The Occurrence of *Staphylococcus aureus* on a Farm with Small-Scale Production of Raw Milk Cheese

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ABSTRACT

In recent years, the small-scale production of raw milk products has increased in Norway, and there is some concern that such foods may pose a risk of staphylococcal food poisoning to consumers. The aim of the study was to evaluate potential sources of contamination of raw milk cheese with *Staphylococcus aureus* on a bovine dairy farm with small-scale production. Samples for bacteriological analyses (n = 144) were collected from the animals, the environment, processing equipments, from humans, and from cheeses at various stages of production. *Staphylococcus aureus* was isolated from 10 of 11 cows, the farmer, equipment, the environment, and the cheese. Seventy-five *Staph. aureus* isolates were genotyped by pulsed-field gel electrophoresis, tested for enterotoxin (SE) production by reversed passive latex agglutination, for SE genes by multiplex polymerase chain reaction, and for penicillin resistance by the cloverleaf method. Five different pulsotypes were identified and SE gene fragments were identified in 11 isolates, but no isolates produced SE or were penicillin resistant. *Staphylococcus aureus* was found throughout the farm, and appeared to be spread with the milk to the environment, equipment, and to products. One pulsotype dominated and was identified from most sample sites on the farm. Raw milk products are vulnerable to contamination with *Staph. aureus*. Strategies to reduce the occurrence of *Staph. aureus* in bulk milk are of particular importance on farms where milk is used for raw milk products.

(Key words: *Staphylococcus aureus*, raw milk cheese, small-scale production, pulsed-field gel electrophoresis)

Abbreviation key: BA = blood agar with washed bovine erythrocytes, BP+RPF = Baird-Parker agar with rabbit plasma fibrinogen, PFGE = pulsed-field gel elec-

trophoresis, PT = pulsotype, SE = staphylococcal enterotoxin.

INTRODUCTION

In recent years, Norwegian agricultural politics has encouraged the production of niche foods including small-scale dairy products. A number of small-scale dairies have been started, and many use raw milk for production. The increasing production of raw milk products in Norway has brought forward certain food safety concerns.

Staphylococcus aureus was recently detected in 75% of 220 samples of bovine bulk milk (Jørgensen et al., 2005). This is of concern because bacteria in raw milk may contaminate raw milk products and create a risk of food poisoning to consumers (Zottola and Smith, 1993; Headrick et al., 1998). Prevalences of 20 to 38% of *Staph. aureus* in Norwegian raw milk products have been reported (Kruse, 1999, 2000; Haugmo, 2001; Jørgensen et al., 2005), and in a Swedish study, coagulase-positive staphylococci were detected in 38% of on-farm manufactured raw goat cheeses (Tham et al., 1990). In France, *Staph. aureus* is reported to be the most frequent cause of foodborne disease from raw milk cheeses (De Buyser et al., 2001), and in Norway, outbreaks of *Staph. aureus* food poisoning have been attributed to raw goat cheese (Aas et al., 1992; Schønberg and Wåltorp, 2001), raw cow cheese (Berg et al., 1996), and potato-mash made with raw milk (Loncarevic and Mathisen, 2004).

Staphylococcus aureus food poisoning is caused by ingestion of food containing preformed enterotoxins (SE). Symptoms have a rapid onset and may include nausea, vomiting, and diarrhea (Jablonski and Bohach, 1997). Eighteen different SE have been described and designated SEA–SEE, SEG–SER, and SEU (Dinges et al., 2000; Fitzgerald et al., 2001; Jarraud et al., 2001; Kuroda et al., 2001; Orwin et al., 2001, 2002; Letertre et al., 2003; Omoe et al., 2003). In favorable conditions, *Staph. aureus* may grow and produce SE in foods, and because the SE are stable with respect to heat and

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storage they may be present in foods where viable *Staph. aureus* are absent (Jablonski and Bohach, 1997).

Dairy animals are probably the main source of contamination of raw milk with *Staph. aureus* (Bone et al., 1989; Gilmour and Harvey, 1990; Asperger, 1994; Vautor et al., 2003). In particular, dairy animals with subclinical *Staph. aureus* mastitis may shed large numbers of *Staph. aureus* into the milk. However, contamination of raw milk and raw milk products from human handling or from the environment during manufacture is also possible.

