Effects of *Bos taurus* autosome 9-located quantitative trait loci haplotypes on the disease phenotypes of dairy cows with experimentally induced *Escherichia coli* mastitis

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ABSTRACT

Several quantitative trait loci (QTL) affecting mastitis incidence and mastitis-related traits such as somatic cell score exist in dairy cows. Previously, QTL haplotypes associated with susceptibility to *Escherichia coli* mastitis in Nordic Holstein-Friesian (HF) cows were identified on *Bos taurus* autosome 9. In the present study, we induced experimental *E. coli* mastitis in Danish HF cows to investigate the effect of 2 *E. coli* mastitis-associated QTL haplotypes on the cows' disease phenotypes and recovery in early lactation. Thirty-two cows were divided in 2 groups bearing haplotypes with either low (HL) or high (HH) susceptibility to *E. coli*. In addition, biopsies (liver and udder) were collected from half of the cows ($n = 16$), resulting in a 2×2 factorial design, with haplotype being one factor (HL vs. HH) and biopsy being the other factor (biopsies vs. no biopsies). Each cow was inoculated with a low *E. coli* dose (20 to 40 cfu) in one front quarter at time 0 h. Liver biopsies were collected at –144, 12, 24, and 192 h; udder biopsies were collected at 24 h and 192 h post-*E. coli* inoculation. The clinical parameters: feed intake, milk yield, body temperature, heart rate, respiration rate, rumen motility; and the paraclinical parameters: bacterial counts, somatic cell count (SCC), and milk amyloid A levels in milk; and white blood cell count, polymorphonuclear neutrophilic leukocyte (PMNL) count, and serum amyloid A levels in blood were recorded at different time points post-*E. coli* inoculation. *Escherichia coli* inoculation changed the clinical and paraclinical parameters in all cows except one that was not infected. Clinically, the HH group tended to have higher body temperature and heart rate

than the HL group did. Paraclinically, the HL group had faster PMNL recruitment and SCC recovery than the HH group did. However, we also found interactions between the effects of haplotype and biopsy for body temperature, heart rate, and PMNL. In conclusion, when challenged with *E. coli* mastitis, HF cows with the specific *Bos taurus* autosome 9-located QTL haplotypes were associated with differences in leukocyte kinetics, with low-susceptibility cows having faster blood PMNL recruitment and SCC recovery and a tendency for a milder clinical response than the high-susceptibility cows did.

Key words: *Escherichia coli*, mastitis, quantitative trait locus haplotype, phenotype

INTRODUCTION

Mastitis, an inflammatory condition of the mammary gland, is the most prevalent disease in periparturient dairy cows (Burvenich et al., 2007; Fox, 2009). Bovine mastitis causes substantial economic losses to the dairy industry because of decreased milk yield, treatment cost, prevention cost, and culling of the affected animals (Petrovski et al., 2006; Bar et al., 2008; Hertl et al., 2011). Moreover, mastitis is considered a painful condition of animal welfare concern (Rasmussen et al., 2011; Fogsgaard et al., 2012). Hence, lowering the risk and incidence, and improving recovery from mastitis through improved breeding is desirable to the dairy industry.

The gram-negative bacterium *Escherichia coli*, belonging to the *Enterobacteriaceae* family, is one of the most common causes of acute clinical mastitis in dairy cows in early lactation (Bradley et al., 2007; Ericsson Unnerstad et al., 2009; Hertl et al., 2011). *Escherichia coli* mastitis is associated with well-characterized clinical signs and subclinical and clinical changes in milk and blood components. The clinical and paraclinical

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changes, termed “evident phenotype” (Detilleux, 2009), are considered the disease phenotypes during mastitis. The common clinical disease phenotypes during *E. coli* mastitis are high fever and anorexia with lowered milk production (Burvenich et al., 2003; Bannerman et al., 2004; Kornalijnslijper et al., 2004). The disease phenotypes expressed in milk are a brief excretion of high numbers of *E. coli*, followed by a dramatic increase in SCC and inflammatory mediators and altered milk appearance (Kornalijnslijper et al., 2004; Vangroenweghe et al., 2004; Vangroenweghe et al., 2005), depending on the severity of the infection (Bannerman et al., 2004; Suojala et al., 2008). Similarly, the important disease phenotypes expressed in blood are leukocyte mobilization with short-term leukopenia, followed by leukocytosis (Vels et al., 2009; Rasmussen et al., 2011) and secretion of large quantities of inflammatory mediators including cytokines (Bannerman et al., 2004) and acute-phase proteins (Jacobsen et al., 2005; Suojala et al., 2008). Among the commonly investigated acute-phase proteins are milk amyloid A (MAA) and serum amyloid A (SAA), which have been suggested to be associated with the degree of infection (Jacobsen et al., 2005; Suojala et al., 2008) and the related tissue trauma (inflammation) (Cray et al., 2009).

Host defense against mastitis is controlled by multiple genes (Pighetti and Elliott, 2011), and the outcome of clinical mastitis may be affected by several host factors, including QTL or QTL haplotype (Lund et al., 2008; Sodeland et al., 2011). In Nordic Holstein-Friesian (HF) cows, the SCS is affected by a QTL located on BTA9 that is partly associated with *E. coli* mastitis, with one haplotype being more susceptible and another being more resistant (Sørensen et al., 2008). Indeed, increasing numbers of studies are investigating the underlying molecular mechanism of *E. coli* mastitis from an immunological point of view (Buitenhuis et al., 2011). Furthermore, numerous studies investigating the effect of SNP in the bovine immunological genes on mastitis susceptibility are emerging (Leyva-Baca et al., 2008; He et al., 2011; Pighetti and Elliott, 2011). Although several QTL affecting mastitis incidence and mastitis-related traits such as SCS exist in dairy cows, only few studies have examined the effect of specific QTL or QTL haplotypes in an experimental setup. Recently, primary bovine mammary gland epithelial cells originating from cows with specific SCS-associated QTL on BTA18 were infected with *E. coli* and investigated for multiple gene expression using quantitative real-time PCR and microarray expression chip technology (Griesbeck-Zilch et al., 2009; Brand et al., 2011). However, so far no one has investigated the direct effect of the BTA9 QTL haplotypes on common clinical, milk, and blood disease phenotypes during experimental *E.*

coli mastitis. Studying the effect of QTL haplotypes on clinical outcome and disease recovery may provide novel insights into the genetic basis of the pathophysiology of bovine *E. coli* mastitis.

