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Theriogenology

Relationship between *Escherichia coli* virulence factors, notably *kpsMTII*, and symptoms of clinical metritis and endometritis in dairy cows

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ABSTRACT. Although *Escherichia coli* is a commensal bacterium of the bovine vaginal microbiota, it is an important pathogenic bacterium that causes diseases of the reproductive tract and subfertility. Recent studies have focused on virulence factors (VFs) of intrauterine *E. coli*; however, actual endometrial VFs have not been clearly identified. The purpose of this study was to identify the VFs of *E. coli* associated with clinical metritis and endometritis. Thirty-two strains of *E. coli* and four mixed *Trueperella pyogenes* (TP) strains were detected in the uterus of 19 Holstein dairy cows with obvious clinical signs (between 8 and 66 days postpartum). The presence of six *E. coli* VFs (*fimH*, *fyuA*, *kpsMTII*, *hra1*, *csgA*, and *astA*) was examined by PCR, and clinical signs and reproductive performance (mixed TP, the percentage of polymorphonuclear neutrophils [PMN%], days to uterine involution, etc.) were evaluated. Four VFs (*fimH*, *hra1*, *csgA*, and *astA*) were detected in all *E. coli* strains, whereas *fyuA* and *kpsMTII* were detected in 94% and 50% of strains, respectively. Cows with *E. coli* strains harboring *kpsMTII* exhibited significantly severe clinical scores (vaginal discharge score, PMN%, uterine involution), suggesting that *kpsMTII* is a key VF for progression of clinical metritis and endometritis. In the present study, we clearly identified six VFs associated with clinical metritis and endometritis. In addition, *E. coli* strains with *kpsMTII* probably play a crucial role in the progression of clinical metritis and endometritis.

KEY WORDS: *Escherichia coli*, *kpsMTII*, postpartum uterine disease, uterine restoration time, virulence factor

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Almost all cows are contaminated or become infected in the uterus immediately after parturition by bacteria ascending from the external environment through the open cervix. Although the uterus harbors a normal flora in approximately 50% of cows by 40 days after parturition and 90% by 60 days after parturition [36], uterine infection can develop in cows in which bacterial clearance does not proceed effectively. Postpartum uterine infections can cause puerperal fever, clinical and subclinical endometritis, and prolong the open period, consequently having a negative impact [6] manifesting as low reproductive performance [16] or increased culling rate [27].

The definition of metritis is described in the report by Sheldon *et al*. [39]. Puerperal metritis is characterized by an enlarged uterus and reddish-brown, moist, watery intrauterine discharge with signs of systemic disease (decreased milk production, dull pain, toxic symptoms) and fever >39.5°C within 21 days of calving [39]. Cows that are not clearly ill but have an abnormally enlarged uterus and purulent uterine discharge detected in the vagina within 21 days after parturition are classified as having clinical metritis [39]. Clinical endometritis is characterized by the presence of purulent or mucopurulent uterine exudate in the vagina after 21 days postpartum, without systemic symptoms [39]. Treatment for endometritis generally involves antibiotics; however, antibiotic therapies can have negative economic impacts due to a suspension of milk production and animal shipping. Preventing genital disease and shifting to the next prosperous lactation period are thus economically important in postpartum cow management.

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Escherichia coli and *Trueperella pyogenes* (TP) are the most widely known bacteria that cause postpartum endometritis [3, 39, 44]. Colonization of the upper reproductive tract by *E. coli* is reportedly associated with severe damage to the endometrium and disruption of ovarian cycle activity, followed by infertility [20, 30, 38, 45]. In the early stages of intrauterine infection, *E. coli* is often the infectious agent, but the source of infection then shifts to bacteria such as TP, with persistent infection leading to chronic uterine disease [12]. Bacterial lipopolysaccharide (LPS) acts on ovarian luteinizing hormone–stimulated theca cells to inhibit steroid production [30]. The presence of *E. coli* and LPS in the lochia during the early postpartum period favors the development of uterine infections by TP and gram-negative anaerobes during the later postpartum period [12]. It is assumed that preventing infection by *E. coli* shortly after parturition prevents the adverse effects associated with LPS and contributes to the prevention of other bacterial infections. For these reasons, it is important for commercial dairies to respond early to infections with *E. coli*.

In uterus, infections can involve diarrheagenic *E. coli* (DEC) and extra-intestinal pathogenic *E. coli* (ExPEC), such as uropathogenic *E. coli* (UPEC), which can infect tissues other than the endometrium. In addition, previously unidentified *E. coli* strains might also infect the endometrium. Specific adhesion to host tissue cells is an essential virulence factor (VF) of most bacterial pathogens. The fundamental processes that determine the ability of bacteria to attach to host cells are mediated by microbial adhesins [25]. Uterine isolates of *E. coli* often lack common VF genes associated with DEC or ExPEC strains and are more likely to adhere to and invade endometrial epithelial and stromal cells, causing more severe endometritis. These particular strains are known as endometrial pathogenic *E. coli* (EnPEC) [40]. Although the evolutionary background of EnPEC is not clear, the ferric yersiniabactin uptake receptor (*fyuA*) gene was found in pelvic inflammatory disease–associated EnPEC but not *E. coli* from the uterus of clinically unaffected animals [19].

