Genome-wide association study for milking speed in French Holstein cows

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ABSTRACT

Using a combination of data from the BovineSNP50 BeadChip SNP array (Illumina, San Diego, CA) and a EuroGenomics (Amsterdam, the Netherlands) custom single nucleotide polymorphism (SNP) chip with SNP pre-selected from whole genome sequence data, we carried out an association study of milking speed in 32,491 French Holstein dairy cows. Milking speed was measured by a score given by the farmer. Phenotypes were yield deviations as obtained from the French evaluation system. They were analyzed with a linear mixed model for association studies. We identified SNP on 22 chromosomes significantly associated with milking speed. As clinical mastitis and somatic cell score have an unfavorable genetic correlation with milking speed, we tested whether the most significant SNP on these 22 chromosomes associated with milking speed were also associated with clinical mastitis or somatic cell score. Nine hundred seventy-one genome-wide significant SNP were associated with milking speed. Of these, 86 were associated with clinical mastitis and 198 with somatic cell score. The most significant association signals for milking speed were observed on chromosomes 7, 8, 10, 14, and 18. The most significant signal was located on chromosome 14 (*ZFAT* gene). Eleven novel milking speed quantitative trait loci (QTL) were observed on chromosomes 7, 10, 11, 14, 18, 25, and 26. Twelve candidate SNP for milking speed mapped directly within genes. Of these, 10 were QTL lead SNP, which mapped within the genes *HMHA1*, *POLR2E*, *GNB5*, *KLHL29*, *ZFAT*, *KCNB2*, *CEACAM18*, *CCL24*, and *LHPP*. Limited pleiotropy was observed between milking speed QTL and clinical mastitis.

Key words: milking speed, mastitis, bovine, genome-wide association study, pleiotropy

INTRODUCTION

Milking speed (MS) measured as the time taken to milk a cow potentially affects the labor cost per cow. Different devices integrated into milking machines [e.g., LactoCorder (Hoefelmayer and Faerber WMB AG, Balgach, Switzerland, 2007), Bou-Matic (Bou-Matic, Madison, WI), or voluntary milking systems (Davis et al., 2008)] provide objective measures of MS, such as average and maximum milk flow rate, and total milking time. Alternatively, MS can be subjectively scored by the farmer. This system is quite reliable in small- to medium-size herds because of the repeated daily milking work. In France, MS is measured subjectively (Rupp and Boichard, 1999) for genetic evaluation purposes.

The relationship between MS with clinical mastitis (CM) and SCS is complex. A previous study in French Holstein reported positive genetic correlations between MS and CM (0.18) and between CM and SCS (0.70; Govignon-Gion et al., 2016). Similarly, a positive genetic correlation between MS and SCC was reported by several authors [0.44 (Rupp and Boichard, 1999), 0.60 (Gäde et al., 2007), and 0.46 (Samoré et al., 2010)], indicating the existence of an unfavorable relationship between MS and mastitis. Genetic correlation between MS and SCS was similar over 2 lactations, with an average correlation of 0.62 (Boettcher et al., 1998). These results support an association between MS and CM. This relationship is plausible because both traits are affected by the anatomy of the teat canal. Animals with a wider teat canal and sphincter on average show a higher milk flow, but the main disadvantage is that teat canals with a greater diameter simultaneously facilitate access to pathogens (Gäde et al., 2007; Sewalem et al., 2011). Another mechanism explaining this positive correlation is that fast milking extracts more alveolar milk rich in somatic cells (Ferneborg and Svennersten-Sjaunja, 2015). But fast milking cows have a complete flush of milk along with bacteria, which may contribute to preventing CM. Slow milking could lead to increased mastitis incidence due to incomplete

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milk-out, or irritated teat ends because of extended milking time (Dodd and Neave, 1951). Milking speed, therefore, should not be too fast or too slow, as both instances would probably lead to an increased incidence of CM; rather, MS should be at an intermediate optimum (Wiggans et al., 2007).

Heritability of MS is moderate: $h^2 = 0.17$ for 305-d farmer scores in French Holstein (Rupp and Boichard, 1999) and 0.14 in Canadian Holstein (Sewalem et al., 2011). It is higher for measured milking time (e.g., 0.38 in German Holstein; Gäde et al., 2007). These values are moderate and high enough for genetic selection to be effective. Nevertheless, little selection has been done on MS due to potential detrimental consequences on mastitis. Mastitis has negative financial effects on the farmer and biological effects on the cow. Financial effects include milk price penalty for high cell counts, treatment cost, loss of milk sales (due to CM and treatment), loss of production over the rest of the lactation, and increased risk of culling. Biological effects on the cow include udder injury and incomplete udder draining, which contribute negatively to cows' welfare. In the breeding objective, it would be desirable to improve MS without increasing mastitis. The QTL with effects not in line with the genetic correlation (i.e., improving MS without affecting mastitis) would be especially useful to reach this objective. In this study, we report on a genome-wide association study (GWAS) for MS, test the SNP associated with MS for associations with CM and SCS, and explore novel MS QTL and candidate genes associated with MS in French Holstein cattle.

MATERIALS AND METHODS

Study Population

A total of 32,491 cows with all phenotypes (MS, SCS, and CM) and genotypes were included in this study. Milking speed score (1 = slow to 5 = fast) is a subjective appraisal given by the farmer and recorded by the type classifier during classification visit. There was only one score for each cow, obtained in the first half of the first lactation. Somatic cell counts were obtained at each monthly test-day. Somatic cell score was defined in the usual way as $SCS = 3 + \log_2(SCC/100,000)$ and averaged over the lactation. The CM events were declared by the farmer and recorded by the technician at each test-day. The phenotype was recorded as one if the cow had at least one CM incidence in the lactation and zero otherwise. Yield deviations (YD, VanRaden and Wiggans, 1991), that is, performances adjusted for non-genetic effect, were obtained from the French national evaluation system (Boichard et al., 2012b). As SCS and

CM might have repeated records, YD was an average over the lactations. Finally, each cow received one phenotype for each trait. Technically, the phenotypes corresponding to MS, SCS, and CM are trait deviations, and not YD (as would be for milk production traits); however, to conform with the original terminology by VanRaden and Wiggans (1991), we use the term YD.