Environmental conditions such as temperature, pH, water activity, salt concentration, and competing microflora influence *Staph. aureus* growth and SE production (Genigeorgis, 1989), and various milk production techniques may be used to prevent the growth of pathogenic bacteria in the products. Nevertheless, it is important that contamination of milk and milk products with *Staph. aureus* is minimized, and a further understanding of the spread of *Staph. aureus* from dairy animals, humans, and the farm environment to milk and raw milk products is needed.

The aim of the present study was to evaluate potential sources of contamination of raw milk cheese with *Staph. aureus* in a typical Norwegian dairy farm with small-scale production.

MATERIALS AND METHODS

The Farm

The study was performed in September 2003 in the summer-farm facility of a Norwegian dairy farm with 11 lactating cows of the Norwegian Red breed. From June through September, the animals pasture at the summer farm where raw milk products are manufactured in a small dairy facility. The facilities are licensed for production and local retail of raw milk products by the food hygiene authorities.

During summer, machine milking is performed in a small cowshed. The milk-room has wash facilities for cleanup and sanitation of milking equipment, and a bulk tank for cooling and storage of milk. Bulk milk is either delivered to a cooperative dairy or used for manufacture of raw milk products on the premises. The dairy consists of a small production room and a room for cheese maturation. Production techniques are largely manual, and the produce includes soured cream and a semihard cheese.

Sampling

Sampling was performed on 2 consecutive days. On d 1, samples were collected from the cowshed and milk room, and on d 2, from the dairy during cheese-making.

Samples were also collected from cheese at different stages of maturation.

Swab samples were collected with sterile cotton swabs (Celsis-Lumac, Newmarket, UK) that were moistened in sterile peptone water, rolled on the test surface, and placed in 4 mL of Voegel-Johnson broth (Oxoid, Basingstoke, UK) with 0.5% agar in sterile glass test tubes. Sterile cotton plugs were used to sample floor drains, test material was absorbed, and the plugs were transferred to sterile polypropylene test tubes (Greiner Bio-one, Frickenhausen, Germany). Fluid and solid samples were collected at volumes of 50 to 100 g in sterile plastic tubes.

The udder and teat skin of each cow were swabbed by moving a swab from the udder skin to the tip of the teat. Quarter milk samples were collected from each cow according to Harmon et al. (1990). The nasal membrane of each cow was swabbed 1 to 5 cm inside one nostril and the vaginal mucosa was swabbed in the vestibular area. Each animal was inspected for teat wounds and wounds or excoriations in other locations, which were swabbed if observed.

From the cowshed and milk-room pre- ($n = 8$) and postmilking ($n = 8$) swab samples were collected from milking equipment (teatcup liners, claw pieces of milk machine clusters, milk filter) and the environment (sink in milk room, tap in milk room, door handles, hosepipe, washing brush). In addition, 2 premilking samples were collected from washing water for udders and teats and from tap water, and a postmilking environmental sample was collected from the milk-room floor drain. The farmer's nose was swab-sampled premilking, and his hands were swab-sampled pre- and postmilking. On d 1, a bulk milk sample was collected during transfer of milk from the farm tank to the milk truck.

On d 2, 80 L of the morning milk was used to produce a semihard cheese. Swab samples were collected pre- ($n = 16$) and postproduction ($n = 10$) from production equipment (cheese molds, vat, plastic scoop, curd cutter, palette knife, strainer, cheese knife, wooden spoon, cheesecloth, cutting board), the environment (wooden shelf in storage room, tap, sink, door handle, window sill, floor in storage room, wash brush). In addition, an environmental sample was collected postproduction from the dairy floor drain. The cheesemaker's nose was swab-sampled preproduction, and her hands were swab-sampled pre- and postproduction.

Cheese making was started approximately 1 h after milking. The cheese-maker wore clean latex gloves during cheese production. Single samples were collected from the cheese at various stages of production. Milk for cheese making (bulk milk from d 2) was sampled from the vat. Mesophilic and thermophilic starter cultures were sampled. Starters had been collected from

commercial dairy products, propagated in the dairy, and maintained in glass jars at 4°C. Rennet was also sampled (Rennet standard 75/25, AB Kemikalia, Lund, Sweden).