The aim of the present study was to test the hypothesis that the BTA9 QTL haplotypes are partly associated with *E. coli* mastitis, with one cow haplotype being more susceptible and another being more resistant to *E. coli* mastitis. The effect of QTL haplotypes on the clinical and paraclinical disease phenotypes associated with *E. coli* mastitis was tested in an experimental in vivo model using a low bacterium dose. We also collected liver and udder biopsies from half of the cows for gene expression analyses. The results from these studies were presented in Buitenhuis et al. (2011) and Jørgensen et al. (2012). As this combined biopsy procedure may have caused additional stress and inflammation, the biopsy procedure was included as a statistical factor in our study. The main effect of the factor biopsy is presented elsewhere (M. Khatun, P. Sørensen, K. L. Ingvarsen, M. Bjerring, and C. M. Røntved, unpublished data), whereas the interaction effect between biopsy and QTL haplotype on the investigated parameters are presented here.

MATERIALS AND METHODS

Selection of Animals

The experiment was performed at Aarhus University, Department of Animal Science (Foulum, Denmark). All procedures involving animals were approved by the Danish Animal Experiments Inspectorate in accordance with the Danish Ministry of Justice Law no. 726 (September 9, 1993) and acts 739 (December 6, 1988) and 687 (July 25, 2003) concerning animal experimentation and the care of experimental animals. Inspection was carried out by members of the Danish Animal Experiments Inspectorate committee during the acute stage of the disease.

The Danish HF breed was used in this study. Potential carriers (bull daughters) of the high-or low-mastitis-resistance QTL haplotype were identified in the Danish National Cattle Database (https://www.landbrugsinfo.dk/Kvaeg/Ryk/Sider/Registreringsblok_web.pdf; [http://www.glr-chr.dk/pls/glrchr/chrmenu\\$.menu](http://www.glr-chr.dk/pls/glrchr/chrmenu$.menu)) by assessing the haplotype status of their sire and grandsire. Daughters were also matched with regard to age, expected calving date (1.5 to 2 mo before expected calving date), and health status record (i.e., free of *Salmonella dublin*, bovine viral diarrhoea virus, group B *Streptococcus*, infectious bovine rhinotracheitis virus, and low antibody titers against *Para tuberculosis* in milk and serum). Forty-two pregnant heifers (20 in

winter and 22 in summer) were purchased from the farmers and transferred to the Aarhus University dairy barn after confirmation of QTL haplotype as described below. The experiment ran for about 3 mo in the spring and 3 mo in the summer of 2007. Prior to experimentally induced *E. coli* mastitis, the general health and udder health status of all cows were evaluated based on rectal temperature, white blood cell (WBC) count, the glutaraldehyde test, the California Mastitis Test (CMT; score 1 to 5; Pyörälä, 2003; Jørgen Kruuse A/S, Marslev, Denmark), and bacteriological examinations of foremilk samples. Only cows with normal body temperature, WBC count, negative glutaraldehyde test, low CMT score (1 or 2), and which were free from major mastitis pathogens were used. Ten cows served as reserves until the post-*E. coli* inoculation (PI) d or h 0, picking the most healthy and least nervous cows when biopsying.

QTL Haplotype Analysis

In a previous study, a QTL affecting clinical mastitis and SCS was mapped on BTA-9 in Danish HF cattle (G. Sahana, Center for Quantitative Genetics, Department of Molecular biology and Genetics, Faculty of Science and Technology, Aarhus University, Foulum, Denmark, personal communication). The QTL explained 2.21% of the genetic variance for clinical mastitis in the HF discovery population.

Four grandsire families and 255 progeny-tested sons were genotyped with microsatellite markers and QTL analysis was performed using linkage analysis and combined linkage disequilibrium and linkage analysis (Lund et al., 2003; Sahana et al., 2008). The linkage analysis had the highest likelihood ratio test at 42.93 cM on chromosome 9, with the QTL interval spanning 22 cM. The maximum likelihood ratio test statistics for both linkage disequilibrium and linkage analysis and linkage disequilibrium analysis alone were located at the same position of the chromosome. A 0.52-cM haplotype consisting of 6 microsatellite markers was identified, which explained the QTL variance within this QTL interval. The founder haplotypes (263; 8 grandsire haplotypes plus 255 dam haplotypes) were grouped into 55 clusters based on identity-by-descent probabilities. The allelic combinations and haplotype effects on clinical mastitis and SCS were estimated for the haplotype clusters with high frequencies. Within-family linkage analysis revealed that 1 grandsire (Lord Lily) was segregating a mastitis-resistance QTL. The 2 haplotypes carried by this grandsire (Lord Lily) were identified. A new group of 345 individuals (106 sires and 235 heifers) was genotyped for these 6 microsatellite markers. The haplotypes were constructed using PHASE software

(Stephens and Donnelly, 2003). The HF heifers carrying 1 of the 2 haplotypes (low susceptibility, **HL** or high susceptibility, **HH**) of the segregating grandsire (Lord Lily) were identified and recruited for the *E. coli* challenge experiment. It was ensured that none of these heifers carried both haplotypes (HL and HH).

Experimental Design

A 2×2 factorial block design was used to study the effect of QTL haplotype on clinical and paraclinical disease phenotypes in *E. coli* mastitis. However, as repeated combined liver biopsies and udder biopsies were collected from half of the cows ($n = 16$) for gene expression analysis, the effect of biopsy and the interaction effect of QTL haplotype with biopsy were also investigated. One group was cows with HL to *E. coli* mastitis and the other group was cows with HH to *E. coli* mastitis. For the interaction effect, cows were divided into 4 groups: the HL group with biopsy (**HLB**) or no biopsy (**HLNB**) and the HH group with biopsy (**HHB**) or no biopsy (**HHNB**). One week (-192 h PI) before *E. coli* inoculation, 24 of the 42 cows were randomly selected for liver biopsy. Thirty-two cows were inoculated with *E. coli* 4 to 6 wk after parturition; this was set as time point 0 h. Sixteen of the 24 biopsied cows continued in the *E. coli* trial on the combined liver and udder biopsy procedure. For convenience, the trial was divided into 4 groups of 8 cows and biopsies were collected from 4 cows in each group. Because of additional disease and delayed milk let-down, 3 cows could not be studied for the entire experimental period and were discarded from the statistical analysis. Hence, 16 HL cows (HLB: $n = 8$; HLNB, $n = 8$) and 13 HH (HHB, $n = 8$; HHNB, $n = 5$) cows were analyzed for the haplotype effect on clinical and paraclinical disease phenotypes. The time points of the experimental treatments and of the clinical, milk, and blood disease phenotype recordings before and after intramammary *E. coli* inoculation at 0 h are presented in Table 1.

