รายงานวิจัยฉบับสมบูรณ์

รูปแบบการดื้อต่อยาปฏิชีวนะและปัจจัยรุนแรงทางพันธุกรรมของเชื้อที่แยกได้จากเยื่อบุโพรงมดลูกที่อักเสบของโคนม

โดย ผศ.ดร. วีระศักดิ์ ปัญญาพรวิทยา

สิงหาคม พ.ศ. 2558

สัญญาเลขที่MRG5580138

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สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย

Abstract

Project Code : MRG5580138

Project Title : Antibiotic resistance profile and virulence factor genes in *Acranobacterium pyogenes* isolated from endometritis dairy cows

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Project Period : 2 years (2 July 2012 to 1 July 2014)

The aims of this study were to identify virulence factor genes that could be associated with clinical finding and to assess antimicrobial agent resistance in *Acranobacterium pyogenes* isolated from uterus of dairy cows with endometritis. Vaginal discharge from 120 dairy cows, 30-90 days postpartum, with abnormal virginal discharge were collected using a trancervical device and the appearance of discharge was scored. Uterine secretions were collected from all cows using transcervical guarded swab with aseptic technique. Bacteriological examination was performed under conventional aerobic conditions. Sensitivity to antimicrobial agents was tested by the disk diffusion method. Genomic DNA fingerprinting was generated using BOX-PCR typing. A conventional PCR was used to determine the presence of four virulence factor genes including *plo, cbpA, nanP* and *fimA*. Result showed that *A. pyogenes* isolates were found in samples from 9 cows (7%) with the same vaginal discharge score (score=2). All isolates were resistant to antimicrobial agents. Most of *A. pyogenes* isolates were resistance to sulfamethoxazole-trimethoprim. Both genes *plo* and *cbpA* were presented in all 9 isolates. The *fimA* gene was found in 2 isolates. The study indicated that virulent gene factors of *A. pyogenes* associated with the clinical finding of uterine discharge. The resistant to antimicrobial agents of *A. pyogenes* was evident.

Keywords : Antibiotic resistance, virulence gene, *Acranobacterium pyogenes,* endometritis, dairy cows

บทคัดย่อ

รหัสโครงการ: MRG5580138

ชื่อโครงการ: รูปแบบการดื้อต่อยาปฏิชีวนะและปัจจัยรุนแรงทางพันธุกรรมของเชื้อที่แยกได้จากเยื่อบุโพรงมดลูกที่อักเสบของโคนม

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การศึกษาครังนี้มีวัตถุประสงค์เพื่อหาปัจจัยความรุนแรงทางด้านพันธุกรรมที่สัมพันธ์กับการแสดงอาการทางคลินิกและหาภาวะการดื้อยาของเชื้อ *Acranobacterium pyogenes* ที่แยกได้จากมดลูกโคที่มีภาวะมดลูกอักเสบ ทำการเก็บตัวอย่างสิ่งคัดหลั่งบริเวณปากช่องคลอดของโคที่มีภาวะมดลูกอักเสบจำนวน 120 ตัวที่อยู่ในช่วงหลังการคลอด 30-90 วันด้วยเครื่องมือเก็บตัวอย่างและทำการบันทึกคะแนนสิ่งคัดหลั่ง ทำการเก็บตัวอย่างสิ่งคัดหลั่งภายในมดลูกด้วยเครื่องมือเก็บตัวอย่างด้วยวิธีที่ปลอดเชื้อ ทำการเพาะเชื้อภายใต้ภาวะที่มีอ็อกซิเจน หาความไวต่อยาปฏิชีวนะของเชื้อด้วยวิธีการที่ใช้แผ่นยาต้านจุลชีพ ทำการหาลายพิมพ์ดีเอ็นเอโดยใช้วิธี BOX-PCR จากนั้นใช้วิธี PCR สำหรับการหาปัจจัยรุนแรงทางพันธุกรรม 4 ชนิดซึ่งได้แก่ plo, cbpA, nanP และ fimA ผลการศึกษาพบว่า *A. pyogenes* ไอโซเลตพบในตัวอย่างมดลูกจากโคทั้งหมด 9 ตัว (7%) ที่มีคะแนนสิ่งคัดหลั่งจากปากช่องคลอด คะแนนเท่ากับ 2 ไอโซเลตของเชื้อที่พบทั้งหมดดื้อต่อยาปฏิชีวนะ พบว่าไอโซเลตของ *A. pyogenes* ดื้อต่อยาซัลฟาเมตท็อกซาโซน-ไตรเมทโทรพริม ไอโซเลตจำนวน 9 ไอโซเลตมีปัจจัยรุนแรงทางพันธุกรรม plo และ cbpA ในขณะไอโซเลตจำนวน 2 ไอโซเลตมี fimA การศึกษาครั้งนี้แสดงให้เห็นถึงความสัมพันธ์ระหว่างปัจจัยรุนแรงทางพันธุกรรมของเชื้อ และพบการดื้อยาปฏิชีวนะอย่างเด่นชัดของเชื้อ *A. pyogenes*

คำหลัก: การดื้อต่อยาปฏิชีวนะ, ปัจจัยรุนแรงทางพันธุกรรม, *Acranobacterium pyogenes,* ภาวะมดลูกอักเสบ, โคนม

บทสรุปโครงการ (Executive Summary)