Phylogenetic analyzes have been used to determine the evolutionary origins of pathogenic *E. coli* strains. Four major phylogenetic groups of *E. coli* have been described to date, designated A, B1, B2, and D [21]. Animals with uterine disease are more likely to harbor group A or B1 bacteria [40]. Several *E. coli* VFs have been suggested as promoting the development of infections with TP, necrotic bacilli, or other organisms [8]. However, although a wide variety of coliforms can infect the uterus, research regarding their VFs is insufficient. A number of recent studies have focused on the pathogenicity of VFs, but these studies have drawn differing conclusions regarding which VFs are decisive factors in determining pathogenesis [8, 9, 17, 24].

Bicalho *et al*. reported that six VFs common to extraintestinal and enteroaggregative *E. coli* and associated with puerperal metritis and clinical endometritis have been identified: *fimH*, *hlyA*, *cdt*, *kpsMTII*, *ibeA*, and *astA*, with *fimH* exhibiting a synergetic relationship with the other five VFs [9]. Another study concluded that only *hra1* and *kpsMTII* are associated with postpartum puerperal metritis [24]. Moreno *et al*. reported that harboring the *fyuA* and *csgA* VF genes may be a risk factor for endometrial disorders [17]. These previous studies suggested an association between *E. coli* VFs and puerperal metritis and clinical endometritis. However, these studies could not analyze *E. coli* VFs isolated from cows diagnosed with puerperal metritis or clinical endometritis because most of the *E. coli* were isolated from the uterus soon after delivery (5–7 days postpartum). Furthermore, no research has focused on the effect of *E. coli* VFs on clinical metritis. Thus, details regarding the characteristics of EnPEC remain unclear, and there have been no studies examining the course of clinical symptoms and the relationship of these symptoms to VFs. The objective of present study, therefore, was to clarify the impact of VFs on clinical metritis and endometritis and the clinical course of infection. The presence of the *fimH*, *fyuA*, *kpsMTII*, *csgA*, *hra1*, and *astA* VF genes was examined in *E. coli* isolates obtained from clinical metritis and endometritis samples from animals with obvious clinical symptoms at three Holstein dairy farms.

MATERIALS AND METHODS

Animals

This study was conducted according to the institutional guidelines for animal experiments of Rakuno Gakuen University (approval no. VH17C10). A total of 19 Holstein Friesian cows from three dairy farms were examined in the study. All animals delivered alone in the calving pen, were milked twice daily, and determined not to have other perinatal disorders without uterine diseases.

Clinical examination

Reproductive examinations were started at 7 days after parturition (± 3 days) and performed weekly (± 3 days) until uterine involution was completed. Any signs of systemic illness were recorded. The uterine involution status of each cow was evaluated by reproductive examinations that included transrectal palpation, vaginoscopy, and ultrasonography (HS-101v; Honda Electronics Co., Ltd., Toyohashi, Japan). Cows that were not ill but had an abnormally enlarged uterus and a purulent uterine discharge from the vagina within 21 days after parturition were determined to have clinical metritis [39]. Clinical metritis and endometritis were also confirmed using ultrasonography (Supplementary Fig. 1). Clinical endometritis was diagnosed by the scoring system of Sheldon *et al*. at ≥22 days postpartum [37] (Table 1). The diameters of the largest part of uterine horn and the cervix were measured by transrectal palpation and ultrasonography and scored on three levels from 0 to 2 as follows: the diameter of the uterine horn was scored 0 for small (<3.5 cm for primiparous cows, <4.0 cm for multiparous cows), 1 for medium (3.5 to 5.5 cm for primiparous cows, 4.0 to 6.0 cm for multiparous cows), and 2 for large (>5.5 cm for primiparous cows, >6.0 cm for multiparous cows). Vaginal discharge was scored based on the amount and characteristics by vaginoscopy. A vaginal discharge score of 0 was given for clear or translucent, 1 for <50 ml with a small amount of thick white flakes, 2 for <50 ml with <50% white or off-white mucosal purulent material, and 3 for exudates of ≥50 ml with ≥50% white or yellow purulent material. A score of 3 was added for foul smell or

Table 1. Scoring system for the assessment of the severity of endometritis

Clinical sign			Score
Diameter of largest uterine horn	Primipara	Multipara	
Large	>5.5 cm	>6.0 cm	2
Medium	3.5–5.5 cm	4.0–6.0 cm	1
Normal	<3.5 cm	<4.0 cm	0
Diameter of cervix	Primipara	Multipara	
Large	>7.5 cm	>7.5 cm	2
Midium	4.5–7.0 cm	5.0–7.5 cm	1
Normal	<4.5 cm	<5.0 cm	0
Vaginal discharge	Caracter	Foul smell	3
		No smell	0
		Bloody	3
	Volume	≥50 ml, pus (≥50%)	3
		<50 ml, pus (<50%)	2
		White clumps	1
		Normal	0

Adapted from [37, 44].

bloody discharge [37, 44]. Cows with total scores of ≥1 were diagnosed as having clinical endometritis. The day of complete uterine involution was defined as the examination day when the total score was 0, the polymorphonuclear neutrophil percentage (PMN%) was normal, and no bacteria were isolated. In the present study, PMN% values of <18%, <10%, and <5% at 21–33 days, 34–47 days, and 48–60 days postpartum, respectively, were defined as normal [16, 44].

