Original article

Trueperella pyogenes and *Escherichia coli* as an etiological factor of endometritis in cows and the susceptibility of these bacteria to selected antibiotics

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Abstract

The aim of this study was to determine the percentage of participation of particular species of microorganisms, isolated from the uterus of cows with endometritis and from cows without inflammatory lesions of the uterus, in the same postpartum period. The aim of the study was also to examine how long after parturition non-treated endometritis persists. Moreover, antibiotic susceptibility tests were carried out of the bacterial isolates dominating in the uterus. Forty cows were included in the study: 20 cows with endometritis (experimental group) and 20 cows without any inflammatory condition of the uterus (control group). The material for cytological and bacteriological tests was collected on the 5th , 26th , 40th and 60th day after parturition, using an intrauterine brush adapted for cows. The total number of collected isolates was 149, including 120 isolates from the uterus of cows with endometritis and 29 isolates from the uterus of cows without endometritis. The following species of microorganisms were isolated from the material collected from cows with endometritis: *T. pyogenes* (49.2%), *E.coli* (22.5%), *F. necrophorum* (11.7%), *Staphylococcus sp.* (6.7%), *B. melaninogenicus* (5.8%), and *Streptococcus sp*. (4.1%). The participation percentage of particular species of bacteria in the material collected from the uterus of cows without endometritis was as follows: *T. pyogenes* (27.6%), *E.coli* (24.2%), *Staphylococcus sp.* (20.7%), *Streptococcus sp.* (20.7%), *B. melaninogenicus* (3.4%) and *F. necrophorum* (3.4%). The highest percentage of T. pyogenes isolates was susceptible to ceftiofur (89.6%); cefoperazone (85.1%) and amoxicillin combined with clavulanic acid (79.1%). E. coli isolates were most susceptible to amoxicillin combined with clavulanic acid (100%), cefoperazone (94.1%) and oxytetracycline (82.3%).

Key words: cows, endometritis, *T. pyogenes*. *E. coli*

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Introduction

Inflammatory conditions of the uterus in herds of dairy cows are a significant economic problem all over the world despite many scientific studies, the introduction of new diagnostic methods, and a variety of different treatment methods. Losses are a result of the treatment of inflammatory conditions of the uterus, fertility disorders, reduced milk production and, finally, culling of animals from the herd. (Gilbert et al. 2005, Lee and Kim 2007, Sheldon et al. 2009, Potter et al. 2010).

Bacterial infections and the mechanisms of local anti-infective immunity of the uterus play an important role in the complex etiology of endometritis. The most frequently isolated pathogens are *Escherichia coli* (*E. coli*) and *Arcanobacterium pyogenes* (*Trueperella pyogenes)* (*T. pyogenes*), which co-operate with anaerobic bacteria such as *Fusobacterium necrophorum* (*F. necrophorum)* and *Prevotella melaninogenicus* (*P. melaninogenicus*), (Foldi et al. 2006, Bicalho et al. 2010, Gautam 2010).

In the elimination of uterine infection, an essential role is played by cell-mediated and humoral mechanisms of local non-specific and specific anti-infective immunity. The first and most important defence mechanism against pathogens is phagocytosis involving phagocytic cells, mainly neutrophils, monocytes and macrophages belonging to innate immune mechanisms, as well as migration from the blood to the inflammation site as a specific response (Beutler et al. 2003, Sheldon et al. 2009, Gautam et al. 2010, Turner et al. 2012). The function of these cells is not only the absorbtion and intracellular killing of pathogens, but also eliminating the dead parts of tissues, which is particularly important in the uterus during the postpartum period.

The available literature shows that the occurrence of inflammatory conditions of the uterus is closely related to the postpartum period, while infections of the reproductive system occurring in this period may persist for a long time after parturition and cause fertility reduction in cows (LeBlanc et al. 2002, LeBlanc 2008, Foldi et al. 2006, Gautam et al. 2010). Sheldon et al. (2004a) confirmed that bacterial infections occurred in 80-100% of cows in the first 2 weeks after parturition. In the following weeks the number of infections decreased, and by 6-8 weeks after parturition pathogens were completely eliminated.

