Relationship between *Escherichia coli* virulence factors and postpartum metritis in dairy cows

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ABSTRACT

The objectives of this study were to report the prevalence of *Escherichia coli* and *Trueperella pyogenes* in the uterus of postpartum dairy cows before the onset of postpartum metritis (PPM) and to quantify their association with subsequent occurrence of PPM, to quantify the association between the presence of genes encoding *E. coli* virulence factors (VF) and PPM, and to determine the accuracy of using early postpartum uterine bacteriology results (bacteria and VF) to identify cows at risk of PPM. A prospective cohort study was conducted on 3 commercial dairy farms. Uterine swabs were collected from 371 Holstein dairy cows (3 commercial herds) at 1 to 7 d in milk and submitted to the laboratory for identification of *E. coli*, *T. pyogenes*, and *E. coli* VF. A total of 40 VF were tested using the radioactive probe hybridization method. Postpartum metritis was defined as the presence of a fetid watery red-brown uterine discharge, associated with fever (rectal temperature >39.5°C), and systemic signs of illness (dullness, reduced appetite, and milk production). Surveillance of PPM was done by trained farmers blinded to laboratory results and cows were followed until 21 d in milk. Statistical analyses were conducted using 2 × 2 tables and mixed logistical regression models. Prevalences of *E. coli*, *T. pyogenes*, and PPM were 42, 34, and 15%, respectively. A total of 32 VF were found in *E. coli* isolates. Most prevalent VF were extraintestinal pathogenic genes such as *fimH* (89%), *hlyE* (87%), and *iss* (70%). Cows positive for intrauterine *E. coli* were 3.2 times more likely to have subsequent PPM compared with bacteriologically negative cows. Cows with VF *hra1* in their uterus were 2.7 times more likely to have PPM than cows positive for *E. coli* and negative for *hra1* and 5.9 times more likely than bacteriologically negative cows. Cows with VF *kpsMTII* in their uterus were 3.2 times more likely to have PPM than

cows positive for *E. coli* and negative for *kpsMTII* and 6.2 times more likely than bacteriologically negative cows. Using *E. coli*, *hra1*, and *kpsMTII* as predictors for subsequent PPM, positive predictive values were 23, 31, and 42%, respectively, whereas the negative predictive values were 91, 80, and 78%, respectively. Overall, these results showed that *E. coli* and some VF were associated with PPM.

Key words: *Escherichia coli*, virulence factors, metritis, dairy cow

INTRODUCTION

A wide variety of bacterial species are present in the uterine lumen of dairy cows during early postpartum period (Jeon et al., 2015; Wagener et al., 2015). These include *Escherichia coli*, *Trueperella pyogenes*, streptococci, staphylococci, *Pseudomonas* spp., *Clostridium* spp., and various gram-negative anaerobes such as *Fusobacterium necrophorum* and *Prevotella melaninogenica* (Williams et al., 2005; Werner et al., 2012; Sens and Heuwieser, 2013; Wagener et al., 2014, 2015). The presence of these bacteria varies over time during the postpartum period and the overall population generally declines during the first 50 d after parturition (Sheldon et al., 2002). However, in a proportion of cows, some of these bacterial species persist in the uterine lumen and can favor the development of postpartum uterine diseases such as metritis and endometritis (Sheldon, 2004).

Postpartum metritis (PPM) is a severe inflammation of all layers of the uterus, (endometrium, submucosa, muscularis, and serosa) and is caused by a bacterial infection in the lumen of the uterus (Bondurant, 1999). The disease generally occurs during the first 21 d after parturition in dairy cattle and is defined by the presence of a fetid watery red-brown vaginal discharge with fever, body temperature being >39.5°C, and systemic signs of illness, such as dullness and anorexia (Drillich et al., 2001). Bacterial species frequently associated with the occurrence of PPM in dairy cows are *E. coli* and *T. pyogenes* (Sheldon et al., 2010; Werner et al.,

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2012; Sens and Heuwieser, 2013; Wagener et al., 2014), and obligate anaerobes such as *F. necrophorum* and *P. melaninogenicus* (Williams et al., 2005). The specific role of each of these bacterial species in the pathogenesis of PPM is still not well understood. However, it has been suggested that *E. coli* could play an important role in this process as it is frequently found in the uterus in early postpartum period, and its presence has been associated with the increased prevalence of other bacterial species (Dohmen et al., 2000; Williams et al., 2007; Bicalho et al., 2012) and the occurrence of severe uterine lesions and subsequent infertility (Sheldon et al., 2002; Williams et al., 2007).

Some recent studies focused on intrauterine *E. coli* and demonstrated that the genes encoding certain *E. coli* virulence factors (VF), such as *cdt*, *astA*, *ibeA*, *hlyA*, *hlyE*, *fyuA*, and *fimH*, are associated with PPM (Bicalho et al., 2010, 2012). It was suggested that the products of these genes may mediate induction of lesions in the uterine mucosa and promote the growth of opportunistic bacterial species, such as *T. pyogenes* and *F. necrophorum*, which would ultimately induce clinical signs of PPM (Bicalho et al., 2012). The disease was associated with negative effects in dairy cows, such as reduced milk production and lower reproductive performances (Toni et al., 2015; Piccardi et al., 2016).

