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ARTICLES

Analysis of gonadocorticoids profile in canine patients diagnosed with ovarian cysts
Zoltán-Miklós GÁL, Ana Hîruța, Alexandru-Raul POP, Alexandra IRIMIE, Ioan Ștefan GROZA

Correlations between hypothyroidism and ovarian cysts in bitches
Zoltán-Miklós GÁL, Alexandru-Raul Pop, Ana Hîruța, Alexandra Irimie and Ioan Ștefan Groza

Presence of subclinical mastitis and economic losses in dairy farms in Serbia
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Praphulla Panta, Umesh Prasad Mandal

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CVJ CLUJ VETERINARY JOURNAL

Table of Contents

ARTICLES

Analysis of gonadocorticoids profile in canine patients diagnosed with ovarian cysts **Zoltán-Miklós GÁL, Ana Hîruța, Alexandru-Raul POP, Alexandra IRIMIE, Ioan Ștefan GROZA**	**2-7**
Correlations between hypothyroidism and ovarian cysts in bitches **Zoltán-Miklós GÁL, Alexandru-Raul Pop, Ana Hîruța, Alexandra Irimie and Ioan Ștefan Groza**	**8-13**
Presence of subclinical mastitis and economic losses in dairy farms in Serbia **Milan Ninković, Nemanja Zdravković and Aleksandra Tasić**	**14-19**
Study on subclinical endometritis in cattle of Kathmandu Valley by using WST (WST) **Praphulla Panta, Umesh Prasad Mandal**	**15-30**

CLUJ VETERINARY JOURNAL

Article

Analysis of gonadocorticoids profile in canine patients diagnosed with ovarian cysts

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Abstract: Veterinarians can obtain a more comprehensive understanding of the progression of ovarian cystic pathology in dogs by assessing hormonal levels. This study aimed to examine the gonadocorticoid profile of dogs diagnosed with ovarian cysts and compare these levels with those of healthy female dogs. The study included 96 female dogs split into two groups: a control group of healthy females and a study group of females with ovarian cysts larger than 4.5–5 mm in diameter and in diestrus or anestrus phase, determined by progesterone measurements. The control group had no ovarian pathologies and matched the estrus cycle phase of the study group. Ovarian cysts were diagnosed using the Esaote MyLab X5 ultrasound machine, while sex hormone levels (testosterone, progesterone, and estradiol) were measured with the Biomerieux MiniVidas analyzer. The analyzer utilizes the Enzyme Linked Fluorescent Assay (ELFA) technique for hormonal assessments. The study group (ovarian cysts) had significantly higher estradiol levels (21.83 pg/ml) compared to the control group (10.70 pg/ml). However, there was no significant difference in progesterone levels between the two groups. The study group females showed significantly higher estradiol levels in both the anestrus phase ($p = 0.0105$) and the diestrus phase ($p = 0.0105$). There was also a significant difference in estradiol levels between control group females in diestrus and anestrus, with significantly higher levels observed in the anestrus phase ($p = 0.0105$). In summary, female dogs with ovarian cysts had higher estradiol levels in both estrus and anestrus phases compared to the control group. The authors recommend assessing hormonal profiles in dogs with ovarian cysts for better treatment planning.

Keywords: canine ovarian cysts; estradiol; progesterone; gonadocorticoids

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1. Introduction

Female canines are a monoestrous, non-seasonal, polytocous species, with an average interestrus period of 6 months, divided into 4 phases. The 4 phases of the sexual cycle for this species are: proestrus, with a duration of approximately 7-10 days, followed by estrus lasting 5-10 days, diestrus lasting 20-90 days, and anestrus, with a variable duration of 15-150 days [1,2].

In canine species, ovarian cysts are clinically significant as they are a major source of hyperestrogenism in bitches, potentially leading to prolonged estrus [3,4]. These follicular cysts are endocrinologically active, secreting estradiol and progesterone. Persistent follicular cysts are believed to induce hyperestrogenism in bitches with estrus extending up to three months [2,3].

The ovarian cysts are classified into the following types: follicular cysts, luteal cysts, cystic corpus luteum, cystic rete ovarii and cysts of the subsurface epithelium [5,6].

Functional ovarian cysts manifest as single or multiple fluid-filled structures of varying sizes, which can be unilateral or bilateral in bitches aged 6-8 years. The pathogenesis is not well understood, but possible causes include insufficient LH surge, changes in gonadotropin receptors within the follicles, and growth factors [6]. Inadequate LH peaks to trigger ovulation of dominant follicles or insufficient receptor responsiveness can lead to ovarian cyst formation. Some researchers suggest that the inhibition of aromatase, an enzyme critical for the conversion of androgens to estrogens, may be an intraovarian disruption linked to the pathophysiology of polycystic ovary syndrome [7].

Clinically, follicular cysts must be differentiated from other ovarian cystic pathologies, including rete ovarii hyperplasia, superficial epithelial cysts, and ovarian neoplasms. Although the life expectancy of different dog breeds varies, there is an increased incidence of ovarian cysts in dogs over 6-8 years of age [6].

The primary endocrine lesion causing anovulation is the central failure to produce timely GnRH pulses and an LH surge, which are essential for final follicular growth and ovulation, or peripheral molecular homeostatic imbalances within the follicles that impair the ovary's response to the preovulatory LH surge. The former hypothesis explains the development of multiple follicular cysts, while the latter is more applicable to the occurrence of solitary follicular cysts [5].

As large cysts lose their structure and the arrangement of cellular layers changes under the pressure exerted by the cystic fluid, identifying the origin of the cyst becomes challenging. Follicular and luteal cysts are the two main types of functional ovarian cysts. Additionally, non-follicular cysts often arise from surface epithelium, underlying cells, or the mesonephric tubules of the ovary [8].

In humans, the etiology of polycystic ovarian syndrome (PCOS) has a strong genetic component. Genes such as CAPN10, the Cytochrome P450 family, the insulin gene, AR, FTO, and FSHR have been implicated [9]. Similarly, in dairy cows, variations in the bovine PCOS-related DENND1A gene have also been identified [10].

The prolonged presence of steroid hormones in the blood, which underlie the formation of ovarian cysts, not only predisposes the organism to uterine pathologies but also affects the ovulation rate, leading to a reduction in the size of the follicles. Histopathologically, the predominant uterine changes include cystic endometrial hyperplasia, periglandular fibrosis, lymphoplasmacytic endometritis, and adenomyosis, with incidences of 19.7%, 14.5%, 4.0%, and respectively 2.6% [11].

By evaluating the hormonal levels, veterinarians can gain a deeper understanding of how this pathology progresses in dogs. The aim of this research was to analyze the gonadocorticoids profile of canine patients diagnosed with ovarian cysts and compare the values with healthy canine females.

2. Materials and Methods

2.1. Study design

The study included a total number of 96 canine females. The dogs were segregated into two following two groups in accordance with the results of the clinical and paraclinical evaluation: control group for the females which were clinically healthy and study group for the females which were diagnosed with ovarian cysts. The study group inclusion criteria required the presence of ovarian cysts with sizes bigger than 4.5–5 mm in diameter, and the subjects needed to be in either the diestrus or anestrus phase. The sexual cycle phase was determined through routine progesterone measurement. For the control group, the inclusion criteria were the absence of pathological ovarian structures and being in the same estrus cycle phase (diestrus or anestrus) as the study group.

