA and a nested set of mRNAs, whereas ToVs lack the leader sequence [17, 18]. Another difference is that the CoVs have a gene encoding an envelope (E) protein that is, in most cases, critical for virus assembly [14], but the E gene is not found in ToVs [17]. In spite of these differences, one prominent similarity between ToV and some beta CoVs [e.g., mouse hepatitis virus (MHV), bovine coronavirus (BCoV), human coronavirus OC43 and others] is the existence of a hemagglutinin-esterase (HE) gene, which is similar to the HE gene of influenza C virus. It is assumed that the HE gene of both ToVs and CoVs was acquired as a result of recombination [4, 13, 18].

Recently, BToV was isolated from a diarrhea specimen of cattle in Japan, and those isolates were grown in cultured cells, thus making it possible to investigate the ToV replication strategy in more detail [1, 12]. The isolate BToV-Aichi exhibited HA activity [7]. Sequence analysis has demonstrated that BToV-Aichi, a tissue-culture-passaged virus with HA activity, lacks a full-length HE gene, and it contains a termination codon midway through the gene

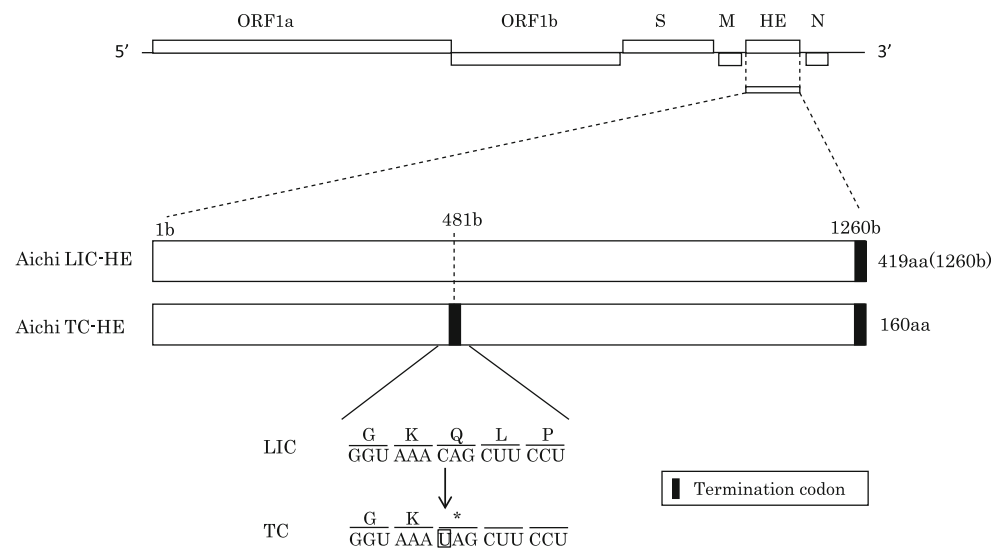
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Fig. 1 Schematic diagram of the BToV genome and the internal termination codon in the HE of BToV-Aichi. The BToV genome is about 25 kilobases in length and has at least seven ORFs. The HE gene consists of 1,260 nucleotides, and the gene amplified directly from specimens from diseased cattle (Aichi LIC-HE) encodes 419 amino acids, whereas tissue-culture-adapted BToV-Aichi (Aichi TC-HE) contains a change (C-U) at nucleotide 481, resulting in termination at amino acid 161



(Fig. 1). The mutation in the HE gene may have arisen during passage in cultured cells, because the full-length HE gene was detected in the initial specimens [1, 12] (Fig. 1). These findings may mean that the HA activity exhibited by BToV-Aichi is mediated by a viral surface protein other than the HE protein. In this study, we focused on the S protein as a candidate HA-active protein and found that the S protein of BToV-Aichi is responsible for the HA activity of the virion.