Some studies have attempted to evaluate the beneficial effect of treating postpartum dairy cows to prevent PPM and avoid the impairment of productive and reproductive performances. Although Drillich et al. (2006) found that treating postpartum cows with metritis did not have a significant effect on milk production and reproductive performance, Risco and Hernandez (2003) revealed that treating postpartum cows affected by retained placenta significantly decreased the prevalence of subsequent PPM but did not improve subsequent reproductive performance. These results suggest that if a treatment is applied before the onset of clinical signs, PPM could potentially be prevented. Therefore, early identification of cows with high risk of developing PPM could help farmers to make a decision on whether or not to treat the cows. As *E. coli* colonizes the uterus immediately after parturition (Dohmen et al., 2000; Bicalho et al., 2012), and some *E. coli* VF have been associated with PPM (Bicalho et al., 2010, 2012), early identification of postpartum cows positive for intrauterine *E. coli* and positive for certain *E. coli* VF could inform on the risk level of cows for developing PPM.

Early postpartum uterine bacteriological analysis, including detection of *E. coli* VF, is readily available to veterinary practitioners and could be used for early identification of high-risk cows. Unfortunately, few data are available to allow the validation of such an

approach. Therefore, the objectives of this study were (1) to report the prevalence of *E. coli* and *T. pyogenes* in the uterus of postpartum dairy cows before the onset of PPM and quantify their association with subsequent occurrence of PPM, (2) to quantify the association between *E. coli* VF and the subsequent development of PPM, and (3) to determine the accuracy of using early postpartum uterine bacteriology results (bacteria and VF) to identify cows at risk of PPM.

MATERIALS AND METHODS

Farm Selection and Sample Collection

A prospective cohort study was conducted from November 2011 to June 2012 on 3 commercial Holstein dairy farms. Convenient farm selection was used and based on motivation of the farmers to diagnose and report uterine diseases, as well as on convenience of being located within 30 km of the bovine ambulatory clinic of the Faculté de médecine vétérinaire, Université de Montréal (St-Hyacinthe, QC, Canada). Participating herds had computerized health event records, and were enrolled on a biweekly (every other week) herd health veterinary program and a monthly testing of dairy herd improvement program. All farms had freestall housing for cows.

Farms were visited weekly by a research technician and a veterinarian. All sampling was done by the same research technician. During farm visits, all cows between 1 and 7 d after parturition that did not yet show clinical signs of PPM were sampled by the research technician. An estimated sample size of 360 cows was targeted for the study. Sample size calculation was based on objective 2 to find a significant difference (error α : 5%; error β : 20%) in PPM prevalence between cows carrying *E. coli* positive for a specific VF (PPM prevalence: 35%) and those not carrying such *E. coli* (PPM prevalence: 15%), and expecting a prevalence of 20% for this VF in the studied population. Enrolled cows were sampled once during the study. For uterine sampling, cows were restrained and the perineum was cleaned and disinfected with 70% ethyl alcohol solution (Isopropyl Alcohol 70% USP; Green Field Inc., Brampton, ON, Canada). A sterile double-guarded uterine swab (Guarded culture swab; Jorvet Inc., Loveland, CO) was introduced in the vagina until it reached the body of the uterus. The swab was then exposed to the dorsal aspect of the uterine wall and pulled back inside its sheath. The swab was then placed in an anaerobic transportation medium (BBL Port-A-Cult Tubes; Becton, Dickinson and Company, Sparks, MD) and kept at 4°C until submission to the veterinary diagnostic laboratory of the Université de Montréal within 12 h of collection.

Procedures of sample collection were approved by the animal care committee of the Université de Montréal.

Bacteria Isolation

For isolation of *T. pyogenes*, uterine swabs were plated on Colombia blood agar (Oxoid, Ottawa, ON, Canada) and incubated aerobically at 35°C for 48 h. Beta-hemolytic, catalase-negative minuscule colonies demonstrating gram-positive coryneform rods identified as *T. pyogenes* were isolated according to the PON-BAC-019 procedure (McVey et al., 2013) commonly used in the veterinary diagnostic laboratory of the Université de Montréal. For isolation of *E. coli*, swabs were plated on blood agar and MacConkey agar (Oxoid) at 37°C. At the World Organisation for Animal Health (OIE) Reference Laboratory for *Escherichia coli* (Faculté de médecine vétérinaire, Université de Montréal), 5 *E. coli* colonies from each positive uterine sample were streaked on blood agar for isolation and further identification. Isolates were submitted to indole spot, Simmons citrate, and motility tests, for confirmation of *E. coli*. *Escherichia coli* isolates were stored in tryptic soy broth containing 30% glycerol at -80°C (Becton, Dickinson and Company) for further analysis.

