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Investigation of stx_2^+ *eae*$^+$ *Escherichia coli* O157:H7 in beef imported from Malaysia to Thailand

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Abstract: To gain insight into the microbiological safety of food products routinely traded across international borders in Southeast Asian countries, beef imported from Malaysia to southern Thailand was examined for contamination with *Escherichia coli* O157 and its subsequent spread into the imported areas. We screened 31 samples exported from Malaysia and 36 domestic Thai samples. Isolation methods including an O157 antigen-targeted immunomagnetic separation technique, screening on CHROMagar O157 medium, and serotype confirmation of *E. coli* isolates by specific agglutination tests were employed. Fourteen strains of *E. coli* O157:H7 were isolated from eight Malaysian samples (25.8%) and six strains from four Thai samples (11.1%). These strains were of the stx_1^- stx_2^+ *eae*$^+$ genotype except one Malaysian strain which was of the stx_1^- stx_2^- *eae*$^+$ genotype. All 19 O157:H7 strains possessing the stx_2 gene produced little or no Stx_2 (reversed passive latex agglutination titer ≤ 4). Of the 19 strains, five Malaysian (38.5%) and two Thai (33.3%) strains exhibited resistance to a set of antibiotics. Finally, the results of two DNA fingerprinting analyses (O157 IS-printing targeted to IS*629* and pulsed-field gel electrophoresis, PFGE) of the O157:H7 strains possessing the stx_2 gene, indicated that the Malaysian and Thai strains are closely related. Therefore, *E. coli* O157:H7 might be transferred from Malaysia to southern Thailand through beef trade.

Keywords: EHEC, *E. coli* O157, beef, O157 IS-printing, PFGE

Introduction

Enterohemorrhagic *Escherichia coli* (EHEC) is an important food-borne pathogen that causes gastroenteritis and systemic diseases such as hemorrhagic colitis and hemolytic uremic syndrome (Karmali *et al*., 1983; Riley *et al*., 1983). The disease was first reported when patients were infected with *E. coli* O157:H7 (Riley *et al*., 1983), the major serotype of EHEC. The resulting disease from EHEC infection is due partly to the production of Shiga toxin (Stx). Stx is divided into two types, Stx_1 and Stx_2, encoded by the stx_1 and stx_2 genes, respectively. Most EHEC strains carry the stx_2 gene. In addition, the *eae* gene, encoding an adherence factor, intimin, and located in the enterocyte effacement (LEE) pathogenicity island is also considered an important virulence factor of EHEC (McKee *et al*., 1995; Schmidt *et al*., 1995; Nataro and Kaper, 1998).

The number of patients infected with *E. coli* O157:H7 has gradually increased in various areas of the world (Willshaw *et al*., 1994; Dundas *et al*., 2001; Effler *et al*., 2001), especially in Japan and the United States (Rangel *et al*., 2005; Muto *et al*., 2008). However, infection by *E. coli* O157:H7 has rarely been reported in developing countries (Nataro and Kaper, 1998), although *E. coli* O157:H7 may be distributed in these countries. A previous study showed that some beef marketed in Malaysia was contaminated with stx_2^+ *eae*$^+$ *E. coli* O157:H7 (Son *et al*., 1998). Southern Thailand shares a partial border with Malaysia, and food products such as beef are exported from Malaysia to Thailand across this border. Beef is popular in this region of Thailand due to the large Muslim population. The food trade across international borders is very active in Southeast Asia. However, it is not clearly understood whether any food-borne pathogens are transferred through the export process and if so, whether the imported pathogens can adapt to the new environment.

To investigate this possibility, we examined the beef exported from Malaysia to Thailand including domestic Thai beef for the presence of *E. coli* O157:H7 and other related strains. To achieve this, we used an isolation method highly sensitive for *E. coli* O157:H7, a newly reported DNA fingerprinting method for *E. coli* O157 (O157 IS-printing), and a conventional pulsed-field gel electrophoresis (PFGE)-based fingerprinting method for the analyses of genetic comparisons.