Uterine smear collection and bacteriology

Intrauterine smears were collected from all cows by endometrial cytology using a sterilized cytobrush instrument (Metricbrush; Fujihira Industry, Tokyo, Japan) [4]. The perineum and vulva were wiped with a dry paper towel and then disinfected with benzalkonium chloride and 70% alcohol. A cytobrush instrument covered with a plastic sheath was introduced into the vagina. The tip of the instrument was guided to the external uterine orifice by rectal palpation. The cytobrush instrument alone was introduced into the uterine body through the cervix after rupturing the tip of the sheath at the external uterine orifice. After inserting the cytobrush into the uterine body, the brush was rotated once to obtain endometrial cells. The instrument was then withdrawn from the uterus and rotated on a sterilized glass slide to prepare a cell smear. All smeared glass slides were stained with modified Giemsa stain, and >400 cells were counted at 200 × magnification using an optical microscope. The ratio of PMNs to all cells on the surface of the endometrium was then calculated (Table 2).

Immediately after smearing, the cytobrush instrument was suspended in a sterile plastic tube containing 1 ml of sterile saline. After stirring the suspension well, 50 µl of the resulting suspension was cultured on 5% sheep blood agar at 37°C for 24–48 hr. After incubation, bacteria were identified based on colony characteristics, Gram stain, and morphology, with *E. coli* the primary bacterium recorded in this study. Mixed infection with TP was also recorded. In addition, the presence of *E. coli* was confirmed by API 20 E biochemical profiling (Sysmex Corp., Kobe, Japan). Finally, 32 samples of *E. coli* were isolated from 19 cows (Table 2) and stored at –80°C in Luria-Bertani broth containing 25% glycerol.

DNA extraction and PCR

Bacterial DNA samples were purified from overnight cultures in LB broth using a GenElute™ Bacterial Genomic DNA kit (Sigma-Aldrich/Merck, Darmstadt, Germany). All reactions were performed in a 20-µl volume using 18 µl of premix containing NEbuffer, dNTP mix (0.2 mM each), 0.5 units of Taq DNA polymerase (New England Biolabs, Ipswich, MA, USA), primer mix (0.5 µM each), and finally, 2 µl of DNA extract (50 ng). All thermal cycling protocols were as follows: 94°C for 1 min, 55–63°C for 1 min (according to each primer set, Supplementary Table 1 [2, 17, 24, 28]), 72°C for 1 min, and a final step of 72°C for 2 min. Negative controls consisting of the PCR mixture without DNA were included in all PCR runs. Amplification products were separated by electrophoresis on a 2% (wt/vol) agarose gel.

Random amplified polymorphic DNA PCR

PCR was carried out in 25 µl containing 50 ng of *E. coli* genomic DNA, 20 pmol of primers, 0.2 mM each dNTP, 1.25 units of Taq DNA polymerase, and standard Taq reaction buffer (New England Biolabs). We used the following primer: 5′-GCGATCCCCA-3′ (primer name: 1283) [1]. The cycling program was 94°C for 1 min, 36°C for 1 min, 72°C for 2 min, and a final step of 72°C for 2 min. Amplification products were separated by electrophoresis on a 2% (wt/vol) agarose gel.

Statistical analysis

The 32 isolated *E. coli* strains were divided into a clinical metritis group (n=10) and endometritis group (n=22) (Table 3). Cows

Table 2. Virulence factor genes of *Escherichia coli* isolated from the postpartum uterus and associated clinical symptoms

