

Short communication

Characterization of bovine coronavirus isolates/from eight different states in the USA

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Abstract

Bovine coronavirus isolates from eight different states of the USA were compared for their antigenic properties and susceptibility to hygromycin B. Antigenic differences were observed among the isolates in a one-way hemagglutination-inhibition (HI) test using a polyclonal antiserum against the Mebus bovine coronavirus isolate. Differences were observed on isoelectric focusing among viral proteins with isoelectric points between 4.45–4.65. Most of the BCV isolates were susceptible to hygromycin B (0.5 mM) whereas a few hygromycin B resistant isolates were also found. © 1999 Elsevier Science B.V. All rights reserved.

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Diarrhea and respiratory diseases are clinically important and cause high mortality in calves (Virtala et al., 1996). Bovine coronavirus (BCV) is an economically important cause of intestinal and respiratory diseases in calves and has also been implicated in winter dysentery of adult cattle (Clark, 1993). Despite differences in clinical disease, it is believed that all BCV isolates belong to a single serotype (Clark, 1993). The genome of coronaviruses is the largest among the RNA viruses (Lai, 1995). Coronavirus has a high frequency of recombination, RNA polymerase lacks proofreading, and defective interfering RNAs are frequently generated in coronaviruses (Lai, 1995). BCV isolates from Canada have been divided into three subgroups on the basis of reactivity with monoclonal antibodies (mAbs) against spike protein of BCV (Michaud and Dea, 1993).

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The current modified, live BCV vaccine is not clinically effective (Thurber et al., 1977) and lack of clinical efficacy of the vaccine may be due to antigenic differences among the vaccine and field isolates of BCV. Minor antigenic and biological differences have been observed among BCV isolates from calf diarrhea and winter dysentery cases from the USA (Tsunemitsu and Saif, 1995) and Canada (Dea et al., 1995). To further understand the molecular epidemiology of BCV, 94 BCV isolates from eight states of the USA were compared. We compared antigenic differences on one-way hemagglutination-inhibition (HI) test, isoelectric focusing of BCV proteins, and relative susceptibility of BCV isolates to hygromycin B.

BCV isolation from clinical samples containing coronavirus-like particles observed by negative contrast electron microscopy has been described before (Kapil et al., 1996). Briefly, human rectal tumor-18 cell line (HRT-18) was inoculated with 10% fecal suspension. Before inoculation, the suspension was filtered through a 0.45 μm filter (Gelman Sciences, Ann Arbor, MI). The confluent monolayers were washed with calcium and magnesium free phosphate buffer saline containing trypsin (5 $\mu\text{g/ml}$). About 0.5 ml of filtered fecal suspension was added on to the confluent monolayers and virus allowed to adsorb for an hour. Minimum essential medium containing trypsin (5 $\mu\text{g/ml}$) (Gibco/BRL, Life Technologies, Gaithersburg, MD) and pancreatin (5 $\mu\text{g/ml}$) (Sigma, MO) was added. The monolayers were examined daily for 3–5 days for cytopathic effects. We successfully propagated 96 BCV isolates in HRT-18 cells (12 from California, 3 from Kansas, 40 from Minnesota, 2 from Nebraska, 1 from North Dakota, 1 from Oklahoma, 8 from Wisconsin, and 29 from Wyoming). All tissue culture propagated BCV isolates were compared at passage 4.

Hemagglutination test was performed in V-bottom plates (Dynatech Laboratories, MA). Two-fold dilutions of BCV samples (25 μl) were prepared in 0.01 M phosphate buffered saline (PBS) containing 0.1% bovine serum albumin (Sigma). After addition of 25 μl of 1% mouse erythrocytes, the plates were incubated at 4°C for 90 min. Hemagglutination titers were expressed as the reciprocal of the highest dilution showing complete agglutination.

For HI test, 72 cell culture propagated BCV isolates (12 from California, 3 from Kansas, 35 from Minnesota, 1 from Nebraska, 8 from Wisconsin and 13 from Wyoming) and 44 wild-type (original fecal samples but not propagated in cell cultures) BCV isolates (7 from California, 1 from Nebraska, 20 from Wisconsin and 16 from Wyoming) were compared by HI test. For 21 of these wild-type samples, cell culture propagated BCV isolates were also tested in HI test.