Identification of *E. coli* VF by Colony Hybridization

Virulence factors were detected in isolates by colony hybridization using radioactively labeled (³²P) DNA probes. Procedures were performed according to the protocol of the OIE Reference Laboratory for *Escherichia coli* as described previously (Maluta et al., 2014; Fairbrother et al., 2015). Briefly, *E. coli* cultures in tryptic soy broth of the isolates were spotted onto Luria-Bertani agar and incubated at 37°C overnight. Colonies were then transferred to Whatman 541 filter paper (Whatman, Piscataway, NJ). The filter papers were processed, hybridized, and visualized by autoradiography.

Probes were derived from *E. coli* control strains by uniplex PCR, using the primers of the tested genes. After amplification, PCR products were purified and concentrated, using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. After purification, probes were marked with phosphate 32 (³²P) using a specific kit (Amersham Ready to go DNA Labeling Beads, GE Healthcare UK Limited, Little Chalfont, UK), according to the manufacturer's instructions.

All selected *E. coli* isolates (5 isolates from each positive uterine sample) were examined for the presence of genes encoding 40 VF using colony hybridization

techniques. Some VF tested were those related to diarrheagenic *E. coli* pathotypes (ETEC, STEC, EPEC, EHEC), and others were related to extraintestinal pathogenic *E. coli* (ExPEC). *Escherichia coli* isolates having the same VF profile in a given sample were considered as the same isolate. The complete list of VF tested is available in Table A1.

PPM Diagnosis

Before the start of the study and every 3 mo during data collection, the definition of PPM was reviewed and standardized with participating farmers. Postpartum metritis was defined as the presence of a fetid watery red-brown uterine discharge, associated with fever (rectal temperature >39.5°C), and systemic signs of illness (dullness, reduced appetite, and milk production) within the first 21 d after parturition (Drillich et al., 2001). One person per farm was assigned the task to clinically diagnose PPM and to report it in individual health records. Farmers were blinded to laboratory results. When PPM was diagnosed, cows were treated based on the usual PPM treatment protocol, which was 5 d of ceftiofur IM SID (2.2 mg/kg; Zoetis Animal Health, Kirkland, QC, Canada) for all farms. If cows were diagnosed with PPM before uterine bacteriological swabbing, cows were not enrolled in the study.

Statistical Analyses

Statistical analyses were performed using SAS (version 9.3; SAS Institute Inc., Cary, NC). The experimental unit of the study was the cow and postpartum metritis was considered as the dependent variable. Individual data, such as parity and date of calving, were exported from the computerized record system into SAS. Cows were classified in 2 categories for parity groups: category 1 for primiparous and category 2 for multiparous. For calving season, cows that calved from November to February were classified as having calved during the winter season, whereas those which calved from March to June were classified as having calved in the spring season. Descriptive statistics were calculated using the FREQ procedure. Significance was declared when $P \leq 0.05$.

Univariable Analyses. Univariable analyses (Chi-squared; FREQ procedure) were performed to screen for associations between (1) bacterial presence (*E. coli* and *T. pyogenes*) and PPM, and (2) *E. coli* VF and PPM. Variables with $P \leq 0.25$ were retained for multivariable modeling.

Association Between Bacterial Presence (*E. coli* and *T. pyogenes*) and PPM. Cows were

grouped according to the presence or absence of each bacterial species. Univariable associations between PPM and bacterial species were tested.

Association Between *E. coli* VF and PPM.

In this analysis, cows were classified in 3 categories: 0 included cows negative for *E. coli*, 1 included cows positive for *E. coli* and absence of VF in all *E. coli* isolates, and 2 included cows culture-positive for *E. coli* and with a VF was present in at least 1 *E. coli* isolate.

Multivariable Analyses. Multivariable logistic regression models (GLIMMIX procedure) were performed to evaluate the associations between prescreened ($P \leq 0.25$) independent variables and PPM. One final model was built for bacterial species. Another final model was built for *E. coli* VF. The reference group for the latter was the group of cows negative for *E. coli*. A backward elimination strategy was used to build the final models until only variables with $P \leq 0.05$ were retained. A post-hoc Tukey-Kramer test was used to identify specific significant associations between all categories (cows negative for *E. coli*, cows positive for *E. coli* and negative for VF, and cows positive for both *E. coli* and VF). All final models accounted for the effect of herd clustering (random effect) and parity group and season (forced variables). Finally, the accuracy of using final model predictors (bacterial species and VF) was calculated individually or in combination for predicting PPM using sensitivity, specificity, and positive and negative predictive values (FREQ procedure).