After the addition of starter cultures, the milk was heated to 32°C and the rennet was added. Coagulation started after 11 min, and the gel of early coagulation was sampled. Curd-cutting was performed manually before heating to 39°C. Curd and whey were sampled separately. The pH of the cheese after 24 h was measured by the cheese maker using Lyphan pH paper (Gerhard Klotz GmbH, Neuhaus am Rennweg, Germany). Soured cream produced in the dairy 2 wk before sampling was also collected. Wedges of cheese (approximately 300 g) were collected after 1 wk and 2 mo of maturation.

A total of 144 samples were collected and transported cooled in polystyrene boxes to the laboratory.

Bacteriological Analyses

Bacteriological analyses were conducted within 5 h of sampling. Milk samples were brought to room temperature (22 to 25°C) and mechanically shaken at 320 rpm for 10 min before plating. Undiluted milk was spread-plated at volumes of 0.1 mL on blood agar with washed bovine erythrocytes (BA; Oxoid) and on Baird-Parker agar with rabbit plasma fibrinogen supplement (BP+RPF; bioMérieux, Marcy-l'Etoile, France). From solid and fluid samples, 10 g was added to 90 g of 0.1% peptone water. Decimal dilutions were spread-plated at volumes of 0.1 mL on BA and BP+RPF. Swabs in Voegel-Johnson media were incubated at 37°C for 4 h before plating to optimize retrieval of stressed cells. Each swab was then plated on BA and BP+RPF. Cotton plugs were added to 50 mL of Voegel-Johnson broth, incubated, and spread-plated as described above. Agar plates were incubated at 37°C for 48 h. Typical colonies were counted after 24 and 48 h of incubation on both types of agar plates. Whenever possible, 5 typical and 5 atypical colonies from each plate were replated on BA and incubated aerobically at 37°C for 24 h for further confirmation of *Staph. aureus*.

All cultures were gram-stained (Difco, Sparks, MD) and tested for the catalase reaction. Cultures of colonies initially picked from BA, and colonies without an opaque halo on BP+RPF were subjected to the tube coagulase test using rabbit plasma with EDTA (Becton Dickinson, Sparks, MD). Colonies with an opaque halo on BP+RPF were considered coagulase-positive. Coagulase-positive isolates were streaked on peptone agar (Difco) supplemented with 7 mg/L of acriflavin (Sigma-Aldrich Chemie, Steinheim, Germany; Roberson et al., 1992) and incubated at 37°C for 24 h. Cultures with

gram-positive, catalase-positive cocci that displayed a positive coagulase reaction and the ability to grow on peptone agar supplemented with acriflavin, were considered *Staph. aureus* (Roberson et al., 1992). Quantitative results were calculated from the mean of the colony counts on BA and BP+RPF (Jørgensen et al., 2005).

Characterization of *Staph. aureus* Isolates

Seventy-five randomly selected *Staph. aureus* isolates were subjected to further characterization. At least one isolate from each *Staph. aureus*-positive sample was included, and additional isolates were selected from samples where contamination from several sources was likely. Five isolates were included from each of the bulk milk samples, and 2 isolates were included from each of the cheese samples taken throughout production. In addition to this, 10 extra isolates were selected from remaining samples.

The selected isolates were characterized by pulsed-field gel electrophoresis (PFGE), reversed passive latex agglutination for SE production, multiplex PCR for SE genes, and for penicillin resistance.

For PFGE, preparation of chromosomal DNA, enzymatic digestion with *Sma*I (Roche, Mannheim, Germany), and electrophoresis were performed essentially as described by Bannerman et al. (1995) with modifications as described by Mørk et al. (2005). Banding patterns from PFGE were compared visually. Different banding patterns were defined as pulsotypes (PT), and designated with uppercase letters. These were compared according to criteria defined by Tenover et al. (1995); PT with 2 to 3 band differences were considered closely related.

Reversed passive latex agglutination (Oxoid) was used to test isolates for production of SEA–SED according to manufacturer's instruction, without quantification.

Multiplex PCR was used to test isolates for the presence of SE genes (Løvseth et al., 2004). The method detects genes encoding SEA–SEEE and SEG–SEJ (*sea–see*, *seg–sej*) and the toxic shock syndrome toxin gene (*tst*). The method includes primers for 16S rRNA for control of DNA isolation, and 4 positive control strains (FRI 913, 3169, R5460, R5010) that include all the genes detected by multiplex PCR. MilliQ water was used as negative control.