Preparation of antiserum and the procedure for HI test have been described before (Sato et al., 1977). For removing nonspecific HI, 200 μl of anti-BCV antiserum (National Veterinary Services Laboratory, Ames, IA) made against Mebus isolate, was diluted with 500 μl of PBS, inactivated at 56°C for 30 min, and mixed with 400 μl of 25% kaolin (Fisher Scientific, Fairlawn, NJ). After shaking for 1 h, kaolin was removed by centrifugation of 100 $\times g$ for 5 min. Clear supernatant was mixed with 200 μl of packed normal mouse erythrocytes to remove nonspecific hemagglutinins. After incubation for 1 h at 37°C, the treated BCV antiserum was separated from mouse erythrocytes by centrifugation. For HI test, four to eight HA units of BCV isolates (in 25 μl) were mixed with 20 μl of treated BCV antiserum. After incubation for an hour at 37°C, 1% mouse

erythrocyte suspension (25 μ l) was added. Plates were reincubated at 4°C for 90 min. The HI activity of BCV antiserum against isolates of BCV was recorded at the reciprocal of the highest dilution of antiserum showing complete inhibition of hemagglutination (Sato et al., 1977). For each virus isolate, back titration was performed at the same time to assure that four to eight HA units of BCV was used. Each sample was repeated three to four times to confirm the results and the test was found to be reproducible.

Bovine coronavirus purification procedure has been described previously (King and Brian, 1982). Infected HRT-18 cells were harvested when approximately 75% of them showed cytopathic effects, such as rounding, detachment and syncytia formation. Briefly, after three freeze-thaw cycles, the cells were scraped and pooled. Cellular debris was removed by centrifugation at 3500 \times g for 20 min, and the clear supernatant was filtered through a 0.45 μ m filter (Gelman Sciences, MI). Polyethylene glycol (PEG-8000) was added at a final concentration of 8% (w/v). After overnight incubation at 4°C, the virus precipitate was pelleted at 10,800 \times g for 20 min. The pellet was saved and resuspended in TNF buffer (pH 7.5). The BCV was purified on a sucrose gradient and all sucrose solutions were made in TNE. The pellet was layered on a 10/60% (w/w) sucrose gradient and centrifuged at 90,000 \times g for 2 h. The virus, at the interphase, was collected and diluted in TNE and layered on 20–60% step gradient (20, 30, 40, 50, and 60% sucrose, 1.5 ml each) and centrifuged at 90,000 \times g (27,500 rpm) overnight. Bands were collected, centrifuged at 90,000 \times g for 2 h to pellet the virus, and stored at –70°C.

For isoelectric focusing, the gel was made with acrylamide-ampholyte solution (40%, Biolyte (Bio Rad) pH 3–10) using ammonium persulfate, riboflavin-5'-phosphate, and TEMED as catalysts for polymerization. After photopolymerization for 45 min on one side, the other side was irradiated for 15 min to eliminate unpolymerized monomer on the gel surface. Purified virus was treated with an equal volume of sample buffer (9.5 M urea, 2% Triton X-100, and biolyte 2%, pH 3–10 at room temperature for 3 h) and then applied (total volume 2 μ l) to the gel with disposable templates. Samples were allowed to diffuse into the gel for 15–25 min. Focusing was done at 100 V for 15 min, then increased to 200 V for 15 min and, to 450 V for 1 h and 30 min. BCV proteins were detected by silver staining (Bio-Rad). The IEF gels on a plastic support were left to dry overnight in a clean area at room temperature.

To assess the susceptibility of BCV isolates, hygromycin B was added at a concentration of 0.5 mM in the cell culture medium. Each sample was propagated in the presence and absence of hygromycin B. After 48 h, the plates were frozen and thawed (three times). The plates were centrifuged and clear supernatant was collected. The amount of BCV in culture supernatant was quantified by a HI test.

On HI test, most wild-type BCV samples (39/44) showed very low reactivity (HI titers 1 : 16, Type III) and only three samples showed high reactivity or inhibition (HI titers 1 : 2048–1 : 4096, Type I). One sample was inhibited at 1 : 128, Type II (Fig. 1). The HI titers of 72 cell culture propagated BCV isolates ranged from 1 : 2–1 : 1024 (Fig. 2). When a sample was compared after cell culture propagation, in most BCV isolates, except WI-17, the HI titers of cell culture propagated BCV were comparatively higher than its wild-type BCV isolate.