RESULTS

Descriptive Statistics

A total of 371 postpartum dairy cows were sampled and enrolled in this study. Samples were collected from 3 freestall farms with farm size ranging from 200 to 240 lactating cows (117, 150, and 104 samples from farms 1, 2 and 3, respectively). Sampled cows were primiparous ($n = 133$; 36%) or multiparous ($n = 238$; 64%) animals. Time after calving at which clinical signs of PPM were observed ranged from 3 to 20 d, with an average of 7 ($SD \pm 3$) days. The overall prevalence of PPM in this study was 15% ($n = 56$); this prevalence was higher in primiparous cows (24%) than in multiparous cows (10%; $P < 0.01$). Cows that calved in the winter season also had higher prevalence of PPM (18%) than cows that calved in the warm season (10%; $P = 0.03$). Overall, 42% ($n = 156$) of uterine samples were positive for *E. coli*, 127 (34%) of the samples were positive for *T. pyogenes*, and 42% ($n = 157$) of uterine samples were bacteriologically negative (Table 1).

A total of 32 VF were identified in the 780 *E. coli* isolates. A total of 214 different VF profiles were identi-

fied from 413 *E. coli* isolates. The number of VF present in any one isolate varied from 1 to 15. Overall, the most prevalent VF among the 413 *E. coli* isolates were *fimH* (89%; $n = 367$) and *hlyE* (87%; $n = 359$), which encode for bacterial adherence and hemolysin toxins, respectively. Other genes encoding for bacterial adhesions, such as *hra1* (21%; $n = 86$) and *papC* (9%; $n = 37$), were also found. Genes encoding virulence factors representing diarrheagenic *E. coli* (ETEC, EPEC, STEC) such as *east1* (17%; $n = 70$), *sta* (9%; $n = 37$), *LT*, *F5*, *eae*, *stx1*, *stx2*, and *stb* (0%) were poorly represented. Isolates were positive for one or more VF representing EPEC, such as *sitA* (23%; $n = 95$), *fepC* (20%; $n = 82$), *irp2* (11%; $n = 45$), *iutA* and *iucD* (15%; $n = 61$), and *cdtB* (12%; $n = 49$). A visual summary of the VF found in the uterine *E. coli* in the present study is shown in Figure 1.

Univariable Associations

Association Between Bacterial Species and PPM. The prevalence of PPM in cows that were positive for *E. coli* (23%) or *T. pyogenes* (21%) at 1 to 7 DIM was higher than in cows bacteriologically negative (10%; $P < 0.01$; Table 2).

Association Between *E. coli* VF and PPM. All 32 VF tested for univariable associations with PM had P -values ≤ 0.25 . All these variables were used for multivariable modeling.

Multivariable Associations

Multivariable Association Between Bacterial Species and PPM. Controlling for farm, season, and parity group effects, *E. coli* was the only bacterium associated with PPM. Cows infected with intrauterine *E. coli* at 1 to 7 DIM had 3.1 times (95% CI: 1.7–5.9; $P < 0.01$) the odds of having PPM compared with cows that were bacteriologically negative at sampling. Least squares means of PPM from this association were 8.7% (SEM = 3.3) and 23.1% (SEM = 7.0).

Multivariable Association Between *E. coli* VF and PPM. Controlling for herd, season, and parity group effects, the VF *hra1* and *kpsMTII* were the only

Table 1. Bacterial species isolated from the uterus of Holstein dairy cows from 3 farms at 1 to 7 DIM

Farm number	Cow (n)	<i>Escherichia coli</i> [n (% by farm)]	<i>Trueperella pyogenes</i> [n (% by farm)]
1	117	50 (43)	34 (29)
2	150	64 (43)	57 (38)
3	104	42 (40)	36 (35)
Total	371	156 (42)	127 (34)

Figure 1. Presence of 32 virulence factors found in intrauterine *Escherichia coli* isolates from 163 Holstein dairy cows sampled at 1 to 7 DIM (figure is sorted by postpartum metritis status). Tp = *Trueperella pyogenes*.

Figure 2. Least squares means ($\pm$ SEM) of postpartum metritis prevalence stratified by bacterial uterine status at 1 to 7 DIM in 371 Holstein dairy cows enrolled in an observational study.

variables associated with PPM that were retained in the final model. Cows positive for intrauterine *E. coli* carrying *hra1* had 2.7 times (95% CI: 1.3–6.4; $P = 0.02$) the odds to be diagnosed with PPM than cows positive for *E. coli* that did not carry *hra1* and 5.9 times (95% CI: 2.7–10.2; $P < 0.01$) the odds compared with cows negative for *E. coli*. Cows positive for intrauterine *E. coli* carrying *kpsMTII* had 3.2 times (95% CI: 1.1–13.3; $P = 0.04$) the odds to be diagnosed with PPM than cows positive for *E. coli* that did not carry *hra1* and 6.2 times (95% CI: 3.1–15.3; $P < 0.01$) the odds compared with cows negative for *E. coli*. Figure 2 presents the least squares means of PPM from this model.