ภาวะมดลูกอักเสบในโคนมเป็นปัญหาสุขภาพที่มีความสำคัญและมีผลก่อให้เกิดความสูญเสียทางเศรษฐกิจอย่างมากในอุตสาหกรรมโคนมทั้งในประเทศไทยและประเทศอื่นๆทั่วโลก ความสูญเสียเหล่านี้มักมาจากความสูญเสียจากสมรรถภาพการสืบพันธ์ที่แย่ลง ค่าใช้จ่ายในการรักษาและอัตราการคัดโคที่มีปัญหามดลูกอักเสบหรือปัญหาระบบสืบพันธุ์ที่เป็นผลตามมาจากมดลูกอักเสบเพิ่มสูงขึ้น ภาวะมดลูกอักเสบในโคนมมักมีสาเหตุจากการติดเชื้อจุลชีพภายในมดลูก ทั้งนี้มีรายงานโดยส่วนใหญ่ว่าเชื้อ *Acranobacterium pyogenes* เป็นเชื้อก่อโรคที่มีความสำคัญมากที่มักพบว่าเป็นเชื้อที่ก่อให้เกิดภาวะมดลูกอักเสบในโคนม การศึกษาในประเทศไทยพบเชื้อนี้มามากกว่า 15 ปี แต่ยังมีการศึกษาเกี่ยวกับเชื้อนี้น้อยมาก ซึ่งปัจจุบันได้มีการใช้ยาปฏิชีวนะที่หลากหลาย ดังนั้นข้อมูลการดื้อยาของเชื้อนี้ควรได้ทำการศึกษา นอกจากนี้ยังไม่เคยมีการศึกษาเกี่ยวกับปัจจัยรุนแรงทางพันธุกรรมของเชื้อนี้ในประเทศไทย ดังนั้นวัตถุประสงค์ของการศึกษานี้ คือ การหาความชุก การดื้อต่อยาปฏิชีวนะของเชื้อและการหาปัจจัยรุนแรงทางพันธุกรรมของเชื้อ *A. pyogenes* ที่เพาะแยกได้จากสิ่งคัดหลั่งในมดลูกโคที่อยู่ในภาวะมดลูกอักเสบ ในเขตจังหวัดเชียงใหม่ ทำการเก็บตัวอย่างสิ่งคัดหลั่งในมดลูกทั้งสิ้น 120 ตัวอย่างด้วยเครื่องมือเก็บตัวอย่างด้วยวิธีที่ปลอดเชื้อ ทำการเพาะเชื้อภายใต้ภาวะที่มีอ็อกซิเจน หาความไวต่อยาปฏิชีวนะของเชื้อด้วยวิธีการที่ใช้แผ่นยาต้านจุลชีพ ทำการหาลายพิมพ์ดีเอ็นเอโดยใช้วิธี BOX-PCR จากนั้นใช้วิธี PCR สำหรับการหาปัจจัยรุนแรงทางพันธุกรรม 4 ชนิดซึ่งได้แก่ plo, cbpA, nanP และ fimA ผลการศึกษาพบว่า *A. pyogenes* ไอโซเลตพบในตัวอย่างมดลูกจากโคทั้งหมด 9 ตัว (7%) ที่มีคะแนนสิ่งคัดหลั่งจากปากช่องคลอดคะแนนเท่ากับ 2 ไอโซเลตของเชื้อที่พบทั้งหมดดื้อต่อยาปฏิชีวนะ และพบว่าไอโซเลตของ *A. pyogenes* ดื้อต่อยาซัลฟาเมตท็อกซาโซน-ไตรเมทโทรพริม ไอโซเลตจำนวน 9 ไอโซเลตมีปัจจัยรุนแรงทางพันธุกรรม plo และ cbpA ในขณะไอโซเลตจำนวน 2 ไอโซเลตมี fimA การศึกษาครั้งนี้แสดงให้เห็นถึงความสัมพันธ์ระหว่างปัจจัยรุนแรงทางพันธุกรรมของเชื้อ และพบการดื้อยาปฏิชีวนะอย่างเด่นชัดของเชื้อ *A. pyogenes*

Introduction to the research problem and its significance

Clinical endometritis is the most common case of uterine infection causes economic losses in dairy industry (Sheldon et al., 2006). This disease associated with lower conception rate, increased calving to conception interval and more cows culled for failure to conceive. *Acranobacterium pyogenes* is considered to be the most relevant bacterium cause endometritis (Sheldon et al., 2004). The microorganism expresses several known virulence factorsassociated with the pathogenicity of this pathogen.

There are only two reports in Thailand that uterine discharge is sampled and prevalence of endometritis is identified (Virakul et al., 1995; Ngarmkum et al., 2001). These studies indicated that the prevalence of endometritis is about 15-22% which is consistent with the finding elsewhere (Gilbert et al., 2005; Sheldon et al., 2006). However, there is no follow up study in Thailand for more than 10 years. Hence, the current epidemiological status is unavailable in literatures. The limitation of such epidemiological information may subsequently effects on the policy making and appropriate strategy to control the disease.

Antibiotic therapy is a common treatment for metritis. However, studies showed that there is a broad and variable antimicrobial resistance profile among *A. pyogenes* microorganisms (Sheldon et al., 2004; Santos et al., 2010). The recent study indicated that *A. pyogenes* is resistance to amoxicillin, ampicillin, chloramphenicol, oxytetracycline and penicillin (Santos et al., 2010). Data in Thailand showed that bacterial isolates collected from uterus from dairy cows in different regions have a different profile of antibiotic resistance (Ngarmkum et al., 2001).This suggests that the resistance of bacterial pathogen to antibiotic in dairy cows is an important issue.

In Thailand, the study of epidemiology and drug resistance profile of *A. pyogenes* causes uterine infection in dairy cow is very limited. Also, there is no report for genetic patterns of this pathogen. This study aims to determine virulence factor genes, drug resistance profile and molecular epidemiology of *A. pyogenes*in dairy cows. The results of this study will provide a better understanding for the pathogen which is important for developing an efficient treatment and control strategy. In additions, the finding of virulence factor genes of such pathogen will be a good source of information for determining the pathogen and host interactions in the follow up study.

Literature review

Epidemiology

Postpartum uterine infection is one of the most important disease causes economic losses in dairy cows (Sheldon et al., 2006;Azawi, 2008). The prevalence of uterine infections varies among studies. The variation is due to the classification of the uterine infection, diagnostic method and the defined period (Azawi, 2008). However, it is estimated that the prevalence of endometritis is about 15-20% (Borsberry and Dobson, 1989; LeBlanc et al., 2002a;Gilbert et al., 2005).

Clinical definitions

The definitions or characterization of the various manifestations of uterine disease either lack precisions or definitions vary among research groups and are not validated. However, the practical definitions particularly for research are proposed (Sheldon et al., 2006). Postpartum uterine diseases are categorized to four groups including puerperal metritis, clinical endometritis, subclinical endometritis and pyometra. Puerperal metritis is an acute systemic illness due to infection of the uterus with bacteria, usually within 10 days after parturition. It is characterized by an enlarged uterus and a red-brown fluid to viscous off-white purulent uterine discharge. Clinical endometritis is characterized by the presence of purulent uterine discharge detectable in vagina 21 days or more postpartum, or mucopurulent discharge detectable in the vagina after 26 days postpartum. Subclinical endometritis is defined as the inflammation of the endometrium that results in a significant reduction in reproductive performance in the absence of sings of clinical endometritis. Subclinical endometritis is determined by the presence of >18% neutrophils in uterine cytology samples collected 21-33 days postpartum or >10% neutrophils at 34-47 days, in the absence of clinical endometritis. Pyometra is characterized by the accumulation of purulent or mucopurulent materials within the uterine lumen and distension of the uterus.

Reproductive and economic consequences of uterine disease

Uterine bacterial infection has a negative effect on reproductive performances of dairy cows. The conception rate is 20% lower for cows with endometritis, the median calving to conception interval longer and there are 3%

animal culled for failure to pregnant (Borsberry and Dobson, 1989; LeBlanc et al., 2002a). Furthermore, cows with a purulent cervical discharge have lower submission rates (McDougall, 2001). The subfertility associated with uterine infection involves disruption of ovarian functions as well as the effects of uterine destruction. In addition, the epidemiological study indicates that uterine infection is as risk factor for delayed ovulation (Odds ratio=4.5) (Opsomer et al., 2000).

Risk factors

Factors that predispose cows to develop inflammation of the uterus are dystocia, retained fetal membranes, twins or stillbirths, abortions and metabolic disorders (Peeler et al., 1994a, b; LeBlanc et al., 2002a).