Cow No.	Parity	Bacterial no.	Days after parturition	PMN (%)	Identified bacteria	Phylogenetic group	*fimH*	*fyuA*	*kpsMT II*	*hra1*	*csgA*	*astA*	Uterine restoration day	Days to first AI	No. of AI	Days open
s2	1	s2-1	14	6.5	*E. coli*	D	+	+	+	+	+	+	40	82	3	152
		s2-2	14	6.5	*E. coli*	B2	+	+	−	+	+	+				
s5	2	s5-1	38	1.4	*E. coli*	D	+	−	−	+	+	+	45	82	1	82
s6	7	s6-1	32	6.8	*E. coli*	B2	+	+	−	+	+	+	39	(−)	(−)	(−)
s7	3	s7-1	6	74.3	*E. coli*	B2	+	+	+	+	+	+	97	169	3	225
		s7-2	13	83.9	*E. coli*	B2	+	+	−	+	+	+				
		s7-3	13	83.9	TP											
		s7-4	21	53.8	TP											
		s7-6	28	79.4	TP											
		s7-7	45	41.5	TP											
		s7-8	48	49.2	TP											
s9	3	s9-1	9	83.7	*E. coli*	B2	+	+	−	+	+	+	40	103	1	(−)
		s9-2	9	83.7	*E. coli*	A	+	+	−	+	+	+				
		s9-3	9	83.7	*E. coli*	B2	+	+	+	+	+	+				
		s9-4	9	83.7	TP											
		s9-5	15	56.9	TP											
s11	1	s11-1	33	6.0	*E. coli*	B2	+	+	+	+	+	+	40	62	1	62
s12	5	s12-1	8	39.6	*E. coli*	B2	+	+	+	+	+	+	60	(−)	(−)	(−)
		s12-2	8	39.6	TP											
		s12-4	15	13.1	TP											
		s12-5	22	16.8	TP											
		s12-6	29	41.8	*E. coli*	B2	+	+	+	+	+	+				
		s12-7	29	41.8	TP											
s13	1	s13-1	16	–	*E. coli*	B2	+	+	+	+	+	+	57	203	3	304
		s13-2	16	–	TP											
		s13-3	22	26.2	TP											
		s13-4	43	4.9	*E. coli*	B2	+	+	−	+	+	+				
s14	1	s14-1	34	7.4	*E. coli*	B2	+	+	+	+	+	+	55	90	4	209
		s14-2	48	0.5	*E. coli*	B2	+	+	−	+	+	+				
s15	1	s15-1	31	0.0	*E. coli*	B2	+	+	+	+	+	+	80	106	3	175
		s15-2	45	9.1	*E. coli*	B2	+	+	+	+	+	+				
		s15-3	66	9.1	*E. coli*	B2	+	+	+	+	+	+				
s16	3	s16-1	20	71.3	*E. coli*	B2	+	+	+	+	+	+	62	98	1	98
s17	6	s17-1	29	10.3	*E. coli*	B2	+	+	−	+	+	+	42	78	1	78
s18	4	s18-1	50	0.01	*E. coli*	B2	+	+	+	+	+	+	36	(−)	(−)	(−)
s21	1	s21-1	30	1.8	*E. coli*	B2	+	+	−	+	+	+	44	60	7	280
		s21-2	30	1.8	*E. coli*	B2	+	+	−	+	+	+				
s23	2	s23-1	33	0.74	*E. coli*	B1	+	−	−	+	+	+	33	99	1	99
s24	2	s24-1	43	4.8	*E. coli*	D	+	+	+	+	+	+	85	90	3	(−)
s25	7	s25-1	38	35.2	*E. coli*	B2	+	+	−	+	+	+	52	(−)	(−)	(−)
s27	1	s27-1	34	10.8	*E. coli*	B2	+	+	−	+	+	+	87	93	2	116
		s27-2	34	10.8	*E. coli*	B2	+	+	+	+	+	+				
		s27-3	34	10.8	*E. coli*	B2	+	+	+	+	+	+				
		s27-4	48	2.8	*E. coli*	B2	+	+	+	+	+	+				
s30	5	s30-1	29	1.6	*E. coli*	B1	+	+	−	+	+	+	29	146	4	(−)

(−) indicates no implementation. PMN; polymorphonuclear neutrophil, AI; artificial insemination, TP; *Trueperella pyogenes.*

Table 3. Clinical metritis and endometritis with *Escherichia coli* diagnosed during and after the puerperium period

Group	Number of *E. coli* strains	Days after parturition	Evaluation of uterine repair			VF prevalence of *E. coli* strains (%)					
			Vaginal mucus score	PMN%	Total score	*fimH*	*fyuA*	*kpsMTII*	*hra1*	*csgA*	*astA*
Clinical metritis	10	11.8 ± 4.3[a]	3.2 ± 2.3[a]	59.2 ± 33.0[a]	6.9 ± 2.4[a]	100	100	60.0	100	100	100
Endometritis	22	37.8 ± 9.3[b]	0.5 ± 0.8[b]	8.1 ± 7.7[b]	1.7 ± 1.3[b]	100	90.9	50.0	100	100	100

Data are shown as mean ± standard deviation. a/b, $P<0.05$. PMN%; the percentage of polymorphonuclear neutrophils, VF; virulence factor.

in the clinical metritis group and endometritis group were within 21 and after 22 days of parturition, respectively. The number of days after parturition, vaginal discharge score, PMN%, and total score were compared between the two groups using the Student's *t*-test after normality testing. The parity, number of days after parturition, vaginal discharge score, PMN%, and reproductive parameters were compared between the *E. coli kpsMTII*-positive group (n=10) and *kpsMTII*-negative group (n=9) using the Student's *t*-test or Welch's *t*-test after normality testing and the *F* test (Table 4). In addition, Fisher's exact test was performed to compare the ratio of TP mixed infections. Data are presented as the mean ± SD. The significance level was set as <5%.