The accuracy of finding *E. coli*, *hra1*, *kpsMTII*, or a combination of these VF to predict subsequent PPM was calculated. Our results showed that 31% of cows positive for intrauterine *E. coli* carrying *hra1* at 1 to 7 DIM developed PPM in wk 2 or 3 postpartum, whereas 80% of cows negative for intrauterine *E. coli* carrying *hra1* did not develop PPM during this time period. For *kpsMTII*, 42% of cows positive for intrauterine *E.*

coli carrying *kpsMTII* developed PPM in the coming weeks, whereas 78% of cows negative for *kpsMTII* did not (Table 3).

DISCUSSION

The results of the present study showed that the presence of *E. coli* was associated with greater odds of developing PPM. More specifically, it showed that the presence of *E. coli* in the uterus of cows at 1 to 7 DIM increased by 3.1 times the odds of having subsequent PPM compared with negative cows in the multivariable analysis after accounting for the effects of farm, season, and parity group. These results were expected and consistent with some previous publications (Sheldon et al., 2010; Werner et al., 2012; Sens and Heuwieser, 2013; Wagener et al., 2014). However, they differ from the ones reported by Santos et al. (2011) and Jeon et al. (2015), who did not find any association between *E. coli* and PPM using metagenomic analysis. Nonetheless, it is important to keep in mind that these 2 studies compared intrauterine bacterial species, including some that are uncultivable in a traditional laboratory from 2 distinct groups of cows, one with PPM and the other without PPM. In our study, standard microbiology culture procedures were used, which may explain this discrepancy with metagenomics studies, as some uncultivable bacteria might have stronger relationships with PPM than *E. coli*. Also, sampling for *T. pyogenes* at only one time point between 1 and 7 DIM might not be ideal because the uterine flora changes over time (Williams et al., 2005, 2007). It is also important to keep

Table 2. Univariable association between the presence of bacterial species in the uterus of Holstein dairy cows at 1 to 7 DIM and the development of postpartum metritis

Bacterial species	n	Postpartum metritis prevalence (%)	P-value
Culture negative	157	10	Referent
<i>Escherichia coli</i> ¹	156	23	0.01
<i>Trueperella pyogenes</i> ¹	127	21	0.01

¹These bacteria could be mixed with other pathogens.

Table 3. Sensitivity (Se), specificity (Sp), positive predictive value (PPV), and negative predictive value (NPV) of finding at 1 to 7 DIM intrauterine *Escherichia coli* and the virulence factors *kpsMTII* and *hra1* or a combination of these virulence factors to predict postpartum metritis in 371 Holstein dairy cows¹

Status	n	Prevalence of postpartum metritis		Se (%)	Sp (%)	PPV (%)	NPV (%)	P-value
		If present (%)	If absent (%)					
<i>E. coli</i>	156	23	9	64	62	23	91	0.01
<i>hra1</i>	49	30	15	43	85	31	80	0.02
<i>kpsMTII</i>	12	42	16	20	97	42	78	0.04
<i>hra1</i> or <i>kpsMTII</i>	58	33	12	34	88	33	90	0.03

¹The concomitant presence of *kpsMTII* and *hra1* was not found in the data.

in mind that *E. coli* isolate identification was based on having the same VF combination in a sample obtained from the same cows. This method is not perfect and cannot allow confirmation with certainty that the isolates were genetically identical. The use of fingerprint PCR would have been a better approach to confirm that 2 isolates were identical. However, it could not be performed in the present study because of financial restriction. It seems unlikely that this situation could have biased our results but it cannot be excluded.

Several studies have attempted to elucidate the etiological role of *E. coli* in the development of PPM. Silva et al. (2009) tested 15 VF on intrauterine *E. coli* and found no association with PPM for any of the VF tested. Sheldon et al. (2010) tested 17 VF, including *kpsMTII*, and found no association with PPM. In contrast, Bicalho et al. (2010) evaluated 32 *E. coli* VF in intrauterine *E. coli* isolates and found that 6 of them (*fimH*, *hlyA*, *cdt*, *kpsMII*, *ibeA*, and *astA*) were associated with PPM. In the present study, all these VF were tested and only *hra1* and *kpsMTII* were associated with PPM in the final model. It was surprising not to find an association between the presence of *fimH* and PPM, as this had already been reported (Bicalho et al., 2010, 2012). Statistical power issues could potentially explain why some of the VF identified in the Bicalho et al. (2010) study were not found in the present study. The prevalence of *fimH* was high (89%) in the present study, and perhaps having a greater number of cows positive for *E. coli* and negative for *fimH* would have given different results. Another possibility could be that VF distribution varies between herds and regions; because all published studies used a smaller number of herds (Silva et al., 2009; Bicalho et al., 2010; Sheldon et al., 2010) for conducting their investigations, it may lead to inconsistent associations with PPM. Conducting such studies in a smaller number of herds is ideal from a study management perspective, as it improves the quality of sampling and PPM disease definition used on farms. However, this strategy could lead to potential biases, such as not having representative samples