Cows were genotyped with different types of SNP chip, so imputation process was required to recover complete genotype information. For imputation, the whole genotyped French Holstein population (males (young and old) and females (with and without phenotypes) was used as a reference. Holstein males and females were genotyped with either Illumina BovineSNP50 Beadchip (50k, Illumina, San Diego, CA), or the customized EuroGenomics SNP chip (LD-chip, Amsterdam, the Netherlands). Two successive imputation steps were carried out. In the first step, 43,800 markers from the 50k were imputed, using all 50k genotypes as a reference. This step was carried out as part of the national evaluation procedure, before this study. In the second step carried out for the present study, additional functional variants were imputed, using animals genotyped for the functional variants included in the LD-chip as a reference. The LD-chip is composed of 2 parts: (1) ~8,000 generic (and supposedly neutral) SNP mainly from BovineLD Genotyping BeadChip (Boichard et al., 2012a) and the 50k chip and (2) a custom part selected from whole-genome sequence variants based on different functional arguments: (1) known genetic variants described in literature, (2) potential regulatory variants located in the promoter regions of genes, (3) nonsynonymous variants with strongly deleterious effect on the function of the encoded protein as predicted by the variant effect predictor (McLaren et al., 2010), (4) breakpoints of structural variants affecting genes as described in Boussaha et al. (2015), and (5) variants corresponding to peaks in GWAS analysis of several economic traits in cattle. All SNP with minor allele frequency (MAF) lower than 0.5%, with a call rate lower than 95%, or deviating from Hardy-Weinberg proportions ($P < 10^{-4}$) were deleted. Four versions of this LD-chip were used, with partial overlap between custom parts. A total of 6,035 variants in LD-chip passed quality checks. Imputation was carried out using FImpute (Sargolzaei et al., 2014), for 187,025 genotyped Holstein animals (males and females, with or without performance records). Imputation errors may affect the 50k SNP not present on the LD-chip, and the candidate variants not present on the 50k. However, this consequence was likely small, as the loss in imputation error rate as expressed by allelic correlation was <0.01 . After imputation, 49,835 SNP distributed over 29 BTA remained.

GWAS and QTL Detection

Genome-wide association studies were performed with GCTA software (Yang et al., 2011). A mixed linear model association analysis was used to test associations between individual SNP and cows' MS, CM, and SCS. Because phenotypes were YD already adjusted for nongenetic effects, for each SNP i , the fitted model per trait was

$$\mathbf{y} = \mathbf{1}\mu + \mathbf{x}\beta + \mathbf{g} + \boldsymbol{\varepsilon},$$

where $\mathbf{y}$ was the vector of YD for n cows, μ was a mean, β was the allele substitution effect of SNP i , $\mathbf{x}$ was the vector of genotype dosages (0, 1, or 2) for SNP i , $\mathbf{g}$ was the vector of polygenic effect with $\mathbf{g} \sim N(\mathbf{0}, \mathbf{G}\sigma_g^2)$, and $\boldsymbol{\varepsilon}$ was a vector of the residual effect with $\boldsymbol{\varepsilon} \sim N(\mathbf{0}, \mathbf{I}\sigma_e^2)$, where $\mathbf{I}$ was an $n \times n$ identity matrix. The variance of $\mathbf{y}$ was $\text{var}(\mathbf{y}) = \mathbf{G}\sigma_g^2 + \mathbf{I}\sigma_e^2$, where $\mathbf{G}$ was the genomic relationship matrix between cows, and σ_g^2 and σ_e^2 were the genetic and residual variances. The relationship between cows j and k was

$$g_{jk} = \frac{1}{w} \sum_{i=1}^w \frac{(z_{ij} - 2p_i)(z_{ik} - 2p_i)}{2p_i(1 - p_i)},$$

where w was the total number of SNP, z_{ij} and z_{ik} were the number of copies of the reference allele for the i th SNP for the j th and k th cow, and p_i was the frequency of the reference allele. To control the type I error rate, and assuming each test was independent, a Bonferroni genome-wide correction was applied. A genome-wide threshold was calculated as the ratio of the α -level to total number of tests, that is, $P(T_i|H_0) \leq \frac{\alpha}{w}$, where T_i is the test, H_0 is the null hypothesis, $\alpha = 1\%$, and $w = 49,835$. In this study, $P = 2.01 \times 10^{-7}$ or $-\log_{10} P = 6.70$. A SNP was therefore considered significant if its $-\log_{10} P$ was greater than 6.70.

The criteria for defining a QTL interval were as follows:

1. Select all SNP with a $-\log_{10} P$ equal to or exceeding the genome-wide threshold $-\log_{10} P \geq 6.70$.
2. For each selected SNP, define a 2-Mb region (1 Mb on each side).
3. Within each 2-Mb window, select the most significantly associated SNP as the lead SNP (m) and $x = -\log_{10} P$.
4. Define a new threshold (d) at LOD drop of 2 (in our case we used $-\log_{10} P$ drop of 2), that is, (d

$= x - 2$) and select all SNP whose $-\log_{10} P \geq d$ inside the 2-Mb region.

5. Discard 2 Mb regions with <4 SNP.
6. To determine the boundaries of the QTL regions, merge 2-Mb regions using the following rules:
 - a. Calculate linkage disequilibrium (**LD**) between the lead SNP of window i and the lead SNP of downstream regions using PLINK (Purcell et al., 2007).
 - b. A new region was defined from the starting point of interval i to the endpoint of the nearest downstream region with $\text{LD} > 0.2$, and the lead SNP of the new regions (i.e., the SNP with the lowest P) was identified.
 - c. Repeat a and b until no LD higher than 0.2 exists between any pair of lead SNP on the chromosome and boundaries of QTL i have been identified.

The procedure is repeated for region $i + 1$ until the end of the chromosome has been reached, implying that QTL region will potentially be >2 Mb wide depending on LD and overlap.

For each QTL peak, the SNP with the highest $-\log_{10} P$ was identified as the lead SNP. The MS lead SNP per chromosome and MS QTL SNP for a QTL peak was tested for their association with CM and SCS with the same model described above for MS. The Bonferroni correction was applied by accounting for the number of tests on these traits (i.e., the number of SNP or the number of QTL significant for MS).

Information on significant SNP was obtained from the National Center for Biotechnology Information database of genetic variations (NCBI dbSNP Build 150; Sherry et al., 2001), functional annotation of genes from BioMart at the Ensembl Genome Browser 90 (Kinsella et al., 2011), Animal QTLdb (Hu et al., 2016), and gene ontology mined through Cytoscape (Shannon et al., 2003). The position of each SNP was defined according to the *Bos taurus* genome assembly UMD3.1 (Zimin et al., 2009).