Pathogenesis

During parturition, microorganisms from the environment invade the birth canal and colonized in the uterus. In general, most healthy cows are able to spontaneously clear this contamination in the next 2-4 weeks (Hussain et al., 1990). However, approximately 10-17% of dairy cows still be infected with pathological bacteria (LeBlanc et al., 2002a). Up to 40% of animals have metritis within the first two weeks of calving and that in 10-15% of these animals infection persists for at least another three weeks causing the chronic uterine disease called endometritis (Sheldon and Dobson, 2004). Common bacteria isolated from cows with acute metritis and chronic endometritis are *A. pyogenes, Prevotella spp., Fusobacterium necrophorum* and *Escherichia coli* (Sheldon et al., 2004; Sheldon and Dobson, 2004). Other studies also found *Staphylococcus spp., Streptococcus spp*. or non-*E.coli* aerobic gram-negative rods(Paisley et al., 1986; Huszenicza et al., 1999). The pathogenic bacteria that are frequently isolated from endometritis cases are *A. pyogenes*and *E. coli*.

The presence of pathogenic bacteria in the uterus causes inflammation and histological lesion of endometrium. The clinical signs of uterine infection depend on number and virulence of the microorganism present and the conditions of the uterus, and its inherent defense mechanism.

Diagnosis of endometritis

The definitive diagnosis of endometritis is made on the basis of histological examination of endometrium biopsies. However, this technique is

costly and time consuming, not clinically accessible in most situations (Sheldon et al., 2006). Hence, the examination of the contents of the vagina for presence of pus is proposed. It is inexpensive and quick procedure and is used in various researches (LeBlanc et al., 2002a; Williams et al., 2005). Several methods of vaginal examination are reported (Sheldon et al., 2006).

The simplest method is to perform a manual examination of vagina and withdraw the mucus for inspection by insertion a clean, lubricated glove hand through the vulva into the vagina. The lateral, dorsal and ventral walls of the vagina and the external cervical os are palpated and the mucous contents of the vagina withdrawn for examination. The second method is to use vaginoscope to inspect of vagina (LeBlanc et al., 2002a). The last method is to use a device for examination of vagina mucous.

Normal discharge from postpartum cows range in color from dark brown to red or white and usually should not be considered abnormal unlessthe fluid is malodorous or other aberrant clinical signs are found (LeBlanc et al., 2002a). In contrast, the clinical signs of endometritis are the presence of a white or whitish-yellow mucopurulent vaginal discharge. The volume of the discharge is variable, but frequently increases at the time of estrus when the cervix dilates and there is abundant vaginal mucus. The cow rarely shows any signs of systemic illness (Jost et al., 2002).

Several scoring systems have been described to estimate the severity of clinical endometritis (Williams et al., 2005;Sheldon et al., 2006). The vaginal can score system as follows: (0) clear or translucent mucus; (1) mucus containing flecks of white or off-white pus; (2) <50 mL exudate containing ≤ 50% white or off-white mucopurulent material; and (3) >50 mL exudate containing purulent material, usually white or yellow, but occasionally sanguineous. The vaginal mucus was also assessed by odour, and given a score 0 for normal odour or a score of 1 if a fetid odour was detected.

Phenotypic and Genomic characteristic of *A. pyogenes*

A. pyogenes is a gram-positive aerobic bacillus that secretes its virulence factors that interact with the immune system of host cells. *A. pyogenes* expresses several virulence factors including a heamolysin with cytolytic activity for immune cells (pyolysin, *plo*), neuraminidases (*nanH* and *nanP*) (Jost et al., 1999), a collagen-binding protein (*CbpA*) and fimbriae (*fimA, fimC, fimE* and

fimG) (Billington et al., 2001). The *fimA* gene is presented in significantly higher frequency in isolated from metritis cows (Santos et al., 2010). The most important virulent factor gene is *plo* gene. It encodes a cholesterol dependent cytotoxinpyolysin.Pyolysin molecules are attracted to cholesterol-rich domains in cell membranes, where the aggregate to form a pore, which leads to osmotic death of the cell (Jost and Billington, 2005).

Antibiotic therapy and antimicrobial resistance of bacteria

Antibiotics therapy is a common treatment for metritis (Azawi, 2008). A variety of antimicrobial agents, administered by intrauterine infusion or parenteral injection, are normally used to treat uterine infection (Gustafsson, 1984). Oxytetracycline is a broad spectrum antibiotic and is indicated for the treatment and control of infections caused by or associated with oxytetracycline sensitive, rapidly growing bacteria. The direct intrauterine administration of oxytetracycline produces immediate therapeutic concentration in the caruncles and endometrium of both healthy and affected animals, and because of its relatively low absorption into the blood stream (Roncada et al., 2000), the therapeutic action is largely confined to the uterine lumen and endometrium (Sheldon et al., 2004b).

To choose an appropriate antimicrobial agent to treat endometritis it is essential to know the susceptibility of the pathogen to antibiotics. Thus, there should be information about drug sensitivity for metritis treatment because currently antimicrobial resistance in pathogenic bacteria has become a common problem. A recent study showed that isolates from uterus of postpartum dairy cows (n=72) exhibit resistance to many antimicrobial agents tested; high levels of resistance are found to amoxicillin, ampicillin, chloramphenicol, oxytetracycline, and penicillin. More than 95% of isolates resistance to at least of the antimicrobial agent tested and multidrug resistance (resistant to at least 3 antimicrobial agents) was observed in 89% of isolates (Santos et al., 2010). This information highlights the problem of antimicrobial resistance in dairy production.

Objectives

1. To determine the prevalence of *A. pyogenes* causes abnormal uterine discharge in dairy cows with 50-80 days postpartum.
2. To determine the antibiotic resistance profile of *A. pyogenes*.
3. To determine the presence of virulent factor genes including *plo*, *cbp*A, *nanP* and *fim*A gene.
4. To determine the association between clinical finding through vaginal mucous score and the presence of *A. pyogenes* in the uterine
5. To determine the association between clinical finding through vaginal mucous score and the presence of virulent factor genes including *plo*, *cbp*A, *nanP* and *fim*A gene.

Methodology

1. Animals

   Holstein-Frisian cows, lactation 1-6, 30-120 days postpartum with abnormal vaginal discharge were enrolled in the study.

2. Case definition

   Clinical endometritis is defined the presence of purulent uterine discharge detectable in vagina 21 days or more postpartum, or mucopurulent discharge detectable in the vagina after 26 days postpartum (Sheldon et al., 2006).

3. Sample size calculation

   Based on the prevalence reported by Virakul et al. (1995), the prevalence of 30% is used for sample size calculation. The population of milking cows in the area proposed to study is 1,000 cows. Accepted error is set at 5% with a 95% confidence level. The sample size is calculated based on the following formula (Thrusfield et al., 2001):

$$n = \left(\frac{t \times SD^2}{L} \right)$$

   Where:

- t is Student's t-value which is 1.96 based on the 95% confidence interval.
- SD = $\sqrt{p(1-p)}$ wherep= prevalence
- L is the accepted absolute error or precision which is 5%.

The calculation was operated by WINEPISCOPE software (Thrusfield et al., 2001). The estimated sample size is at least 81. In this study, a total of 90 cows from approximately 70 dairy farms in Chiang Mai province were included.