BToV was isolated and passaged several times in cultured HRT-18 cells (established from a human rectal tumor) and was designated as BToV-Aichi [12]. We infected HRT-18 cells with the virus, and supernatant of the infected cells was used to measure HA activity. HRT-18 cells were maintained as described previously [12]. Erythrocytes for the HA test were collected from 2-month-old Wistar rats (SJD, Shizuoka, Japan) and also from other animals. The antiserum against BToV was prepared in guinea pigs by immunizing them with virions of BToV-Hokkaido [7]. BToV-Hokkaido was revealed to have a truncated HE protein similar to that of BToV-Aichi [7]. The antiserum was used for the HA inhibition (HI) test, the hemadsorption (HAD) inhibition (HADI) test, and immunofluorescence (IF). The HA, HI and IF tests were conducted as described previously [7, 16]. For the HAD test, HRT-18 cells infected with BToV-Aichi and COS-7 cells expressing either S or HE protein were overlaid with 0.25 % rat erythrocytes suspended in phosphate-buffered saline (PBS), pH 7.2 at room temperature for 1 h. Cells were then washed with PBS, and HAD was monitored by microscopy. For the HADI test, the cells were treated with anti-BToV-Hokkaido serum at room temperature for 1 h and an erythrocyte suspension was overlaid. For the expression of HE and S proteins, each gene was amplified from virion RNA by reverse transcription, followed by PCR as described elsewhere [16]. As PCR forward primers

for the HE and S genes, we used HE-for (5' CCTAATAA CTAATAAAC 3') and S-for (5' ATAAGTTATGTATA GTGCCTAA 3'), respectively, covering their initiation codons, and as reverse primers for HE and S, we used HE-rev (5' GATGCTGAGTTTAATATTC 3') and S-rev (5' GATCAGGGAAGGGCCAATAA 3'), covering their termination codons. Two different HE genes were isolated and cloned into a plasmid; one was from a diarrhea specimen (HE-LIC), and the other was from BToV-Aichi passaged in cultured HRT-18 cells (HE-TC). PCR products were then inserted into an expression vector (pTarget), as described elsewhere [16]. By nucleotide sequencing, clones containing the correct viral gene were selected and used for expression. An HE gene with an HA tag at the 3' end was also produced with a pair of primers, HE-for and HE-rev tagged with the HA sequence (5' TATCCATATG ATGTT CCAGA TTATGCT TAA 3') at its 3' end, and inserted into the expression vector. The proteins were expressed in COS-7 cells using Trans COS transfection reagent (Mirus Bio.).

We first confirmed the HA activity of BToV-Aichi using erythrocytes from different animals, such as rat, mouse, chicken, cow, horse, turkey and guinea pig. Of those samples, only rat and mouse erythrocytes were agglutinated, and the efficiency of rat cell agglutination was four- to eightfold higher than that of the mouse erythrocytes. Thus, in the subsequent experiments, we used rat erythrocytes. We first examined the HI activity of serum of guinea pigs immunized with BToV-Hokkaido. The HA activity of BToV was fully inhibited by this antiserum at a dilution of 20,000–40,000, whereas normal control guinea pig serum failed to inhibit hemagglutination (HA), and these findings are in good agreement with the previous observations [7]. We also examined the HAD activity of virus-infected cells as another marker of virion HA activity. As shown in

Fig. 2, virus-infected cells showed clear HAD activity (Fig. 2a), and antigen-positive cells were also observed using the anti-BToV serum (Fig. 2b). The HAD activity of infected cells was blocked when the infected cells were treated with the anti-BToV serum before addition of rat erythrocytes (Fig. 2c), while control serum failed to inhibit HAD activity (Fig. 2d). These results suggested to us that viral proteins present on virions, as well as on infected cells, showed HA activity, which was completely inhibited by the anti-BToV serum. We then examined the reactivity of this antibody to HE and S proteins expressed independently in COS-7 cells by IF. Antibodies in the serum could bind to virus antigen in infected cells (Fig. 2b) and also the singly expressed S protein (Fig. 3k), but not with HE protein (Fig. 3m) expressed in COS-7 cells, indicating that the HI activity of the antibody is most likely attributable to its interaction with the S protein rather than the HE protein. Furthermore, we found that COS-7 cells expressing the S protein alone showed HAD activity (Fig. 3a), and this HAD activity was inhibited by the anti-BToV antibody (Fig. 3f), whereas COS-7 cells expressing full-length HE protein exhibited strong HAD activity (Fig. 3c) that was not inhibited by the antibody (Fig. 3h). Since the HE protein expressed in COS-7 cells failed to be detected by the anti-BToV antibody, which implies that HE proteins might not be sufficiently expressed in COS-7 cells, we expressed HE protein with a tag at the C-terminal end and compared the HAD activity of cells transfected with the HE gene without the tag. As shown in Fig. 3c and d, cells transfected with either the HE gene or the HE + tag gene showed robust HAD activity, and the latter cells were stained with anti-tag serum (Fig. 3n), indicating that the full-length HE protein possesses HA activity. Truncated HE protein was also expressed, but it did not exhibit HAD activity (Fig. 3b). Since it was not known whether the HE protein with the truncation was successfully expressed or not, we expressed the HE protein with a tag upstream from the termination site, examined the transfected cells by IF using serum against tag, and found that the truncated HE protein was not detected (data not shown). At present, we have no idea why the truncated HE protein was not detected in the infected cells. It is possible that the protein was excreted from cells very fast or that it is labile and thus easily degraded. The above results strongly suggest that it is the S protein and not the HE protein that possesses the HA activity of BToV-Aichi. This is the first report to show that the S protein of BToV possesses the HA activity.