Heritability and Correlations

Heritability coefficients were estimated for the 3 traits from both SNP and pedigree information. The SNP-based variances were calculated using GREML as implemented in the GCTA software, whereas pedigree-based variances were estimated by fitting a polygenic model as implemented in the Bayz software (Janss, 2014). In both instances, the model can be described as

$$\mathbf{y} = \mathbf{1}\mu + \mathbf{a} + \mathbf{e},$$

where $\mathbf{y}$ is the $(n \times 1)$ vector of YD and $n = 32,491$, μ is a common mean, $\mathbf{a}$ is a $(n \times 1)$ vector of additive genetic effects of the individuals, and $\mathbf{e}$ is a vector of random residuals. $\text{Var}(\mathbf{a})$ was assumed to be proportional to the pedigree-based or SNP-based genetic relationship matrix, according to the model assumed.

Genetic correlations were obtained with Bayz in a 3-trait model.

RESULTS

The SNP-based heritability estimate for 3 traits was $h_{MS}^2 = 0.37$, $h_{CM}^2 = 0.02$, and $h_{SCS}^2 = 0.38$. The pedigree-based heritability estimate for the 3 traits was $h_{MS}^2 = 0.44$, $h_{CM}^2 = 0.04$, and $h_{SCS}^2 = 0.40$. For SCS, these high values are in the nature of YD, which are means of repeated measures adjusted for permanent environmental effects (VanRaden and Wiggans, 1991). The pedigree-based genetic correlations were $r_{MS/CM} = 0.16$, $r_{MS/SCS} = 0.47$, and $r_{CM/SCS} = 0.75$.

The SNP array scans for MS revealed significantly associated markers in French Holstein cattle (Figure 1). A total of 971 SNP on 22 chromosomes showed genome-wide significant associations with MS (Supplemental Table S1; <https://doi.org/10.3168/jds.2017-14067>). The associated genomic regions and their lead SNP for MS for 22 chromosomes are presented in Table 1. The lead SNP for associated regions on BTA3, 5, 7, 8, 9, 14, 16, 17, 18, 20, 22, 26, 28, and 29 were located within genes and for BTA2, 10, 11, 12, 13, 19, 21, and 25 in intergenic regions. The top 3 strongest association signals were observed on BTA14 (rs110425867; $P = 1.77 \times 10^{-117}$; *ZFAT* gene), BTA7 (rs41622861; $P = 1.07 \times 10^{-29}$; *POLR2E* gene), and BTA18 (Chr18: 57534701; $P = 2.19 \times 10^{-26}$). The 5 most significant SNP on BTA18 were in the following positions (bp): 57,516,245 (rs110801791), 57,516,702 (rs110572377), 57,517,094 (rs109371542), 57,534,701 (rs381815997), and 57,536,322 (rs378859840).

The most significant SNP per chromosome for CM is presented in Table 2. The 2 most significant SNP

Figure 1. Manhattan plots of whole-genome association analysis for milking speed in French Holstein cattle. The x-axis represents chromosomes. The y-axis represents Bonferroni-corrected $\log_{10}(P)$. Dotted line indicates genome-wide significance threshold at $P < 2.01 \times 10^{-7}$, and different colors represent the QTL region.

Table 1. Lead SNP from significantly associated genomic regions for milking speed in French Holstein cattle

BTA	QTL interval ¹		Lead SNP								
	Start (bp)	End (bp)	Position ² (bp)	MAF ³	Effect allele	Effect size ⁴	SE of effect size	P ⁵	Rsnp ID ⁶	Closest gene	Sequence variant
2	84,734,002	86,110,460	85,362,275	0.16	G	-0.1	0.01	6.55×10^{-12}	rs209624660	<i>HECW2</i>	Intergenic variant
3	119,093,276	121,151,076	120,122,176	0.12	G	0.13	0.01	1.44×10^{-16}	rs42818933	<i>OTOS</i>	Downstream gene variant
5	105,562,079	107,038,059	106,223,457	0.28	G	-0.15	0.02	7.25×10^{-18}	rs135992016	<i>TIGAR</i>	3' Untranslated region variant
7	45,187,871	45,199,967	45,196,738	0.42	T	0.11	0.01	1.07×10^{-29}	rs41622861	<i>POLR2E</i>	Downstream gene variant
8	84,482,438	86,807,838	85,645,138	0.13	T	0.14	0.01	8.86×10^{-23}	rs110752781	<i>IPPK</i>	Intron variant
9	85,806,111	91,133,711	88,469,911	0.04	T	-0.16	0.02	6.11×10^{-8}	rs42760519	<i>PPP1R14C</i>	Intron variant
10	53,836,132	54,043,420	53,949,424	0.12	A	0.16	0.01	1.56×10^{-27}	rs41572676	<i>MNS1</i>	Intergenic variant
11	80,245,328	83,128,297	81,666,608	0.37	G	-0.08	0.01	5.09×10^{-14}	rs110874568	<i>FAM49A</i>	Intergenic variant
12	32,984,636	37,663,436	35,324,036	0.4	C	0.06	0.01	5.66×10^{-7}	rs110566968	<i>SGSG</i>	Intergenic variant
13	59,393,140	62,066,140	60,729,640	0.27	A	0.08	0.01	3.29×10^{-9}	—	<i>ANGPT4</i>	—
14	8,166,449	8,304,533	8,264,685	0.07	C	0.42	0.02	1.77×10^{-117}	rs110425867	<i>ZFAT</i>	Intron variant
16	2,836,923	3,001,315	2,876,485	0.21	C	-0.09	0.01	6.77×10^{-11}	rs109050466	<i>TMCC2</i>	Downstream gene variant
17	73,270,448	75,926,548	74,598,498	0.39	A	-0.07	0.01	9.74×10^{-11}	rs110101282	<i>DGCR2</i>	Upstream gene variant
18	56,561,695	57,557,142	57,534,701	0.03	G	0.27	0.02	2.19×10^{-26}	—	<i>CEACAM18</i>	Downstream gene variant
19	51,525,148	51,829,647	51,581,082	0.15	T	-0.12	0.01	3.13×10^{-16}	rs41924210	<i>MAFG</i>	Synonymous variant
20	46,768,356	47,992,701	47,410,881	0.02	G	0.23	0.03	2.15×10^{-9}	rs110506918	—	Intergenic variant
21	1,442,962	3,937,362	2,690,162	0.15	A	0.08	0.01	7.67×10^{-9}	rs109922786	<i>ATP10A</i>	Intergenic variant
22	58,398,580	61,026,580	59,712,580	0.07	A	0.11	0.02	3.97×10^{-7}	rs109296410	<i>GP9</i>	Downstream gene variant
25	33,293,815	35,212,826	34,596,606	0.39	T	0.08	0.01	6.01×10^{-13}	rs109291657	<i>CCL24</i>	Intergenic variant
26	43,211,435	44,915,718	44,539,739	0.36	C	-0.08	0.01	9.03×10^{-13}	rs109153955	<i>LHPP</i>	Intron variant
28	23,352,482	26,004,682	24,678,582	0.41	G	-0.08	0.01	2.90×10^{-13}	rs42138898	<i>HERC4</i>	Intron variant
29	49,686,981	50,029,423	49,779,067	0.33	A	-0.08	0.01	1.52×10^{-9}	rs42193049	<i>TRPM5</i>	Upstream gene variant