Use of a particular method for the diagnosis of inflammatory conditions of the uterus in cows depends primarily on the time of occurrence and severity of the inflammatory process. Diagnosis of extensive disease processes such as metritis and pyometra does not require the use of diagnostic methods other than the clinical; however, the diagnostics of endometritis, and especially the evaluation of the severity of the disease process, requires the use of additional methods, such as ultrasonographic, vaginoscopic, cytological, bacteriological and histopathological tests (Bonett et al. 1993, Sheldon et al. 2006, Barlund et al. 2008). One of the basic methods for diagnosis of endometritis in cows is the cytological test, which specifies the percentage of leukocytes in material collected from the uterus. Involution of the uterus is completed between 21 and 33 days post partum (DPP); however histological reversion to the pre-pregnancy condition takes place about 6 weeks after parturition (Sheldon et al. 2006). Therefore, in a cytological test carried out between 21 and 33 DPP, the acceptable number of leukocytes will be up to 18%, while between 34 and 47 DPP the number of leukocytes should not exceed 10% (Sheldon et al. 2006, Sheldon et al. 2009). In the period between 40 and 60 DPP, the number of leukocytes in cytology should not exceed 5% (Gilbert et al. 2005). Exceeding these thresholds for particular periods of time indicates the occurrence of endometritis.

The above-mentioned publications do not clearly indicate how long endometritis subclinica may persist postpartum. Few data are available specifying which microbes play a dominant role in the development and persistence of endometritis in cows in Poland. Therefore, in the study below, the condition of the reproductive system of cows from 5 to 60 days after parturition was monitored, based on cytological and bacteriological examination. The aim of the study was to determine both the etiological agent as well as the duration of the endometritis in cows, and to determine the participation percentage of particular species of microorganisms, isolated from the uterus of cows with endometritis and from cows without endometritis, in the same period after parturition. Additionally, antibiotic susceptibility tests were carried out of the bacterial isolates dominating in the uterus.

Materials and Methods

Experimental animals

The study was conducted on 40 cows of the Holstein-Friesian breed (HF) aged from 3 to 6 years, after second or third parturition. The animals were maintained in the same environmental conditions, and were fed and housed in identical manners. The feeding was based on the TMR (Total Mixed Ration). The condition of cows was assessed as being good or very good (Body Condition Score 3.5-4.0), and in the pre-

vious lactation the cows had produced over 7500 liters of milk.

All cows were subject to routine veterinary control in the post partum period. Apart from a general animal examination, the control included detailed examination of the reproduction system, based on vaginoscopic examination, rectal uterus examination together with a USG which was performed using a Honda HS – 1500 apparatus (Honda Electronics CO., LTD, Toyohashi, Japan), with a dual frequency 5.0/7.5 MHz intrarectal transducer, and uterus cytology. In addition, bacteriological cultures of smears from the uterus were made. The first test was carried out on day 5 after parturition and showed significant enlargement of the uterus (uterus inaccessible). In most cows the general symptoms of uterine inflammation did not occur; however, 9 cows were diagnosed with *metritis puerperalis,* with increased core body temperature >39.5°C and symptoms of toxemia. These animals were excluded from further studies and subjected to suitable treatment. In the remaining cows, the second test was performed on day 26 after delivery. Based on a uterine cytological test (neutrophil count above 18%), 20 cows with subclinical endometritis (*endometritis subclinica)* were selected; these represented the experimental group. There was no discharge from the reproductive tract, and a vaginoscopy examination showed no pathological secretion. In this group of animals another cytological and bacteriological test was performed on days 40 and 60 after delivery. The control group consisted of 20 cows without endometritis on day 26 postpartum (neutrophil count below 18%). Similarly to the experimental group, other tests were performed on the control animals on days 40 and 60 after delivery. During the study neither group of animals exhibited disorders of other body systems (metabolic disorders, mastitis, displaced abomasum, etc.) which could have influenced the results. In animals of both groups, diagnostic cytological tests were performed; their purpose was to confirm or rule out endometritis.

Material

The material for bacteriological and cytological tests consisted of uterine smears collected with intrauterine brushes adapted for cows (Jiangsu Yada Technology Group Co., Ltd, Jiangsu, China). The procedure for material collection was performed according to the literature (Kasimanickam et al. 2004, Sheldon et al. 2006). Within an hour, the biological material collected for laboratory tests was delivered to the laboratory.