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Materials and Methods

Isolation of bacteria

Malaysian and Thai beef were purchased from local markets in Hat Yai City, Southern Thailand, from March to September 2008. Fifty grams of each sample was incubated at 37°C in 450 ml TSB supplemented with 20 µg/ml novobiocin. After 6 h, 1 ml of the culture was added to 10 ml of TSB and incubated at 42°C for 2 h. Afterwards, 1 ml of the culture was mixed with anti-*E. coli* O157 Dynabeads (DYNAL BIOTECH ASA, Oslo, Norway), and the beads were rinsed and suspended following the manufacturer's specifications. The beads were streaked on Chromagar O157 (CHROMagar, Paris, France) and incubated at 37°C for 24 h. Mauve colonies were screened for virulence genes (stx_1, stx_2, *eae*), as described below, and examined for the O157 antigen by an agglutination test using anti-O157 antiserum (*E. coli* O antiserum O157, Denka Seiken Co., Ltd., Tokyo, Japan).

Virulence genes and a 60 MDa plasmid

The presence of the stx_1, stx_2 and *eae* genes was investigated by the PCR method using EVT-1 and EVT-2, EVS-1 and EVS-2 and AE19 and AE20 primer pairs, respectively, as described previously (Vuddhakul *et al*., 2000). The ability to produce bundle-forming pili (BFP) and the presence of the locus of enterocyte effacement (LEE) were assessed by detecting the *bfp* and *esc*V genes, respectively, using the PCR method as described previously (Müller *et al*., 2006). The presence of a 60 MDa plasmid was examined by the alkaline plasmid extraction method using the Wizard SV mini prep kit (Promega Corp., Wisconsin, USA), followed by 0.7% agarose gel electrophoresis.

Stx_2 production

The nucleotide sequence partly responsible for no or low-level production of Stx_2 in certain *E. coli* O157 strains was detected by the toxin-non-producing PCR (TNP-PCR) technique as described previously (Koitabashi *et al*., 2006). Briefly, four separate PCR reactions designated as TNP-A, TNP-B, TNP-C and TNP-D targeting the *Q* gene and its surrounding regions of the chromosome were performed using primer pairs TNPf1 and TNPr1, TNPf2 and TNPr2, TNPf3 and TNPr2, and TNPf4 and TNPr3, respectively. Amplicon sizes of 458 bp (TNP-A), 694 bp (TNP-B), 268 bp (TNP-C) and 549 bp (TNP-D) were detected by 1.5% agarose gel electrophoresis. *E. coli* O157:H7 Thai-12 was used as a positive control. Relative levels of Stx_2 production were examined by the reverse passive latex agglutination assay (RPLA) using the VTEC-RPLA kit (Denka Seiken Co., Ltd., Tokyo, Japan), according to the manufacturer's instructions. Briefly, the test isolate was grown in casamino acid-yeast extract medium with shaking at 37°C overnight. After centrifugation, the supernatant was collected, and Stx_2 was detected using the RPLA kit. The highest dilution of supernatant giving a positive agglutination was defined as the RPLA titer. The *E. coli* EDL933 strain was used as a positive control.

O:H serotyping

Serotyping the O antigen (lipopolysaccharide) and the H antigen (flagellar) of motile strains was performed using *E. coli* antisera set 1 and set 2 (Denka Seiken Co.) according to the agglutination test.

Antibiotic susceptibility tests

All strains of stx^+eae^+ *E. coli* O157 was examined for antibiotic susceptibility using the disk diffusion method (NCCLS, 2000). Twelve antibiotic disks were used, cephalothin (30 µg), ceftriaxone (30 µg), ampicillin (10 µg), trimethoprim/sulfamethoxazole (1.25 µg), norfloxacin (10 µg), colistin (10 µg), amikacin (30 µg), ciprofloxacin (5 µg), ceftazidime (30 µg), cefepime (30 µg, gentamicin (10 µg) and imipenem (10 µg). *E. coli* ATCC 25922 was used as a control strain.

O157 IS-printing

DNA profiles of test strains of *E. coli* O157 were determined by O157 IS-printing using the multiplex PCR technique, as reported previously (Ooka *et al*., 2009b). Briefly, DNA of the test strain was extracted by the alkaline boiling method. PCR was performed using the O157 IS-printing system kit (Toyobo Co. Ltd, Osaka, Japan), according to the manufacturer's specifications. Two sets of primers were included using the same conditions; pre-heating at 96ºC for 2 min, followed by 20 cycles of 96ºC for 20 sec, 64ºC for 30 sec, and 68ºC for 1 min. Amplification products were electrophoresed in a 3.0% gel NuSieve agarose gel (Takara Biochemicals) at 90 volts for 1.5 h. DNA profiles were analyzed using the Fingerprinting II program (Bio-Rad, Hercules, California, USA).