4. Vaginal mucous examination, collection and scoring

For examination, cows were inspected for the presence of fresh discharge on the vulva, perineum, or tail. If discharge was not visible externally, cows were examined by using vaginoscope. The vulva was wiped clean with damp paper towels, and lubricant was applied to vaginoscope. The vaginoscope was inserted into the vagina up to the level of the external os of the cervix. Inspection of the cervix and vagina was performed with illumination from a penlight (LeBlanc et al., 2002b).

Metricheck (Metricheck, Simcro, New Zealand) device was used for vaginal mucous collection. When using the Metricheck tool, the device is introduced into the vagina up to the level of the external os of the cervix and the discharge was scooped for evaluation after exteriorization of the device (McDougall, 2001).

The vaginal mucous was scored using the scoring system described previously (LeBlanc et al., 2002b). However, for analysis, the score 1-3 will be group together and assigned to be a new group because two previous reports in Thailand (Virakul et al., 1995; Ngarmkum et al., 2001) indicated that abnormal vaginal mucous collected from dairy cows in Thailand can be divided into 2 group including turbidity mucous and mucous with yellow pus. Then the modified scoring system includes score W (equal to score 1 to 3 described by LeBanc et al., 2002b) and score Y (equal to score 4 described by LeBanc et al., 2002b).

5. Collection of uterine endometrium discharge

The animals were restrained and cleaned perineum and vulva with saline irrigate solution, disinfectant solution and then dried. The collection of uterine endometrium discharge were operated using technique described previously (Virakul et al., 1995). In brief, a transcervical guarded swab were used for swabbing cervical discharge. The swab comprised a 0.3 cm diameter cotton wool tip sheathed in a long sterilized stainless steel 45 cm long, 0.6 cm

diameter with 1.5x0.5 cm hole at 0.5 cm from the blocked tip and wrapped with plastic breeding sheath with the hole at the same position. To prevent contamination, this guard tube was covered with sanitary sheath before inserted through the vagina, push the guard tube pierce through the sanitary sheath when reach external cervical through the cervical canal into the lumen of the uterus, guided by palpation per rectum. In the uterine lumen, the swab was extruded from the guarded tube to swab from the endometrium. Withdraw the swab into the guard tube and removed from the uterus then transfered the swab to the transport media.

6. Microbiological examination

Each swab will be streaked on blood agar and incubated at 37°C 24-48 hours. *A. pyogenes* was identified on the basis of the colony, Gram-stain, morphology, heamolysis, bio-chemical profile and other standard tests (Cowan and Steel, 1974). All *A. pyogenes* isolates will be stocked at -80°C in Mueller-Hinton broth containing 20% glycerol until further testing.

7. Antimicrobial sensitivity test

The antimicrobial sensitivity of *A. pyogenes* to different antibiotics was tested by disk diffusion method in Mueller-Hinton agar. The antimicrobial agents included penicillin G, oxytetracycline, cephalexin, cephazolin, gentamicin, amoxicillin, ampicillin, streptomycin and amoxicillin with clavulanic acid. Interpretation of the test results included sensitivity (S), intermediate sensitive (I) and resistant (R) were based on Clinical and Laboratory Standards Institute criteria (CLSI, 2008).

8. Genomic DNA isolation

Colonies of *A. pyogenes* were scraped off the agar plates and suspended in 500 µl of SET buffer (75 mMNaCl, 25 mM EDTA, 20 mMTris; pH 7.5). The genomic DNA was isolated as described previously (Pospiech and Neumann, 1995). Extracted DNA was suspended in 100 µl of TE buffer and stored at 4°C until use. Concentration and purity of DNA was evaluated by optical density (at 230, 260 and 280 nm) and the integrity was assessed through a 0.8% agarose gel electrophoresis.

9. Clonal analysis of *A.pyogenes*

Genomic DNA fingerprinting was generated by BOX-PCR technique as described previously (Silva et al., 2008). The BOX-PCR technique is a repetitive element sequence based PCR (Rep-PCR) typing method that uses an oligonucleotide primer (BOXAIR) directed for the bacterial conserved BOX elements (Tyler et al., 1997). Briefly, the genomic DNA fingerprint by PCR using the BOXA1R primer was generated and then amplified in a Thermal Cycler. An initial denaturation step (95 °C, 2 min) was followed by; denaturation 34 cycles (95°C, 1 min), annealing (53°C, 1 min) and extension (72°C, 5 min), with a single final extension cycle at 72°C for 10 min. in all PCR runs, a negative control consisting of the same PCR mixture without DNA was included. The PCR products was electrophoresed through a 1.5% agarose gel at 70 V for 4 hours and stained with ethidium bromide to determine the band sizes. DNA banding patterns were photographed.

10. Screening of genes encoding known and putative virulence factors of *A.pyogenes*

Presence of four known and putative virulence factor genes including *plo, cbpA, NanP and fimA*in *A. pyogenes* isolates were assessed by PCR (Santos et al., 2010). The PCR primers used to amplify four *A. pyogenes* virulence factor genes are shown in Table 1. Amplification products was separated by electrophoresis through 1.5% agarose gel and stained with ethidium bromide. DNA template of a reference strain of *A. pyogenes* was used as a positive control. Positive results was considered to be amplicons with expected molecular sizes.

11. Statistical analysis

A chi-square test will be used to test 1) the association between vaginal mucous score (score W and score Y) and the presence of *A. pyogenes* in the uterus and 2) the association between the presence of virulent factor genes and vaginal mucous score. In case of the data is not met assumption for the Chi-square test, the Fisher's exact test will be used. The level of significant is set at α=0.05. All statistical analysis will be performed using R statistical software.

Table 1. Details of PCR primers (Silva et al., 2008) used to amplify *Arcanobacterium pyogenes* virulence factors and appropriate combinations of conditions to generate gene-specificamplicons.

Target gene	Primer sequence (5'–3')	Annealing temperature (ºC)	Amplicon size (bp)
plo	Fw-tcatcaacaatcccacgaagag Rv-ttgcctccagttgacgcttt	60	150
nanP	Fw-ttgagcgtacgcagctcttc Rv-ccacgaaatcggccttattg	60	150
cbpA	Fw-gcagggttggtgaaagagtttact Rv-gcttgatataaccttcagaatttgca	60	124
fimA	Fw-cactacgctcaccattcacaag Rv-gctgtaatccgctttgtctgtg	57	605

Results

Prevalence

Ninety cows out of 198 lactating cows from 38 farms were diagnosed as clinical endometritis cows therefore the prevalence of endometritis was 45.45%. For such endometris cows, the percentage of cows with vaginal discharge score 1, 2 and 3 were 4.44%, 83.33%, 12.22% respectively.

Table 2. The bacterial type, number of isolates and percentage of finding.

Gram	Bacterial identification	No. of Isolates	Percentage by group	Percentage by total finding
Positive	*Corynebacterium spp.*	26	34.21	21
	Bacillus spp.	18	23.68	14
	Streptococcus spp.	14	18.42	11
	Arcanobacterium pyogenes	9	11.84	7
	Staphylococcus spp.	8	10.53	6
	Micrococcus spp.	1	1.32	1
Total		76	100	60
Negative	*Escherichia coli*	30	58.83	24
	Pasteurella spp.	7	13.73	6
	Citrobacter spp.	3	5.88	2
	Proteus spp.	2	3.92	1.6
	Enterobacter spp.	2	3.92	1.6
	Achromobacter spp.	2	3.92	1.6
	Klebsiella spp.	2	3.92	1.6
	Actinobacillus spp.	1	1.96	0.8
	Erysipelothrix spp.	1	1.96	0.8
	Hemophilus spp.	1	1.96	0.8
Total		51	100	40
	Total findings	127		100

Bacteriological findings

There were 127 bacterial isolates from 90 uterine samples. The major pathogens were *E.coli* and *Corynebacterium spp.* There were 9 isolated diagnosed as *A. pyogenes*. The detail for bacterial species was shown in Table 2.