of VF for the general population of dairy herds. The fact that PPM diagnosis in the present study was done by trained farmers and not confirmed by veterinarians could also have influenced the study results. This is certainly a weakness of the current study, but doing otherwise was unrealistic when conducting a field study involving multiple herds. Only one person per farm was trained and charged with doing PPM surveillance to reduce this effect. But, veterinarians or not, it has also been shown that intra- and interobserver reliability for smell, color, and viscosity is relatively poor and that sensorial assessment of vaginal discharge is subjective (Sannmann and Heuwieser, 2015). Future studies should investigate VF in large-scale studies involving a large number of herds, using trained veterinarians to diagnose PPM, and trying to quantify intra- and interobserver agreement if more than one person is involved in the diagnostic process. Therefore, it is important to keep in mind that our study results do not deny the previously reported importance of *fimH* in the pathogenesis of PPM as *fimH* encodes for type 1 pili containing the *fimH* adhesion, which is present in the majority of *E. coli* isolates (Langermann et al., 1997). Sheldon et al. (2010) also concluded that endometrial pathogenic *E. coli* were expressing the *fimH* gene because mannose treatment of *E. coli* isolates decreased their ability to adhere to endometrial cells.

As previously described, *kpsMTII* was associated with PPM in the present study. This gene encodes for the capsular proteins K1 or K5, allowing bacteria to escape phagocytosis. The presence of *kpsMTII* has been associated with cellulitis in chickens (de Brito et al., 2003) and urinary tract infections in women (Moreno et al., 2005, 2009). Recently, the presence of *kpsMTII* was also found to be associated with PPM in dairy cows (Bicalho et al., 2010); therefore, it was not surprising to find an association between this VF and PPM in our study. More specifically, *kpsMTII* was found in 13% of cows that developed PPM in the present study, being comparable to the prevalence of 12.5% reported by Bicalho et al. (2010).

Another VF associated with PPM in the present study was *hra1*, which encodes for the heat-resistant-agglutinin 1 located on the outer membrane of the bacteria. Although this finding is novel in the dairy cow, it is not surprising considering its potential effect in humans. Its role in colonization and its virulence was shown in neonatal meningitis *E. coli* strain RS218 (Fagan and Smith, 2007). The presence of this gene is sufficient to confer agglutination of human erythrocytes, bacterial autoaggregation, enhanced biofilm formation, aggregative adherence, and invasion of epithelial cells (Mancini et al., 2011). Recently, *hra1* was also found in enteroaggregative *E. coli* isolated from humans (Bhargava et al., 2009). It seems highly plausible that *hra1* allows bacterial autoaggregation, leading to the formation of large aggregates of bacteria that cannot be phagocytized. This gene could also enhance formation of biofilms that persist on the uterine mucosa. Further studies are needed to confirm this hypothesis.

Although *kpsMTII* and *hra1* were associated with PPM, the detection of these genes in the early postpartum period only conferred relatively low positive predictive values (<42%), which may seem inappropriate if using this gene-detection strategy to identify cows that will develop subsequent PPM. However, our results suggest that not finding these 2 VF during the first week postpartum can provide high specificity and high negative predictive values. It is important to keep in mind that PPM is a complex multifactorial disease in which several factors are involved, including uterine bacterial contamination but also immune response to this infection. For farms located relatively close to a diagnostic laboratory, sampling all cows 1 d after calving and submitting this sample to the laboratory for determining whether *E. coli*, *hra1*, *kpsMTII*, or other VF identified by previous studies are present could potentially help farmers to identify early and quite accurately cows at low risk of PPM. Subsequently, the rest of the cows could be considered at higher risk of PPM and a surveillance strategy for diagnosis of PPM could be implemented. In the short term, the presence of *E. coli* in the uterus of dairy cows could be identified early using on-farm mastitis bacteriological culture kits. In the long term, development of a specific diagnostic kit for VF can be envisaged, but future research and technology development need to be done in this area. It is also important to keep in mind that finding cows early at high risk for PPM would be even more helpful to farmers if a proven prevention treatment strategy could be used. Current literature on this specific approach is currently lacking, but a study investigating a treatment prevention strategy for PPM reported some benefits when using retained placenta as the criteria for identi-

fying cows at high or low risk (Risco and Hernandez, 2003). Future research focusing on disease prevention and also on prudent use of antibiotics should be done before implementing such an approach. Future research should also confirm the test accuracy performance found in the present study, as the numbers of cows positive for *hra1* and *kpsMTII* were relatively small.

CONCLUSIONS

The presence *E. coli* in the uterine lumen of dairy cows during early postpartum period was associated with greater odds of subsequent PPM in dairy cows. Similarly, some *E. coli* VF, such as *hra1* and *kpsMTII*, were also associated with greater odds of PPM. Although using the presence of these bacteria or VF for predicting subsequent PPM in dairy cows yielded relatively low positive predictive values, their absence from the uterus was more accurate to identify cows at low risk of PPM.