Since equine ToV (EToV) and BCoV HE protein, as well as porcine transmissible gastroenteritis virus (TGEV) S protein, bind sialic acid on red blood cells (RBCs) [4, 15, 21], we examined whether BToV-Aichi S protein binds sialic acid on rat RBCs. Neuraminidase-treated rat RBCs

failed to be agglutinated by BCoV, as reported previously [20], and also by BToV-Aichi, implying that sialic acid is utilized by both BCoV and BToV-Aichi for binding to rat RBCs (data not shown). EToV HE protein binds to sialic acid, yet it is not a functional receptor expressed on permissive cells, since even EToV with the HE protein deleted can infect cells [4, 17]. The sialic acid utilized by the TGEV S protein is unlikely the functional receptor, since porcine aminopeptidase N is generally accepted to be an authentic, functional receptor for TGEV [14]. We also examined whether or not treatment of HRT-18 cells permissive to BToV reduced its infectivity and found that it did not affect the efficiency of BToV infection, suggesting to us that sialic acid is not involved in the infection of cultured HRT-18 cells. In coronavirus infection in general, sialic acid could be a virus-binding molecule, but it is not likely to be the functional receptor that allows the infection in permissive cells.

Another membrane-associated protein of ToV is the integral membrane (M) protein [17]. Since a small portion of the N-terminal region of the M protein is located outside the envelope, the M protein could be responsible for HA activity. M protein expressed in COS-7 cells can be detected with anti-BToV serum; however, COS-7 cells expressing M protein failed to show HAD activity, implying that M is not responsible for the HA activity of the virus (data not shown).

The anti-BToV serum used in the present study failed to react with the HE protein but successfully reacted with the S protein. It also reacted with M and N proteins expressed individually in cultured cells, as demonstrated by IF. The virus used for immunization, BToV-Hokkaido, was revealed to have a truncated HE protein similar to that of BToV-Aichi (data not shown). Thus, BToV-Hokkaido also may fail to stimulate anti-HE antibody production.

Although the HA activity displayed by most CoVs is mediated by the HE protein, the S proteins of avian infectious bronchitis virus (IBV), porcine TGEV, and BCoV have been reported to have HA activity [3, 11, 15]. The first two of these CoVs have no HE protein. BCoV has an HE protein, and it is still controversial whether the S or HE protein is responsible for the HA activity of the virus [10, 15]. The S1 subunit of the IBV S protein has been reported to be involved in HA activity [3]. It is of interest that TGEV mutants with an HA-negative S protein, isolated from the HA-positive, wild-type virus, exhibit reduced virulence for animals, which may be indicative of the importance of binding of the virus to sialic acid for high virulence [11]. The isolation of a BToV variant with an HA-negative S protein could be helpful for understanding whether the sialic-acid-binding ability of BToV is also important for its high virulence, if the mutant shows reduced virulence. The establishment of an animal model