Antibiotic resistance profile

Antimicrobial susceptibility profiles of overall, gram-positive and gram-negative bacterial isolates were shown in figure 1, 2 and 3, respectively. Overall, the most resistant antimicrobial agent was Oxytetracycline. Similarly, Oxytetracycline was the most resistant antimicrobial agent for gram-positive bacterial isolates. For gram-negative bacteria, streptomycin was the most resistant antimicrobial agent.

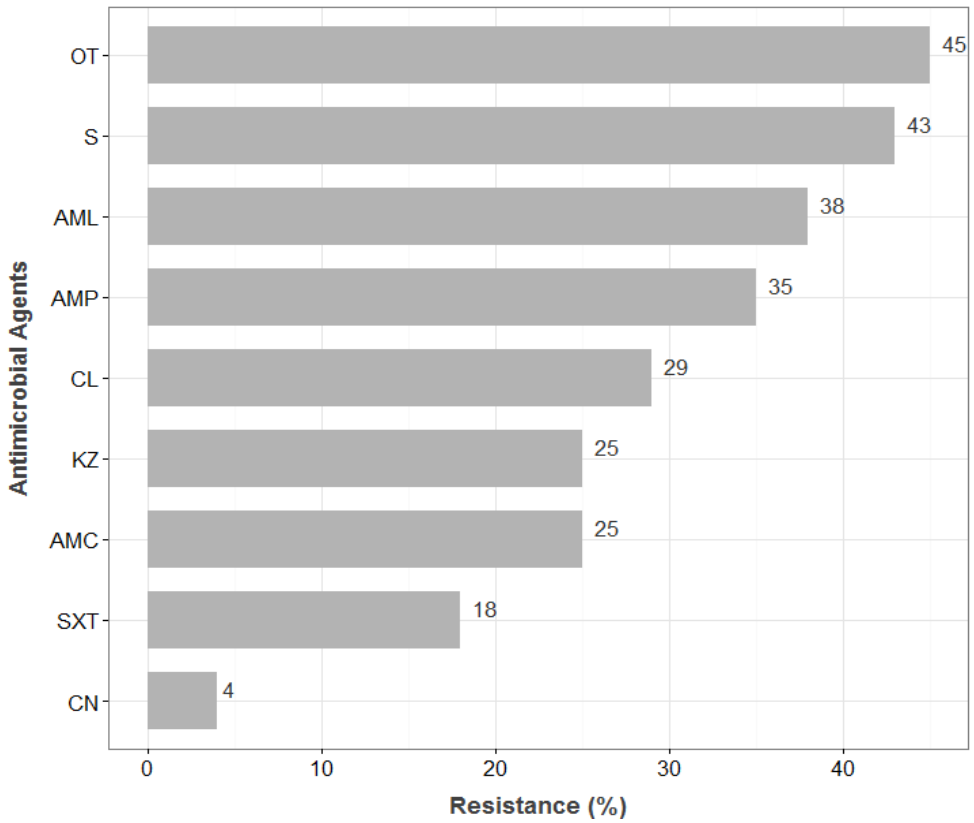

Figure 1. Antimicrobial susceptibility of overall bacterial isolates for 9 antibiotics: AMC = amoxicillin with clavulanic acid, AML = amoxicillin, AMP = ampicillin, CL = cephalexin, CN = gentamicin, KZ = cefazolin, OT = oxytetracycline, S = streptomycin and SXT = sulfamethoxazole-trimethoprim.

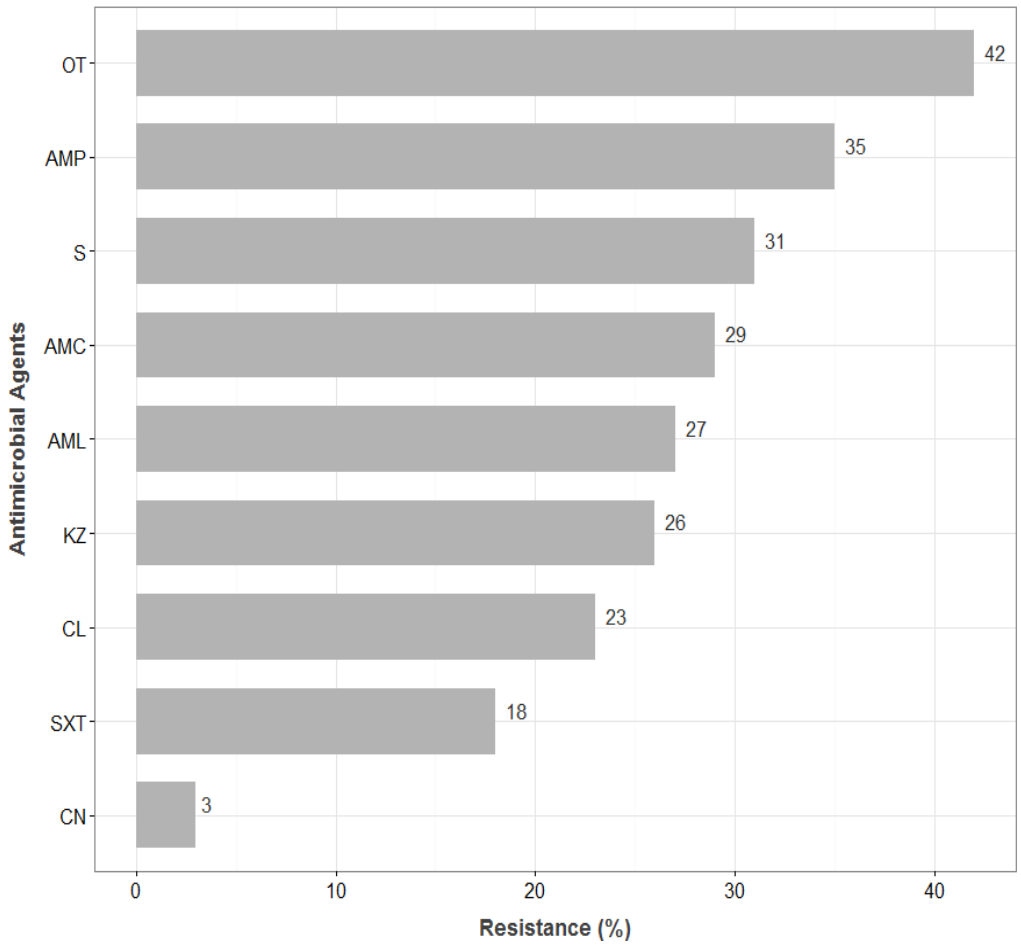

Figure 2. Antimicrobial susceptibility of 76 gram-positive bacterial isolates for 9 antibiotics: AMC = amoxicillin with clavulanic acid, AML = amoxicillin, AMP = ampicillin, CL = cephalexin, CN = gentamicin, KZ = cefazolin, OT = oxytetracycline, S = streptomycin and SXT = sulfamethoxazole-trimethoprim.