Isolates were tested for inactivation of penicillin (β -lactamase activity) by the cloverleaf method as described by Bergan et al. (1997). *Staphylococcus aureus* ATCC 25923 was used as indicator strain. A penicillin-sensitive strain (Sk256d) and a penicillin-resistant strain (Sk259a) were used as negative and positive controls, respectively.

Table 1. *Staphylococcus aureus* found in different sample sites in 11 dairy cows. Isolates were tested by pulsed-field gel electrophoresis; different banding patterns were defined as pulsotypes (PT) A–D.

Sample site	No. of sampled animals	No. of samples	No. of <i>Staph. aureus</i> -positive samples	No. of animals with <i>Staph. aureus</i> -positive samples	No. of isolates tested	<i>Staph. aureus</i> PT (No. of isolates)
Quarter samples	11	42	9	4	12	A (8), B (1), C (3)
Udder/teat skin	11	11	10	10	10	A (9), C (1)
Nostril	11	11	0	0	0	
Vulva	11	11	2	2	2	A (1), C (1)
Teat wounds	4	4	4	4	4	A (4)
Skin wounds	1	2	1	1	1	A (1)

RESULTS

Bacteriological Findings

Staphylococcus aureus was isolated from 50 (34.7%) of the samples. One or more samples from 10 of the cows were *Staph. aureus*-positive (Table 1). Four cows had *Staph. aureus*-positive quarter milk samples at levels between 5 and 1250 cfu/mL. One, 1, and 2 of these cows shed *Staph. aureus* from 1, 2, and 3 mammary quarters, respectively. Bulk milk from d 1 was *Staph. aureus*-positive.

Results from samples collected in the cowshed and milk room are presented in Table 2. With the exception of a hosepipe in the milk room, *Staph. aureus* was not isolated from the premilking samples from the cowshed and the milk-room. Five of the postmilking samples from milking equipment (a teatcup liner, the claw-pieces of 2 clusters, and a milk filter) and the environment (the milk room floor drain) were *Staph. aureus*-positive.

Results from samples collected in the dairy are shown in Table 3. *Staphylococcus aureus* was not isolated from preproduction samples, but 5 of the postproduction equipment samples (a plastic scoop, cheesecloth, the vat, and a wooden spoon) and environmental samples (a

floor drain in the production room) were *Staph. aureus*-positive. Concentrations of *Staph. aureus* in the milk for cheese-making, cheese ingredients, and cheese during production are shown in Table 4.

All the isolates identified as *Staph. aureus* displayed β -hemolysin on BA, except the 2 isolates from the cheese producer and 1 isolate from the dairy floor-drain.

pH in the Cheese

The pH in the cheese after 24 h was between 4.9 and 5.2.

PFGE

By PFGE, 5 different banding patterns (PT A to E) were identified among the 75 *Staph. aureus* isolates (Figure 1). Pulsotypes A, B, C, D, and E included 57 (76%), 6 (8%), 8 (10.7%), 1 (1.3%), and 3 (4%) isolates, respectively (Tables 1 to 4). Isolates belonging to PT A were found on the animals, in bulk milk, cheese ingredients, and cheese. Pulsotype B was found in a quarter sample, bulk milk, milking equipment, and environmental samples from the milk-room and dairy. Pulsotype C was found in quarter samples, on the animals, in milk for cheese production, and in the cheese. Two

Table 2. *Staphylococcus aureus* found in different sample sites in the cowshed and milk-room. Isolates were tested by pulsed-field gel electrophoresis; different banding patterns were defined as pulsotypes (PT) A–D.

Sample site	No. of samples	No. of <i>Staph. aureus</i> -positive samples	No. of isolates tested	<i>Staph. aureus</i> PT (No. of isolates)
Samples premilking				
Farmer (nose)	1	1	2	A (2)
Farmer (hands)	1	0	0	
Milking equipment	3	0	0	
Environmental samples	5	1	1	A (1)
Tap water	1	0	0	
Washing water	1	0	0	
Samples postmilking				
Farmer (hands)	1	1	2	A (2)
Milking equipment	5	4	4	A (3), B (1)
Environmental samples	4	1	2	A (1), B (1)

Table 3. *Staphylococcus aureus* found in different sample sites in the dairy. Isolates were tested by pulsed-field gel electrophoresis; different banding patterns were defined as pulsotypes (PTs) A–D.