The diagnosis of females with ovarian cysts, as well as the collection of samples for endocrine profiling of these patients, was conducted at the Faculty of Veterinary Medicine in Cluj-Napoca within the Department of Reproduction, Obstetrics, and Reproductive Pathology (small animal clinic), as well as at the specialized veterinary practice Quantas Repro Vet in Cluj-Napoca.

The clinical history of patients suspected of having ovarian cysts primarily included infertility (repeated mating without successful conception), irregular heat cycles (prolonged estrus with shortened anestrus periods), pregnancy checks, routine gynecological examinations, essential vaginal hemorrhages, as well as other reproductive tract pathologies such as cystic endometrial hyperplasia, pyometra, mammary gland tumors, or vaginal tumors.

The imaging diagnosis of ovarian cystic formations was performed using a stationary Esaote MyLab X5 ultrasound machine with a microconvex probe. Subsequently, the cystic structures were measured using the ultrasound machine's software. In some cases, the diagnosis was confirmed through macroscopic examination of the ovaries, for the patients following ovariohysterectomy for therapeutic purposes or routine ovariohysterectomy (at the owner ´s request). It is noteworthy that in these females, clinical signs were absent, and preoperative clinical examination revealed that the patients were clinically healthy.

2.2 Sample Collection

Blood samples were collected from all patients for routine blood work. The blood samples were centrifuged at 5000 rpm for 10 minutes and the serum was stored at −18° C until the evaluations were done. The Biomerieux MiniVidas analyzer was used to measure sex hormones such as testosterone, progesterone, and estradiol. This automatic analyzer uses the Enzyme Linked Fluorescent Assay (ELFA) technique for hormonal assays.

All owners provided informed written consent, granting permission for sample collection and its use in research, as well as for the anonymous publication of the results.

2.3. Statistical analysis

The obtained data were examined for normality using the Shapiro-Wilk test. For the normal distribution results comparison, the independent samples t-test was applied. In case the normality was rejected for the examined data, the Mann-Whitney test was applied. Values of $p < 0.05$ were considered as statistically significant. The statistical analysis was performed using MedCalc® Statistical Software version 22.032 (MedCalc Software Ltd, Ostend, Belgium).

3. Results

From the total of 96 canine females, 48 were included in the control group (healthy females) and 48 in the study group (ovarian cysts). Within the control group, 26 females were in the diestrus phase and 22 were in the anestrus phase. In the study group, 23 females were in the diestrus phase and 25 were in the anestrus phase.

The values resulting from the statistical analysis are presented in Table 1. The results for the testosterone levels were not included since all the females had values < 0.05 ng/ml, except for one female from the study group which had 0.09 ng/ml. The females from the study group (ovarian cysts) had significantly higher estradiol values ($p < 0.0001$) with an arithmetic mean of 21.83 pg/ml (95% CI: 14.65–25.37), compared with the control group, which had a median of 10.70 pg/ml (95% CI: 9.86–12.30). For the progesterone values comparison between the control group (median: 7 ng/ml) and the study group (median: 2.96 ng/ml), there wasn't a statistically significant difference ($p = 0.6735$), hence no additional comparisons involving progesterone were done. Estradiol values in diestrus and anestrus were further compared between and within the study and control groups.

Table 1. Statistical analysis results for the estradiol and progesterone levels in the control and study groups

Hormones	Estral phase	n	Mean ± SD	Median	Min.	Max.	Confidence Interval for Mean 95%
Estradiol study group	Diestrus	23	23.75 ± 13.41	23.17	9	55.86	17.95–29.55
	Anestrus	25	24.79 ± 20.10	21.19	9	95.40	16.49–33.09
	Overall	48	24.29 ± 17.05	21.83	9	95.40	19.34–29.25
Estradiol control group	Diestrus	26	10.51 ± 2.01	9.63	8.21	15.5	9.69–11.32
	Anestrus	22	13.24 ± 3.50	12.95	8.47	21.08	11.69–14.79
	Overall	48	11.76 ± 3.08	10.7	8.21	21.08	10.86–12.66
Progesteron study group	Diestrus	23	36.75 ± 23.69	38.26	4.07	80.00	26.50–47.00
	Anestrus	25	1.38 ± 0.92	0.95	0.25	2.98	1.00–1.77
	Overall	48	39.6 ± 22.78	2.96	0.25	80.00	11.32–25.34
Progesteron control group	Diestrus	26	37.57 ± 19.76	37.19	3.08	80.00	29.59–45.56
	Anestrus	22	1.22 ± 0.77	1.05	0.21	2.57	0.88–1.56
	Overall	48	20.91 ± 23.30	7.00	0.21	80.00	14.15–27.68

Legend: SD - standard deviation; n – number of individuals. The estradiol values are expressed pg/ml. Progesteron levels are expressed as ng/ml.

For the comparison between the estradiol values in the anestrum phase between the control group and the study group, it was noticed that there was a statistically significant difference. The females from the study group had a significantly higher ($p = 0.0105$) estradiol value in the anestrum phase with a median of 21.19 pg/ml (95% CI: 13.44–26.09), compared with the control group which had a median level of 12.95 pg/ml (95% CI: 10.46–15.72).

For the comparison between the estradiol values in the diestrum phase between the control group and the study group, it was noticed that there was a statistically significant difference. The females from the study group had a significantly higher ($p = 0.0105$) estradiol value in the diestrum phase with a median of 23.17 pg/ml (95% CI: 11.75–28.77), compared with the control group which had a median level of 9.63 pg/ml (95% CI: 9.02–10.92).

For the comparison of estradiol values between the control group females in diestrus and anestrus, it was noticed that there was a statistically significant difference. The females in the anestrus phase had a significantly higher ($p = 0.0105$) estradiol value with a median of 12.95 pg/ml (95% CI: 10.46–15.72), compared with the female in the diestrus phase which had a median level of 9.63 pg/ml (95% CI: 9.02–10.92).

For the comparison of estradiol values between the study group females (ovarian cysts) in diestrus and anestrus, it was noticed that there wasn't a statistically significant difference ($p = 0.8607$). The females in the anestrus phase had a median value for estradiol of 21.19 pg/ml (95% CI: 13.44–26.09), compared with the females in the diestrus phase which had a median level of 23.17 pg/ml (95% CI: 11.74–28.77).

4. Discussion

Female dogs exhibiting estrus behavior with a proestrus or estrus period extending beyond 30 days should undergo ovarian imaging using ultrasonography to determine the presence of follicular cysts. Follicular cysts are typically large (8-12 mm in diameter), with thin walls and containing an anechoic fluid. These cysts must be carefully differentiated from normal follicles, cavitary corpora lutea, and parovarian cysts. The consequences of untreated follicular cysts are unclear; however, they may lead in most cases to pyometra and neoplasms such as mammary gland tumors and vaginal tumors, in addition to that in some cases, bone marrow suppression may occur due to persistently elevated estrogen levels. Consequently, in most cases, attempts are made to induce luteinization using HCG (500 IU per bitch) administered three times. In cases unresponsive to treatment, progesterone administration was attempted to be used to achieve cyst regression (though progesterone increases the risk of pyometra in a uterus influenced by estrogen) or an ovariectomy may be performed [12].

In examining the prevalence of cystic structures among 400 specimens, one study found a diverse distribution of cyst types. Follicular cysts were identified in 41 specimens (10.3%), corpus luteum cysts were present in 9 specimens, representing 2.3%, more than 360 specimens, or over 90%, had rete ovarii cysts and cysts of subsurface epithelial structures were found in 20 specimens, comprising 5.0% of the total. [13]

The endocrine potential of each ovarian cyst can be determined by analyzing the concentrations of estradiol-17ß and progesterone in the cystic fluid. In a study, the levels of estradiol-17ß in cystic fluid ranged from 2.0 to 568,000.0 pg/ml (median 545.0 pg/ml), while progesterone concentrations ranged from 0.1 to 20,138.0 ng/ml (mean 31.0 ng/ml). Additionally, hormone levels vary among different types of cysts present on the same ovary [14].