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Appendix

Table A1. List of genes encoding 40 *Escherichia coli* virulence factors investigated in a study looking at the relationship between virulence factors and the occurrence of postpartum metritis in 371 Holstein dairy cows from 3 commercial herds

Virulence factor	Function	DNA sequence (5'→3')	Amplified product (bp)	Control strains	Reference
Adhesins					
<i>Aida</i>	Adhesin involved in diffuse adherence,	Forward (For): ACAGTATCATATGGAGCCA Reverse (Rev): TGTGCGCCAGAACTATTA	585	ECL1033	Ngeleka et al., 2003
<i>bmaE</i>	M-agglutinin major subunit	For: ATGGCGCTAACTTGCCATGCTG Rev: AGGGGGACATATAGCCCCCTTC	507	ECL12344	Chapman et al., 2006
<i>clpG-31a</i>	CS31A Coli surface-associated	For: GCACAATTACTGCTGATGCCG Rev: GTTATAAGTTACTGCCACGTTTC	710	31a	Girardeau et al., 1991
<i>Eae</i>	Intimin	For: CATTATGGAACGGCAGAGGT Rev: ATCTTCTGCGTACTGCGTTCA	791	ECL6611	Beaudry et al., 1996
<i>FimH</i>	Type 1 fimbriae	For: TGCAGAACGGATAAGCCGTGG Rev: GCAGTCACCTGCCCTCCGGTA	581	EDL933	Chapman et al., 2006
<i>F5 (K99)</i>	F5 fimbrial adhesin	For: TCGGACTACCAATGCTTCTG Rev: TATCCACCATTAGACGGAGC	450	ECL13316	Ojeniyi et al., 1994
<i>F17</i>	F17a pili major sub-unit	For: TATCCTTGGAATACTGGCGG Rev: CCAGTGGTGTAATCCGTGTT	300	ECL13256	Cid et al., 1999
<i>hra1</i>	Hemagglutinin - non fimbrial adhesin	For: TCACTTGCAGCCAGCGTTTC Rev: GTAACCTCACACTGCTGTCACCT	537	ECL1033	Ewers et al., 2007
<i>Paa</i>	Porcine A/E-associated gene	For: ATGAGGAACATAATGGCAGG TCTGGTCAGGTCGTCAATAC	360	ECL7805/ECL6611	An et al., 1999
<i>papC</i>	Pilus assembly, pap operon	For: GACGGCTGTACTGCAGGGTGTGGCG Rev: ATATCCTTTCTGCAGGGATGCAATA	328	ECL13256	Daigle et al., 1994
<i>sfaA</i>	Type S fimbriae major sub-unit	For: CGGAGGAGTAATTACAAACCTGGCA Rev: CTCCGGAGAACTGGGTGCATCTTAC	410	94-279-171	Hilali et al., 2000
Invasins					
<i>Afa</i>	Dr antigen-specific adhesin operons	For: GGCAGAGGGCCGGCAACAGGC Rev: CCCGTAACGCGCCAGCATCTC	594	ECL18162 (2H16)	Goluszko et al., 1997
<i>afaD8</i>	Invasin, AFA-8	For: GTTGAACCTGAGTCTTAATACCACTG Rev: TGAGCATTTCTCCGCTAACTGATAAT	280	ECL13455	Hilali et al., 2000
<i>ibeA</i>	Invasion protein	For: AGGCAGGTGTGCGCCGCGTAC Rev: TGGTGCTCCGGCAAACCATGC	170	ECL12312	Ewers et al., 2007
Toxins					
<i>cnf1</i>	Cytotoxic necrotizing factor 1	For: TTATATAGTCGTCAAGATGGA Rev: CACTAAGCTTTACAATATTGAC	634	ECL13455	Oswald et al., 1994
<i>Cnf2</i>	Cytotoxic necrotizing factor 2	For: GTCACAATAGACAATAATTTTCCG Rev: TATCATACGGCAGGAGGAAGCACC	1240	S5	Oswald et al., 1994
<i>cdtB-1</i>	Cytolethal distending toxin I (b subunit)	For (a1): AAATCACCAAGAATCATCCAGTTA Rev (a2): AAATCTCCTGCAATCATCCAGTTTA For (s1): GAAAGTAAATGGAATATAAATGTCCG Rev (s2): GAAAATAAATGGAACACACATGTCCG	430	ECL9632	Chapman et al., 2006
<i>cvaC</i>	Colicin V, conjugative plasmids	For: CACACACAAACGGGAGCTGTT Rev: CTTCCCGCAGCATAGTTCCAT	680	ECL12364	Chapman et al., 2006
<i>East1</i>	EaggEC heat-stable enterotoxin	For: TCGGATGCCATCAACACAGT Rev: GTCGCGAGTGACGGCTTTGTAG	125	ECL7805	Savarino et al., 1993
<i>hlyA</i>	Alpha-hemolysin (chromosome)	For: AGCCGGAACAGTTCTCTCAG Rev: CCAGCATAACAGCCGATGTA	526	ECL7805	Sandhu et al. 1997
<i>HlyE</i>	Hemolysine E	For: GAAACCGCAGATGGAGCATT Rev: CGCCCGCAGCAATAGAATAG	543	EDL933	Silva et al., 2009