	No. of samples	No. of <i>Staph. aureus</i> -positive samples	No. of isolates tested	<i>Staph. aureus</i> PT (No. of isolates)
Samples preproduction				
Cheese maker (nose)	1	1	2	E (2)
Cheese maker (hands)	1	0	0	
Production equipments	9	0	0	
Environmental samples	7	0	0	
Samples postproduction				
Cheese maker (hands)	1	1	1	E (1)
Production equipment	6	4	6	A (6)
Environmental samples	5	1	2	B (1), D (1)

PT unique to single sources were isolated from the dairy floor drain (PT D) and the cheese maker (PT E). The banding-patterns of PT A and PT B differed by 2 bands and were considered closely related.

Enterotoxin Production, Enterotoxin Genes, and Penicillin Resistance

None of the 75 *Staph. aureus* isolates tested by reversed passive latex agglutination were positive for SE production (SEA–SED).

Eleven (14.7%) of the isolates tested by multiplex PCR were positive for SE genes. Eight of the SE gene-positive isolates contained *seg*, and 3 isolates were positive for *sei*, *seg*, and *tst*. Isolates assigned to the same PT had the same SE-gene profile. Isolates belonging to PT A, B, and D were SE gene-negative; isolates belonging to PT C were positive for *seg*, and isolates belonging to PT E were positive for *seg*, *sei*, and *tst*. No isolates inactivated penicillin on the cloverleaf test.

DISCUSSION

Staphylococcus aureus was found throughout the farm, and was isolated from cows, humans, processing equipment, the environment, cheese at various stages of production, and from soured cream produced 2 wk earlier.

The number of animals with *Staph. aureus*-positive mammary quarters was high (36%) compared with a previous study, in which 22% of tested Norwegian dairy cows had *Staph. aureus*-positive udder secretions (Sølverød and Østerås, 2001). In the present study, the dairy cow body site most often colonized with *Staph. aureus* was the udder and teat skin, which agrees with reports by others (Davidson 1962; Schalm et al., 1971). The bacterium was also isolated from wounds and the vaginal mucosa of some animals, but no nasal swabs were positive.

Cleaned equipment for milking and for cheese production were *Staph. aureus*-negative, but samples collected from the same equipment postmilking and post-

Table 4. Concentrations of *Staphylococcus aureus* in samples of bulk milk, and in raw milk products during manufacture. Isolates were tested by pulsed-field gel electrophoresis; different banding patterns were defined as pulsotypes (PT) A–D.

Sample	<i>Staph. aureus</i> concentration (cfu/mL or cfu/g)	No. of isolates tested	<i>Staph. aureus</i> PT (No. of isolates)
Bulk milk on d 1	355	5	A (3), B (2)
Milk for cheese-making ¹	240	5	A (3), C (2)
Mesophilic starter culture	20	2	A (2)
Thermophilic starter culture	20	2	A (2)
Rennet	0	0	
Gel of early coagulation	2000	2	A (2)
Whey	200	2	A (2)
Curd at cheese pressing	15,000	2	A (2)
Cheese after 7 d maturation	6000	2	A (1), C (1)
Cheese after 10 wk maturation	0	0	
Soured cream ²	100	2	A (2)

¹Bulk milk from the second day of sampling.

²From previous production, 2 wk old.

Figure 1. Pulsed-field gel electrophoresis pulsotypes (PT) observed among 75 isolates of *Staphylococcus aureus*. Lanes: L = lambda ladder, A–E = PT A through E.

production were generally contaminated. It appears that the bacteria were spread with the milk and product material to the equipment and the environment during milking and cheese production. The sanitation process seemed effective in removal of *Staph. aureus*.

Bulk milk from both sample days were acceptable with respect to present microbiological criteria for raw milk intended for raw milk products (<500 cfu/mL; European Commission, 1992). Increasing concentrations of *Staph. aureus* were identified from bulk milk, the gel of early coagulation, and the cheese curd. The increase in *Staph. aureus* levels from bulk milk to the gel of early coagulation reflects *Staph. aureus* growth. However, the much greater concentration of *Staph. aureus* in curd compared with whey may be due to the physical entrapment of organisms in the curd (Tatini et al., 1971), and agrees with previous observations (Stecchini et al., 1991; Vernozy-Rozand et al., 1998).