Bacteriological tests

Bacteriological tests were performed on standard substrates: Columbia blood agar, Schaedler blood agar and McConkey agar (BioMerieux SA, Marcy L'Etoile, France), intended for the culture of aerobic and anaerobic bacteria (Malinowski et al. 2011). The cultures were incubated at 37°C for 24-48 hours under aerobic and anaerobic conditions. After the incubation, any microorganisms present were initially identified based on the time of growth, morphology of colonies and evaluation of Gram-stained microscopic specimens. Isolated strains of microorganisms were subjected to further identification by biochemical characterization kits, using API tests (Appareils et Procédés d'Identification: BioMérieux SA, Marcy L'Etoile, France) (Malinowski et al. 2011).

Cytological tests

After preparing cultures from the bacteriological test swabs, imprint cytological specimens were performed, which were fixed and stained using the Haemacolour method after thorough drying (Merck KGaA, Darmstadt, Germany). Slides for cytological examination were prepared by rolling the uterobrush onto a clean microscope glass slide. Specimens were evaluated under a microscope (Olympus CX 41 – Olympus Corporation, Tokyo, Japan) at 1000x magnification. Cytological examination of the specimens was based on an evaluation of the number of neutrophils in the smears. At least 100 cells (endometrial cells, leukocytes) visible in various fields of view in particular specimens were subject to counting. The percentage of neutrophils was established among them.

Determination of susceptibility of bacteria to selected antibiotics

Susceptibility of microorganisms to antibiotics was evaluated using the disk diffusion method on Mueller-Hinton agar. Susceptibility of Trueperella pyogenes (*T. pyogenes*) and Escherichia coli (*E. coli*) isolatates to the following antibiotics was tested: amoxicillin combined with clavulanic acid (30 μg), ampicillin (10 μg), cephapirin (30 μg), cefoperazone (75 μg), ceftiofur (30 μg), gentamicin (10 μg), cloxacillin (5 μg), neomycin (30 μg), oxytetracycline (30 μg) and rifampicin (5 μg).

Table 1. Uterine cytology in cows with endometritis (experimental, n=20) and in cows without endometritis (control, n=20).

Type of test		Day 5 after parturition	Day 26 after parturition	Day 40 after parturition	Day 60 after parturition
Neutrophils (%)	experimental	65.05[a] ± 6.36	38.90[b**] ± 5.99	29.85[c**] ± 5.94	13.95[d**] ± 3.83
	control	64.35[a] ± 6.39	13.60[b**] ± 3.41	6.35[c**] ± 2.41	2.15[d**] ± 1.95

** – statistical significance at p≤0.001 with respect to the control.
a-d – Statistical differences between the results for the material collected at different times in the group for probability *P*<0.01.

Table 2. Microorganisms isolated from the uterus of cows with endometritis for particular test dates (days after parturition).

Days after parturition	*T. pyogenes* (59)		*E. coli* (27)		*F. necroph.* (14)		*Staph. sp.* (8)		*P. melani.* (7)		*Strept. sp.* (5)	
	n	%	n	%	n	%	n	%	n	%	n	%
5	7	11.9	14	51.9	1	7.2	4	50.0	1	14.3	3	60.0
26	12	20.3	8	29.6	3	21.4	3	37.5	2	28.6	2	40.0
40	17	28.8	4	14.8	5	35.7	1	12.5	3	42.8	0	0.0
60	23	39.0	1	3.7	5	35.7	0	0.0	1	14.3	0	0.0

Table 3. Microorganisms isolated from the uterus of cows without endometritis for particular test dates (days after parturition).

Days after parturition	*T. pyogenes* (8)		*E. coli* (7)		*Staph. sp.* (6)		*Strept. sp.* (6)		*F. necroph.* (1)		*P. melani.* (1)	
	n	%	n	%	n	%	n	%	n	%	n	%
5	5	62.5	7	100.0	4	66.7	6	100.0	1	100.0	1	100.0
26	2	25.0	0	0.0	2	33.3	0	0.0	0	0.0	0	0.0
40	1	12.5	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0
60	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0

Table 4. Percentage of particular species of microorganisms isolated from the uterus of cows with endometritis and cows without endometritis.