¹QTL interval = QTL interval between the start and the end of the region in one BTA may include more than one 2-Mb region.

²Position = base pair position in the UMD3.1 assembly of the most significant SNP per chromosome.

³MAF = minor allele frequency.

⁴Effect size = allele substitution effect of the SNP.

⁵P = Bonferroni-corrected P.

⁶Rsnp ID = reference SNP identification. The most significant SNP per chromosome.

were on BTA11 (rs109293663; $P = 3.44 \times 10^{-12}$; *KLHL29* gene) and BTA8 (rs109145510; $P = 1.08 \times 10^{-7}$; *NUDT2* gene). The most significant SNP for SCS are presented in Table 3. Twenty-eight lead SNP were significant and 14 of these mapped within genes. The 3 most significant SNP were on BTA15 (rs42396627; $P = 1.03 \times 10^{-26}$; intergenic), BTA20 (rs29016097; $P = 3.54 \times 10^{-20}$; *HMGCS1* gene), and BTA16 (rs109540385; $P = 3.30 \times 10^{-18}$; *ACOT7* gene). None of the lead SNP were lead SNP for more than one of the 3 traits. When we compared proximity of lead SNP with each other between 3 traits (Table 4), we observed 5 chromosomes where the lead SNP were located within 3 Mb of each other. These were (1) MS/CM: BTA12 (1.02 Mb), BTA16 (2.26 Mb), BTA25 (2.18 Mb), and BTA29 (1 Mb); and (2) MS/SCS: BTA25 (2.88 Mb). The allele substitution effects were generally low for all 3 traits for BTA 12 (MS: $b = 0.06$; CM: $b = -0.07$), and BTA29 (MS: $b = -0.08$; CM: $b = -0.05$; SCS: $b = 0.05$). The genes where these lead SNP for SCS mapped included the *TNFSF11* gene on BTA12 (rs109845266, $P = 2.05 \times 10^{-14}$) and the *ME3* gene BTA29 (rs384263462, $P = 7.71 \times 10^{-7}$).

Eleven novel MS QTL mapped by 483 significant SNP were observed on BTA7, 10, 11, 14, 18, 25, and 26 (Table 5). Ten QTL lead SNP mapped within genes, whereas one QTL lead SNP on BTA25 was in an intergenic region >10 kb from nearest gene. Of the 10 lead SNP that mapped to a gene, rs110425867 on BTA14 was the most significant [$P = 1.77 \times 10^{-117}$, MAF = 0.07, effect size = 0.42(0.02), *ZFAT* gene]; the second was rs41622861 on BTA7 [$P = 1.07 \times 10^{-29}$, effect size = 0.11(0.01), MAF = 0.42, *POLR2E* gene]; and the third was rs42843551 on BTA18 [$P = 2.19 \times 10^{-26}$, effect size = 0.28(0.02), MAF = 0.03, *CEACAM18* gene].

The 3 widest MS QTL intervals were on BTA7 (19,220,954, 6.3 Mb with 35 SNP), BTA7 (45,196,738, 4.9 Mb with 37 SNP), and BTA7 (63,533,331, 4.6 Mb with 31 SNP).

The 3 smallest QTL intervals were on BTA14 (38,248,644, 1.5 Mb with 27 SNP), BTA18 (57,534,701, 1 Mb with 13 SNP), and BTA10 (58,318,595, 0.4 Mb with 39 SNP; Figure 2). Two QTL lead SNP selected from imputed whole genome sequence variants in dairy cows mapped directly to gene: *PDGFRB* and *CEACAM18*. Distribution per chromosome of the 11 QTL was such that BTA7 had 3 QTL; BTA10 had 1 QTL; BTA 11 had 1 QTL; BTA14 had 2 QTL; BTA18 had 1 QTL; BTA 25 had 2 QTL; and BTA26 had 1 QTL.

To check for pleiotropy between the MS QTL and significant SNP with SCS and CM, we compared significant GWAS SNP for MS for their effects on SCS and CM. First, we checked if the 971 genome-wide

significant SNP associated with MS were also significant in CM and SCS. One hundred ninety-eight showed association with SCS, and 86 with CM (Figure 3). Second, we calculated the ratio of standardized allele substitution effects (i.e., allele substitution effect of the SNP divided by the genetic standard deviation of the trait) for the MS QTL lead SNP to SCS and CM. We compared the direction of this ratio to the genetic correlation of the traits. Because MS/SCS has a stronger genetic correlation ($r_{MS/SCS} = 0.47$), we focused on ratios for these 2 traits. Of the 11 MS QTL, 4 had ratios not in line with the genetic correlation. These include QTL on BTA7, BTA10, and BTA25 (Table 6). In general, considering all QTL significant for MS, we observed limited pleiotropic effect of MS QTL SNP with CM or SCS (Figure 3).