Similar protein bands could be detected in all 11 BCV isolates (CA-1, ND-3467, WI-1-SK, WI-2, WI-9, WI-10, WI-11, WI-17, WI-18, WY-28, and WY-29) studied by SDS-

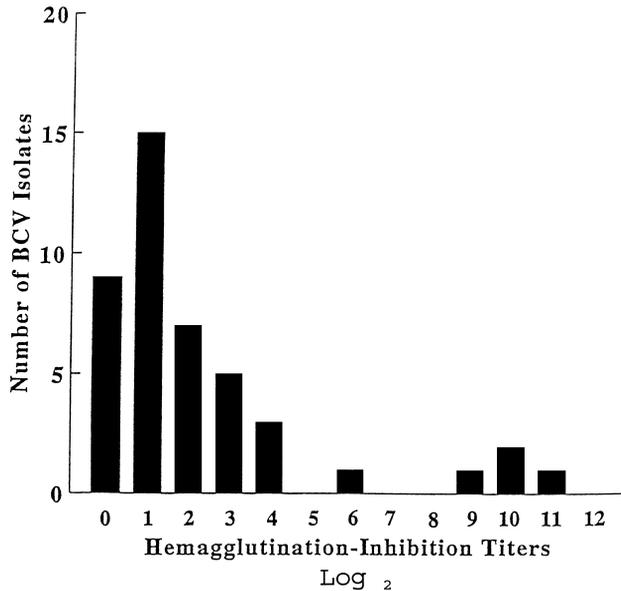


Fig. 1. Hemagglutination-inhibition (HI) activity of an anti-bovine coronavirus (Mebus isolate). Antiserum against wild-type bovine coronavirus isolates: X-axis denotes the HI titers and the numbers indicate superscripts (Log₂). On the basis of a one-way HI test, only 4 out of 44 isolates were inhibited up to 1 : 1024–4096, Type I; 1 was inhibited up to 1 : 128, Type II; 30 were inhibited up to 1 : 16 and 9 isolates were not inhibited, Type III. Y-axis indicates number of BCV isolates inhibited at a particular HI titer.

PAGE analysis (data not shown). No protein bands were detected in uninfected HRT-18 cells processed in a similar manner. The identity of BCV proteins was confirmed by a Western blot analysis or radio immunoprecipitation. Polyclonal antibody, bovine anti-BCV antiserum, detected all the BCV proteins: spike dimer, spike subunits, nucleoprotein, hemagglutinin-esterase proteins (dimer and subunits), and membrane proteins. The molecular weight of hemagglutinin-esterase protein was the same in all the BCV isolates studied.

On the basis of seven Wisconsin BCV isolates studied, we concluded that most bands were invariable between different BCV isolates and sharply focused on IEF gels. However, bands towards the anodic end of the IEF profile (*pI* 4.45–4.65) showed variation between different isolate and even different fractions of the same isolate. A double-band migrated at *pI* 4.65 in most BCV isolates but in WI-1-SK, fraction b, and WI-9, fraction 140, it migrated at *pI* 4.45. (Fig. 3). To demonstrate the differences, all samples were repeated at least three times after the IEF protocol was standardized to ensure the reproducibility of the differences between BCV isolates.

Relative susceptibility of 32 BCV isolates to hygromycin B (0.05 M) was studied (Table 1). A drop in hemagglutination titer by 4-fold or higher was considered significant and the BCV isolate was considered susceptible to hygromycin B. On the basis of this criteria, four BCV isolates (MN 9, MN 29, MN 30 and MN 36) were considered resistant to hygromycin B; however, the other 28 BCV isolates were susceptible to hygromycin B.