A study involving a retrospective examination of ovaries removed during ovariohysterectomy performed for the treatment of pyometra over a 12-month period revealed that 20.7% (17/82) of bitches had follicular cysts. The presence of ovarian cysts showed no significant association with cystic endometrial hyperplasia in Fisher's exact test ($P = .78$). Approximately 35.3% of bitches with ovarian cysts also had pyometra, while 58.8% had pyometra without cystic endometrial changes. Only 5.9% of females developed ovarian cysts without uterine pathology [15]. Although hyperestrogenism is less common in bitches with follicular cysts, the development of the cystic endometrial hyperplasia-pyometra complex is frequent, leading to polyuria and polydipsia, mucoid or purulent vaginal discharge, vomiting, abdominal distension, abdominal stress, or pain [16]. Clinical signs associated with hyperestrogenism syndrome include pancytopenia, anemia, granulocytopenia, thrombocytopenia, or hemorrhagic vulvar discharge. In chronic cases, females may develop typical bilateral symmetrical alopecia, lichenification, hyperkeratosis, and bone marrow suppression [17].

In females, testosterone is primarily produced by the ovaries and the adrenal glands. However, as this study suggests, the ovaries do not produce significant amounts of testosterone in females with ovarian cysts or in healthy females.

The pathogenesis, diagnosis, and treatment of cystic ovarian diseases in bitches remain unclear. Therefore, it is crucial to establish an early diagnosis and implement appropriate treatment strategies promptly to prevent disease progression [6].

5. Conclusions

In conclusion, estradiol levels were significantly elevated in female dogs with ovarian cysts during both the diestrus and anestrus phases compared to the control group. In female dogs without ovarian cysts, estradiol levels were notably higher during the anestrus phase than in the diestrus phase, likely due to increased ovarian responsiveness to gonadotrophins in late anestrus.

The ovaries do not typically produce significant amounts of testosterone in females with ovarian cysts or in healthy females.

The presence of ovarian cysts does not alter significantly serum progesterone levels during either the diestrus or anestrus phases. Progesterone mainly regulates the estrous cycle in this species and is a reliable marker for identifying the estrous period in female dogs, even when cystic formations are present. This is corroborated by the observation that active corpora lutea can coexist with cysts in canines, particularly during the diestrus phase. The authors recommend hormonal profile determination in canine patients with ovarian cysts, for a better understanding regarding the evolution of this pathology and for a more accurate treatment plan. This proactive approach not only aims to improve the health and well-being of the affected dogs but also enhances the overall outcomes of treatment strategies.

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Conflicts of Interest: The authors declare no conflict of interest.

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Article

Correlations between hypothyroidism and ovarian cysts in bitches

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Abstract: This study involved 48 canine individuals with ovarian cystic formations larger than 4–5.5 mm in diameter, as identified by ultrasound, who were in the diestrus or anestrus phase of the sexual cycle. Both T4 and fT4 levels were measured in all 48 cases. The imaging diagnosis of ovarian cystic formations was performed using the stationary ultrasound equipment Esaote MyLab X5, and with the owners' consent, samples were collected and the obtained data processed. Peripheral venous blood samples were collected in coagulation activator-lined tubes. Hormone assays were performed using the Biomerieux MiniVidas hormone analyzer. The results are automatically calculated by the machine using stored calibration curves and are expressed in µg/dL for T4 and in ng/dL for fT4. The objective of this study is to determine the prevalence and extent of hypothyroidism in polycystic ovarian syndrome in bitches by measuring the levels of both T4 and fT4 in 48 patients with ovarian cysts. Considering clinical symptoms, borderline values were classified as indicative of hypothyroidism. 27% (n = 13) were diagnosed with hypothyroidism, while 73% (n = 35) had euthyroidism. Statistically significant differences were found in the results of both T4 and fT4 between the euthyroid and hypothyroid groups. This study found a high prevalence of hypothyroidism in female dogs with ovarian cysts. Given the impact of hypothyroidism on ovulation in women and its potential effects in dogs, thyroid function testing is recommended for female dogs with infertility.

Keywords: cysts, hypothyroidism, infertility, bitch

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1. Introduction

Polycystic ovarian syndrome (PCOS) is the leading global cause of infertility in women, associated with a high level of comorbidities. This syndrome is linked to excessive ovarian and/or adrenal androgen hormone secretion, anovulation, and often insulin resistance and other associated metabolic disorders [1]. PCOS is thus a heterogeneous condition involving changes in the reproductive and cardiovascular systems, as well as metabolic and oncological implications, with serious health consequences [2].

Thyroid dysfunction in women can impact fertility through various mechanisms, resulting in anovulatory cycles, luteal phase defects, elevated prolactin levels (PRL), and disruptions in sex hormone balance. As a result, proper thyroid function is essential for fertility, pregnancy, and the maintenance of a healthy pregnancy, even in the early stages [3].

Thyroid hormones influence the function of nearly every organ in the body; therefore, canine hypothyroidism can present a wide range of clinical signs. One factor in determining the effects of the disease is the challenge of confirming a diagnosis of hypothyroidism in dogs. Establishing the diagnosis is hindered both by the lack of specificity of thyroxine (T4) analysis and the insensitivity of thyrotropin testing. Given that purebred dogs are intentionally bred, the impact of hypothyroidism on reproduction holds significant clinical importance. While a wide range of abnormalities occur in women with this disorder, there is limited

knowledge about the effects of thyroid hormone deficiency on reproductive performance in female dogs [4].

The metabolically active fraction of thyroxine (T4), known as free T4 (fT4), is widely acknowledged to more closely reflect the thyroid status compared to T4. Determining fT4 through equilibrium dialysis has been proposed as an alternative, non-invasive method, and is considered by some authors to be more sensitive and specific than measuring T4. This is because fT4 represents the amount of thyroid hormone available for use by the body's cells, whereas the total T4 level can be influenced by binding proteins [5]. In the same reference the authors state that the specificity of the low fT4 is between 93–94%.

In small animal breeding programs, infertility is one of the most critical issues that poses a challenge to successful management. Fertility involves achieving conception, followed by a pregnancy through implantation and successfully carrying the pregnancy to full term.

This study aims to establish the prevalence and degree of involvement of hypothyroidism in polycystic ovarian syndrome in the canine species, by testing the levels of both T4 and fT4 in 48 patients with infertility.

2. Materials and Methods

In this study, 48 individuals belonging to the female canine species were included, in which ovarian cystic formations larger than 4–5.5 mm in diameter were identified by ultrasound, and they were in the diestrus or anestrus phase of the sexual cycle. In all 48 cases both T4 and fT4 levels were measured.

The diagnosis of females with ovarian cysts, as well as the collection of samples for thyroid hormone measurements, were conducted at the Faculty of Veterinary Medicine in Cluj-Napoca within the Reproduction, Obstetrics, and Reproductive Pathology Department (small animal clinic), as well as at the specialized private veterinary clinic Quantas Repro Vet in Cluj-Napoca.

The imaging diagnosis of ovarian cystic formations was conducted using the stationary ultrasound equipment Esaote MyLab X5, with a microconvex probe, followed by measuring the cystic structures using the ultrasound machine's software. After diagnosing cases of ovarian cysts, with the owners' consent to collect samples and process the obtained data, peripheral venous blood samples were collected in coagulation activator-lined tubes.