DISCUSSION

Milking speed is here characterized by farmers' subjective appraisal of time taken to milk a cow on a scale of 1 (slow) to 5 (fast). The trait is recorded and routinely evaluated under the French cattle genetic evaluation system for type traits. Relatively high genomic (0.37) and pedigree heritability (0.44) estimates show that a subjective score based on repeated milking is very accurate under French conditions (i.e., with medium-sized herds). The genetic correlation between MS and CM was low (0.16), whereas the genetic correlation between MS and SCS was moderate (0.47) and high between CM and SCS (0.75). This is similar to those reported by Govignon-Gion et al. (2016) and Rupp and Boichard (1999) in the same population. Correlation between either MS and CM or MS and SCS is unfavorable as both instances increase the incidence of udder infection. Finding MS QTL whose SNP exhibit limited pleiotropic effects with CM and SCS could be beneficial to selecting for faster milking cows. An objective QTL selection logic is therefore paramount. In contrast to a study by Lipkin et al. (2016) that considered P -values only, our QTL selection logic accounted for both P -values and the long-range LD (r^2) usually observed in dairy cattle. In our study, we report 11 MS QTL, of which 4 QTL are in limited pleiotropy with CM and SCS, suggesting that faster milking cows can be selected for, at least with QTL reported here, with only minimal risk of increases in SCS or CM.

Gene ontology (Shannon et al., 2003) on QTL SNP mapped several genes. Two interesting QTL were on BTA14 and mapped *ZFAT* gene, and *KCNB2* gene: (1) *ZFAT* (zinc finger and AT-hook domain containing) is a protein that functions as an immune-related transcriptional regulator (Doi et al., 2011). It has been reported to promote fibroblast survival in model or-

Table 2. Lead SNP from significantly associated genomic regions for clinical mastitis in French Holstein cattle

BTA	Pos ¹ (bp)	MAF ²	Effect allele	Effect size ³	SE of effect size	P ⁴	Rsnp ID ⁵	Gene	Sequence variant
1	37,209,389	0.27	G	-0.06	0.01	3.21×10^{-3}	rs109564354	—	Intergenic variant
2	91,061,862	0.13	C	-0.08	0.01	2.14×10^{-5}	rs110681542	<i>KIAA2012</i>	Intron variant
7	63,935,103	0.29	A	-0.06	0.01	4.12×10^{-5}	rs109359663	—	Intergenic variant
8	77,078,725	0.20	G	0.07	0.01	1.08×10^{-7}	rs109145510	<i>NUDT2</i>	Downstream gene variant
9	102,930,778	0.12	T	-0.07	0.01	1.99×10^{-3}	rs110974178	<i>RPS6KA2</i>	Splice region variant and synonymous variant
11	75,586,338	0.33	C	-0.07	0.01	3.44×10^{-12}	rs109293663	<i>KLHL29</i>	Intron variant
12	33,306,946	0.17	G	-0.07	0.01	5.47×10^{-5}	rs110978166	—	Intergenic variant
13	10,663,054	0.49	G	-0.05	0.01	7.86×10^{-4}	rs110227221	—	Intergenic variant
14	23,421,933	0.17	C	-0.07	0.01	2.86×10^{-5}	rs41722312	—	Intergenic variant
16	4,138,735	0.47	C	-0.04	0.01	5.50×10^{-3}	rs109110164	<i>IKBKE</i>	Downstream gene variant
18	22,728,849	0.49	C	-0.05	0.01	5.40×10^{-4}	—	—	Intergenic variant
20	51,449,833	0.33	C	0.05	0.01	7.56×10^{-5}	rs41948047	—	Intergenic variant
22	41,109,447	0.47	G	0.05	0.01	5.83×10^{-5}	rs42009425	—	Intergenic variant
23	40,703,070	0.33	C	-0.05	0.01	5.82×10^{-4}	rs41641410	—	Intergenic variant
24	12,874,615	0.08	T	-0.08	0.02	6.76×10^{-3}	rs110235797	—	Downstream gene variant
25	31,473,299	0.14	C	0.06	0.01	6.93×10^{-3}	rs109867781	—	Intergenic variant
26	38,169,833	0.46	C	-0.05	0.01	2.06×10^{-3}	rs109408844	<i>EMX2</i>	3' Untranslated region variant
27	29,893,059	0.37	C	-0.05	0.01	5.46×10^{-3}	rs41578732	—	Intergenic variant
29	49,779,067	0.33	G	-0.05	0.01	6.80×10^{-3}	rs42193049	—	Upstream gene variant

¹Position = base pair position in the UMD3.1 assembly of the most significant SNP per chromosome.

²MAF = minor allele frequency.

³Effect size = allele substitution effect of the SNP.

⁴P = P of lead SNP $\leq 10^{-3}$.

⁵Rsnp ID = reference SNP identification. The most significant SNP per chromosome.

Table 3. Lead SNP from significantly associated genomic regions for SCS in French Holstein cattle