Isolated microorganisms	Isolates from cows with endometritis (120)		Isolates from cows without endometritis (29)		Total isolates (149)	
	n	%	n	%	n	%
T. pyogenes	59	49.2	8	27.6	67	44.9
E. coli	27	22.5	7	24.2	34	22.8
F. necroph.	14	11.7	1	3.4	15	10.1
Staph. sp.	8	6.7	6	20.7	14	9.4
P. melani.	7	5.8	1	3.4	8	5.4
Strept. sp.	5	4.1	6	20.7	11	7.4

Statistical analysis

The Shapiro – Wilk test was used in order to confirm the normal distribution of the examined features. The results of the cytological examination were compared between the groups in order to determine their statistical significance using Student's t test. Statistical differences between particular material samplings in both experimental groups were calculated using one-factor analysis of variance (One-way ANOVA) as well as post-hoc Turkey's and Duncan's tests, for a probability value of p<0.01.

Table 5. Susceptibility of T. pyogenes and E. coli to antibiotics.

Used antibiotics	T. pyogenes						E. coli					
	S		I		R		S		I		R	
	n	%	n	%	n	%	n	%	n	%	n	%
Amp	28	41.8	18	26.9	21	31.3	24	70.6	2	5.9	8	23.5
Amx	56	83.6	3	4.5	8	11.9	30	88.2	4	11.8	0	0.0
Cfp	43	64.2	14	20.9	10	14.9	23	67.6	9	26.5	2	5.9
Cpr	21	31.4	23	34.3	23	34.3	0	0.0	17	50.0	17	50.0
Clox	4	6.0	2	3.0	61	91.0	0	0.0	0	0.0	34	100.0
G	17	25.4	17	25.4	33	49.2	18	53.0	8	23.5	8	23.5
Cft	58	86.6	2	3.0	7	10.4	16	47.1	6	17.6	12	35.3
Neo	11	16.4	15	22.4	41	61.2	0	0.0	17	50.0	17	50.0
Oxyt	13	19.4	9	13.4	45	67.2	20	58.8	8	23.5	6	17.7
Rifam	18	26.9	17	25.4	32	47.8	0	0.0	0	0.0	34	100.0

S – sensitive; I – intermediately sensitive; R – resistant. Amp – ampicillin, Amx – amoxicillin with clavulanic acid; Cfp – cefoperazone; Cpr – cephapirin; Clox – cloxacillin; G – gentamycin; Cft – ceftiofur; Neo – neomycin; Oxyt – oxytetracycline; Rifam – rifampicin.

Results

Results of cytological tests

The cytological test results from cows with endometritis are presented in Table 1. The neutrophil percentage in both examined groups was similar 5 days post partum (DPP) (65.05% for the experimental group and 64.35% for the control group; Table 1). The average leukocyte count in both groups decreased significantly ($p<0.01$); the lowest values were reached on 60 DPP. There were significant differences ($p<0.001$) between the experimental and the control groups. A significantly higher percentage of neutrophils was observed in cows with endometritis on 22, 40 and 60 DPP ($p<0.001$; Table 1).

Results of bacteriological tests

From the material collected during 4 subsequent tests (days 5, 26, 40 and 60 after parturition), 149 isolates were obtained, including 120 from the uterus of cows with endometritis and 29 from the uterus of cows without endometritis (80.5 and 19.5% respectively). The most frequently isolated microorganism was *T. pyogenes* both in the pure culture and in the mixed cultures with other bacteria (*Fusobacterium sp., Prevotella sp., Staphylococcus sp., Streptococcus sp.*), and represented 44.9% of all isolates (Table 4). Significantly higher percentages of this bacterium were isolated in the group of cows with endometritis than in the control animals (49.2% and 27.6% respective-

ly), and it was a dominant microorganism isolated on 60 DPP from the uterus of sick cows (Table 1). In the control group, over half of the T. pyogenes isolates (62.5%) were observed on day 5 after parturition, while in the last test no growth of this bacteria was observed (Table 3).