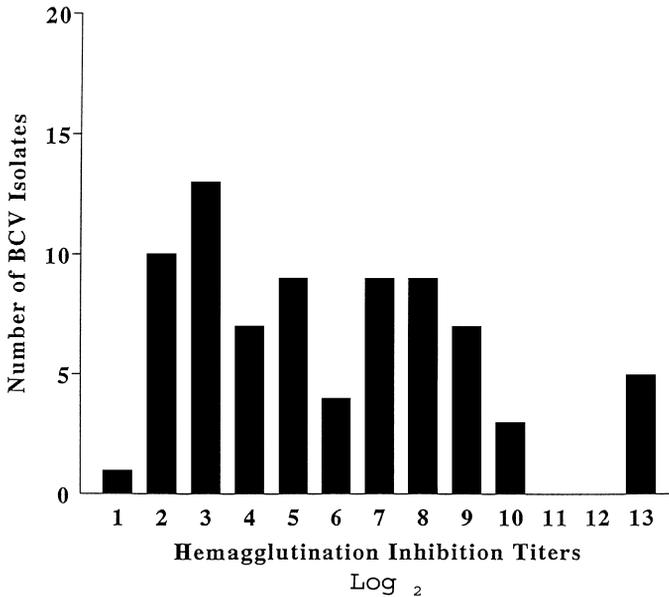


Fig. 2. Hemagglutination-inhibition activity of an anti-bovine coronavirus (Mebus isolate). Antiserum against cell culture propagated bovine coronavirus isolates: on the basis of a one way HI test, 77 BCV isolates were inhibited from 1 : 2 up to 1 : 1024. X-axis denotes the HI titers and the numbers indicate superscripts (Log 2). Y-axis indicates number of BCV isolates inhibited.

Isolates of BCV from calf diarrhea and winter dysentery differ in cytopathology, some isolates are non fusogenic while others are weakly or highly fusogenic (Dea et al., 1995).

Cytopathic effects produced by BCV isolates have no correlation with the clinical condition but depends on the presence of trypsin in the medium (Dea et al., 1995). Similarly, no differences were observed in CPE among calf diarrhea and winter dysentery isolates by another group (Tsunemitsu and Saif, 1995). We observed some differences in cytopathic effects among the calf diarrhea isolates; however, passage number, plaque purification, and the presence of trypsin and pancreatin in the cell culture medium greatly affected the cytopathology of BCV isolates. It is possible that some isolates of BCV are fastidious and do not grow on HRT-18 cell line but will grow in organ cultures (Kapil et al., 1996). Plaque variation has also been observed among BCV isolates and the isolates differ in size and shape of the plaques (Kapil et al., 1995).

It is believed that BCV isolates are antigenically related (Clark, 1993). BCV isolates do not differ significantly in reactivity on indirect immunofluorescent test (Tsunemitsu and Saif, 1995). Either slight or no differences were observed on virus neutralization tests (Dea et al., 1995; Tsunemitsu and Saif, 1995). Because spike protein is the major immunogen that elicits antibodies detected by neutralization and indirect fluorescent antibody tests (Saif, 1993) and elicits a bulk of cross reactive antibodies among BCV isolates, spike protein may not be very useful to differentiate BCV isolates. Minor antigenic variation has been observed among BCV isolates from neonatal calf diarrhea and winter dysentery cases by a one-way test (Dea et al., 1995). Our results in this study