Housing, Feeding, and Milking

Prior to calving, the heifers were housed in a freestall barn. After calving, the cows were transferred to a straw-bedded tiestall barn. Eight days before the experimentally induced *E. coli* mastitis, the cows were moved so that they were kept with an empty tiestall between them during the remaining study period. One week before disease induction, the cows had a mean BW of 547 ± 74 kg for the HL group versus 547 ± 46 kg for the HH group. Moreover, a BCS of 3.0 ± 0.3 versus 3.0 ± 0.4 was recorded for the HL and HH groups, respectively (Troels Kristensen's 1 to 5 scale;

Table 1. Time points (hour and day) of the experimental treatments and disease phenotype recordings before and after intramammary *Escherichia coli* inoculation in dairy cows at 0 h

Treatment and recording	Time	
	Hour	Day
Vein catheter for blood collection	−36	−1.5
Liver biopsy	−144, 12, 24, 192	−6, 0.5, 1, 8
Udder biopsy	24, 192	1, 8
Clinical phenotypes		
Feed intake		−7 to 10
Milk yield		−7 to 10
Body temperature	0, 3, 6, 12, 18, 24, 36, 48, 60, 72, 84, 96, 108, 120, 132, 144, 156, 168, 180, 192, 204, 216, 228, 240	0 to 10
Heart rate	0, 3, 6, 12, 18, 24, 36, 60, 84, 132, 180, 228	0 to 10
Respiration rate	0, 3, 6, 12, 18, 24, 36, 60, 84, 132, 180, 228	0 to 10
Rumen motility	0, 3, 6, 12, 18, 36, 60, 84, 132, 180, 228	0 to 10
Paraclinical milk phenotypes		
Bacterial counts	0, 3, 6, 12, 18, 24, 36, 48, 60, 72, 84, 96, 108, 120, 132, 144, 156, 168, 180, 192, 204, 216, 228, 240	0 to 10
SCC, MAA ¹	0, 3, 6, 12, 18, 24, 36, 60, 84, 132, 180, 228	0 to 10
Paraclinical blood phenotypes		
WBC count, ² PMNL, SAA ³	0, 3, 6, 12, 18, 24, 36, 60, 84, 144, 192, 240	0 to 10

¹MAA = milk amyloid A.²WBC = white blood cells.³SAA = serum amyloid A.

Kristensen, 1986). A TMR diet based on corn silage plus minerals and vitamins was fed ad libitum twice per day in equal portions at about 0800 and 1530 h. The diet and mineral supplementation were formulated to fulfill the nutrient requirements for dairy cows according to Danish standards. Feed intake was measured twice per day, and the cows were ensured free access to water. The cows were milked twice per day at 0600 and 1700 h.

Preparation of Bacteria and IMI

The procedures for collection, preparation, and IMI of the bacteria were described by Buitenhuis et al. (2011). Danish field isolate *E. coli* strain (k2bh2) isolated from a cow with severe, acute mastitis was used in this experiment. Each cow was inoculated with 10 mL pyrogen-free 0.9% sodium chloride solution containing approximately 20 to 40 *E. coli* cfu after the evening milking (time 0 h), following the inoculation procedure described in Vels et al. (2009). The front quarter with the lowest SCC (<100,000 cells/mL) was used for the *E. coli* inoculations.

Collection of Liver and Udder Biopsies

Four liver biopsies were taken as described by Vels et al. (2009) at −144, 12, 24, and 192 h PI; 2 udder biopsies were taken as described by Buitenhuis et al. (2011) at 24 h and 192 h PI. Liver biopsies were collected after local anesthesia with 10 mL of 2% lidocaine (Skander-

borg Apotek, Skanderborg, Denmark). Udder biopsies were collected after the liver biopsies from cows sedated by i.v. administration of Domosedan vet. (10 mg/mL; detomidine; Orion Pharma, Espoo, Finland) at 0.1 mL per 100 kg of BW and were given local anesthesia on the udder skin using xylocaine (1% lidocaine; Astra Zeneca A/S, Albertslund, Denmark). After udder biopsy, a prophylactic antibiotic treatment against infection with gram-positive bacteria was administered to all biopsy cows by i.m. injection of 30 mL of Penovet vet. (300,000 IU benzylpenicillin procaine/mL; Boehringer Ingelheim Danmark A/S, Copenhagen, Denmark).

Additional Medical Treatment During the Disease Trial

During the acute stage of mastitis, 1 cow (HHB), who responded severely to the *E. coli* mastitis was treated with Finadyne vet. (2.2 mg/kg, i.v.; Intervet/Schering-Plough, flunixin meglumine; 50 mg/mL), Norodine vet. (16 mg/kg; ScanVet Animal Health A/S, Fredensborg, Denmark; containing sulfadiazine at 200 mg/mL and trimethoprim at 40 mg/mL), Calciject 40 vet. (0.5 to 1 mL/kg; ScanVet Animal Health A/S; calcium borogluconate containing 30 mg of Ca/mL), and 2 L of hypertonic sodium chloride solution [Vetivex 20; sodium chloride 7.2% (wt/vol); Jørgen Kruuse A/S] from 48 to 96 h PI. Another cow (HLB) developed a minor leg injury 72 h after *E. coli* challenge and was given antibiotic treatment for 3 d of 1 daily injection of Penovet vet. (5 mL/100 kg, i.m.). No other cows were given

medical treatment during the trial except for the treatment associated with the combined biopsies. Because of the relatively late medical treatment compared with the acute experimental disease stage, the 2 medically treated cows were retained in the statistical analysis.