A second dominant species in the test was E. coli (22.8% of all isolates) (Table 4). 51.9% of all E.coli isolates from the group of cows with endometritis was observed in the first test (Table 2). In the following tests the percentage of E. coli isolates had decreased gradually to 29.6% by day 26, 14.8% by day 40 and 3.7% by day 61 after parturition in cows with endometritis (Table 2). In the control group, E.coli isolates were observed only in the first test. In the next 3 tests carried out on the material collected from the uterus of cows from the control group, no growth of E. coli was observed (Table 3).

The participation of other species of microorganisms: *F. necrophorum*, *Staphylococcus sp.*, *Streptococcus sp.* and *P. melaninogenicus* was less significant (Table 4). These bacteria, in most cases, were associated with the dominant pathogens (mixed flora), so susceptibility to antibiotics was determined only for *T. pyogenes* and *E. coli*.

Results of susceptibility of bacteria to selected antibiotics

The data in Table 5 indicates that the highest percentage of *T. pyogenes* isolates was susceptible to ceftiofur, cefoperazone and amoxicillin combined with

clavulanic acid, taking together the sensitive (S) and intermediately sensitive (I) isolates. Susceptibility to ampicillin and cephapirin was significantly lower. A significant number of *T. pyogenes* isolated appeared to be resistant to cloxacillin, oxytetracycline and neomycin.

E. coli isolates were most susceptible to amoxicillin combined with clavulanic acid (100%), cefoperazone and oxytetracycline. A high percentage of isolates susceptible to gentamicin, ampicillin and ceftiofur was also observed. The least efficient were cloxacillin and rifampicin (100% of resistant isolates). Half of the *E. coli* isolates were resistant to neomycin and cephapirin.

Discussion

In these studies, bacteria from the uterus of cows with normal or disturbed postpartum periods were isolated, and the species and antibiotic susceptibility of dominant bacteria were determined. The results of personal research is consistent with those of other authors, which indicates that, in the bacteriological test of the smears from the uterus with endometritis, most frequently isolated are: *Trueperella pyogenes*, *Escherichia coli*, *Pseudomonas sp.*, *Streptococcus sp.*, *Staphylococcus sp.*, *Pasteurella multocida*, *Clostridium sp.*, *Fusobacterium sp*. and *Bacteroides sp.* (Bondurant 1999, Zerbe et al. 2001, LeBlanc 2008). The results are also consistent with a view presented by Gautam et al. (2010) that, in the uterus of cows in the early postpartum period, the same microorganisms are present, regardless of the occurrence of endometritis at a later period. It has not been fully recognized why some of the cows are not able to eliminate the microorganism, resulting in the development of infection, while some are, even though the presence of the same microorganisms was confirmed in bacteriological tests. Some authors claim that during normal uterine functioning, microorganisms entering the uterus are promptly eliminated by mechanisms of local anti-infective immunity, while in the case of their reduced activity endometritis develops (Foldi et al. 2006, LeBlanc et al. 2011).

From all of the experimental cows, pathogenic microorganisms were isolated between day 5 and day 40 after parturition. In 3 cows, despite an increased number of leukocytes in the cytological tests, there was no bacterial growth confirmed in bacteriological tests until day 60. In the available literature there are studies describing elevated levels of neutrophils, indicating an inflammatory condition not related to the presence of bacteria in bacteriological testing (McDougall et al. 2011, Barański et al. 2012). It is not known what may be the cause of the development and persistence of the inflammatory condition without confirmation of pathogen presence. Only Sheldon et al. (2009) suggest that this condition may persist for different periods of time after eliminating pathogens from the uterus due to the disregulation of the prostaglandin secretion mechanism. Studies conducted by Gabler et al. (2009) confirm this theory and underline that the amount of intracellularly-produced PGE in the endometrial cells is lower in cows with endometritis than in healthy cows, which may contribute to early embryonic death. The results of our studies conducted in this field confirm that in only 3 cows out of the 20 cows tested (15%) bacteria from the uterus were spontaneously eliminated. It also shows that spontaneous elimination of bacteria in the course of endometritis is possible without treatment; however, this concerns only a small percentage of cows. At the same time, the results of the tests clearly indicate that the development of inflammatory conditions and initial inflammations occur during the early postpartum period, and may persist for a very long time after delivery in the form of endometritis. This is consistent with literature data, which confirm the long-term persistence of the postpartum infections of the uterus in the form of endometritis (LeBlanc et al. 2002, Kasimanickam et al. 2004, LeBlanc 2008). According to the authors, the presence of pathogenic microorganisms in the uterus at a specified time after delivery does not have to be related with the occurrence of endometritis. Effective mechanisms of the local anti-infective resistance initially do not allow for intensive development of pathogens, and later lead to their complete elimination. An example may be cows in the control group. Only on day 5 after delivery were bacteria isolated from all cows. By the next test, the number of isolated bacteria had decreased, and by day 60 after delivery a lack of microorganisms in the bacterial cultures was observed. This is also consistent with data presented by many authors where, during the normal postpartum period, pathogens are eliminated gradually from the uterus, so that 6 weeks after delivery uterine cultures should show no growth of microorganisms (Foldi et al. 2006, Gautam et al. 2010).