The collected samples were centrifuged at 5000 rotations per minute to obtain serum. The liquid fraction was transferred into 1 ml Eppendorf tubes and stored at a freezing temperature of –18 degrees Celsius to conduct the thyroid profile. In most cases, the thyroid profile was conducted for diagnostic purposes, so the owners agreed to have T4 and fT4 levels measured. In this scenario, the processing of the samples was done directly after collection. Processing samples stored in the freezer involves first allowing them to thaw at room temperature for 15 minutes. After thawing, the samples need to be homogenized before testing. Hormone assays were performed using the Biomerieux MiniVidas hormone analyzer. This is an automated analyzer that utilizes immunoassay techniques for testing infectious markers (viral, bacterial), tumor markers, hormones, markers for cardiovascular conditions and hemostasis pathology, drug assays, allergology, etc. Special kits from the supplier are required for each assay.

The results are automatically calculated by the machine using stored calibration curves and are expressed in nmol/L. Samples with concentrations greater than 320 nmol/L need to be retested after dilution with 1/2 in T4-free serum (1 volume of sample and 1 volume of T4-free serum) and retested in the VIDAS T4 test. The results for fT4 are automatically calculated by the machine using stored calibration curves and are expressed in pmol/L.

The obtained fT4 and T4 data were examined for normality using the Shapiro-Wilk test. For the normal distribution results comparison, the independent samples t-test was applied. In case the normality was rejected for the examined data, the Mann-Whitney test was applied. Values of $p < 0.05$ were considered as statistically significant. The statistical analysis was performed using MedCalc® Statistical Software version 22.032 (MedCalc Software Ltd, Ostend, Belgium).

3. Results

In the following table the levels of both T4 and fT4 in the studied cases are presented (Table 1). The prevalence of hypothyroidism among female dogs diagnosed with ovarian cysts and who underwent thyroid hormone testing (n = 48) was 12% (n = 6). In 15% of cases (n = 7), thyroid hormone levels were at the lower limit. Such cases close to the lower limit were included in the borderline group and were considered along with those with hypothyroidism, as the clinical history revealed signs of thyroid insufficiency. From a clinical perspective, considering that these levels should be interpreted in conjunction with the clinical symptoms, we classified the borderline values as indicative of hypothyroidism. Therefore, 27% (n = 13) of cases were diagnosed with hypothyroidism and a percentage of 73% (n = 35) of cases were diagnosed with euthyroidism.

Table 1. The levels of the T4 and fT4 in the studied cases

NR.CRT	Breed	Age	T4 µg/dl	FT4 ng/ml	T4 REF. µg/dl	FT4 REF. ng/ml
1	Central Asian Shepherd	3	1.56	1.11	1.50–4	0.80–3
2	Romanian Bocovina Shepherd	4	2.4	1.35	1.50–4	0.80–3
3	Labrador Retriver	11	1.57	1.05	1.50–4	0.80–3
4	Bernese Mountain Dog	6	1.37	0.86	1.50–4	0.80–3
5	American Bully	2	3.34	3.28	1.50–4	0.80–3
6	Tibetan Mastiff	3	1.31	0.91	1.50–4	0.80–3
7	French Bulldog	7	2.63	2.21	1.50–4	0.80–3
8	Central Asian Shepherd	8	1.99	2.79	1.50–4	0.80–3
9	Malinois Shepherd	7	2.05	2.11	1.50–4	0.80–3
10	Siberian Husky	2	2.77	1.89	1.50–4	0.80–3
11	Central Asian Shepherd	8	4.03	2.8	1.50–4	0.80–3
12	Bernese Mountain Dog	8	1.32	0.74	1.50–4	0.80–3
13	Cane Corso Italiano	4	1.79	1.83	1.50–4	0.80–3
14	Belgian Shepherd	4	3.56	2.81	1.50–4	0.80–3
15	Belgian Shpeherd	4	2.67	1.85	1.50–4	0.80–3
16	American Bully	4	0.93	0.7	1.50–4	0.80–3
17	Pero de Pressa Canario	9	1.78	1.35	1.50–4	0.80–3
18	Akita Inu	10	1.26	1.10	1.50–4	0.80–3
19	Middle Mixed breed	12	0.74	0.75	1.50–4	0.80–3
20	Malinois Shepherd	5	2.48	2.31	1.50–4	0.80–3
21	Pug	4	2.59	1.79	1.50–4	0.80–3
22	German Shepherd	9	2.52	1.74	1.50–4	0.80–3
23	German Shepherd	3	1.55	0.77	1.50–4	0.80–3
24	Bull Terrier	9	2.39	1.85	1.50–4	0.80–3
25	Middle Mixed Breed	12	1.23	1.02	1.50–4	0.80–3
26	Malinois Shepherd	7	1.32	1.85	1.50–4	0.80–3
27	American Bully	2	1.97	1.6	1.50–4	0.80–3
28	Bichon Maltese	8	2.23	1.72	1.50–4	0.80–3
29	American Bully	1	4.46	1.67	1.50–4	0.80–3
30	American Bully	3	2.25	1.23	1.50–4	0.80–3
31	English Bulldog	1	2.39	1.64	1.50–4	0.80–3
32	Caucasian Shepherd	7	1.7	1.3	1.50–4	0.80–3
33	Bernese Mountain Dog	4	1.48	0.87	1.50–4	0.80–3
34	Wired Dachshund	7	0.57	0.37	1.50–4	0.80–3
35	American Bully	1	2.25	1.91	1.50–4	0.80–3
36	American Bully	1	1.31	0.87	1.50–4	0.80–3

NR.CRT	**Breed**	**Age**	**T4 µg/dl**	**FT4 ng/ml**	**T4 REF. µg/dl**	**FT4 REF. ng/ml**
37	Beagle	11	2.28	1.34	1.50–4	0.80–3
38	Yorkshire Terrier	6	2.74	1.4	1.50–4	0.80–3
39	American Bully	1	1.12	1.07	1.50–4	0.80–3
40	Central Asian Shepherd	5	0.87	0.7	1.50–4	0.80–3
41	Middle Mixed Breed	16	2.31	1.14	1.50–4	0.80–3
42	Mini Bullterrier	1	3.2	1.57	1.50–4	0.80–3
43	American Bully	2	3.49	2.23	1.50–4	0.80–3
44	German Shepherd	4	2.69	1.41	1.50–4	0.80–3
45	Alaskan Malamut	6	1.3	1.16	1.50–4	0.80–3
46	American Bully	4	2.03	1.25	1.50–4	0.80–3
47	Wired Dachshund	4	1.18	0.91	1.50–4	0.80–3
48	French Bulldog	1	2.69	1.87	1.50–4	0.80–3

The T4 values for canine females diagnosed with ovarian cysts were compared in the euthyrotic (n = 35) and hypothyrotic (n = 13) groups. Since the normality of the T4 values in both groups was accepted, the independent sample t-test was applied. The results indicated a statistically significant difference ($p = 0.001$) in T4 values between the euthyrotic group and the hypothyrotic group.

The fT4 values for canine females diagnosed with ovarian cysts were compared in the euthyrotic (n = 35) and hypothyrotic (n = 13) groups. Since the normality of the data was rejected for the fT4 in the hypothyrotic group, the Mann-Whitney test was applied. The results indicated a statistically significant difference ($p = 0.0001$) in fT4 values between the euthyrotic group and the hypothyrotic group.