RESULTS

E. coli isolated from the postpartum uterus and phylogenetic distribution

A total of 32 strains of *E. coli* were isolated from 19 cows, of which 4 had mixed infection with TP. The data of the examinations in which *E. coli* were detected are summarized in Table 2. All 32 *E. coli* strains obtained by culturing on blood agar medium were confirmed as *E. coli* by PCR (Fig. 1). Almost all of the isolates were confirmed as different strains based on RAPD PCR results (Supplementary Fig. 2). To determine the phylogenetic distribution of these strains, the genes *chuA*, *yjaA*, and TSPE4C2 were examined by PCR and classified into four phylogenetic groups, A, B1, B2, and D, using the method of Clermont *et al*. [10, 21] (Table 2 and Fig. 2). Eighty-one percent of the strains were classified as group B2, whereas groups A, B1, and D accounted for 3, 6, and 9%, respectively. Strains lacking both the *fyuA* and *kpsMTII* genes were other than B2 and classified as group B1 or D (Table 2).

Table 4. Comparison of reproductive parameters between cows with *kpsMTII*-positive and *kpsMTII*-negative *Escherichia coli*

Group	n	Parity	Days after parturition	Ratio of mixed infection with TP (%)	Evaluation of uterine repair			Days to uterine involution	Days to 1st AI	Days open in pregnant cows
					Vaginal mucus score	PMN%	Total score			
Detected	12	2.2 ± 1.4[a]	30.6 ± 17.3	33.3 (4/12)	1.7 ± 1.7[a]	24.5 ± 29.7[a]	3.4 ± 2.8	61.6 ± 21.1[a]	109.6 ± 42.8	167.6 ± 78.0
Non-detected	7	4.3 ± 2.6[b]	32.7 ± 3.9	0 (0/7)	0.1 ± 0.4[b]	8.3 ± 12.4[b]	2.9 ± 1.3	40.6 ± 7.7[b]	93.0 ± 32.7	134.8 ± 97.3

Data are shown as mean ± standard deviation. a/b, $P<0.05$. AI; artificial insemination, PMN%; the percentage of polymorphonuclear neutrophils.

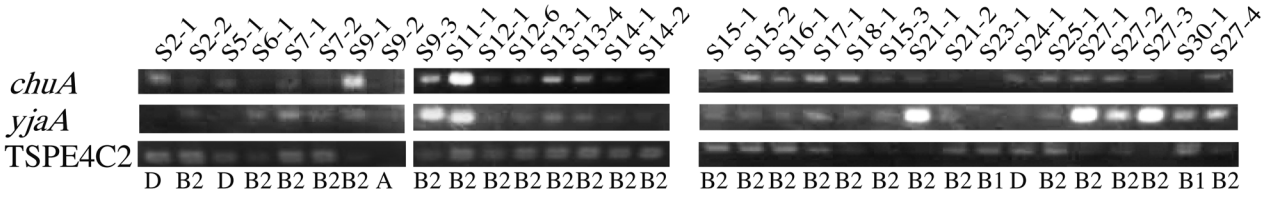

Fig. 1. PCR amplification of the *chuA* and *yjaA* genes and DNA fragment TSPE4-C2 specific for *E. coli* phylogenetic groups. PCR fragments of *chuA*, *yjaA*, and TSPE4-C2 were confirmed as 279-, 211- and 152-bp fragments, respectively. These amplification patterns allowed phylogenetic group strain determination (Table 2).

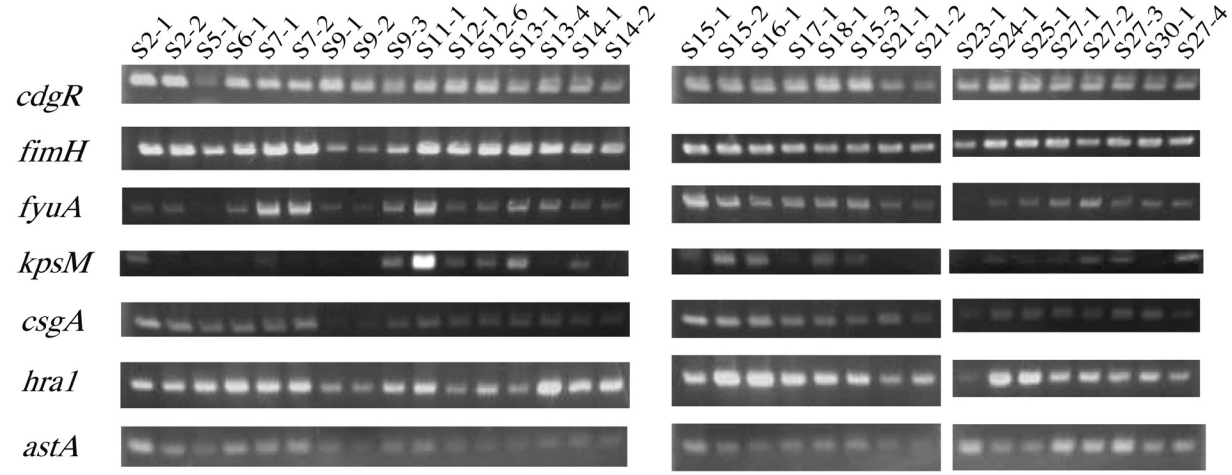

Fig. 2. PCR amplification of six Virulence Factor genes from *Escherichia coli* strains isolated from endometritis samples. PCR fragments of *fimH*, *fyuA*, *kpsMTII*, *csgA*, *hra1*, and *astA* were confirmed. The data are summarized in Table 2.