BTA	Pos ¹ (bp)	MAF ²	Effect allele	Effect size ³	SE effect size	P ⁴	Rsnp ID ⁵	Gene	Sequence variant
1	147,605,954	0.42	A	0.06	0.01	1.72×10^{-4}	rs110784822	<i>C1H21ORF56</i>	Intron variant
2	71,779,928	0.38	T	0.06	0.01	1.00×10^{-11}	rs108999073	<i>CFAP221</i>	Intron variant
3	90,495,998	0.47	A	0.05	0.01	5.45×10^{-9}	rs41603623	—	Intergenic variant
5	20,900,211	0.09	T	-0.09	0.01	6.58×10^{-5}	rs135435016	<i>CER1</i>	Upstream gene variant
6	89,059,253	0.46	G	0.05	0.01	3.90×10^{-4}	rs110326785	<i>NPFPR2</i>	Missense variant
7	18,707,985	0.43	A	-0.04	0.01	2.59×10^{-6}	rs109543015	—	Downstream gene variant
8	18,086,724	0.21	T	0.06	0.01	5.00×10^{-9}	rs29011096	—	Intergenic variant
9	59,692,848	0.39	A	-0.05	0.01	1.10×10^{-7}	rs43605692	—	Intergenic variant
10	90,484,606	0.15	G	0.08	0.01	1.99×10^{-14}	rs41596528	—	Intergenic variant
11	45,893,893	0.40	C	-0.05	0.01	1.16×10^{-9}	rs41664535	—	Intergenic variant
12	12,750,252	0.27	A	0.07	0.01	2.05×10^{-14}	rs109845266	<i>TNFSF11</i>	Intron variant
13	25,681,327	0.14	G	0.08	0.01	1.38×10^{-12}	rs110720761	<i>KIAA1217</i>	Intron variant
14	16,481,804	0.29	C	0.07	0.01	2.78×10^{-16}	rs42228525	<i>NSMCE2</i>	Intron variant
15	29,113,608	0.34	A	0.08	0.01	1.03×10^{-26}	rs42396627	—	Intergenic variant
16	47,851,773	0.47	G	-0.06	0.02	3.30×10^{-18}	rs109540385	<i>ACOT7</i>	Intron variant
17	63,541,690	0.38	A	-0.05	0.01	6.79×10^{-11}	rs29014800	<i>TPCN1</i>	Intron variant
18	60,854,919	0.13	A	0.06	0.01	5.97×10^{-7}	rs42576394	—	Intron variant
19	7,561,633	0.48	G	-0.05	0.01	2.34×10^{-10}	rs133975572	—	Intergenic variant
20	31,468,943	0.24	G	-0.08	0.01	3.54×10^{-20}	rs29016097	<i>HMGCS1</i>	Intron variant
21	49,533,074	0.37	A	-0.05	0.01	3.41×10^{-10}	rs41643764	<i>SEC23A</i>	Intron variant
22	14,782,972	0.24	T	-0.05	0.01	5.53×10^{-7}	rs42002063	—	Intergenic variant
23	42,621,004	0.35	C	-0.07	0.01	7.53×10^{-16}	—	—	Intergenic variant
24	48,164,849	0.34	G	0.05	0.01	2.35×10^{-8}	rs110994144	<i>ZBTB7C</i>	Synonymous variant
25	36,472,030	0.34	T	0.05	0.01	2.95×10^{-7}	rs110312059	<i>ACTL6B</i>	Downstream gene variant
26	16,536,774	0.14	G	-0.06	0.01	1.13×10^{-5}	rs42091720	—	Intron variant
27	43,851,804	0.45	T	0.05	0.01	1.30×10^{-9}	rs110135286	—	Intergenic variant
28	16,124,333	0.49	C	0.04	0.01	1.34×10^{-3}	rs109325381	—	Intergenic variant
29	9,060,499	0.21	T	0.05	0.01	7.71×10^{-7}	rs384263462	<i>ME3</i>	Intron variant

¹Position = base pair position in the UMD3.1 assembly of most significant SNP per chromosome.

²MAF = minor allele frequency.

³Effect size = allele substitution effect of the SNP.

⁴P = P of lead SNP $\leq 10^{-3}$.

⁵Rsnp ID = reference SNP identification. The most significant SNP per chromosome.

Table 4. Proximity of milking speed lead SNP position compared with clinical mastitis (CM) and SCS in French Holstein

BTA	Lead SNP Pos ¹ (bp)			Effect size ²			P ³		
	MS ⁴	CM	SCS	MS	CM	SCS	MS	CM	SCS
2	84,362,275	91,061,862	71,779,928	−0.1	−0.08	0.06	6.55×10^{-12}	3.21×10^{-3}	1.00×10^{-11}
3	119,122,176		90,495,998	0.13		0.05	1.44×10^{-16}		5.45×10^{-9}
5	105,223,457		20,900,211	−0.15		−0.09	7.25×10^{-18}		6.58×10^{-5}
7	44,196,738	63,935,103	18,707,985	0.11	−0.06	−0.04	1.07×10^{-29}	2.14×10^{-5}	2.59×10^{-6}
8	84,645,138	77,078,725	18,086,724	0.14	0.07	0.06	8.86×10^{-23}	4.12×10^{-5}	5.00×10^{-9}
9	87,469,911	102,930,778	59,692,848	−0.16	−0.07	−0.05	6.11×10^{-8}	1.08×10^{-7}	1.10×10^{-7}
10	52,949,424		90,484,606	0.16		0.08	1.56×10^{-27}		1.99×10^{-14}
11	80,666,608	75,586,338	45,893,893	−0.08	−0.07	−0.05	5.09×10^{-14}	3.44×10^{-12}	1.16×10^{-9}
12 ⁵	34,324,036	33,306,946	12,750,252	0.06	−0.07	0.07	5.66×10^{-7}	5.47×10^{-5}	2.05×10^{-14}
13	59,729,640	10,663,054	25,681,327	0.08	−0.05	0.08	3.29×10^{-9}	7.86×10^{-4}	1.38×10^{-12}
14	7,264,685	23,421,933	16,481,804	0.42	−0.07	0.07	1.77×10^{-117}	2.86×10^{-5}	2.78×10^{-16}
16 ⁵	1,876,485	4,138,735	47,851,773	−0.09	−0.04	−0.06	6.77×10^{-11}	5.50×10^{-3}	3.30×10^{-18}
17	73,598,498		63,541,690	−0.07		−0.05	9.74×10^{-11}		6.79×10^{-11}
18	57,534,701	22,728,849	60,854,919	0.27	−0.05	0.06	2.19×10^{-26}	5.40×10^{-4}	5.97×10^{-7}
19	50,581,082		7,561,633	−0.12		−0.05	3.13×10^{-16}		2.34×10^{-10}
20	46,410,881	51,449,833	31,468,943	0.23	0.05	−0.08	2.15×10^{-9}	7.56×10^{-5}	3.54×10^{-20}
21	1,690,162		49,533,074	0.08		−0.05	7.67×10^{-9}		3.41×10^{-10}
22	58,712,580	41,109,447	14,782,972	0.11	0.05	−0.05	3.97×10^{-7}	5.83×10^{-5}	5.53×10^{-7}
25 ⁵	33,596,606	31,473,299	36,472,030	0.08	0.06	0.05	6.01×10^{-13}	5.82×10^{-4}	2.95×10^{-7}
26	43,539,739	38,169,833	16,536,774	−0.08	−0.05	−0.06	9.03×10^{-13}	6.76×10^{-3}	1.13×10^{-5}
28	23,678,582		16,124,333	−0.08		0.04	2.90×10^{-13}		1.34×10^{-3}
29 ⁵	48,779,067	49,779,067	9,060,499	−0.08	−0.05	0.05	1.52×10^{-9}	6.80×10^{-3}	7.71×10^{-7}

¹Lead SNP = most significant SNP per BTA. Pos (bp) = bp position in the UMD3.1 assembly.

²Effect size = allele substitution effect; SE ≤ 0.01 .

³P = P of lead SNP $\leq 10^{-3}$.

⁴MS = milking speed.