Blood and Milk Sampling

Thirty-six hours before *E. coli* inoculation, sterile Micro-Renathane polyvinyl catheters (type S-54-HL; Norton Performance Plastics Co., Akron, OH) were inserted into the jugular vein of each cow and flushed with a sterile 0.9% sodium chloride solution containing 50 to 200 IU of Na-heparin (Løvens Kemiske Fabrik, Ballerup, Denmark). Two sets of blood samples were drawn aseptically from the catheters with a syringe at the time points presented in Table 1. One set of blood samples was collected in 4-mL K₃EDTA blood tubes (Vacuette blood collection tubes; Greiner Bio-One, Hettich Labinstrument ApS, Hillerød, Denmark) and was analyzed for total and differential WBC count on a hemocytometer (Cell-Dyn 3500; Abbott Laboratories A/S, Copenhagen, Denmark). The other set was collected into 9-mL blood tubes stabilized with Na-heparin and immediately placed on ice for SAA analysis. The blood tubes were centrifuged at $2,000 \times g$ at 4°C for 20 min. Plasma was collected and stored at -20°C until SAA analysis.

Milk samples were collected as foremilk samples before milking at the time points shown in Table 1. Sterile 10-mL foremilk samples were collected for bacteriological investigations from the *E. coli*-inoculated quarter. In addition, the negative control quarter was sampled before udder biopsy at 24 h and 192 h PI, or if the CMT test indicated the presence of mastitis in quarters other than the inoculated quarter. Samples for SCC and MAA analysis were collected after the samples for bacteriological analysis. This milk was collected in 50-mL sterile tubes and filtered through a 100- μ m filter to remove major pus aggregates before it was distributed into smaller tubes. Somatic cell count analysis was conducted immediately after sampling; all other samples were kept on ice and then frozen at -20°C.

Recordings of Clinical Parameters

The clinical response parameters included feed intake (kg/d), milk yield (L/d), body temperature (°C), heart rate (beats/min), respiration rate (breaths/min), and rumen motility (contractions/3 min). Feed intake was calculated as the allowance in the morning and afternoon minus the total leftover. The daily feed intake and milk yield data were collected from the barn records twice per day. The udder condition (inflammation) in

the *E. coli*-inoculated gland was scored as described in Rasmussen et al. (2011; data not shown). Together, the production parameters and clinical responses were defined as the clinical disease phenotypes.

Recordings of Paraclinical Parameters

The milk parameters, defined as the milk disease phenotype, included bacterial counts, SCC, and MAA concentration. In addition, the CMT test was used to screen foremilk samples from all 4 quarters to monitor the general udder health during the trial (data not shown).

Escherichia coli numbers (cfu/mL) were quantified as described in Buitenhuis et al. (2011). Furthermore, at several time points, 10- μ L aliquots of foremilk from the *E. coli*-infected quarter as well as the negative control quarter used for udder biopsy were cultured on blood agar and tryptic soy agar for 48 h at 37°C to test for the presence of mastitis pathogens other than *E. coli*. The SCC (cells/mL) of milk samples was measured in the barn using a portable DeLaval cell counter (DCC; DeLaval Holding AB, Tumba, Sweden; range 10 to $4,000 \times 10^3$ cells/mL).

Milk samples collected just before udder biopsy at 24 h contained coagulase-negative *Staphylococcus* spp. in the negative control quarter of 3 cows, with 2 cows showing elevated SCC from 500,000 cells/mL to 650,000 cells/mL. Otherwise, no mastitis pathogens other than *E. coli* were identified.

Milk amyloid A concentrations were measured using a commercially available ELISA kit (Tridelta Development Ltd., Bray Co., Wicklow, Ireland) according to the manufacturer's instructions. The milk samples were diluted 1:2,500 and tested in duplicate. Test samples outside the range of the standard curve were further diluted up to a maximum of 1:10,000. The interassay and intraassay coefficients of variation for the ELISA were <12% of the positive control (107.2 μ g/mL) at dilution 1:50,000 and <20% of the positive control (428.5 μ g/mL) at dilution 1:50,000. The detection limit of the ELISA was 0.1 μ g/mL.

The blood parameters, defined as blood disease phenotype, included WBC, PMNL, and SAA. White blood cell and leukocyte differential counts were calculated using a hemocytometer (Cell-Dyn 3500; Abbott Laboratories A/S). Only the WBC (10^6 cells/mL) and PMNL (10^6 cells/mL) data are reported in this study.

Plasma concentrations of SAA (ng/mL) were measured using a commercially available ELISA kit (Tridelta Development Ltd.) as described in Vels et al. (2009). The plasma samples were initially diluted 1:500 and tested in duplicate. Test samples outside the range of the standard curve were further diluted to a

maximum of 1:2,000. The interassay and intraassay coefficients of variation for the ELISA were <9.5% of the positive control (16.1 µg/mL) at dilution 1:500 and <10% of the positive control (155.1 µg/mL) at dilution 1:2,000. The detection limits of the ELISA were 9.5 to 150 µg/mL.

Statistical Analysis

The data were analyzed using the nlme (package 3.1–96) function in R (version 2.12.1, <http://www.r-project.org/>). The clinical, milk, and blood disease phenotypes were considered response variables to test the effect of the QTL haplotype. Phenotypic observations at different time points (Table 1) were analyzed using a linear mixed model. The following models (M) were used in the analyses:

$$\text{M1: } y_{ijkl} = t_i + qtl_j + b_l + t_i \times qtl_j + t_i \times b_l + qtl_j \times b_l + t_i \times qtl_j \times b_l + a_k + e_{ijkl};$$

$$\text{M2: } y_{ijkl} = t_i + qtl_j + b_l + t_i \times qtl_j + t_i \times b_l + a_k + e_{ijkl};$$

$$\text{M3: } y_{ijkl} = t_i + qtl_j + b_l + t_i \times b_l + a_k + e_{ijkl};$$

$$\text{M4: } y_{ikl} = t_i + b_l + t_i \times b_l + a_k + e_{ikl}.$$

In the above models, y_{ijkl} was the response variable (disease phenotype), t_i was the i th time point measured in days for feed intake and milk yield and in hours for the other disease phenotypes, qtl_j was the QTL haplotype effect of the j th haplotype (HL or HH), b_l was the biopsy effect of the l th biopsy (B or NB), a_k was the random effect of the k th cow, and e_{ijkl} was the random error associated with the measurement at the i th time point for the j th haplotype, l th biopsy, and k th cow. Measurements at different time points (Table 1) for the same animal were assumed to be correlated using a first-order autoregressive structure.