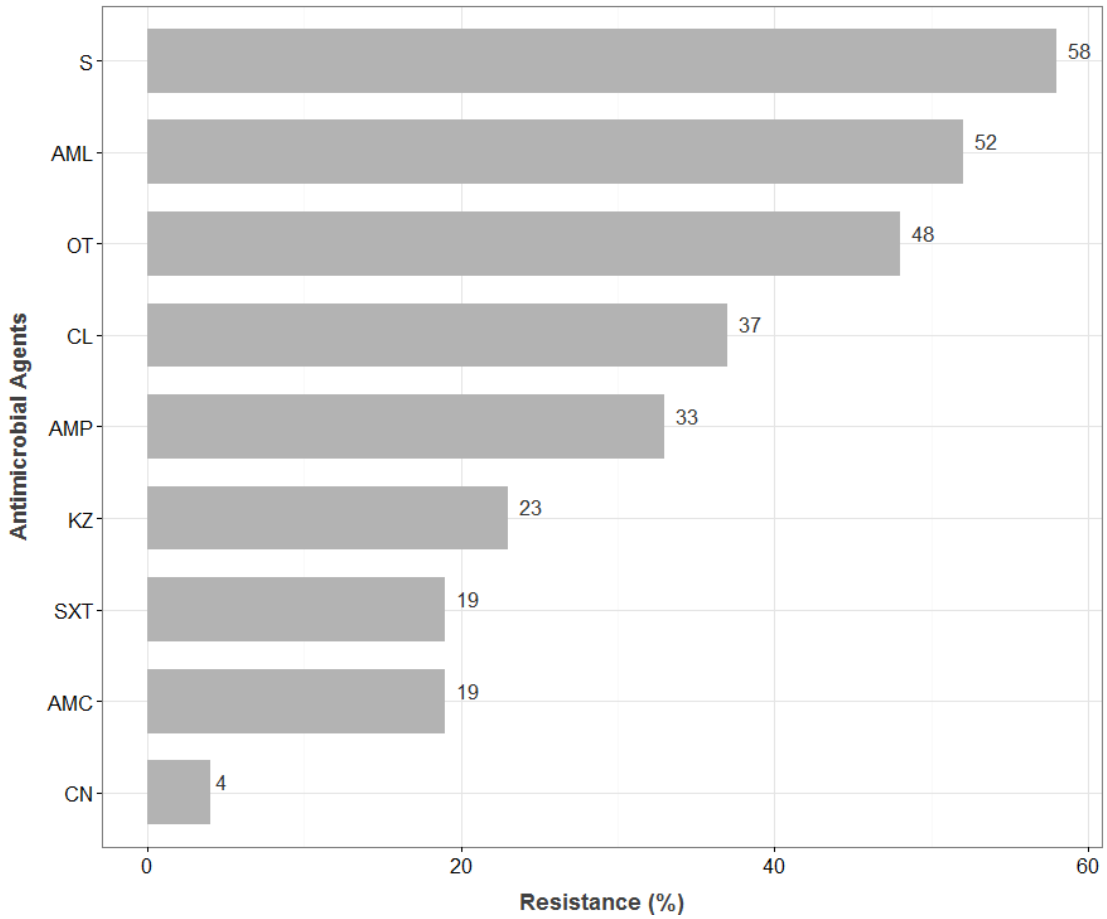

Figure 3. Antimicrobial susceptibility of 51 gram-negative bacterial isolates for 9 antibiotics: AMC = amoxicillin with clavulanic acid, AML = amoxicillin, AMP = ampicillin, CL = cephalexin, CN = gentamicin, KZ = cefazolin, OT = oxytetracycline, S = streptomycin and SXT = sulfamethoxazole-trimethoprim.

Virulent factor genes

The genes *plo* and *cbpA* were presented in all 9 isolates of *A. pyogenes*. The *fimA* gene was only detected in 2 isolates (Table 3).

Association between clinical finding, mucous score and the presence of *A. pyogenes*

All *A. pyogenes* were isolated from metritis cows with score=2 only (Table 3). Therefore, the association between mucous score and the present of *A. pyogenes* could not be statistically determined because the zero sample size from cows with metritis score other than score =2.

Table 3. Vaginal discharge score, antimicrorbial resistance pattern and virulence gene finding of nine *A. pyogenes* isolates.

Isolate number	Vaginal discharge score	Antimicrobial resistance pattern	Virulence gene finding
I	2	CL	*plo, cbpA*
II	2	-	*plo, cbpA*, *fimA*
III	2	AMC AML AMP KZ S	*plo, cbpA*
IV	2	SXT	*plo, cbpA*
V	2	CL KZ SXT	*plo, cbpA*
VI	2	CN S SXT	*plo, cbpA*
VII	2	-	*plo, cbpA*
VIII	2	-	*plo, cbpA*, *fimA*
IX	2	SXT	*plo, cbpA*

Clonal types

DNA banding patterns from BOX-PCR technique was shown in figure 4. All nine *A. pyogenes* isolates were from different farms and had different clonal types.

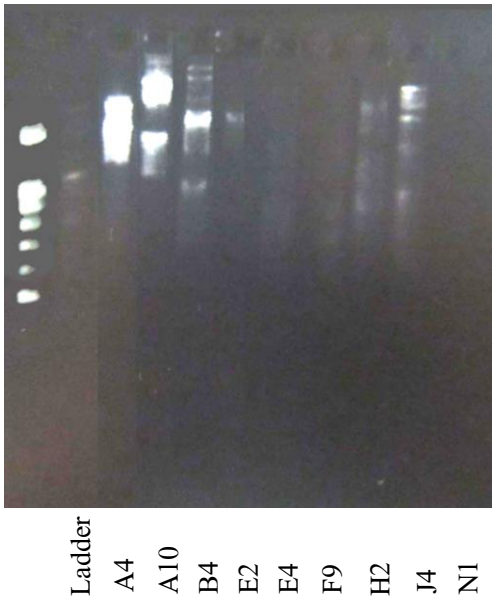

Figure 4. DNA banding patterns obtained for *A. pyogenes* isolated by BOX-PCR fingerprinting.

Discussions

The prevalence of endometritis in dairy cows in this study was higher than the previous study shown that endometritis prevalence was 15-22% (Virakul et al., 1995; Ngarmkum et al., 2001). However, the prevalence of *A. pyogenes* isolated from endometritis cows was lower than the previous reports although we sampled more cows than the previous studies. The different finding may be from a different in sampling period and cow condition due to the dissimilar in farm managements between dairy farms in central and northern area.