Continued

Table A1 (Continued). List of genes encoding 40 *Escherichia coli* virulence factors investigated in a study looking at the relationship between virulence factors and the occurrence of postpartum metritis in 371 Holstein dairy cows from 3 commercial herds

Virulence factor	Function	DNA sequence (5'→3')	Amplified product (bp)	Control strains	Reference
<i>Sta</i>	Thermo stable toxin a	For: TCCCCTCTTTAGTCAGTCAACTG Rev: GCACAGGCAGGATTACAACAAAGT	163	ECL7805	So and McCarthy, 1980
<i>Stb</i>	Thermo stable toxin b	For: GCAATAAGGTTGAGGTGAT Rev: GCCTGCAGTGAGAAATGGAC	368	ECL7805	Lortie et al., 1991
<i>LT</i>	Thermo labile toxin	For: TTACGGCGTTACTATCCTCTCTA Rev: GGTCTCGGTCAGATATGTGATTC	275	ECL7805	Furrer et al., 1990
<i>stx1</i>	Shiga-like toxin I	For: TTAGACTTCTCGACTGCAAAG Rev: TGTTGTACGAAATCCCCTCTG	531	ECL6611	Woodward et al., 1992
<i>stx2</i>	Shiga-like toxin II	For: CTATATCTGCGCCGGGTCTG Rev: AGACGAAGATGGTCAAAACG	327	ECL6611	Woodward et al., 1992
Capsule synthesis <i>kpsM-II</i>	Polysialic acid transport proteins, group II (K1, K4, K5, K7, K12, K30, K42, K92)	For: GCGCATTGCTGATACTGTTG Rev: CATCCAGACGATAAGCATGAGC	272	O1:K1	Chapman et al., 2006
Iron acquisition <i>fepC</i>	Ferric enterobactin transport	For: TACCTGGATAATGCTGTCTGG Rev: ATGGTGTTGATGGGGCTGGC	347	EDL933	Ye and Xu, 2001
<i>fyuA</i>	Yersinia siderophore receptor (ferric yersiniabactin uptake)	For: TGATTAACCCCGCGACGGGAA Rev: CGCAGTAGGCACGATGTTGTA	880	ECL8985	Chapman et al., 2006
<i>irp1</i>	Protein for biosynthesis of yersiniabactin (peptide/polypeptide synthetase)	For: TGAATCGCGGGTGTCTTATGC Rev: TCCCTCAATAAAGCCCCACGCT	240	ECL12421	Karch et al., 1999
<i>irp2</i>	Iron-repressible protein (yersiniabactin synthesis)	For: AAGGATTCTGCTGTTACCGGAC Rev: TCGTCGGGCAGCGTTTCTTCT	286	ECL12364	Ewers et al., 2007
<i>iroN</i>	Novel catecholate siderophore	For: AAGTCAAAGCAGGGGTTGCCCCG Rev: GACGCCGACATTAAGACGCAG	665	ECL12021	Chapman et al., 2006
<i>iucD</i>	Aerobactin (siderophore) synthesis and receptor	For: AAGTGTCTGATTTTATTGGTGTA Rev: CCATCCGATGTCAGTTTTCTG	778	ECL3110/ECL17088	Herrero et al., 1988
<i>iutA</i>	Ferric aerobactin receptor (iron uptake/transport)	For: GGCTGGACATCATGGGAACTGG Rev: CGTCGGGAACGGGTAGAAATCG	300	ECL1106	Chapman et al., 2006
<i>sitA</i>	Putative iron transport gene	For: AGGGGGCACAACTGATTCTCG Rev: TACCGGGCCGTTTTCTGTGC	608	ECL12344	Rodriguez-Siek, et al., 2005
Additional virulence factors					
<i>iss</i>	Increased serum survival	For: ATCACATAGGATTCTGCCG Rev: CAGCGAGTATAGATGCCA	309	ECL12364	Ewers et al., 2007
<i>malX</i>	PTS system for maltose and glucose	For: GGACATCCTGTTACAGCGCGCA Rev: TCGCCACCAATCACAGCCGAAC	922	ECL12021	Ewers et al., 2007
<i>neuC</i>	p7 protein (polysialic acid synthesis)	For: GGTGGTACATTCCGGGATGTC Rev: AGGTGAAAAGCCTGGTAGTGTG	676	ECL12421	Ewers et al., 2007
<i>Tsh</i>	Temperature sensitive hemagglutinin (hemoglobin protease)	For: GGTGGTGCAGTGGAGTGG Rev: AGTCCAGCGTGATAGTGG	640	ECL3110	Dozois et al., 2000
<i>Usp</i>	Uropathogenic specific protein	For: ACATTACCGGCAAGCCTCAG Rev: AGCGAGTTCCTGGTGAAAGC	440	ECL12021	Bauer et al., 2002