VF genes and clinical symptoms in cows

To determine which VFs are associated with which clinical symptoms, six VF genes were investigated by PCR for each *E. coli* strain isolated (Table 2 and Fig. 1). A total of 10 and 22 *E. coli* strains were isolated from the clinical metritis and endometritis groups, respectively (Table 3). The evaluation of uterine involution in the clinical metritis group revealed a severe level of disease in these cows (Table 3). Five VF-encoding genes (*fimH*, *fyuA*, *hra1*, *csgA*, *astA*) were detected in all 10 *E. coli* strains isolated from the uterus of clinical metritis cows (Table 3). The prevalence of these five VFs in *E. coli* strains isolated from the uterus of endometritis cows was as high as 90.9–100% (Table 3). Of the 6 VFs investigated in the study, the gene encoding *fyuA* was detected in all strains isolated from the endometritis group except strains S5-1 and S23-1 (Table 2). The prevalence of *kpsMTII* in *E. coli* isolated from the clinical metritis and endometritis groups was 60 and 50%, respectively (Table 3). In addition, strains lacking *fyuA* also lacked *kpsMTII* (Table 2).

VF genes and reproductive performance

Reproductive performance was compared between the *kpsMTII*-positive and *kpsMTII*-negative groups (Table 4). Significant differences were found between the two groups in terms of parity, vaginal discharge score, and PMN% ($P<0.05$) (Table 4). The rates of mixed infection with TP in the *kpsMTII*-positive and *kpsMTII*-negative groups were 33.3 and 0% (Table 4), respectively, with all mixed infections with TP occurring in the *kpsMTII*-positive group (Table 2). Although the number of days of uterine involution in the *kpsMTII*-positive group was greater than that in the *kpsMTII*-negative group, there was no significant difference in the number of days to first artificial insemination (AI) and days open (Table 4).

DISCUSSION

In this study, the association between clinical symptoms and VFs of strains isolated from cows with clinical metritis and endometritis was investigated. Although *E. coli* has been reported as a member of the normal microbiota of the bovine reproductive tract in healthy heifers [35], data indicate it also plays a crucial role in the establishment of metritis and endometritis [15]. These contradictory results may be due to the diversity of *E. coli* strains, namely, the diversity of specific strains carrying VF genes. To evaluate which VFs are associated with particular clinical symptoms, six VF genes were investigated by PCR for each *E. coli* strain isolated from cows with obvious clinical metritis or endometritis (Table 2 and Fig. 1).

In our study, there was a difference in the rates of phylogenetic groups compared with previous reports. Duriez *et al*. reported that ExPEC strains primarily belong to phylogenetic group B2 and harbor several VF genes. In contrast, most commensal *E. coli* strains belong to groups A and B1 and harbor few VF genes compared with the corresponding pathogenic strains [13]. Clermont *et al*. also clearly demonstrated that strains harboring *chuA*, a gene required for heme transport in O157:H7 and other clinical *E. coli* strains, are distributed in groups B2 and D. However, some reports of phylogenetic classification studies have shown that *E. coli* isolated from the postpartum uterus (of cows with metritis and endometritis) belong mainly to groups A and B1 [17, 40]. Those results may reflect commensal strains isolated from the uterus of postpartum dairy cows. In this study, most of the strains isolated from animals with severe clinical manifestations of both clinical metritis and endometritis belonged to group B2 (Table 2 and Fig. 2), suggesting that they were ExPEC. These data suggest that ExPEC is involved in the pathogenesis of clinical metritis and endometritis, and we speculate that VFs encoded by genes other than the three that determine phylogenetic group are involved in the pathogenicity of ExPEC.

VF genes were detected at a higher rate in the present study compared with previous reports [9, 17, 24, 40]. Kassé *et al*. investigated as many as 40 VFs and concluded that statistically, only *hra1* and *kpsMTII* are associated with postpartum puerperal metritis, but they did not find a clearly associated VF [24]. Bicalho *et al*. reported that six VFs common to extraintestinal and enteroaggregative *E. coli*, such as *fimH*, *hlyA*, *cdt*, *kpsMTII*, *ibeA,* and *astA*, are associated with puerperal metritis and clinical endometritis, with a synergistic relationship between *fimH* and the other five VFs [9]. Moreno *et al*. reported that vaginal *E. coli* populations harboring the *fyuA* and *csgA* VF genes may represent a risk factor for endometrial disorders; specifically, those that also possess *kpsMTII* may have pathogenic potential to cause repeat breeder syndrome [17]. In all of these reports, the percentage of detected VFs was not as high as in our study, probably because a relatively higher rate of commensal *E. coli* strains without VFs were isolated in these previous studies. This difference is also consistent with the results of phylogenetic analyzes, in which a low percentage of B2 isolates was reported in previous studies, in contrast to a high percentage of B2 isolates in our study.