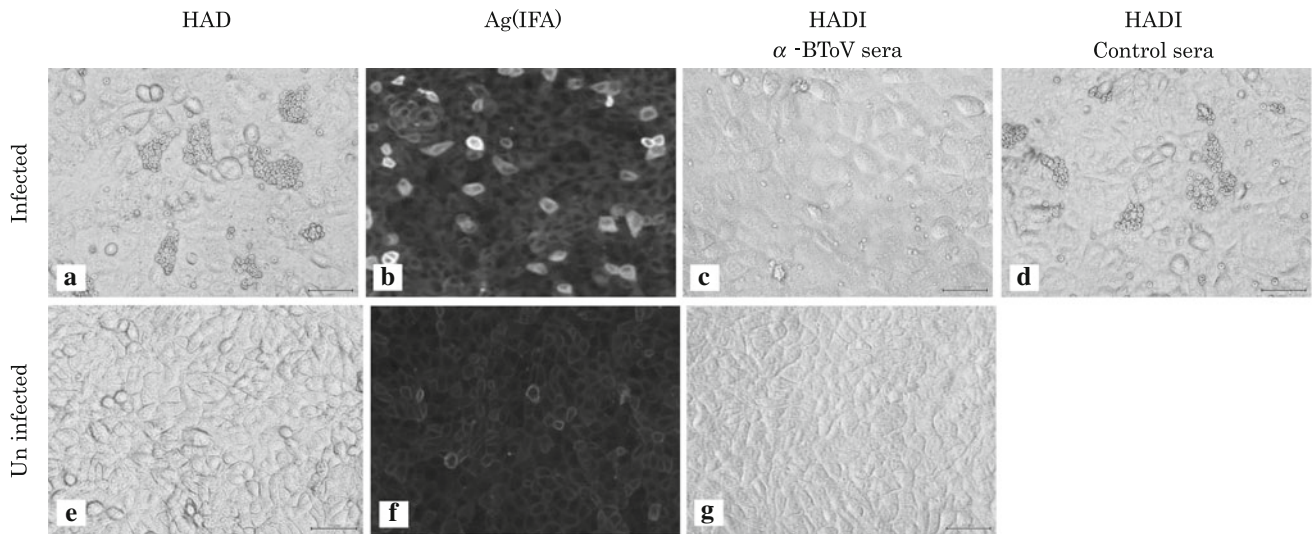


Fig. 2 HAD activity of BTov-Aichi-infected cells, HRT-18 cells cultured in 24-well plates were infected (**a–d**) or mock-infected (**e–g**) with BTov-Aichi at 0.05 TCID₅₀/cell and incubated for 3 days. HAD was then examined using rat erythrocytes (**a**, **e**). Virus and mock-infected cells were visualized by IF with anti-BToV-Hokkaido

guinea pig serum (**b**, **f**). To see the HADI activity of the anti-BToV-Hokkaido serum, infected cells were pretreated with that serum (**c**, **g**) or with normal guinea pig serum (**d**) at room temperature for 1 h, and rat erythrocytes were added to each well to see whether HAD was blocked or not