Pulsed-field gel electrophoresis (PFGE)

PFGE was performed as previously described (Izumiya *et al*., 1997). Briefly, chromosomal DNA of the test strain was cleaved with *Xba*I restriction enzyme (TOYOBO Co. Ltd., Osaka, Japan) at 37°C for 3 h. The digested DNA fragments were separated in 1% Pulsed-Field Certified agarose (Bio-Rad

Table 1. Characteristics of *E. coli* strains isolated from Malaysian and Thai beef

Serotype of isolated strains	Beef sample		No. of isolates (strain name)	Virulence gene pattern
	Origin	Designation		
O157:H7	Malaysia	9	1 (M 1)	$stx_1^- stx_2^+ eae^+$
		10	1 (M 2)	$stx_1^- stx_2^- eae^+$
		11	1 (M 3)	$stx_1^- stx_2^+ eae^+$
		13	3 (M 4-M6)	$stx_1^- stx_2^+ eae^+$
		17	2 (M8-M9)	$stx_1^- stx_2^+ eae^+$
		29	1 (M10)	$stx_1^- stx_2^+ eae^+$
		30	3 (M 11-M13)	$stx_1^- stx_2^+ eae^+$
		31	2 (M 14-M15)	$stx_1^- stx_2^+ eae^+$
	Total no.	8	14	
	Thai	5	1 (T1)	$stx_1^- stx_2^+ eae^+$
		6	1 (T2)	$stx_1^- stx_2^+ eae^+$
		24	1 (T3)	$stx_1^- stx_2^+ eae^+$
		29	3 (T4-T6)	$stx_1^- stx_2^+ eae^+$
	Total no.	4	6	
O116:H31	Malaysia	13	1 (M7)	$stx_1^+ stx_2^+ eae^-$
	Total no.	1	1	

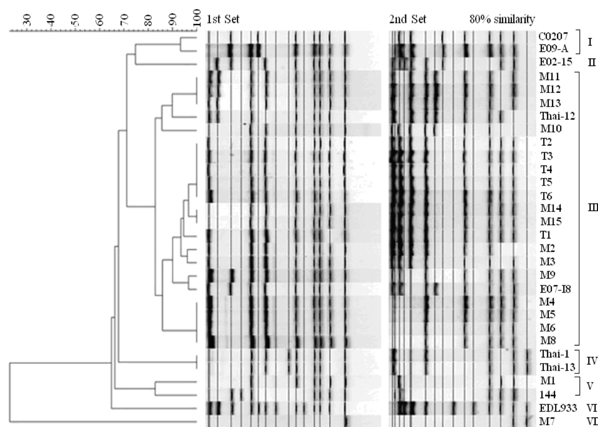

Figure 1. O157 IS printing-based dendrogram of *E. coli* isolates from Malaysian and Thai beef

Table 2. Antibiograms of stx_2^+ eae^+ *E. coli* O157:H7 strains from Malaysian and Thai beef

Antibiogram[a]	No. (strain name in parenthesis) of strains from beef originating in:	
	Malaysia	Thailand
KF	1 (M8)	0
CR	0	1 (T4)
KF CR	1 (M14)	1 (T3)
AMP CR	1 (M15)	0
KF AMP AK	1 (M9)	0
KF CR AMP	1 (M12)	0
Resistant/total strains (percent resistance)	5/13 (38.5 %)	2/6 (33.3 %)

[a] The antibiotics examined were cephalothin (KF), ceftriaxone (CR), ampicillin (AMP), trimethoprim/sulfamethoxazole, norfloxacin, colistin, amikacin (AK), ciprofloxacin, ceftazidime, cefepime, gentamicin and imipenem. Both resistant and intermediate reactions are judged as resistant in this study, and only these reactions are listed in these antibiograms.