In the present study, *Staph. aureus* concentrations in the cheese did not reach levels considered a risk with respect to staphylococcal food poisoning (>10⁵/g; Jablonski and Bohach, 1997). Active lactic starter cultures in cheese are generally inhibitory of *Staph. aureus* growth (Stadhouders et al., 1978), and the low pH observed in the cheese after 24 h reflects activity of the starter culture. In agreement with previous studies, the levels of *Staph. aureus* decreased during cheese maturation (Stecchini et al., 1991; Vernozy-Rozand et al., 1998). Although *Staph. aureus* dies during maturation

and storage of cheese, SE may retain its biological activity (Genigeorgis, 1989). Consequently, fully matured cheeses negative for viable *Staph. aureus* are not necessarily safe with respect to staphylococcal food poisoning. It seems advisable that if cheeses are to be tested for *Staph. aureus*, testing should be performed as soon as possible after cheese production.

Five *Staph. aureus* PT, with different distributions, were recognized in the present study. The predominant type, PT A, was retrieved from most sample sites. The presence of isolates belonging to this PT in soured cream produced 2 wk earlier indicates that it is a continuous problem in the studied farm. This is supported by the finding of *Staph. aureus* isolates belonging to PT A and PT B in quarter samples collected from the same farm in 2000 (unpublished data). The finding of *Staph. aureus* belonging to PT A in both starter cultures most likely indicates contamination during storage in the dairy. Pulsotype C was also isolated from the animals, milk, and cheese, but not from other sources.

It is likely that the farmer's hands were contaminated with PT A during milking. Previous studies indicated that milkers' hands can be fomites in the spread of *Staph. aureus* in dairy herds (Fox et al., 1991). However, the identification of PT A from the farmer's nose premilking suggests that he was colonized with PT A, and thus, indicates that he is a potential source of this strain on the farm. On the other hand, human variants of *Staph. aureus* generally do not produce β -hemolysin (Hajek and Marsalek, 1969; Schalm et al., 1971; Devriese, 1984), and PT A was recognized previously in mastitis samples from cows, goats, and sheep from several regions of Norway (unpublished data). For these reasons, and because PT A was present in 10 of 11 cows, the animals are considered its primary reservoir.

Isolates of PT E were found only on the cheese maker, and colonies did not display β -hemolysin phenotypically on BA, nor did the colony of PT D. It is possible that these strains are human variants of *Staph. aureus*; and, because they were not discovered in milk or cheese, human contamination of cheese during production seemed of lesser importance in the studied farm. The use of gloves by the cheese maker may have prevented contamination of the cheese.

In the present study, SE genes were infrequently found in the studied *Staph. aureus* isolates, and *seg* and *sei* were the only SE genes identified. Previously, 55% of the tested *Staph. aureus* isolates from Norwegian bovine bulk milk were SE gene positive (Jørgensen et al., 2005). The involvement of SEI and SEG in food poisoning is unclear; in fact, SEI may have reduced emetic potential (Munson et al., 1998). However, *sei* and *seg* have been found in isolates of *Staph. aureus* implicated in food poisoning outbreaks (McLauchlin et

al., 2000). At present, no commercial immunological tests are available to identify SEI or SEG in foods; thus, the products were not tested for the presence of SE.

The spread of antibiotic resistant bacteria with milk and milk products is a concern (Sato et al., 2004). In this study, all isolates tested were penicillin sensitive, but the spread of *Staph. aureus* throughout the production chain is suggestive of a potential for resistant bacteria to spread with raw milk products.

In the present study, *Staph. aureus* in bulk milk appeared to be the main source of contamination of equipment, the environment, and of raw milk products. Although various milk production techniques may reduce or prevent growth of *Staph. aureus* and production of SE in raw milk products, the microbiological quality of the milk is of particular importance. The present study was limited to one sampling event and to a small number of cows. Nevertheless, it demonstrates a frequent occurrence of *Staph. aureus* in the animals and a large potential for bacteria to spread throughout the production chain in small-scale dairies.

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