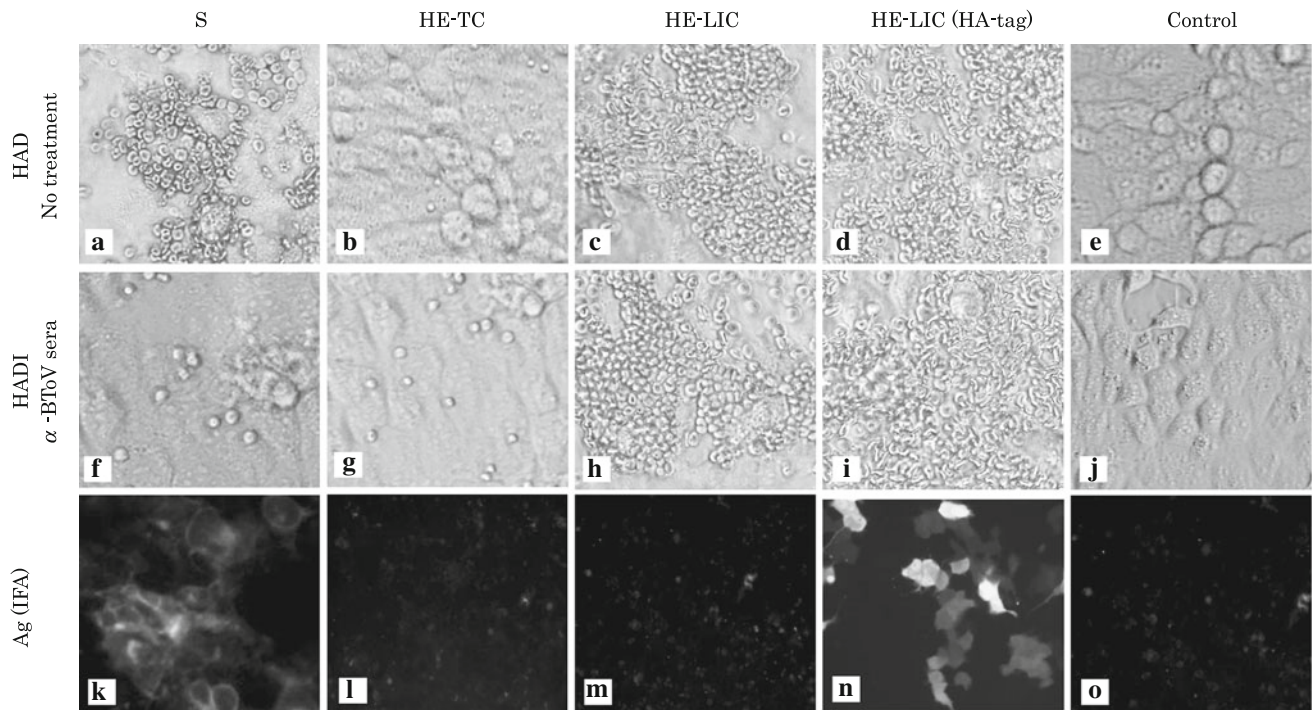


Fig. 3 HAD examined by transiently expressed S and HE proteins of BTovs. COS-7 cells cultured in 24-well plates were transfected with a plasmid containing the S gene (**a**, **f**, **k**), the HE gene derived from BTov-Aichi (HE-TC) (**b**, **g**, **l**), the HE gene isolated from diseased cattle (HE-LIC) (**c**, **h**, **m**), HE-LIC with an HA tag in its C-terminus (**d**, **i**, **n**) or plasmids without any viral genes (**e**, **j**, **o**). Three days after transfection, the HAD of the cells was examined using rat

erythrocytes (**a–e**). To see the HADI of COS-7 cells transfected as described above using anti-BToV-Hokkaido serum, transfected cells were treated with the serum at room temperature for 1 h, and HAD of rat erythrocytes was then examined (**f–j**). The expression of each protein was observed by IF with anti-BToV-Hokkaido (**k**, **i**, **m**, **o**) or anti-HA monoclonal antibody (**n**)

for BToV infection using mice is of great importance in the study of the pathogenesis of BToV.

It is interesting that HE proteins failed to be expressed in tissue-culture-passaged viruses. EToV Berne, which can multiply in cultured cells, also has an HE gene with a termination codon in the middle of the gene [18, 21]. In our case, the original virus in the specimens from cattle with diarrhea has an HE gene without an internal termination codon, which may mean that the virus in diseased animals has a membrane-associated HE protein. However, the HE gene found in the virus passaged several times in cultured cells contained the termination codon, as seen in BToV-Aichi [12]. These findings indicate that the HE protein is not indispensable for virus replication in cultured cells but may play an important role in the pathogenesis of diarrhea caused by this virus. We have not yet clarified why the virus with a truncated HE protein is selected after several passages in cultured cells. However, BToV may have to be protected by a variety of host defense mechanisms such as innate immunity to infect animals or to break the structural barrier of mucus found in intestines. Possibly, the esterase activity of the HE protein has the ability to destroy mucus, thus making it easier for the virus to attach to the receptor of epithelial cells lacking a mucus coat. However, those barriers are not present in cultured cells. In such an environment, the virus does not need to cope with the host defense mechanism and may discard the unnecessary component from virions, resulting in more efficient replication. To study the biological role of the HE protein in animals, we need BToV with full-length HE protein that can be grown in cultured cells. BToV-Aichi was passaged through cultured cells several times after isolation. However, the viruses with a lower passage history could have the full-length HE gene. We are, at the moment, trying to obtain BToV with a full-length HE protein from among the viruses with a shorter passage history in cultured cells. The comparison of ToVs with HE and without HE protein will allow us to investigate the biological role of the HE protein in infected animals.

Beta CoVs generally have an HE protein, although a prototype strain of MHV, MHV-A59, lacks this protein [13]. This virus has a pseudogene corresponding to an HE gene lacking an initiation codon. Nevertheless, MHV-A59 grows quite efficiently in cultured cells and causes hepatitis and encephalitis in mice [6, 13]. It has also been reported that the HE protein is not critical for interaction of the virus with permissive cells [5]. These observations indicate that the MHV HE protein is not absolutely critical for infection in cells and in animals. However, there are several variants of JHMV, a highly neurotropic strain of MHV, that have an HE protein; some are extremely neurovirulent, while others are moderately neurovirulent [19]. Such neurovirulence has been reported to be influenced by the amount of HE protein

in the virus, indicating the possible significance of the HE protein in pathogenesis [9, 23].

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