⁵Rows where the lead SNP are <2.5 Mb of each other.

ganisms (Koyanagi et al., 2008; Fujimoto et al., 2009). Fibroblasts are predominant components of mammary stroma (Darcy et al., 2000; Chen et al., 2016) and play crucial roles in the development and involution of the bovine mammary gland (Sladek and Rysanek, 2010). (2) *KCNB2* (potassium voltage-gated channel subfamily B member 2) is a protein-coding gene. The protein is a member of a complex class of voltage-gated ion channel that mediates transmembrane potassium transport in excitable membranes of smooth muscle (Vasan et al., 2007). Other QTL SNP mapped within several genes. These included QTL on BTA7: *PLOR2E2* [MAF = 0.4, allele substitution effect (b) = 0.11(0.01)], *PDGFRB* [MAF = 0.29, b = 0.11(0.01)], QTL on BTA14: *LRRC6* [MAF = 0.08, b = 0.29(0.02)], and QTL on BTA18: *CEACAM18* [MAF = 0.03, b = 0.28(0.02)]. The MAF for some of the SNP mapping to genes were low, and allele substitution effects were high. For instance, rs42843551 was a lead SNP in a QTL with 13 significant SNP within a 1-Mb region. Among its mapped genes include *IL4I1* gene and *CEACAM18* gene (carcinoembryonic antigen-related cell adhesion molecule). The *CEACAM18* gene is of interest because it is a primordial member of the carcinoembryonic antigen family and reported to serve as a pathogen receptor in cattle (Kammerer et al., 2004), part of the

immune system. The main immune system cell types are lymphocytes (Janeway et al., 2001). Several studies using model organisms and cattle data have shown that bovine peripheral blood lymphocytes express only immunoglobulin receptor family tyrosine-based inhibitory motifs containing *CEACAM18* isoforms, and upregulate their expression upon stimulation, suggesting an inhibitory function in these cells (Donda et al., 2000; Markel et al., 2002). One such factor that plays a major role in regulating milk secretion in cattle (Wilde et al., 1997) and goats (Li et al., 1999) is a feedback inhibitor of lactation found in milk. Feedback inhibitor of lactation is thought to be produced by the mammary cells as they synthesize and secrete milk (Wilde et al., 1997; Janeway et al., 2001). Accumulation of feedback inhibitor of lactation in the milk-producing alveoli results in feedback inhibition of milk synthesis and secretion (Wilde et al., 1990), thus increasing the time taken to milk a cow. The *IL4I1* gene (interleukin 4 induced 1) on BTA18: 56,691,667–56,725,849 is a protein-coding gene whose related pathways include tyrosine metabolism. Tyrosine regulates mammary epithelial cell proliferation by activating the AKT serine/threonine kinase 1 (*AKT1*) gene at the transcriptional level in mammary glands of dairy cows (Crompton et al., 2014; Hou et al., 2016). The *ATK1* gene is important for the mam-

Table 5. Eleven novel QTL in chromosome 7, 10, 11, 14, 18, 25, and 26 for milking speed in French Holstein

BTA ¹	Pos ² (bp)	Effect allele	MAF ³	Effect size ⁴	Effect size SE	P ⁵	SNP in region	Start (bp)	End (bp)	Gene ⁶
7	19,220,954	G	0.47	0.09	0.01	2.55 × 10 ⁻²⁷	27	19,188,895	19,381,937	TUBB4A
7	45,196,738	T	0.4	0.11	0.01	1.07 × 10 ⁻²⁹	15	45,187,871	45,199,967	POLR2E
7 ⁷	63,533,331	A	0.29	0.11	0.01	6.34 × 10 ⁻²³	17	63,407,820	63,549,703	PDGFRB
10	58,318,595	G	0.1	0.17	0.02	7.34 × 10 ⁻²⁶	32	58,026,405	58,324,001	GNB5
11	75,329,413	T	0.26	-0.08	0.01	3.19 × 10 ⁻⁰⁹	11	74,895,799	75,623,195	KLHL29
14	8,264,685	T	0.07	0.42	0.02	1.77 × 10 ⁻¹¹⁷	5	8,166,449	8,304,533	ZEAT
14	38,248,644	A	0.4	-0.1	0.01	4.93 × 10 ⁻²²	6	38,021,172	38,454,345	KCNB2
18 ⁷	57,534,701	CCA	0.03	0.28	0.02	2.19 × 10 ⁻²⁶	13	56,561,695	57,557,142	CEACAM18
25	6,292,680	T	0.38	-0.08	0.01	2.81 × 10 ⁻¹²	13	5,728,001	6,686,788	RBFOX1
25	34,596,606	C	0.39	0.08	0.01	6.01 × 10 ⁻¹³	13	33,293,815	35,212,826	CCL24
26	44,539,739	T	0.36	-0.08	0.01	9.03 × 10 ⁻¹³	18	43,211,435	44,915,718	LHPP

¹BTA = *Bos taurus* autosome UMD3.1 assembly.
²Position = base pair position in BTA.
³MAF = minor allele frequency.
⁴Effect size = allele substitution effect of the SNP.
⁵P = P of lead SNP.
⁶Gene = closest gene mapped by QTL lead SNP.
⁷Rows where the lead SNP was selected from whole-genome sequence data.

mary remodeling process in dry cows as well as for increasing persistency of lactation in lactating cows (Hou et al., 2016).

General GWAS results as presented in Figure 1 and Table 1 show clear peaks on BTA5 (*TIGAR* gene), BTA8 (*IPPK* gene), and BTA10 (*MNS1* gene). Similarly, Jardim et al. (2018) reported QTL for MS on several chromosomes including BTA5, BTA8, and BTA10. These QTL overlapped with the significantly associated regions identified in our study. The TP53 induced glycolysis regulatory phosphatase (*TIGAR*) is a p53-regulated gene. The p53 is a stress-induced transcription factor that controls various cellular response mechanisms including cell cycle arrest and apoptosis (Li and Jögl, 2009). Its expression can influence the release of oxytocin response during pregnancy and lactation in model organisms (Soloff and Wieder, 1983) and oxytocin is released in cows for the letdown of milk (Watters et al., 2015). The timing of oxytocin release relative to milk removal is an important factor affecting milk ejection and persistency during lactation (Nostrand et al., 1991). *POLR2E* and *IPPK* are protein-coding genes. They are associated with the canonical pathway glucocorticoid receptor signaling. This is of interest because glucocorticoid receptor signaling exerts anti-inflammatory effects on mammary cells during injury, as would be caused by a bacterial infection (Smoak and Cidlowski, 2004). Based on all these known functions, these genes can be considered as functional candidate genes.