In the full model (M1), the main and interaction effects of the QTL haplotype and biopsy were assumed to be time dependent. Model 2 (M2) included the main effects of QTL haplotype and biopsy that were both time dependent. Model 3 (M3) included the main effect of QTL haplotype that was additive and the main effect of biopsy that was time dependent. Model 4 (M4) included the main effect of biopsy that was time dependent (this is the most reduced model). The models were compared using a likelihood ratio test. We tested M1 against M2 (test for time-dependent interaction effects between QTL haplotype and biopsy), M2 against M3

(test for time-dependent effect of QTL haplotype), and M3 against M4 (test for effect of QTL haplotype that is consistent over time).

All tests were considered significant if $P < 0.05$. Because of large variations for the SCC, MAA, PMNL, and *E. coli* values in milk, these parameters were $\log_2$ transformed before statistical analysis.

RESULTS

Intramammary *E. coli* inoculation resulted in acute clinical *E. coli* mastitis in 29 of the 30 inoculated cows (statistical values not shown). After 72 h, half of the cows (16 of 29) had cleared the *E. coli* infection. After 180 h, all cows had cleared the infection, except for 3 cows that remained intermittently positive for *E. coli* during the remaining study period.

The QTL haplotype factor affected the paraclinical disease phenotypes SCC (consistent effect) and PMNL (time-dependent effect). Furthermore, the HH group tended to exhibit a higher clinical disease phenotype response (body temperature and heart rate) than the HL group. The biopsy factor showed statistically significant interactions with the QTL haplotype effects on body temperature, heart rate, and PMNL (Table 2).

Effect of QTL Haplotype on Clinical Disease Phenotypes

Escherichia coli mastitis lowered feed intake, milk yield and rumen motility, but increased body temperature, heart rate, and respiration rate in all infected cows (Figures 1 and 2).

Intramammary *E. coli* infection decreased feed intake by 47.2% for HL and 37.9% for HH (Figure 1A) and milk yield by 31.4% for HL and 35% for HH (Figure 1B). However, no significant differences were found between the HL and HH groups for feed intake and milk yield under either the interaction or the additive model (Table 2).

Quantitative trait locus haplotype had no consistent effect on body temperature, but the HH group tended to have higher body temperatures than the HL group ($P = 0.07$) during the acute disease stage (Table 2; Figure 2A). All 29 cows developed fever ($>39^\circ\text{C}$) within 12 to 18 h of infection, with mean peak temperatures of 40.2°C at 12 h PI and 39.9°C at 18 h PI in groups HH and HL, respectively. Mean body temperature remained above preinfection values (38.5°C) until 18 h PI for the HL group, but remained elevated for an additional 6 h until 24 h PI in the HH group.

The mean heart rate was relatively high during the whole trial (>70 beats/min) and it followed the pattern of the body temperature. The heart rate was increased

Table 2. Effect of *Escherichia coli*-associated QTL haplotype on the clinical, milk, and blood disease phenotypes of experimentally induced *Escherichia coli* mastitis in dairy cows

Parameter ¹	<i>P</i> -value ² (M1 ³ vs. M2 ⁴)	<i>P</i> -value ⁵ (M2 ⁴ vs. M3 ⁶)	<i>P</i> -value ⁷ (M3 ⁶ vs. M4 ⁸)
Clinical phenotype			
Feed intake	0.36	0.51	0.14
Milk yield	0.94	0.97	0.70
Body temperature	0.004**	0.07†	0.42
Heart rate	0.01*	0.08†	0.71
Respiration rate	0.77	0.72	0.95
Rumen motility	0.33	0.76	0.92
Paraclinical milk phenotype			
Bacterial count	0.82	0.99	0.58
SCC	0.19	0.80	0.02*
MAA	0.40	0.85	0.15
Paraclinical blood phenotype			
WBC count	0.30	0.27	0.88
PMNL	0.03**	0.02*	0.95
SAA	0.36	0.84	0.77

¹MAA = milk amyloid A; WBC = white blood cells; SAA = serum amyloid A.

²Likelihood ratio test for time-dependent interaction effects between QTL haplotype and biopsy.

³M1 = model 1, in which the main and interaction effects of the QTL haplotype and biopsy are assumed to be time dependent (full model).

⁴M2 = model 2, which includes the main effects of QTL haplotype and biopsy that are both time dependent.

⁵Likelihood ratio test for time-dependent effect of QTL haplotype.

⁶M3 = model 3, which includes the main effect of QTL haplotype that is additive and the main effect of biopsy that is time dependent.

⁷Likelihood ratio test for effect of QTL haplotype that is consistent over time.

⁸M4 = model 4, which includes the main effect of biopsy that is time dependent.

†*P* < 0.1; **P* < 0.05; ***P* < 0.01.

in both groups, which attained peak levels at 12 h PI, with 105 and 102 beats/min in the HH and HL groups, respectively (Figure 2C). The HH group had a higher heart rate than the HL group between 36 and 132 h PI; this was close to being statistically significant under the interaction model (*P* = 0.08).

The respiration rate was also relatively high during the trial (>30 breaths/min). Faster respiration rates were found in the HH group than in the HL group over time but this was not statistically significant. Mean respiration rates peaked at 12 h PI in the HH group (40.2 breaths/min) and at 18 h PI in the HL group (38.6 breaths/min), and then again at 132 h PI (42 and 40.5 breaths/min in the HH and HL groups, respectively; Figure 2E).

No significant differences were found between the 2 QTL haplotypes with regard to rumen motility (Figure 2F). The lowest mean values for rumen contractions were observed at 12 h PI, with the HL group having 2.7 contractions/3 min and the HH group having 3.1 contractions/3 min; both were significantly lower than the motility recorded before inoculation.

Effect of QTL Haplotype on Paraclinical Disease Phenotypes

Quantitative trait locus haplotype had no time-dependent or consistent effect on *E. coli* shedding (Table

2; Figure 3A). The kinetics of the *E. coli* shedding were almost similar in the 2 cow groups, with a sharp primary peak around 24 h PI. This was followed by a minor secondary peak at 48 h PI, but only in the HH group. Numerically, we did observe that the HH group had a higher *E. coli* bacterial count in the early acute phase from 6 h to 18 h PI than the HL group. However, *E. coli* levels in this time period were not statistically significantly different (*P* = 0.16). *Escherichia coli* levels also appeared numerically higher at the same time points in the HH group than the HL from 24 h PI to 48 h PI. In the recovery phase (60 to 240 h PI), 50% of cows in the HL group had 1 or more milk samples that were positive for *E. coli* at a given time point compared with 62% of cows in the HH group.