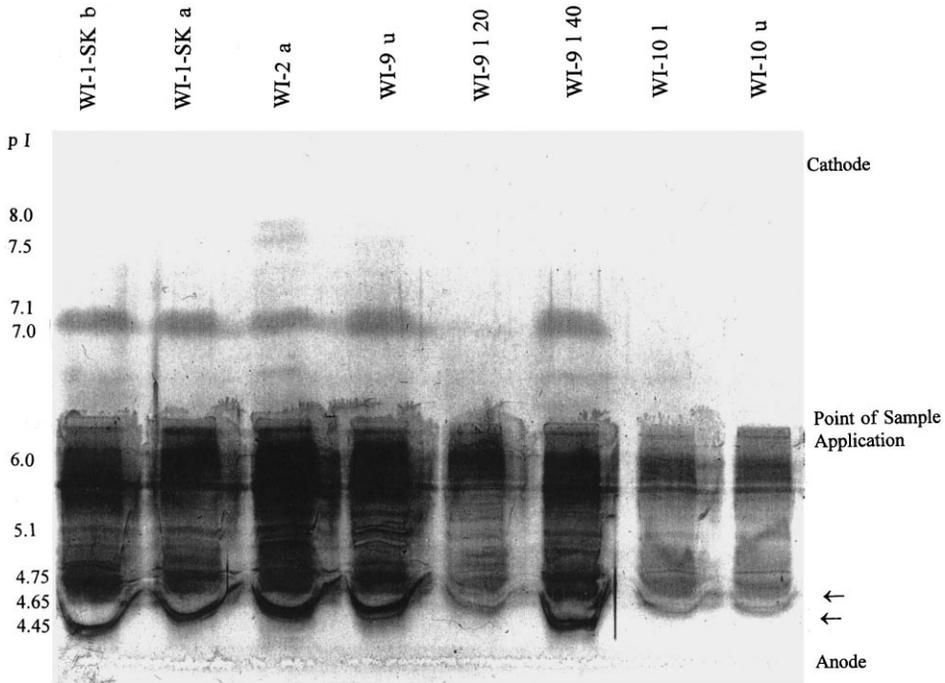


Fig. 3. Polypeptide profile of bovine coronavirus by isoelectric focusing: *pI* of the proteins was calculated by comparing with a IEF standard *pI* 4.45–8.0 (Bio Rad). A double band towards the anodic end of the gel was found to differ (either 4.45 to 4.65; shown with arrows) between different isolates and different fractions of the same isolate.

support and extend the previously reported findings on HI test using a large number (94) of American isolates collected from eight different states of the USA. None of the previous studies (Dea et al., 1995; Tsunemitsu and Saif, 1995) reported HI results on wild-type BCV or the virus in fecal samples. Results on wild-type isolates may need cautious interpretation because the presence of colostral IgG1 and local IgA in feces may interfere with results. On the other hand, passaging of BCV also affects HI. Passage of BCV in cell culture may also affect the antigenic composition (Hussain et al., 1991), hemagglutination activity (Storz et al., 1992), and intestinal replication of BCV (Kapil et al., 1990). It is important to study all isolates at low passage (up to passage 3 or 4) and at the same passage. In the only other American study, samples were studied at different passages (Tsunemitsu and Saif, 1995).

We tried to develop a polyclonal antiserum against CA-1, a Type III BCV isolate. The Type III antiserum was not high titered and we could not study the two-way HI relationship among BCV isolates. For most BCV isolates, the cell culture passaged virus showed higher HI titers compared to its wild-type virus in fecal samples. When cell culture-propagated and wild-type versions of BCV (WI-17) were compared, they showed HI titers of 1 : 128 and 1 : 2048, respectively. This may be because the hyperimmune serum used in the HI test was produced with a BCV isolate that was highly adapted in cell

Table 1
Relative susceptibility of bovine coronavirus isolates to hygromycin B

Isolate	Hygromycin B (HAU) (–)	Hygromycin B (HAU) (+)
MN-1	256	<2
MN-2	2048	8
MN-5	4096	16
MN-6	2048	<2
MN-7	2048	16
MN-8	4096	16
MN-9*	4096	4096
MN-10	256	<2
MN-11	4096	16
MN-12	4096	4
MN-13	512	128
MN-15	32	4
MN-16	512	8
MN-17	512	16
MN-19	2048	16
MN-20	4096	4
MN-21	2048	4
MN-22	1024	8
MN-23	128	32
MN-24	512	<2
MN-25	256	<2
MN-26	512	8
MN-27	1024	16
MN-29*	64	128
MN-30*	256	128
MN-32	256	8
MN-33	256	4
MN-34	32	<2
MN-36*	256	128
MN-37	128	<23
MN-39	256	64
CA-1	256	64

Hygromycin B was added at a final concentrations of 1 mM in tissue culture fluid. A 4-fold or higher drop in HAU was considered significant.

(–): No hygromycin B; (+): Hygromycin B added in tissue culture medium.

* Hygromycin B resistant isolates. HAU (in 25 µl) as the inverse of highest dilution showing complete agglutination.

culture or it could be due to the presence of some antibodies in feces or it may be a unique property of the WI-17 isolate. Typing of wild-type BCV isolates by HI may be useful to study the epidemiology of BCV. Hemagglutination and receptor destroying activities have no correlation with the clinical source of BCV isolates (Tsunemitsu and Saif, 1995).