This study was the first report for identifying bacterial isolation from metritis cows in the northern part of Thailand. The major pathogens causing metritis in this study were *E. coli* and *Corynebacterium spp.* The result was similar to previous report (Virakul, et al., 1995) shown that *E. coli* and *Corynebacterium spp* were the major pathogen causing metritis in dairy cows from dairy farms in the central of Thailand. Our finding provides useful information for dairy farmers and veterinarians for making a prevention and control program. Because the common sources of *E. coli* and *Corynebacterium spp.* are environmental sources such as floor, manure and soil therefore the contamination from environment sources to cow will be a major potential route of transmission. Dirty and wet floor can yield a number of environmental bacteria. Most of dairy farms in this study are tied-stall farms hence cows are tied all time in their own small area. As the area for calving is the same area for milking and living, unclean floor will be a risk factor for such contamination causing endometritis. To prevent the disease, the rigorous hygiene around calving and post calving time in the barn should be implemented for high metritis incidence dairy farm. The farmer should pay attention on the cleanliness of floor and keep it dry most of the time. Moreover, the farmer should pay attention about reproductive problem around post-calving such as retained placenta because this condition is the important risk for endometritis (Peeler et al., 1994a, b; LeBlanc et al., 2002a).

The antimicrobial resistance pattern of bacterial isolates were observed in the study. Overall, bacterial isolates were most resistant to oxytetracycline, ampicillin and streptomycin (Figure 1). In contrast, the most susceptible antimicrobial were gentamicin. Regarding to our knowledge, oxytetracycline was

the common antibiotic used by artificial insemination technician dairy farmers in this study area. The highly resistance to oxytetracycline may be the result of using oxtetracycline as a routine therapy for uterine infection especially for intrauterine infusion. Furthermore, some farmers injected all post-calving cows with oxytetracycline intramuscularly without observing the clinical sing of endometrisis. Thus, a vastly used for such antimicrobial agents may be the important factor causing the resistance of bacteria to antimicrobial agents. Although ampicillin and streptomycin are not the common antibiotics for treatment metritis, these antibiotics are widely used for other purpose such as treatment for mastitis or other bacterial infection. This study suggested that the imprudent use of antibiotic may related with the resistant.

All *A. pyogenes* isolates were from different farms and had different clonal types (Figure 4) suggesting that the identified clonal types were herd-specific as described in previous study (Silva et al., 2008). In the current study, there were no risk for *A. pyogenes* transmission from one farm to another farm because this pathogen is mostly localized in uterine. The chance of contamination of *A. pyogenes* from farm to farm is extremely low in term of contamination from artificial insemination equipment or veterinary equipment used for uterine therapy as this pathogen cannot survive for a long time at dry environment. In contrast, if the strain of *A. pyogenes* were the similar strains, likely to occurred within farm, one of possible mode of transmission is the contamination from the environmental sources to cow. For instance, the metritis cows with *A. pyogenes* may drop the vaginal discharge on the bedding or floor and other cows contact with the discharge and be infected.

The association between vaginal discharge score and the finding of *A. pyogenes* was not able to determine because we did not have *A. pyogenes* isolates from all different vaginal score (Table 3). The pathogens were only found from uterine swab samples from cows having vaginal discharge score equal 2 (Score2Cow). The finding that *A. pyogenes* were not found in other cows might be the situation that Score2Cow were the highest proportion of cows in the study. Therefore, we could not conclude that *A. pyogenes* were more likely to find in Score2Cow due to the limitation of sample size.

This was the first study about virulence gene of *A. pyogenes* causing endometritis from dairy cows in Thailand. The *plo* and *cbpA* genes were presented in all isolates, which is related to previous study found 100% of both genes in *A. pyogenes* isolates. All isolates exhibited the characteristic *A. pyogenes* β-hemolysis on blood agar plates and carried genes *plo* and *cbpA*. Pyolysin (*plo*) is a primary virulence factor of *A. pyogenes* with the potent extracellular toxin. Pyolysin is a hemolysin capable of lysing red blood cells of a variety of animal species, and is responsible for the characteristic beta-hemolysis exhibited by *A. pyogenes* grown on blood-containing media (Pascual et al., 1997). The finding that *cbpA* was presented in all *A. pyogenes* isolates suggesting that the ability to bind collagen may be an important characteristic of these strains, promoting adhesion to uterine tissue (Silva et al., 2008). In the current study, 2 out of 9 of *A. pyogenes* isolates had *fimA* virulent gene. The important of *fimA* is that Fimbriae (pili) are filamentous, proteinaceous surface appendages that are key components in the cell-to-surface or cell-to-cell adherence of numerous bacterial pathogens. Jost and Billington (2005) reported *fimA* was present in approximately 94% of *A. pyogenes* isolates. Perhaps the differences observed in our results could be explained by inherent variations between different strains and the simple presence of those virulence factors in the genome of the bacterium is not sufficient to explain its pathogenicity (Silva et al., 2008). In addition, the degree of postpartum bacterial contamination of the uterus, host intrinsic uterine defense mechanisms, synergistic action with other bacteria and probably differential virulence genes expression are likely to play a relevant role in the establishment and persistence of disease (Santos et al., 2010).

In conclusion, the study showed that the prevalence of endometritis in dairy cow was moderate high. However, *A. pyogenes* was not a major pathogen found in uterine samples. The virulent gene factors of *A. pyogenes* associated with the clinical finding of uterine discharge. The resistant to antimicrobial agents of *A. pyogenes* was evident.