Somatic cell count was significantly affected by QTL haplotype, which had a consistent additive effect on the SCC level (Table 2; M3 vs. M4). The SCC of the 2 cow groups followed the same kinetics. Although it cannot be seen in Figure 3B, the SCC level already started to differ from 6 h PI, and tended to differ significantly between the 2 groups in the time period from 6 to 18 h PI (*P* < 0.10). Somatic cell counts peaked in both groups from 18 h to 24 h PI, where the upper limit of the DCC measuring capability was reached for most cows. After the SCC peak from approximately 36 h PI, the HH group continued to have a consistently higher

Figure 1. Effect of *Bos taurus* autosome 9 (BTA9) QTL haplotypes on clinical disease phenotypes related to production in 2 groups of dairy cows challenged with *Escherichia coli* mastitis. The results are reported as LSM and show the difference between each group by time. A: feed intake (kg/d); B: milk yield (kg/d). HL = low-susceptibility QTL haplotype; HH = high-susceptibility QTL haplotype. Liver biopsies (■) were collected at -6, 0.5, 1, and 8 d postinoculation (PI). Udder biopsies (▼) were collected at 1 and 8 d PI. The SEM were 1.7 and 1.8 to 1.9 for feed intake, and 1.4 to 1.5 and 1.5 to 1.6 for milk yield, for the HL and HH groups, respectively. Quantitative trait loci haplotypes had no effect on feed intake or milk yield ($P > 0.05$).

SCC than the HL group did ($P < 0.05$). Hence, the HL group had a lower SCC at the end of the study period and a faster recovery, based on SCC.

Milk amyloid A levels were not affected by QTL haplotype group. The peak MAA levels in both the HL and HH groups occurred at approximately 60 h PI. At this time point we also observed the greatest numerical difference in MAA levels between the 2 groups (Figure 3C). The MAA level remained numerically higher in the HH group until approximately 132 h PI, after which time it returned to almost the same level in the 2 groups.

Although the WBC mean values differed between the HL and HH groups in the acute disease stage and the recovery stage (Figure 4A), WBC count was not significantly affected by QTL haplotype at any time point (Table 2). The WBC levels decreased to a minimum at 18 h PI (leukopenia), which was followed by an increasing trend (leukocytosis) that peaked at 36 and 60 h PI in the HL and HH groups, respectively (Figure 4A).

The PMNL count mirrored the kinetics of the WBC count. Although no consistent effect was observed of haplotype on PMNL numbers, a time-dependent haplotype effect was found between HL and HH groups

(Table 2; M2 vs. M3). The PMNL count decreased to a minimum at 12 to 18 h PI, but was followed by a marked increase. The PMNL count peaked at 36 and 60 h PI in the HL and HH groups, respectively, with the HL group peaking more than 24 h before the HH group (Figure 4B). The peak PMNL level in the HL group was 5.7×10^6 cells/mL and that in the HH group was 5.4×10^6 cells/mL.

The SAA blood concentrations started to increase after *E. coli* inoculation and reached peak levels from 24 to 60 h PI. Subsequently, SAA levels declined markedly and reached base levels at approximately 192 h PI in both groups (Figure 4D). The SAA level and kinetics were not significantly different between the 2 groups under either the interaction model or the additive model (Table 2).

Interaction Between QTL Haplotype and Biopsy Effect

The main effects of combined liver and udder biopsies on the clinical and paraclinical parameters of dairy cows with experimental *E. coli* mastitis will be published elsewhere (M. Khatun, unpublished data). The interaction effects of QTL haplotype group, biopsy, and sampling time are presented in Table 2 (M1 vs. M2). Interaction effects were found for body temperature, heart rate, and PMNL count. In the acute disease stage, biopsying increased body temperatures in both the HLB and HHB groups, and the HHB group exhibited higher temperatures than the HLB group did ($P < 0.01$; Figure 2B). Heart rate followed the same pattern: the HHB group exhibited higher heart rates than the HLB group did ($P < 0.05$; Figure 2D). After peaking at 36 h PI, PMNL count in the HLB group followed the kinetics of the HHB and HHNB groups, which remained significantly higher than that of the HLNB group throughout the study ($P < 0.05$; Figure 4C). No interaction effects were found between QTL haplotype group and biopsying for the other clinical and paraclinical parameters.

DISCUSSION

Genetic selection based on SCS is commonly used in breeding programs because of the high heritability of SCS and the availability of SCC data in well-organized field recording systems (Detilleux, 2009). Previous studies in dairy cows have demonstrated that SCS in Nordic HF cows is affected by QTL (Lund et al., 2008). Moreover, BTA9 QTL haplotype effects were demonstrated for pathogen-specific mastitis when investigating clinical mastitis recordings from the Danish cattle database (Sørensen et al., 2008). Likewise, QTL haplotypes

Figure 2. Effect of *Bos taurus* autosome 9 (BTA9) QTL haplotypes on clinical disease phenotypes in 2 groups of dairy cows challenged with *Escherichia coli* mastitis. The results are reported as LSM and show the difference between each group by time. A: body temperature response (°C) due to haplotype effect; B: body temperature response (°C) due to QTL haplotype and biopsy interaction effect; C: heart rate (beats/min) due to haplotype effect; D: heart rate (beats/min) due to haplotype and biopsy interaction effect; E: respiration rate (breaths/min); F: rumen motility (contractions/3 min). HL = low-susceptibility QTL haplotype; HH = high-susceptibility QTL haplotype; B = biopsied cows; NB = nonbiopsied cows. Liver biopsies (■) were collected at -144 (not shown), 24, and 192 h postinoculation (PI). Udder biopsies (▼) were collected at 24 and 192 h PI. The SEM for body temperature were 0.1 and 0.1; for heart rate, 2.6 to 4.9 and 2.9 to 5.6; for respiration rate, 2.5 to 4.1 and 2.8 to 4.1; and for rumen motility, 0.3 and 0.3 to 0.4, for the HL and HH groups, respectively. A time-dependent effect of QTL haplotype was observed for body temperature and heart rate ($P < 0.1$). The 2 clinical disease phenotypes also varied due to a time-dependent interaction effect between QTL haplotype and biopsy ($P < 0.05$).