Six cases of hypothyroidism have been identified in females with ovarian cysts, and 7 cases with thyroid parameter values at the lower limit, borderline, classified as hypothyroidism. The cases of hypothyroidism and diagnosed cystic formations (n = 13) were from the categories of giant breeds (n = 5), large breeds (n = 2), medium breeds (n = 4), and small breeds (n = 2). Thus, we observed a prevalence of 38.5% in giant breed individuals, 30.8% in medium-sized females, and 15.4% in patients of large and small sizes.

4. Discussion

Fertility issues are typically categorized into one of four groups: abnormal estrous cycles, normal estrous cycles, unsuccessful breeding, or failure to carry a litter to full term. This classification system helps in creating a list of potential causes and conducting a systematic evaluation of all possibilities [6].

Thyroid hormones influence the function of nearly every organ in the body; therefore, canine hypothyroidism can present a wide range of clinical signs.

In women, hypothyroidism is linked to a wide range of reproductive disorders, from abnormal sexual development to menstrual irregularities and infertility. The importance of this disorder on the menstrual cycle, in women, has been studied and know since 1950s [7]. Hypothyroidism disrupts the normal physiological secretion of GnRH, which is essential for normal follicular development and ovulation. A delay in LH response can result in insufficient progesterone secretion by the corpus luteum [7]. Both gonadotropins and thyroxine seem to be essential for achieving optimal fertilization rates and blastocyst development [7]. On a cellular level, thyroid hormones work together with FSH to directly stimulate granulosa cell functions, including morphological differentiation. Thyroid hormones assist in FSH-induced LH/HCG receptor activation and progesterone secretion. Therefore, insufficient availability of thyroid hormones at the ovarian level may contribute to gonadal dysfunction [8]. In a study, the author observed primary and secondary infertility in 6.2% of 16 overtly hypothyroid women. This prevalence was similar, 4.8% in the euthyroid group with goiter and 2.4% in normal control women (unknown thyroid function) [9].

The clinical signs of canine hypothyroidism include low metabolic rate, dermatological conditions (dermatological abnormalities are reported in 60–80% of hypothyroid dogs), reproductive abnormalities (prolonged interestrus, silent estrus, lack of cyclicality, spontaneous abortion, small size puppies or low postpartum weight compared to the breed characteristics, uterine inertia, and weak or stillborn puppies);

however, evidence for this association is limited. If hypothyroidism is a cause of female reproductive dysfunction, it seems to frequently pass undiagnosed in veterinary practice. For example, a single case of hyperprolactinemia in a dog with primary hypothyroidism has been reported in the veterinary literature [10].

A study assessed the influence of short-term induced hypothyroidism on fertility, gestation, parturition, and neonatal health in female dogs. There was no difference in the estrous cycle interval, litter size, or gestation length between the hypothyroid group and the control dogs. The duration of uterine contractions was longer, but the strength of contractions was weaker in the hypothyroid group compared to the control dogs; however, the interval between puppies was not affected. Postpartum puppy mortality was significantly higher among females with hypothyroidism [4]. It is not clear why the female dogs in this study did not develop the common reproductive abnormalities associated with hypothyroidism in women. One possible reason could be the relatively short duration of the present study and, consequently, of the hypothyroidism, for reproductive abnormalities associated with hypothyroidism to manifest. It is possible that thyroid insufficiency gradually develops in the case of spontaneous hypothyroidism, compared to the sudden induction of severe hypothyroid status for experimental purposes discussed in this study. Therefore, prolonged hypothyroidism may occur in natural disease as it progresses from subclinical to more severe manifestations, becoming evident over time [4].

Infertility and insulin resistance are the result of glucose metabolism abnormalities in women with Polycystic Ovary Syndrome (PCOS). PCOS is the most common cause of anovulatory infertility, while hyperinsulinemia in women leads to increased androgen production and the presence of insulin resistance (IR). Patients with IR and PCOS may have elevated plasma levels of homocysteine, which can influence both short-term reproductive function and long-term cardiovascular complications associated with insulin-resistant PCOS [11]. Patients with hypothyroidism frequently experience insulin resistance, which results in increased serum gonadotropin (LH) levels. This stimulates the ovaries to produce excessive androgens, thus triggering or worsening PCOS [12]. A study discovered that 22.5% of PCOS patients also had subclinical hypothyroidism (SCH), in contrast to only 8.3% of the normal control group [13] compared to another study that the prevalence of subclinical hypothyroidism (SCH) in PCOS patients was 43.6% [12]. Xing et. Al [14] reported an increased prevalence of SCH in PCOS women at 26.97%. In our investigation, among all the cases diagnosed with ovarian cysts and infertility, 27% had T4 and fT4 values indicative of hypothyroidism, which aligns with the previous mentioned studies. Statistically, there was a significant difference in results for both T4 and fT4 between the euthyroid and hypothyroid groups. On the canine species we did not find studies that indicate a correlation between these 2 disorders. The limitations of our study are comparable to those of Fatima et al.'s study [12], such as the absence of a temporal understanding between the two disorders, clinical outcomes following hypothyroidism treatment, and the correlation with other disorders like diabetes or obesity.

5. Conclusions

Given the recognized significance and impact of hypothyroidism on the physiological process of ovulation in women, and its lesser-studied effects in canine species, there should be increased consideration of this disorder in the pathological evaluation of infertility in female dogs. This study revealed a notable prevalence of hypothyroidism in female dogs with ovarian cysts. Specifically, one-third of the canine females in the research group with ovarian cysts were also diagnosed with hypothyroidism, indicating a significant correlation between the two conditions. As ovarian cystic pathology can lead to infertility, this study suggests testing thyroid functions in female dogs with a clinical history of infertility. Another noteworthy finding in this study is that middle and giant breeds are more prone to hypothyroidism. Therefore, there is a high likelihood that hypothyroidism could be the underlying cause of infertility in these breeds. Cases exhibiting clinical signs such as infertility, skin lesions, obesity tendency, high blood pressure, etc., along with borderline values of the T4 and fT4 indicators, should be regarded as hypothyroidism cases.

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Article

Presence of subclinical mastitis and economic losses in dairy farms in Serbia

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Abstract: The objective of the study was to determine the presence of subclinical mastitis in smallholder dairy farms in Serbia. We also aimed to show economic losses due to the presence of subclinical mastitis on the farms. The physical-chemical parameters of bulk tank milk samples were analysed: fat content, protein content, lactose and non-fat dry matter content. The total number of somatic cells ranged from 21000-4690000 in pooled milk samples. From 2020 to 2022, based on the number of somatic cells in the milk, there was a statistically significant ($p<0.05$) increase in the number of collective bulk tank milk samples that did not meet the requirements for Class I milk. Economic losses due to the reduction of milk quality 0.033 euro/l lead to economic losses of approximately 20 euro per cow every month due to the reduced price of milk based on the classification of milk into classes based on the number of somatic cells.