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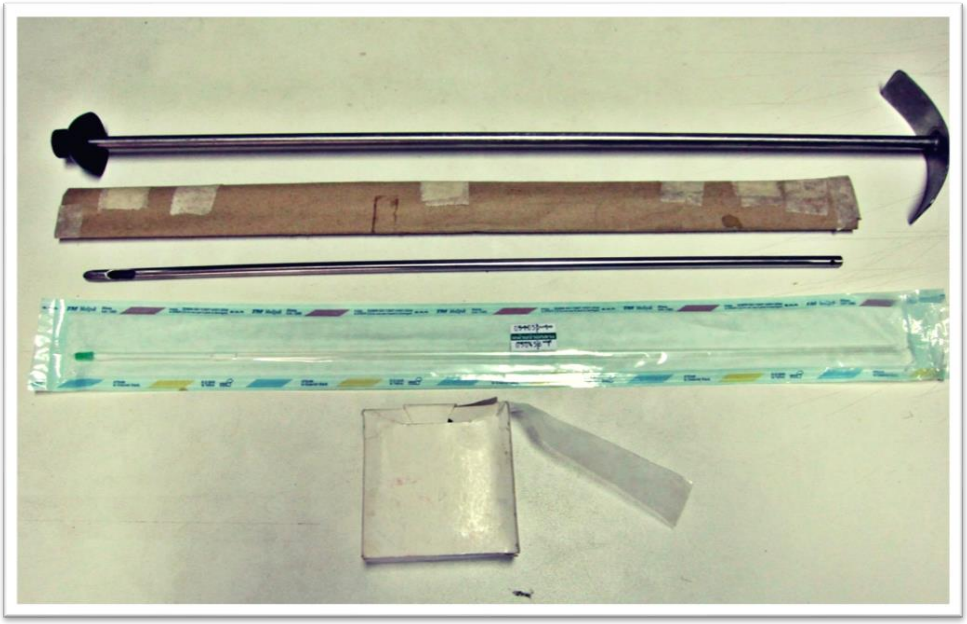
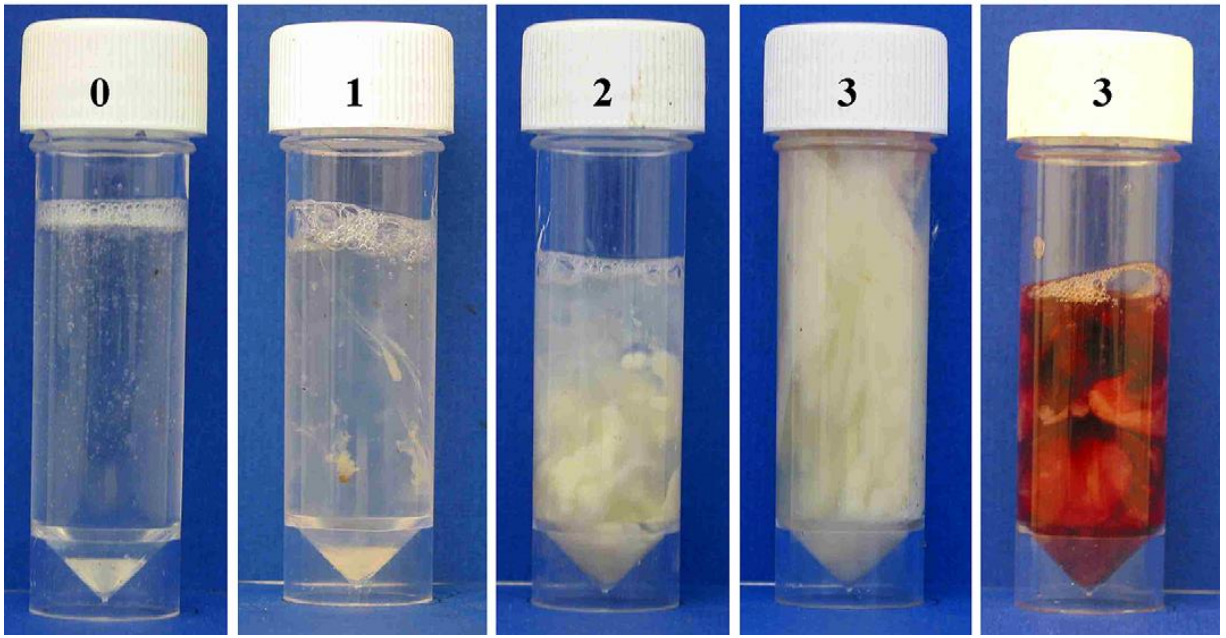
Appendices

Appendix A

Materials and methods



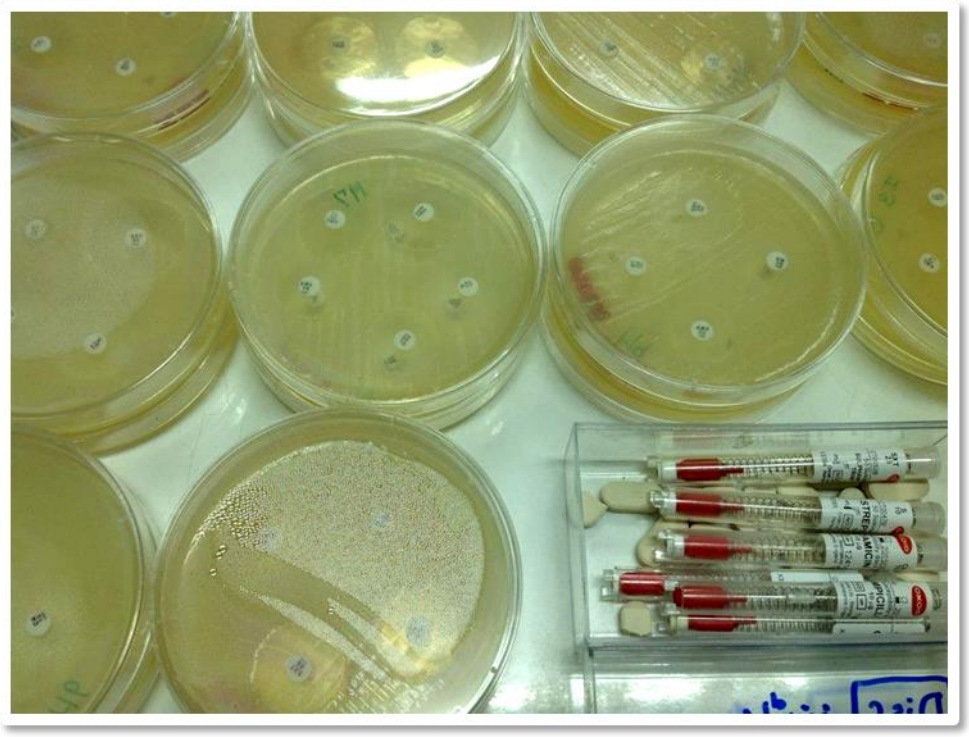
Sampling instruments: A = Metricheck; B = long sterilized stainless steel 45 cm long, 0.6 cm diameter with 1.5x0.5 cm hole at 0.5 cm from the blocked tip; C = 0.3 cm diameter cotton wool tip sheathed in plastic breeding sheath with the hole at the same position; D = sanitary sheath



Vaginal discharge scoring (Williams et al., 2005): 0 = clear or translucent mucus; 1 = mucus containing flecks of white or off-white pus; 2 = <50 mL exudate containing ≤50% white or off-white mucopurulent material; 3 = >50 mL exudate

Sampling methods: Collecting uterine discharge by aseptic technique. In the uterine lumen, the swab was extruded from the guarded tube to swab from the endometrium. Withdrew the swab into the guard tube and removed from the uterus then transferred the swab to the transport media and cultured within 3-4 hours after collection.

Microbiological examination: Bacteriological samples were cultured for aerobic bacteria on sheep blood agar and enriched in brain heart infusion (BHI) medium for 24 h at 37°C. The broths were streaked onto sheep blood agar plates and cultured for a second time 24 h at 37°C. Bacteria were identified on the basis of the characteristics of the colony, Gram stain, morphology, hemolysis, biochemical profile with TSI agar and MIL medium, and other standard tests such as citrate, catalase, urease and growth on MacConkey agar.



Antimicrobial susceptibility test: The antimicrobial susceptibility was tested by the disc diffusion method and performed according to Clinical and Laboratory Standards Institute (CLSI) guidelines in Mueller-Hinton agar (Malinowski et al., 2010). The following antibacterial agents (Oxoid) were used: amoxicillin with clavulanic acid (AMC; 30 µg), amoxicillin (AML; 10 µg), ampicillin (AMP; 10 µg), cephalexin (CL; 30 µg), cefazolin (KZ; 30 µg), gentamicin (CN; 10 µg), oxytetracycline (OT; 30 µg), streptomycin (S; 10 µg) and sulfamethoxazole-trimethoprim (SXT; 25 µg). Interpretation of the test results were sensitivity (S), intermediate sensitive (I) and resistant (R) were based on CLSI criteria.