Furthermore, lead SNP among MS, CM, and SCS were different (Table 4). The allele substitution effects were similar for the 2 lead SNP: *TNFSF11* gene on BTA12 (rs109845266) and *ME3* gene on BTA29 (rs384263462). The *TNFSF11* is a protein-coding gene with main gene ontology biological functions including calcium ion homeostasis, mammary gland alveolus development, bone resorption, calcium-mediated signaling, and immune response (Yates et al., 2016). The *ME3* gene is a malic enzyme involved in pyruvate metabolism. Previous studies have shown that pyruvate accumulation caused by inhibition of lipid metabolism can cause hypoxia signaling in mastitis in cattle (Genini et al., 2011). The *TNFSF11* had positive substitution effects for SCS and MS, whereas *ME3* had a positive substitution effect for SCS only.

CONCLUSIONS

Increased MS is of economic interest in cows because milking is a major component of the workload of the farmer. But increased MS should be obtained without deteriorating mastitis resistance, or even better while improving mastitis resistance. In the French Holstein,

Figure 2. Eleven novel QTL for milking speed in French Holstein cattle as observed in chromosome 7, 10, 11, 14, 18, 25, and 26. Color version available online.

MS accounts for 5% of the total merit index, whereas udder health (SCS and CM) accounts for 18%. The GWAS SNP from whole genome sequence variants as incorporated in the EuroGenomics chip and 50k SNP data identified several variants that likely influence MS in French Holstein cattle. We identified several candi-

date genes for MS including *HMHA1*, *POLR2E*, *GNB5*, *KLHL29*, *ZFAT*, *KCNB2*, *CEACAM18*, *CCL24*, *LHPP*, *PDGFRB*, *TIGAR*, and *IL4I1*. We also identified 11 MS QTL, of which 4 may allow for selecting for increased MS without increased incidence of CM. Finding quantitative trait nucleotides is challenging, as there

Figure 3. The plot of standardized allele substitution effects for significant genome-wide association study SNP associated with milking speed (MS), clinical mastitis (CM), and SCS. Left indicates a change in CM; right indicates a change in SCS. Color version available online.

Table 6. Comparison of direction of effect size of milking speed QTL lead SNP against SCS and clinical mastitis (CM)

BTA ¹	Pos ² (bp)	Effect size ³			Standardized effect size ⁴			Ratios of effect size		Genetic correlation	
		MS ⁵	CM	SCS	MS	CM	SCS	MS/CM	MS/SCS	MS/CM	MS/SCS
7	19,220,954	9.46 × 10 ⁻²	1.12 × 10 ⁻³	2.43 × 10 ⁻²	2.70 × 10 ⁻¹	1.86 × 10 ⁻²	4.86 × 10 ⁻²	14.5	5.56	0.16	0.47
7	45,196,738	1.11 × 10 ⁻¹	-1.95 × 10 ⁻²	-1.53 × 10 ⁻³	3.18 × 10 ⁻¹	-3.25 × 10 ⁻¹	-3.05 × 10 ⁻³	-0.98	-104	0.16	0.47
7	63,533,331	1.10 × 10 ⁻¹	8.91 × 10 ⁻³	-2.32 × 10 ⁻⁴	3.15 × 10 ⁻¹	1.49 × 10 ⁻¹	-4.65 × 10 ⁻⁴	2.12	-677	0.16	0.47
10	58,318,595	1.75 × 10 ⁻¹	3.85 × 10 ⁻²	-3.31 × 10 ⁻²	5.00 × 10 ⁻¹	6.42 × 10 ⁻¹	-6.61 × 10 ⁻²	0.78	-7.56	0.16	0.47
11	75,329,413	-7.69 × 10 ⁻²	-2.25 × 10 ⁻²	-2.02 × 10 ⁻²	-2.20 × 10 ⁻¹	-3.75 × 10 ⁻¹	-4.04 × 10 ⁻²	0.59	5.44	0.16	0.47
14	8,264,685	4.17 × 10 ⁻¹	-1.44 × 10 ⁻²	4.92 × 10 ⁻²	1.19 × 10 ⁻⁰	-2.39 × 10 ⁻¹	9.84 × 10 ⁻²	-4.98	12.1	0.16	0.47
14	38,248,644	-9.87 × 10 ⁻²	1.35 × 10 ⁻²	-9.64 × 10 ⁻³	-2.82 × 10 ⁻¹	2.25 × 10 ⁻¹	-1.93 × 10 ⁻²	-1.25	14.6	0.16	0.47
18	57,534,701	2.81 × 10 ⁻¹	-8.45 × 10 ⁻²	5.57 × 10 ⁻²	8.02 × 10 ⁻¹	-1.41 × 10 ⁻⁰	1.11 × 10 ⁻¹	-0.57	7.20	0.16	0.47
25	6,292,680	-7.95 × 10 ⁻²	8.24 × 10 ⁻³	1.56 × 10 ⁻²	-2.27 × 10 ⁻¹	1.37 × 10 ⁻¹	3.12 × 10 ⁻²	-1.65	-7.28	0.16	0.47
25	34,596,606	7.85 × 10 ⁻²	9.59 × 10 ⁻³	3.80 × 10 ⁻²	2.24 × 10 ⁻¹	1.60 × 10 ⁻¹	7.61 × 10 ⁻²	1.40	2.95	0.16	0.47
26	44,539,739	-7.98 × 10 ⁻²	9.60 × 10 ⁻³	-2.96 × 10 ⁻³	-2.28 × 10 ⁻¹	1.60 × 10 ⁻¹	-5.92 × 10 ⁻³	-1.43	38.5	0.16	0.47

¹BTA = *Bos taurus* autosome UMD3.1 assembly.

²Position = base pair position in BTA.

³Effect size = allele substitution effect of the SNP.

⁴Standardized effect size = allele substitution effect of the SNP divided by the genetic SD of the trait.

⁵MS = milking speed.

are many regions of LD and small-effect QTL. The QTL reported on this study can be used to augment the dairy cattle QTL database for better prediction accuracy in routine genomic selection. Further study is needed to find an optimum MS to avoid any negative effect on mastitis incidence.

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