The *fimH* gene encodes one of the most extensively studied adhesins, which binds directly to D-mannose, which in turn is bound to a carrier protein. The fimH protein is understood to be one of the most important VFs because it is uniquely involved in determining receptor specificity and can be a stepping stone to infection [26]. Typically, type I pili including the fimH adhesin are involved in mediating the attachment of *E. coli* to mammalian cells [23]. The inhibition of *E. coli* adhesion to uterine endometrial cells by D-mannose as described by Sheldon *et al*. indicates that fimH is involved in adhesion to uterine endometrial cells. In previous studies, *fimH* has consistently been the most frequently detected VF in *E. coli* isolated from the uterus [9, 17, 24]. In addition, *fimH* is often detected in healthy vagina and also in the healthy uterus after parturition [9, 17, 24]. Therefore, *fimH* may be necessary for enabling *E. coli* to remain in the uterus, although further studies are needed to confirm this hypothesis.

The heat-resistant agglutinin 1, *hra1*, was originally described as an autoaggregating and hemagglutinating protein from an O9:H10:K99 *E. coli* strain pathogenic in piglets and lambs [29]. In addition, *hra1* was first reported by Kassé *et al*. as a VF associated with postpartum puerperal metritis in dairy cows [24]. In strains of *E. coli* that are exceptional colonizers, *hra1* is an integral outer membrane protein that confers the ability to adhere to human epithelial cells, resulting in bacterial autoaggregation.

Although *hra1* is also found in non-pathogenic *E. coli*, its presence enhances colonization by diarrheagenic *E. coli* such as enteroaggregative *E. coli* (EAEC) and of invasive *E. coli* [7, 14, 31]. The bacterial autoaggregation mediated by *hra1* can lead to the formation of large bacterial aggregates that are not phagocytosed and may be involved in the formation of biofilms that enable the bacteria to remain in the uterine mucosa [24]. The *astA* gene encodes EAEC heat-stable toxin 1 (EAST1), which has been implicated in the development of human diarrhea [43]. Bicarho *et al*. reported that EAST1 may play an important role in the development of postpartum uterine infections and that the VF gene *astA* is the second most important predictor of puerperal metritis after *fimH* [9].

Both *fyuA* and *csgA* are VFs involved in biofilm formation [19, 41]. The *fyuA* gene encodes a ferric scavenger receptor with a high prevalence in UPEC, which is among the most prevalent agents of urinary tract infections [18]. It is one of the most upregulated genes, and in biofilm, the ferric yersiniabactin uptake receptor fyuA plays an important role in iron uptake and biofilm formation during urinary tract infections caused by *E. coli* [19]. The *csgA* gene encodes a major structural subunit of Curli, the biofilm-forming amyloid. Curli is expressed on the surface of gram-negative enterobacteria and known to interact with a wide range of proteins that contribute to bacterial virulence. Curli mediates host cell adhesion and invasion and is a potent inducer of the host inflammatory response [5, 42]. The onset of CsgA aggregation is delayed by stoichiometric concentrations of fibrinogen, which inhibits the initial events of CsgA assembly [41]. Moreno *et al*. reported that *E. coli* of phylogenetic group B1, which possess the *fyuA* and *csgA* genes, are highly motile, express Curli fimbriae and cellulose, and have the ability to form biofilms, may be widely involved in the development of postpartum disorders [17].

In our study, surprisingly, four VFs (*fimH*, *hra1*, *csgA*, and *astA*) were detected in all 32 *E. coli* strains isolated from the postpartum uterus, even though they were isolated from cows diagnosed with clinical metritis or endometritis lacking systemic symptoms (Table 3). All of these VFs are reportedly associated with uterine infections [3, 13, 16]. Although the relationship between bovine uterine infections and these VFs of *E. coli* has not been elucidated, the data suggest these six VFs play important roles in uterine infection. Identifying the VFs associated with the development of uterine infections will require the isolation of *E. coli* from the uterus of cows that have not shown clinical symptoms in a large population, followed by characterization of the differences in *E. coli* VFs between cows diagnosed with puerperal, clinical metritis, or clinical endometritis and evaluation of the association of *E. coli* VFs with systemic symptoms. Of the 32 strains isolated in the present study, only two strains lacking *fyuA* were identified (Table 2). These strains, which also lacked *kpsMTII* and produced relatively mild clinical symptoms, belonged to phylogenetic groups B1 and D (Table 2). Although the VFs were clearly associated with clinical metritis and endometritis, whether the differences in pathogenicity of the isolates were due to the presence or absence of particular VFs or to differences in phylogenetic group has not been determined.

The mechanism associated with clinical symptoms affecting the postpartum genital tract could involve *fimH*-mediated adhesion to the endometrium, *hra1*-promotion of colony formation, *fyuA*- and *csgA*-mediated promotion of biofilm formation, and *astA* functioning as a toxin. The detailed mechanisms by which each VF causes infection and inflammation of the uterus should be the subject of future research.