Keywords: bulk tank milk, cows, milk components, somatic cells, subclinical mastitis

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1. Introduction

Control of raw milk from the bulk tank is essential to monitor the presence of subclinical and clinical mastitis on the farms. Subclinical mastitis is common in dairy cows and is mainly caused by infectious and non-infectious agents. Subclinical mastitis leads to a decrease in the amount of milk, a decrease in the production of milk components, and economic losses due to the decline in milk quality, manifested by an increase in the number of bacteria and somatic cells [1]. A higher somatic cell count is an indicator of udder health [2]. The number of somatic cell counts is an indicator of subclinical mastitis. Level of somatic cells in milk >250,000 cells/mL and indicates subclinical mastitis in herds [3]. Determining the number of somatic cells in collected milk can indicate the presence of subclinical mastitis and help timely detection of subclinical mastitis to prevent economic losses due to a reduction in the amount of milk and milk components altered [4]. National regulation of the Republic of Serbia for milk quality prescribes the following conditions for milk, based on the results of testing the quality of raw milk in an authorized laboratory. Raw cow's milk is, depending on the total number of microorganisms and somatic cells, classified into: Class I milk - contains up to 100,000 cfu/ml (colony forming unit per ml) of the total number of microorganisms and the total number of somatic cells up to 400,000/ml; cow's raw milk has at least 3.2% milk fat, has at least 3.8% protein, has at least 8.5% dry matter without fat; Class II milk - contains from 100,001 to 400,000 cfu/ml of the total number of microorganisms and the total number of somatic cells up to 400,000/ml; Class III milk - contains more than 400,000 cfu/ml of the total number of microorganisms and the total number of somatic cells up to 400,000/ml [5] (Serbian Regulation of quality raw milk 106/2017). The appearance of subclinical mastitis is associated with changes in the contents of milk components such as milk protein, fat, lactose, fatty acid, and pH value [6]. Bulk tank milk with a high SCC (somatic cell count) affects the shelf life of milk products [7]. The presence of subclinical mastitis is associated with udder injuries, mastitis pathogens, defective milking equipment, and awareness of milkmen. The average contents of milk components in milk cows are as follows: protein 2.9-5.0%, milk fat 2.5-6.0%, lactose 3.6- 5.5%, minerals 0.6-0.9% and water 85.5%-89.5%. The main factors that affect the contents of milk components are breed, stage of lactation, diet, parity, age, health status, and occurrence of mastitis and metabolic diseases [6 ,8, 9]. The presence of inflammatory reactions in the udder might cause disorders in the contents of milk components [10, 11].

To our knowledge, there are no scientific reports on information presented on subclinical mastitis and milk quality from Serbian smallholder dairy farms in bulk tank milk analysis. The aim of this study was to determine the presence of subclinical mastitis based on the number of somatic cells which determine the milk quality of raw milk from bulk tank milk samples originating from Serbian smallholder dairy farms with a capacity of up 20 cows and from this, to determine the economic losses due to the lower price of raw milk due to a decrease in milk quality.

2. Materials and Methods

In total, 161 bulk tank milk samples were collected from 2020 to 2022 from smallholder's farms from three Republic of Serbia districts (Mačvanski, Kolubarski, and Sremski district). All farms were considered smallholder dairy farms, with an average of 16 Simmental or Holstein dairy cows in lactation and a milk yield average of 19.3kg/cow/day or approximately 5,886 kg/cow/year. Milk samples were taken from farms of up to 20 cows. Samples were collected from September to November during 2020, 2021 and 2022. The diets had similar contents: corn silage, alfalfa hay, and concentrate with about 16% protein in the meal. Samples were collected in 50 ml sterile screw-cap tubes. All analyses were performed within 24 h of collection at the farm. Physical-chemical parameters of milk were analysed: fat content, protein content, lactose and non-fat dry matter content (NFDM). Standard methods used were the acid butirometric method for fat content - SR EN ISO 1211:2010 (Funke DR.N-Gerber Labortechnik GmbH (Nova-Safety), the Kjeldahl method for protein content - SR EN ISO 8968-1-4:2016 using an automatic distillation unit Buchi K-350 (BUCHI Labrotechnik AG, Switzerland) and oven drying at 105°C for the total dry substance – SR ISO 6731:96 (ISCO, NTC 9000 (temperature range 25-300°C)). High-performance liquid chromatography (HPLC) was used to determine the lactose content in raw milk samples, using a Waters (Milford, USA) HPLC system, with a Waters 2414 refractive index detector (RID). A lactose standard was supplied by DR Ehrenstorfer (LGC, Germany) with a certified purity of 99.0%. A Fossomatic 5000, Foss-electric (Hillerød, Denmark) was used to determine the number of somatic cells in milk samples.

Milk quality parameters were analysed using a GraphPad® Prism®6 and SPSS ver. 22 software for descriptive statistic parameters and ANOVA using the Tukey post hoc test. For SCC and protein data, which were not normally distributed, Kruskal Wallis and Mann-Whitney post hoc nonparametric tests were conducted.

3. Results

A total of 161 bulk tank samples were evaluated for each the components milk fat, protein non-fat dry-matter and somatic cell counts of milk samples from Serbian smallholder farms. Contents of milk components in each year are shown in (Table 1).

Table 1. Contents of milk components in each year

Year	Parameter	Observation	Min.	Max.	Mean	Median	Std. Deviation	Coefficient of variation
2020	**Somatic cell count**	57	27000	688000	172456	132000	149890	86.9%
	Milk fat	57	3.2	7	4.08	3.9	0.683	16.7%
	Protein	57	2.65	3.86	3.34	3.34	0.281	8.42%

	NFDM	57	5.4	10	8.85	9.0	0.944	10.7%
2021	**Somatic cell**	44	31000	1429000	396614	320000	324285	81.8%
	Milk fat	44	2.4	7.8	3.93	3.62	1.15	29.2%
	Protein	44	2.74	4.04	3.25	3.14	0.3	9.23%
	NFDM	44	7.6	15	9.25	9.20	1.07	11.6%
2022	**Somatic cell**	60	21000	4690000	504350	248000	760752	151%
	Milk fat	60	2.4	7.9	4.45	4.25	1.35	30.3%
	Protein	60	2.92	3.99	3.22	3.20	0.248	7.69%
	NFDM	60	7.7	10	8.77	8.71	0.535	6.11%

The average content of milk fat, protein, NFDM and the number of somatic cells were 4.15%, 3.27%, 8.96% and 357.807 cells/ml, respectively, across the three years. The quality of milk based on the number of somatic cells in shown in (Table 2).

Table 2. Distribution of milk samples according to requirements milk quality

	2020	2020	2021	2021	2022	2022
0-400,000	54	33.5%	24	14.9%	36	22.4%
400,000–1,000,000	3	1.9%	18	11.2%	19	11.8%
>1,000,000	0	0.0%	2	1.2%	5	3.1%

During 2020 to 2022, it was found that on average 24.8% of herds had a number of somatic cells >400,000 cells/ml and so did not meet the requirements for class I milk quality. Based on the number of somatic cells in the milk, there was a significant ($p<0.05$) increase in the number of BTM (bulk tank mil) samples that did not meet the requirements for class I quality (Figure 1).

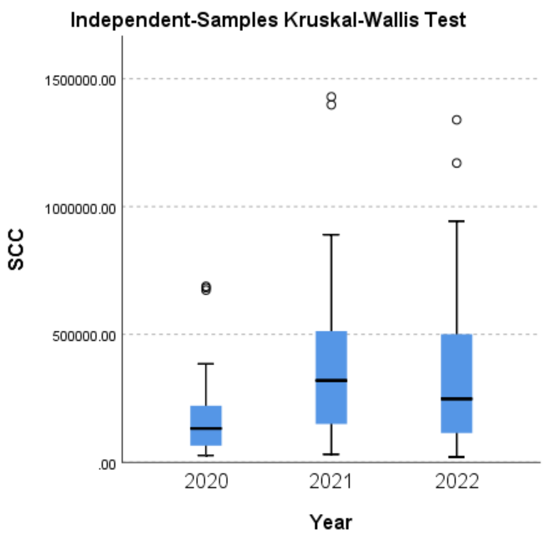

Figure 1: Somatic cell counts over the period 2020 - 2022 ($\bar{X}$±SD).