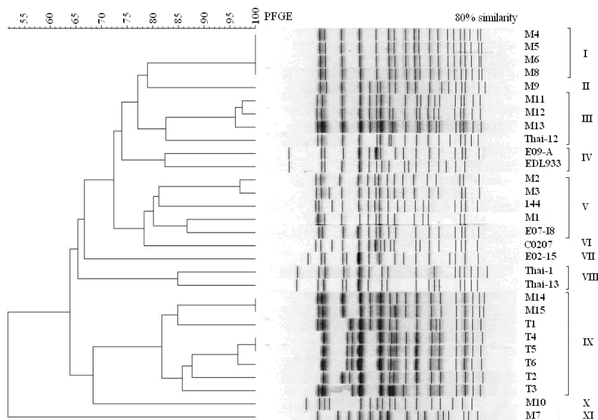

Figure 2. PFGE-based dendrogram of *E. coli* isolates from Malaysian and Thai beef

Table 3. Reference strains of stx_2^+ eae^+ *E. coli* O157:H7 used in DNA fingerprinting analyses

Strain name	Origin	Year of isolation	Presence of gene:			TNP-PCR	Literature
			stx_1	stx_2	*eae*		
Thai-1	Beef, Thailand	1998	+	+	+	+	Vuddhakul *et al*., 2000
Thai-12	Beef, Thailand	1998	-	+	+	+	Vuddhakul *et al*., 2000
Thai-13	Bovine feces, Thailand	1998	+	+	+	+	Vuddhakul *et al*., 2000
144	Human, Japan	2003	-	+	+	+	Koitabashi *et al*., 2006
C0207	Human, Japan	2003	-	+	+	+	Koitabashi *et al*., 2006
E02-15	Beef, China	2004- 2005	-	+	+	+	Koitabashi *et al*., 2008
E07-I8	Beef, China	2004- 2005	-	+	+	+	Koitabashi *et al*., 2008
E09-A	Beef, China	2004- 2005	-	+	+	+	Koitabashi *et al*., 2008
EDL933	Human, USA	1982	+	+	+	-	Riley *et al*., 1983

Laboratories) using 0.5× TBE buffer in the CHEF-DRIII system (Bio-Rad). Electrophoresis was performed at 6 V/cm with a field angle of 120º at 14ºC. The pulse times were 2.2 to 54.2 s for 19 h. After electrophoresis, the gel was stained with ethidium bromide, and DNA profiles were analyzed using the Fingerprinting II program (Bio-Rad Laboratories, Tokyo, Japan).

Results

In this study, 31 beef samples from Malaysian exports to Thailand and 36 beef samples from Thai (domestic) products were examined. We employed a method to effectively isolate *E. coli* O157 strains. After the initial screening, 15 strains were isolated from eight Malaysian samples, and six strains were isolated from four Thai beef samples. However, the results of subsequent repeated agglutination tests indicated that one of the Malaysian isolate belongs to serotype O116:H31, and the remaining isolates belong to O157:H7 (Table 1). Therefore, we isolated 20 strains of the O157:H7 serotype from 12 beef samples (one to three strains per each sample). Sample 13 yielded three O157:H7 strains and one O116:H31 strain. All O157:H7 strains but one was of the stx_1^- stx_2^+ eae^+ virulence gene type; the exceptional strain exhibited stx_1^- stx_2^- eae^+ genotype (Table 1, strain M2). Accordingly, 13 Malaysian and 6 Thai strains were *E. coli* O157:H7 possessing the stx_2 and *eae* genes.

The O116:H31 strain, M7, was of the stx_1^+ stx_2^+ eae^- genotype. All the strains listed in Table 1 possessed the *esc*V gene, suggesting that they harbored the LEE pathogenicity island. Plasmid analysis revealed that all O157:H7 strains contained a 60 MDa plasmid unique to EHEC (data not shown).

The 19 strains of stx_2^+ eae^+ *E. coli* O157 were examined for the inability to produce Stx_2 by the TNP-PCR method. All isolates were positive in all four PCR tests (TNP-A, TNP-B, TNP-C and TNP-D) (data not shown). This indicated their inability to produce Stx_2 due to the *Q* gene and surrounding nucleotide sequences unique to the stx_2 positive but Stx_2 non-producing strains (Koitabashi *et al*., 2006). Examination of Stx_2 production from the same test strains using the RPLA method confirmed that most of the strains did not produce Stx_2. Strain M10 exhibited an RPLA titer of only 1:4 and the titer of the other 18 strains were below the detection limit (< 2) whereas the titer of a positive control, strain EDL933, was > 1:128 (data not shown).