Figure 3. Effect of *Bos taurus* autosome 9 (BTA9) QTL haplotypes on paraclinical disease phenotypes related to milk in 2 groups of dairy cows challenged with *Escherichia coli* mastitis. The results are reported as LSM and show the difference between each group by time. A: *E. coli* level in milk (cfu/mL); B: SCC (cells/mL); C: milk amyloid A (MAA, ng/mL). HL = low-susceptibility QTL haplotype; HH = high-susceptibility QTL haplotype. Liver biopsies (■) were collected at -144 (not shown), 12, 24, and 192 h postinoculation (PI). Udder biopsies (▼) were collected at 24 and 192 h PI. The SEM were 0.6 to 0.7 and 0.7 to 0.8 for *E. coli* level, 0.3 and 0.3 for SCC level, and 0.6 and 0.7 for MAA level, for the HL and HH groups, respectively. Somatic cell counts differed consistently over time ($P < 0.05$).

partly associated with *E. coli* mastitis were identified, with one QTL haplotype being more susceptible to this mammary gland infection and another QTL haplotype being more resistant to the infection (Sørensen et al., 2008). In the present study, we hypothesized that SCC and other clinical and paraclinical disease phenotypes of dairy cows associated with *E. coli* mastitis could be affected by these QTL haplotypes on BTA9. Hence, we established an experimental disease model in dairy cows to illustrate these QTL haplotype differences using SCC and other clinical and inflammatory parameters. Because high doses of *E. coli* would lead to serious mastitis (severe pathogen effect) regardless of haplotype and lower doses tend to result in large individual variations in disease response (Kornalijslijper et al., 2004; Vangroenweghe et al., 2004), we chose a low-dose model (20 to 40 cfu/mL in 1 quarter). *Escherichia coli* infection was successfully established in 29 of 30 inoculated cows. The infected cows developed acute *E. coli* mastitis that ranged from mild to severe mastitis in both QTL haplotype groups and followed the disease kinetics and response described by others (Burvenich et al., 2003; Vangroenweghe et al., 2005). Likewise, *E. coli* mastitis lowered feed intake, rumen motility, and milk yield, but increased body temperature, heart rate, and respiration rate and the concentrations of milk- and blood-related inflammatory mediators in the infected cows. In healthy cows exposed to experimental *E. coli* mastitis, the infection is brief in the majority of cows because of active pathogen clearance by the host (Bannerman et al., 2004; Kornalijslijper et al., 2004; Suojala et al., 2008). Spontaneous recovery from *E. coli* mastitis will occur within 7 to 14 d without any medical treatment. This was also the case in our study. As was our intention, the low *E. coli* dose resulted in large individual variations with regard to *E. coli* number and shedding period. Major individual variations were found for both QTL haplotypes, and were highest in the HH group. Although we did observe a numerically higher *E. coli* count from 24 to 48 h PI, no statistical effect of QTL haplotype on *E. coli* levels could be documented in either the early and acute disease stage in which the *E. coli* levels peaked, or in the recovery period. Our finding is in contrast to that reported by Bonnefont et al. (2011), who demonstrated an effect on *Staphylococcus aureus* shedding in the dairy Lacauene sheep line bred for low and high SCC in second lactation, but in accordance with the same study, which was unable to document an effect on *Staphylococcus epidermidis* shedding in first lactation. Also, in their study, sheep were inoculated with 1,000 cfu/mL in 1 quarter. In our study, we only used primiparous cows for the *E. coli* mastitis challenge, which are known to have a lower clinical response and a faster recovery

Figure 4. Effect of *Bos taurus* autosome 9 (BTA9) QTL haplotypes on paraclinical disease phenotypes related to blood in 2 groups of dairy cows challenged with *Escherichia coli* mastitis. The results are reported as LSM and show the difference between each group by time. A: white blood cell (WBC) count (10^6 cells/mL); B: PMNL response (10^6 cells/mL); C: PMNL response due to haplotype and biopsy interaction effect (10^6 cells/mL); D: serum amyloid A level (SAA; ng/mL). HL = low-susceptibility QTL haplotype; HH = high-susceptibility QTL haplotype; B = biopsied cows; NB = nonbiopsied cows. Liver biopsies (■) were collected at -144 (not shown), 24, and 192 h postinoculation (PI). Udder biopsies (▼) were collected at 24 and 192 h PI. The SEM were 0.6 and 0.6 for WBC count, 0.1 and 0.1 for PMNL level, and 31.9 to 33 and 35.1 to 36.6 for SAA level, for the HL and HH groups, respectively. A time-dependent effect of QTL haplotypes was observed for PMNL level ($P < 0.05$). The PMNL level also varied due to a time-dependent interaction effect between QTL haplotype and biopsy ($P < 0.05$).

from *E. coli* mastitis than multiparous cows (Burvenich et al., 2003). Whether the use of multiparous cows or a different *E. coli* dose would have influenced the results remains to be investigated. Nevertheless, we were able to demonstrate that the QTL haplotype affected other

paraclinical disease phenotypes induced by the intramammary *E. coli* infection, including SCC and PMNL.

Whereas Bonnefont et al. (2011) were unable to document an effect on SCS between the resistant and susceptible sheep lines, either before or after the chal-

lence with the 2 *Staphylococcus* species, we succeeded in demonstrating a BTA9 QTL haplotype effect on SCC level in HF cows after *E. coli* challenge. Indeed, the IMI with *Staphylococcus* spp. and our *E. coli* strain resulted in increased SCC and bacteria counts in both ruminant species. However, compared with Bonnefont et al. (2011), who sampled their sheep 3 to 4 times within a 48-h period postinoculation, we sampled the dairy cows 11 times for a period of 228 h postinoculation. Still, as *E. coli* is a gram-negative bacterium and *Staphylococcus* spp. are gram-positive bacteria, major differences are found in their disease pathogenesis and immune response when cows are infected (Bannerman et al., 2004; Schukken et al., 2011). Therefore, one should be careful comparing the 2 infection models with regard to SCC and leukocyte recruitment to the mammary gland.