Most authors indicate that *E. coli* is a predominant etiological factor of uterine infection during the first week after parturition (Williams et al. 2005, Földi et al. 2006, LeBlanc 2008, Sheldon et al. 2009). Our studies confirmed the findings of the earlier authors, which show that during the first week after delivery in cows with *metritis*, *E. coli* is a dominant species of bacteria isolated from the uterus of cows in the experimental and control group. It is even assumed that this microorganism acts as a promoter, and promotes

the development of uterine infections caused by different pathogens (Földi et al. 2006, Bicalho et al. 2010, Gautam et al. 2010). In the following tests carried out on cows with endometritis, the percentage of *E. coli* isolates decreased gradually to the advantage of *T. pyogenes* and anaerobic bacteria (*Fusobacterium sp.*, *P. melaninogenicus*). Our results confirm the findings of other authors, which show that in the etiopathogenesis of bacterial infections of the uterus, cooperation between *T. pyogenes* and *F. necrophorum* as well as *P. melaninogenicus* is significant and increases the possibility of the occurrence of uterine inflammatory conditions and the intensification of disease symptoms (Ruder et al. 1981, Olson et al. 1984, Sheldon et al. 2009). It was even determined that *T. pyogenes* produces a substance called growth factor for *F. necrophorum* and is able to produce pyolysin, which damages endometrial epithelial cells, and perhaps phagocytic cells as well. The anaerobic bacteria are also able to produce substances that impair the mechanisms of uterine local immunity. *F. necrophorum* produces leukotoxin and *P. melaninogenicus* produces a substance which inhibits phagocytosis, which allows them to inhibit cell-mediated and humoral immunity, or both. It promotes the development of infections, and microorganisms may persist in the uterus for an extended length of time (Miller et al. 2007, Williams et al. 2008a,b, Sheldon et al. 2010).

Antibiotics used in our studies were characterized by different levels of efficacy. The highest in vitro activity against *T. pyogenes* and *E. coli* isolates showed cephalosporins and amoxicillin combined with clavulanic acid. Other authors also confirm the high efficiency of cephalosporins against aerobic microorganisms isolated from the uterus of cows (Sheldon et al. 2004a,b, Galvão et al. 2009, Malinowski et al. 2011). In the case of anaerobic bacteria, no positive effect on metritis treatment with ceftiofur was observed (Jeremejeva et al. 2010).

Significant differences were observed in *T. pyogenes* and *E. coli* susceptibility to oxytetracycline. Isolates resistant to this antibiotic were observed in other studies (Cohen et al. 1995, Cohen et al. 1996, Farca et al. 1997, Malinowski et al. 2011). Different results obtained by Silvo and Lobato (1998) showed the high efficiency of oxytetracycline against *T. pyogenes*. In our studies, the percentage of *T. pyogenes* isolates resistant to this antibiotic was about 68%, while the majority of *E. coli* isolates appeared to be susceptible to oxytetracycline (about 83%).

In the group of antibiotics used in the experiment, the lowest in vitro activity occured with cloxacillin and rifampicin. Literature data and our own studies show that besides tetracyclines and cephalosporines, rifampicin is one of the most frequent antibiotics used in

Poland to treat metritis in cows. However, their efficiency is hard to assess due to the different doses of these drugs used by veterinarians (Malinowski et al. 2011).

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