There was a drop class I quality BT samples from 33.5%, over 14.9%, to 22.4% in year 2020, 2021 and 2022 respectively. Analysis of milk components such as milk fat , NFDM and SCC showed a statistically significant differences in observed period $p<0.05$ (Table 3).

Table 3. Milk components analysis of bulk tank samples

Parameters	Period	Significant	Adjusted P Value
Somatic cell count	2020 vs. 2022	Yes	0.002
Somatic cell count	2020 vs. 2021	Yes	0.001
Milk fat	2021 vs. 2022	Yes	0.049
NFDM	2021 vs. 2022	Yes	0.015

4. Discussion

The appearance of subclinical mastitis is associated with an increase in the number of somatic cells in milk, which affects the quality of raw milk as well as the price of milk. The most common non-infectious cause of the increase in the number of somatic cells is pain, which can occur due to mechanical trauma, inadequate pressure in the milking machine, lameness, and the presence of other diseases that can affect the retention of milk in the mammary gland [12]. Many factors that affect the number of somatic cells include udder infection, stage of lactation, parity, individual response to the infection, inadequate microclimate conditions, milking equipment, metabolic disease, and lameness [13, 14]. Determining the level of SCC in bulk tank samples is a useful tool for monitoring udder health and detecting contagious mastitis agents such as *Staphylococcus aureus*, *Streptococcus agalactiae*, *Mycoplasma bovis* [2, 15]. The increased somatic cell counts lead to reduced cheese production and affect the sustainability of cheese [16]. For manufacturing high-quality dairy products, raw milk must have an adequate composition (e.g., protein and fat levels), be free from unpleasant taste and smell, free from detectable drug residues, added water and have low SCC. In our study, the average bulk tank somatic cell count (BTSCC) from 2020 to 2022 was 339,536 cells/ml, and these somatic cell numbers are very similar to those of the study by [17]. The numbers of somatic cells obtained in our study are close to the threshold value of 400,000 cells/ml, indicating the presence of subclinical mastitis in these smallholder farms. However, the study by [18] reported that an average BTSCC was 521,583 cell/ml and indicated widespread udder health in Northern Cyprus. Our results are similar to [19] in Holland, which reported a BTSCC of 392,220 cells/ml. Many authors have reported an increased number of somatic cells associated with an increase in milk fat, protein and non-fat dry matter [4]. The presence of mastitis affects the yield of milk and the contents of milk components [8, 20]. However, compared with our results, [21] reported an average content of milk fat of 3.39%, protein content 3.13%, lactose 4.27%, non-fat dry matter 8.13% and 615,500 somatic cells/ml which were significantly lower values of the content of components, as well as a significantly higher number of somatic cells.

Economic losses due to reduced milk quality can be calculated on the basis of the chemical and hygienic composition of milk. In most dairies in Serbia, the price of raw milk is based on the classification of milk into classes [5], with a difference of about 4 dinars (0.033 euros) per 1L milk between classes based on the quality of raw milk. If all the milk production for small farms (up to 20 cows) was found to be in Class II instead of Class I, the average economic losses calculated on the basis of a yearly production of *ca* 6000 L would be 24,000 dinars (about 200 euros per year) or 16.67 euros per cow/month due to the increased number of somatic cells in milk, together with the economic cost of reduced milk production. Our study provides information on raw milk quality from bulk tank samples from smallholder farms in Serbia and is a measure of udder health to prevent the occurrence of subclinical mastitis and showed typical economic losses due to decreased milk quality. Determining bulk tank somatic cell numbers is the first step in detecting subclinical mastitis, after which the California mastitis test would be important to detect simply and easily cows that have an increased numbers of somatic cells.

5. Conclusions

A reduction of milk quality worth 0.033 euro/L would lead to economic losses of nearly 20 € per cow on a monthly basis due to the reduced price of milk based on the classification of milk into classes according to the number of somatic cells and the content of milk components. This research shows the importance of surveillance of bulk tank milk to detect subclinical mastitis in farms and to prevent economic losses from lower milk prices due to reduced production of milk components, leading to reduced profitability.

Author Contributions: "Conceptualization and methodology, M.N.; software, N.Z.; formal analysis and investigation , A.T.; data curation, N.Z.; writing—original draft preparation, M.N.; writing—review and editing, N.Z and A.T.; All authors have read and agreed to the published version of the manuscript".

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Article

Study on subclinical endometritis in cattle of Kathmandu Valley by using WST (WST)

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Abstract:

The current investigation aimed to identify the different levels of nonspecific bacterial infection in the genitalia of repeat-breeding cattle of Kathmandu District using White Side Test (WST). A total of 307 crossbred Holstein Friesian cows were considered for this purpose, out of which 246 repeat-breeding cattle were under treatment and 61 cattle with normal cycle (control group) were artificially inseminated at their first service. Cervical mucus samples were collected 8 to 12 hours after the first signs of behavioral estrus, and subjected to a WST (WST) and bacteriological examination. From the results of WST, it has been inferred that only 9(14.75%) animals in the control group were positive and the remaining 52(85.24%) were negative. However, the majority of repeat-breeding animals were positive 190 (77.22%)) and only 56 (22.76%) of animals were found negative. Microbiological examination of 190 samples revealed *E. coli* 54(28.42%), *Staphylococcus spp.* 39(20.52%), *Streptococcus spp*. 26(13.68%), *Corynebacterium spp*. 10(5.263%), *Pseudomonas spp*. 7(3.68%), *Klebsiella spp.* 9(4.73%), and mix bacterial growth 44(23.15%). The remaining 56 repeat-breeding cattle were negative with WST, possibly due to the absence of bacteria. Other causes may be viruses, placental retention, ovarian cysts, dystocia, etc. The findings of the present study divulge that WST may be utilized in the field to detect subclinical endometritis caused by bacteria.

Keywords: WST, Subclinical endometritis, estrus cervical mucus, Repeat Breeding

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1. Introduction

Uterine inflammation is a serious problem in Nepal's breeding dairy cows, causing considerable financial losses [1]. Uterine infections include endometritis, metritis, mucometra, and pyometra. Among this endometritis is one of the major gynecology problems affecting reproductive efficacy and the economy of milk production in dairy animals [1].

Among different types of endometritis, subclinical or occult endometritis poses a serious threat to fertility since it is extremely difficult to identify by standard clinical-gynecological examination. Subclinical endometritis is currently being considered as a significant factor in dairy cows' lower conception rates [2]. Endometrial inflammation modifies the uterine environment, impairing the ability to conceive or sustain an embryo. In Nepal, subclinical endometritis has been identified as the most frequent reason why bovines are unable to conceive. The occurrence of endometritis has been associated with a weak uterine defense mechanism (UDM) in females [3] mainly due to inadequate husbandry and sanitation practices and exposed of genital organs to microbial invasion either at parturition or during estrus.