Sensitivity of the 19 strains of stx_2^+ eae^+ *E. coli* O157:H7 to 12 commonly used antibiotics was examined. The majority of strains were similar in that they were sensitive to all 12 antibiotics tested. However, two of six (33.3%) Thai strains were resistant to one or two antibiotics and five of thirteen (38.5%) Malaysian strains were resistant to one to three antibiotics (Table 2). One Malaysian (M14) and one Thai (T3) strain shared the same pattern of antibiotic resistance.

The DNA fingerprints of the 19 strains of stx_2^+ eae^+ *E. coli* O157:H7 isolated in this study were examined and compared. Included in the DNA fingerprinting analyses for comparison were stx_2^- eae^- *E. coli* O157:H7 (M2) and stx_2^+ eae^- *E. coli* O116: H31 (M7) isolated in this study; stx_2^+ eae^+ TNP-PCR-positive strains of *E. coli* O157 isolated from beef, bovine feces, and humans in Thailand, Japan, and China in previous studies (Table 3); and a standard *E. coli* O157 strain, EDL933. The DNA fingerprints were obtained, first, by O157 IS-printing. Two sets of primers were used showing similar results. The DNA profiles revealed 19 distinct patterns that could be classified into seven groups, I to VII, with the O116: H31 strain (M7) being located in the most distantly related group VII (Figure 1). All the current Thai strains of stx_2^+ eae^+ *E. coli* O157:H7 (T1-T6) were in group III and showed 95 - 100% similarity, whereas high degrees of heterogeneity (25 - 100% similarity) were observed among the current Malaysian strains of stx_2^+ eae^+ *E. coli* O157:H7 (M1-M15). However, most of the current Malaysian strains were also found in group III.

Next, the DNA fingerprints of the same set of the test strains were obtained by digestion of the chromosome with *Xba*I followed by separation of generated DNA fragments using the PFGE method. This technique generated 25 distinct DNA patterns that were clustered into 11 groups designated as groups I to XI with the O116: H31 strain (M7) located in the most distantly related group XI (Figure 2). The tendency of the distribution of the test strains was similar to that of the O157 IS-fingerprinting result, but the PFGE analysis had higher resolution. All current Thai strains of stx_2^+ eae^+ *E. coli* O157:H7 (T1 - T6) were in group IX (82 - 100% similarity), and the DNA profiles of two of the six strains (T4 and T5) were identical. The current Malaysian strains of stx_2^+ eae^+ *E. coli* O157:H7 showed more heterogeneity (52 - 100% similarity), and 15 of the 19 strains were classified into seven groups (groups I, II, III, V, IX, X and XI). Thus, only two, M14 and M15, of the current 19 Malaysian strains were in the same group (group IX) as the current Thai strains. The results indicated that all current Thai strains of stx_2^+ eae^+ *E. coli* O157:H7 were similar to some of the current Malaysian strains of stx_2^+ eae^+ *E. coli* O157:H7.

Discussion

E. coli O157:H7 was isolated from eight (25.8%) of 31 samples of Malaysian beef exported to Thailand and four (11.1%) of 36 samples of Thai beef. The isolated *E. coli* O157:H7 strains were 20 in total and 19 harbored the stx_2 and eae genes (Table 1). The successful isolation of these O157 strains from food can be attributable, in part, to the selective isolation procedure including an immunomagnetic separation technique followed by selection using the CHROMagarO157 agar medium.

IS*629* is most frequently detected in *E. coli* O157 strain (Ooka *et al*., 2009a). O157 IS-printing is a simple and rapid technique for the detection and differentiation of *E. coli* O157:H7 (Ooka *et al*., 2009a; Ooka *et al*., 2009b). The technique is based on the variability in the genomic location of IS*629* among the *E. coli* O157:H7 strains. Two sets of primers were designed, and each set generates polymorphisms at 16 loci of IS*629* in the chromosome and plasmid of *E. coli* O157:H7. This technique is useful because primers for specific detection of the eae and *hly*A genes can also be included in the first set of primers, and primers for detection of the stx_1 and stx_2 genes can be added to the second set of primers. They are used to confirm the test strains exhibit the important genotypes of *E. coli* O157:H7 (Tables 1; the hly^+ result is not included in the tables). In this study, the