Although the SCC levels in our study followed the same kinetics in the 2 cow groups, the SCC already started to differ in the early acute phase (from around 6 h) before the SCC peak. Somatic cell count levels also differed in the late inflammatory phase and in the recovery period, where the level remained consistently higher in the HH group than in the HL group. Correspondingly, we observed a shorter SCC recovery (<150,000 cells/mL) period in the HL group compared with the HH group. However, no differences were found between the 2 groups for the SCC peak level within the 2 phases (from 18 to 24 h). One possible explanation could be the method used to analyze the SCC. We used the handy DCC for bulk milk and cow-side testing (Røntved et al., 2005). This instrument is optimized to count somatic cells in the range 10,000 to 4,000,000 cells/mL. However, it plateaus at 5,000,000 cells/mL, which results in high-cell-count samples yielding a similar SCC and a comparatively lower SCC than they would have by the Coulter count method or the Fossomatic cell counter (Foss Electric A/S, Hillerød, Denmark; Røntved et al., 2005). Indeed, this SCC level was reached for the majority of our milk samples from 18 to 24 h.

The finding of a significant difference in SCC level between the 2 QTL haplotype groups provides further evidence of genetic influence on SCC and confirms previous findings for BTA9 (Lund et al., 2008; Sørensen et al., 2008). Clearly, *E. coli* number and SCC kinetics are associated with each other, as the colonization and multiplication of *E. coli* induces secretion of inflammatory mediators and tissue damage that mobilize leukocytes, especially PMNL to the infection site. Hence, genes related to SCC level and recruitment in the acute stage are expected to play a role in the pathogenesis. In contrast, the comparatively higher SCC in the HH group in the recovery stage may be associated with

genes responsible for a higher inflammatory host response during the acute stage or a weakened ability to heal, or both.

In an older study, healthy dairy cows in early lactation were shown to have a high heritability of blood PMNL numbers (Dettileux et al., 1994). In this study, similar numbers of PMNL were found in blood in the 2 QTL haplotype groups both before and during *E. coli* mastitis (although influenced by biopsy), whereas milk PMNL numbers were not investigated. Hence, these 2 PMNL phenomena (numbers and kinetics) seem to be independent of each other.

Because of blood-derived leukocyte migration toward the udder during mastitis, WBC count will be decreased, resulting in short-term leukopenia in the acute disease stage, followed by leukocytosis in the recovery stage. In our *E. coli* study, both the WBC and PMNL counts followed this pattern during mastitis, which suggests that, in both QTL haplotype groups, these leukocytes were later replaced by newly produced leukocytes from the bone marrow. Interestingly, we observed a much faster recruitment of PMNL (24 h) in the HL group than in the HH group. Whether this PMNL recruitment is associated with the faster recovery in HL cows in terms of a lower SCC remains to be documented. Indeed, a faster recovery could be associated with less tissue trauma caused by lower numbers of PMNL that migrate through the secretory tissue into the milk, and lower concentrations of inflammatory mediators [for instance, tumor necrosis factor α (TNF- α) and proteolytic enzymes released from activated or dead PMNL].

The effects of QTL haplotype on the 2 cow groups' disease phenotype responses were also observed for 2 clinical parameters, as the HH group tended to exhibit a higher body temperature and heart rate than the HL group. Hence, the paraclinical parameters SCC and PMNL supported the clinical observation of a milder clinical response in the HL group. Fever, heart rate, and leukocyte mobilization are induced by proinflammatory mediators such as TNF- α , IL-1, and IL-6 released during the acute-phase response (Vels et al., 2009). Hence, these mediators could be interesting to investigate in a future study. In relation to the acute-phase response in this study, we measured the acute-phase proteins SAA and MAA. Milk amyloid A, also known as SAA isotype 3, is primarily produced in the udder during mastitis (Jacobsen et al., 2005; Molenaar et al., 2009). Serum amyloid A and MAA concentrations are associated with the degree of tissue damage in response to bacterial infection (Cray et al., 2009; Molenaar et al., 2009), where MAA can be further enhanced by udder biopsy (M. Khatun, P. Sørensen, K. L. Ingvarsen, M. Bjerring, and C. M. Røntved, unpublished data). In our study, large variation existed among individual

cows with respect to both SAA and MAA concentrations in blood and milk, respectively. In accordance with our *E. coli* findings we were not able to document a QTL haplotype effect on *E. coli*-induced tissue damage in the mammary gland.

In addition to the factor QTL haplotype, we also investigated the factor biopsy. Biopsy did affect the clinical and paraclinical disease response transiently, including milk yield, *E. coli* shedding, and MAA concentration (M. Khatun, unpublished data). Further, we showed that a biopsy interaction with the QTL haplotype affected body temperature, heart rate, and PMNL, where the effect of QTL haplotype on these disease phenotypes was further increased by liver and udder biopsies in the HH group.

The immune response directed against *E. coli* in the bovine mammary gland primarily involves factors belonging to the innate immune system (Schukken et al., 2011). Recently, we have shown that genes and pathways associated with PMNL migration, such as pathogen-associated molecular pattern (PAMP) receptors, inflammatory cytokines, chemokines, chemokine receptors, and adhesion molecules, are significantly up-regulated in the mammary gland during acute *E. coli* mastitis (Buitenhuis et al., 2011). Further, combining the transcriptome with the phenome and genome, we presented a list of 10 ranked genes likely to be related to mastitis susceptibility for the QTL (Jiang et al. 2012). The list includes factors directly or indirectly associated with PMNL recruitment, such as interferon γ receptor 1, vanin 1, vanin 2, IL-22 receptor α 2, IL-20 receptor α , and TNF α -induced protein 3.

Presently, we are investigating PMNL-related immunological factors and pathways, with altered expression profiles during *E. coli* mastitis that could be linked to the *E. coli*-associated QTL haplotypes. In Danish HF dairy cows, these BTA-9-associated factors are potential genetic targets for future selection of individuals with a milder clinical responsiveness and faster SCC recovery to *E. coli* mastitis.

CONCLUSIONS

Using an experimental *E. coli* mastitis model, we confirmed that QTL haplotypes on BTA9 in Danish HF cows are associated with SCC level during and after *E. coli* mastitis. Cows with the low-susceptibility QTL haplotype had a significantly faster SCC recovery and PMNL recruitment to the circulatory system than the high-susceptibility-QTL-haplotype cows did. Moreover, the low-susceptibility-QTL-haplotype cows tended to show milder a clinical response than the high-susceptibility-QTL-haplotype cows did; the latter group also had an augmented clinical response to *E. coli* mastitis

in the presence of the additional stress of repeated liver and udder biopsying. Knowledge of QTL haplotype effects on clinical and paraclinical mastitis phenotypes provides avenues to gain insight into the genetic influences on *E. coli* mastitis.

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