Interestingly, in our study, *kpsMTII* was detected at a lower rate than the other VFs examined (Table 4). *kpsMTII* encodes capsular protein K1 or K5 [24]. These proteins play a role in evading or counteracting host nonspecific immunity by interacting with the bacterial surface and complement system in the early stages of infection [22]. The presence of *kpsMTII* has been linked to cellulitis in chickens [11] and urinary tract infections in women [33, 34]. Bicalho *et al*. reported that cows in which the uterus is contaminated with *E. coli* carrying the *kpsMTII* gene are 9.2 times less likely to become pregnant than cows with an uncontaminated uterus [9]. Kassé *et al*. reported that cows harboring *E. coli* with *kpsMTII* detected in the uterus at 1–7 days in milk are 6.2 times more likely than cows with no *E. coli* detected and 3.2 times more likely than cows with *E. coli* lacking *kpsMTII* to subsequently develop postpartum puerperal metritis [24]. Moreno *et al*. reported that the presence of *E. coli* harboring *kpsMTII* can cause repeat breeder syndrome [17]. Thus, previous reports consistently indicate that *kpsMTII* causes postpartum uterine problems, and we hypothesize that *kpsMTII* is also associated with clinical metritis and endometritis.

In the present study, the presence of *kpsMTII* was found to significantly worsen vaginal discharge score and PMN% compared to its absence (Table 4). However, the higher vaginal discharge score and PMN% in cows with fewer days postpartum is a physiologic phenomenon, and it is therefore difficult to compare these parameters between cows. Groups must be compared according to the number of days postpartum using a larger number of samples. In addition to the five VFs associated with clinical metritis and endometritis mentioned above, *kpsMTII* was found to further aggravate the condition. The present study demonstrated that *kpsMTII* is associated with a delay in postpartum uterine involution in dairy cows. The postpartum uterine involution time for cows in the *kpsMTII*-positive group was 61.3 days, whereas it was 40.6 days for cows in the *kpsMTII*-negative group, indicating that uterine involution required a significantly longer amount of time in the *kpsMTII*-positive group (Table 4). This may be the result of a system in which the K1 or K5 proteins encoded by *kpsMTII* evade immune recognition by interacting with the bacterial surface and complement system and by employing molecular mimicry [22]. While *kpsMTII* was found to delay uterine involution, no significant difference was observed in number of days to first AI or days open. This may be due to other factors affecting ovarian function or to environmental factors that collectively preserve sperm activity in the uterus between the time of uterine involution and conception. Furthermore, as reproductive examinations were conducted weekly, the absence of significant differences could have been due to the fact that as soon as all conditions other than uterine involution were achieved, conception could have occurred immediately. During the clinical course, some cows in which *kpsMTII* was not detected exhibited improvement in symptoms, and some cows in which *kpsMTII* was detected exhibited stable or worsening of symptoms; more extensive research will be needed to explain this difference.

Although a statistically significant difference was not demonstrated, the rates of mixed infections with TP in the *kpsMTII*-positive and *kpsMTII*-negative groups were 33.3 and 0%, respectively (Table 3). From all cases of mixed infection, *E. coli* with *kpsMTII* were detected in the early stage (Table 2). As the presence of *E. coli* and LPS in lochia in the early postpartum period predisposes to uterine infections caused by TP and gram-negative anaerobic bacteria in the late postpartum period [12], the VF *kpsMTII* may play a role in this transition.

In addition, cases of mixed infection with *E. coli* and TP are common in young animals (Table 4). Moreover, the animals with *E. coli* harboring *kpsMTII* in this study exhibited significantly lower parity (Table 4). To date, there have not been any reports on the relationship between VFs in *E. coli* and TP. The possibility that TP may have an effect on the delay in uterine involution mediated by *kpsMTII* cannot be denied. Although it is clear that the presence of *kpsMTII* delays uterine involution, further large-scale studies are needed to clarify whether TP is involved in this delay. Moore *et al*. reported detecting the s16 rRNA gene sequence of TP in the uterus of virgin heifers [32]. These data are consistent with the fact that TP infections (umbilical cord inflammation, abscesses, and mastitis, etc.) that we encounter in clinical practice are more common in calves, heifers, and younger cows. These data may provide an opportunity to study the relationship between age and TP infection and between VFs in *E. coli* and TP infection. Prior to the present study, VFs associated with postpartum uterine infection had not been clearly identified, but we did so by carefully isolating bacteria from animals clearly exhibiting clinical symptoms.

In summary, we demonstrated that at least six VFs (*fimH*, *fyuA*, *hra1*, *csgA*, *astA*, and *kpsMTII*) are strongly associated with the development of clinical metritis and endometritis and that *kpsMTII* in particular is involved in prolonged uterine involution and worsening of symptoms. In addition, a possible relationship between *E. coli* VFs and TP infection in the uterus was identified. Further studies are required to clarify the mechanisms through which these factors mediate the pathogenesis of postpartum uterine infections and to examine the relationship between *E. coli* and TP in mixed infections. This study thus enhances understanding of the pathogenicity of *E. coli* VFs.

CONFLICT OF INTEREST. The authors declare no conflicts of interest associated with this manuscript.

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