A number of studies have been published in which American (Hussain et al., 1991), French (Vautherot and Laporte, 1983), Canadian (Michaud and Dea, 1993), English (El-Ghorr et al., 1989), and Scottish (Clark et al., 1990) isolates of BCV have been antigenically compared using a panel of mAbs. All of the previous studies, except the Canadian study, have failed to classify the BCV isolates into subgroups. A monoclonal

antibody (S2/1) neutralizes some BCV isolates (S2, Scottish isolate, Mebus isolate, and PQ, a Canadian isolate) but does not neutralize other isolates of BCV (English isolate, CK and Scottish isolate, S1). However, in a Canadian study, 13 Quebec isolates, collected between 1987–1990, have been classified into three antigenic subgroups (Michaud and Dea, 1993) on the basis of reactivity with a panel of mAbs.

We observed minor differences in the protein profile of BCV isolates by SDS-PAGE analysis, Western blot analysis and radio immunoprecipitation of BCV isolates (data not shown). Similarly, no differences were observed among neonatal calf diarrhea and Winter dysentery isolates from Quebec by Western blot analysis (Dea et al., 1995). However, minor differences were observed in isoelectric focusing of BCV proteins (Fig. 3). Different serotypes of infectious bronchitis virus (Sadasiv et al., 1991) and temperature sensitive mutants of mouse hepatitis virus (Oleszak et al., 1992) differ on isoelectric focusing of spike proteins. Isoelectric focusing is a tedious procedure and needs a lot of careful standardization in research laboratories. In our studies, a suitable point of application of samples was determined by applying purified BCV at three different places: 1.5, 2.7, and 3.9 cm from the cathode. Sample application at 1.5 cm from the cathode was not suitable because the bands were wavy. Application of samples at 2.7 cm from the cathode was found to be suitable for IEF analysis (Fig. 3). We tried three different shapes of the template for the application of BCV protein samples: squares (■), horizontal slits or dashes (-) and vertical slits (|). In our opinion, dashes gave the most clearly visible bands.

We observed varying levels of susceptibility of BCV isolates to hygromycin B. The mechanism of action of hygromycin B is not known; however, differential susceptibility of BCV isolates points to direct action of hygromycin B on BCV RNA replication. Because most isolates of BCV were found to be susceptible to low concentrations of hygromycin B (0.5 mM) and because hygromycin has no reported toxicity in cattle, hygromycin B may be a possible treatment for scours caused by BCV. Moreover, relative susceptibility of BCV isolates to hygromycin B provides an easy epidemiological marker to differentiate BCV isolates, into susceptible and resistant categories and can be easily adopted by diagnostic laboratories trying to differentiate BCV isolates. Susceptibility of BCV isolates to hygromycin B can be adopted by most veterinary diagnostic laboratories that have facilities for cell culture. First, BCV is isolated in HRT-18 in the presence of trypsin and pancreatin (Kapil et al., 1996). The virus is then propagated in the presence of hygromycin B to assess the susceptibility and the titer of the virus is measured by a HI.

Hemagglutinin-esterase genes of a winter dysentery isolate and two neonatal calf diarrhea isolates from Quebec, Canada, have been compared to Mebus isolate of BCV. No frame shifting, deletion or insertion changes were observed (Dea et al., 1995). When hemagglutinin-esterase cDNAs of highly virulent bovine coronavirus (LY138) and avirulent bovine coronavirus (L9) were compared, four amino acid substitutions occurred (Zhang et al., 1991). Similarly, we have observed only minor changes among two hemagglutinin esterase cDNAs sequenced so far (Zhang and Kapil, unpublished results). Thus, differences observed in HI tests cannot be explained at the level of nucleic acid sequence (Dea et al., 1995).

Some wild-type BCV isolates could not be inhibited by the BCV antiserum against the Mebus isolate (Fig. 1) indicating the presence of antigenically different BCV isolates. We

speculate that a multivalent vaccine containing different serotypes (Types I, II, and III) for BCV selected on the basis of HI patterns might offer better protection than the current monovalent modified live virus vaccine that is not clinically effective (Deleeuw and Tiessink, 1985). Some of the techniques described in this paper can be useful to diagnostic laboratories. Typing of BCV isolates can be easily adopted by most veterinary diagnostic laboratories. BCV isolates can be differentiated by HI and susceptibility or resistance to hygromycin B.

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