DNA profiles of all 19 strains of *stx*$_2^+$ *eae*$^+$ *E. coli* O157:H7 isolates from Thai and Malaysian beef and reference strains were determined by O157 IS-printing (Table 3). The O157 IS-printing generated seven DNA profile groups. The DNA profiles of all *E. coli* O157:H7 isolates including the reference strains were closely related (65 - 70% similarity) and were classified into groups I – VI, whereas the DNA profile of *E. coli* O116 (strain M7) was classified into group VII that showed only about 25% similarity to the O157 strain (Figure 1). This suggests that O157 IS-printing specifically differentiates *E. coli* O157 from other *E. coli* strains. All DNA profiles of the TNP-PCR-positive O157 isolates from Asia (groups I - V) were similar and distinguishable from a TNP-PCR-negative strain isolated in the United States (EDL933), indicating that these *E. coli* O157 strains may be prevalent and transferred among Asian countries.

In this study, DNA fingerprints of the test strains were also analyzed using the PFGE method. The result was consistent with that of O157 IS-printing. However, the PFGE method exhibited higher discriminatory power than O157 IS-printing. The *E. coli* O157:H7 isolates from Malaysian beef were classified into six groups by the PFGE method, whereas they were separated into only two groups by O157 IS-printing (Figures 1 and 2). In addition, PFGE could differentiate 3 Malaysian isolates (M11, M12 and M13), 3 Thai isolates (T2, T3 and T6) and 2 Thai control strains (Thai 1 and Thai 13) whereas IS-printing failed to do this. The PFGE method had a higher resolution than O157 IS-printing because DNA profiles were generated from the entire genome of the tested organisms, but DNA profiles from O157 IS-printing were created from only IS*629* and clustered in the 1.4 Mb Sakai specific sequences (Ooka *et al*., 2009a).

As described above, DNA profiles of the Malaysian strains were diversified. This may be because beef was imported from different sources in Malaysia to maintain a constant supply to a large Muslim population. The PFGE analysis demonstrated a wide range of similarity (65 - 90%) among the Malaysian and Thai isolates (Figure 2). Of these strains M11, M12 and M13, isolated from Malaysian beef, and Thai 12, isolated previously from bovine feces in Thailand (a reference strain), were classified into group III. Two Malaysian strains, M14 and M15, and all Thai strains (T1 - T6) isolated in the present study were classified into group IX. The close similarity between particular Malaysian and Thai strains suggested a possible transfer of *E. coli* O157:H7 from Malaysia to Thailand.

The antibiograms of two strains of *stx*$_2^+$ *eae*$^+$ *E. coli* O157:H7, M14 from Malaysia and T3 from Thailand, were identical (Table 2, KF CR). This phenotypic observation supports the result of the DNA fingerprinting analyses, suggesting that these two strains are closely related because they were classified in the same group by O157 IS-printing (Figure 1, group III) and PFGE (Figure 2, group IX).

Koitabashi *et al*. (2006) showed that most of *E. coli* O157:H7 isolated from Asia produced no or low levels of Stx_2 because they had a unique *Q* gene producing a weakly active antitermination Q protein and had a defect of their *stx*$_2$ promoter causing little or no production of Stx_2. Those strains were named as Stx_2-negative strains. The results of TNP-PCR and RPLA indicated that all of the *E. coli* O157:H7 strains isolated from Thai and Malaysian beef in the present study were assigned to the Stx_2-negative group (Table 3). However, the production of Stx_2 *in vivo* was needed to confirm that these strains were not harmful to humans.

In conclusion, the data obtained in this study suggested the possibility of transfer of *stx*$_2^+$ *eae*$^+$ *E. coli* O157:H7 from Malaysia to Thailand through beef trade. Although the *E. coli* O157:H7 strains isolated in this study were of the Stx_2-negative type, some of the Stx_2-negative strains were reported to produce low levels of Stx_2 *in vitro* (Koitabashi *et al*., 2006). Trans-border transfer of food contaminated by pathogens and the practically borderless state in Southeast Asia is a public health concern.

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