Bovine genital tract infections can be specific or non-specific, with the latter being the most important in causing endometritis. Some of the specific pathogens such as *Campylobactor fetus* and *Trichomonas fetus* develop infection without any predisposing cause, whereas non-specific pathogens residing in the genital tract as saprophytes and set infection under favorable conditions. The postpartum bovine uterus contains wide range of bacteria, both Gram-positive and Gram-negative, aerobes and anaerobes [4] Numerous bacteria have also been found and grown from the uterus of cattle with endometritis, including *Streptococci*, *Staphylococci*, *Corynebacterium spp*., *Klebsiella spp*., and *E. coli.*

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One of the emerging businesses in Nepal that offers a reliable source of income is livestock farming. Farmers favor more productive cattle over less productive cattle due to the market's rising need for milk, particularly cross Holstein Friesian (with good blood levels) livestock since they produce milk in greater quantities. From India to Nepal, a sizable number of crossbred cattle are imported. Cattle with various diseases and reproductive disorders have been reported to be imported as a result of improper inspection during quarantine or illegally importing cattle from open borders. Some of the most significant issues farmers today are dealing with include the retention of the placenta, repeat breeding, extended calving intervals, infertility, prolapse, dystocia, anestrus, cervicitis, cystic ovary, and endometritis. This research was undertaken with the intention of improvising these issues by addressing all of the aforementioned challenges. Early detection of metritis or endometritis will help to reduce future treatment costs and complications brought on by this condition.

WST has been described as a test for the diagnosis of subclinical endometritis [5]. The normal oestrus discharge has too low number of leucocytes to bring about a positive color change on doing WST [5]. The subclinical discharges have moderate number of leucocytes causing a moderate color change compared to the clinical discharge having very high number causing intense color reaction. The relative efficacy of Whiteside test is 77.5% [6].

Therefore, it was intended for the current study to assess the validity of the WST in detecting genital infection in repeat-breeding cattle raised in the Kathmandu District. Repeat breeders are cows with apparently healthy genitalia, normal estrous, has no abnormal vaginal discharge but failed to conceive in three or more consecutive inseminations [7]. To further demonstrate that the results of the WST for endometritis are accurate under field conditions, bacterial culture was performed.

WST is use for diagnosis of subclinical uterine infection in repeat breeding cows [8]. The investigators classify the positive samples as slight, moderate, and intense subclinical infections based on the color reaction, such as light yellow, yellow, and dark yellow. The test revealed 92.85%of cervical mucus positive from repeat breeding cows indicating subclinical uterine infection. Out of the total, 44.75% were slightly positive, 42.50% were moderate, whereas, 15-38 % were detected to have an intense uterine infection.

WST is interpreted as negative, mild, moderate, and severe as recorded 16.67, 66.67, 09.26, and 7.41 percent color pattern of no color, light yellow, yellow, and dark yellow type, respectively in repeat breeder crossbred Jersey cows. On culture 60 (75.00 %) of the 80 repeat breeding animals were found positive and 20 (25.00 %) were free of bacteria. The different bacterial isolates from repeat breeding cows were *Staphylococcus spp.* 16 (21.05%), *E. coli* 14 (18.42%), *Bacillus spp.* 10 (13.16%), Corynebacterium spp. 10 (13.16%), Pseudomonas spp. 8 (10.53%), *Proteus spp.* 8 (10.53%), *Klebsiella spp.* 6 (7.89%), and *Streptococcus spp.* 4 (5.26%). [9]

2. Materials and Methods

2.1 Site of Study

The study was conducted in a Kathmandu district located in Kathmandu Valley, Bagmati Province of Nepal. It covers an area of 413.69 km2 (159.73 sq mi) with 11 municipalities [10]. Total Population of cattle in Kathmandu District is about 52,470 [11].

The Kathmandu district is surrounded by:

East: Bhaktapur District and Kavrepalanchok District

West: Dhading District and Nuwakot District

North: Nuwakot District and Sindhupalchok District

South: Lalitpur District and Makwanpur District

2.2 Sample size

For estimating the minimum sample size for an unknown population, the formula estimated by ***Cochran*** was [12]

$$\boldsymbol{N}_0 = \frac{z^2 \times p(1-p)}{e^2}$$

z = critical value of the desired level of confidence (i.e. 95%) its z-value is 1.96

e = margin of error i.e., is 5%

p = estimated proportion of an attribute that is present in the population (that can be a maximum of 50%)

n_0 = sample size/proportion of unknown population

now,

$$\boldsymbol{N}_0 = \frac{z^2 \times p(1-p)}{e^2}$$

$$= \frac{(1.96)^2 * 0.5(1-0.5)}{(0.05)^2} = \sim 385 \text{ cattle's}$$

First, calculate the proportion and then use the formulation for the population correction factor to calculate the exact sample size (formula)

$$\boldsymbol{N} = \frac{n_0 \times N}{n_0 + (N-1)}$$

n = sample size of known population

n_0 = proportion of the unknown population

N = known population

Total Population of cattle in Kathmandu District is about 52,470

Now,

$$\boldsymbol{n} = \frac{n_0 \times N}{n_0 + (N-1)}$$

$$= \frac{385 \times 52470}{385 + (52470-1)} = \sim 383 \text{ cattle's}$$

Therefore, the total sample size is 383 but due to the pandemic of Lumpy Skin Disease (LSD), only 307 samples were collected.

2.3 Sampling Technique

Simple random sampling was adapted as it favors the unbiased selection of animals [12] and a total of 307 samples were collected from cattle at the heat from different farms in the Kathmandu district.

Out of total 307 samples, 12 (Budhanilkantha Municipality), 18 (Chandragiri Municipality), 10 (Dakshinkali Urban Municipality), 35 (Kageshwori Manahara Municipality), 6 (Kathmandu Metropolitan City), 123 (Kirtipur Municipality), 10 (Nagarjun Municipality), 9 (Shankharapur Urban Municipality), 11(Tarkeshwor Municipality), 56 (Tokha Municipality), were collected. (Table 1)

Table 1: No. of sample collection from different municipalities of Kathmandu District

Municipality of Kathmandu District	Total number of samples collected
Budhanilkantha Municipality	12
Chandragiri Municipality	18
Dakshinkali Municipality	10
Gokarneshwor Municipality	35
Kageshwori Manahara Municipality	17
Kathmandu Metropolitan City	6
Kirtipur Municipality	123
Nagarjun Municipality	10
Shankharapur Municipality	9
Tarkeshwor Municipality	11
Tokha Municipality	56
Total	307

2.4 Experimental Design

The present study was conducted at different farms of Kathmandu District during the period from May 1 to September 1 2023. Several 307 crossbred Holstein Friesian cattle of 246 repeat breeding cows presented for treatment and 61 clinically normal cows presented for artificial insemination at their first service, were selected for sample collection (shown in supplementary figure S1).

All of the animal's estrus cervical mucus was collected 8 to 12 hours after the first signs of behavioral estrus. Animals were properly restrained for this purpose. For rectal feces evacuation, a full-sleeve gloved left hand was inserted per rectum and lubricated (with sterile liquid paraffin/oil). The vulva and the perineum were both properly cleaned with soap and water, dried with soft absorbent cotton, and then disinfected with 70% alcohol. The vagina had been penetrated with a 10 mL sterilized pipette and a 20 mL disposable syringe was linked to the pipette's exterior pointed end.

The pipette was guided through the vaginal canal to the cervical os or mid cervix and was grabbed by the left hand already introduced per rectum then cervical mucus was aspirated from the cervical os or mid cervix and then transferred to a sterilized test tube.

For WST, 1 mL of estrus cervical mucus was heated with an equal volume of 5% sodium hydroxide up to boiling point (shown in supplementary figure S2) and after cooling the intensity of color changes was studied and graded as normal (no color), mild infection (light yellow color), moderate infection (yellow color) and severe infection (dark yellow color) [13]. (shown in supplementary figure S3)

In animals that were positive for the WST, the cervical mucus was sent for bacteriological culture, and isolation and identification of the organisms were carried out based on cultural, morphological, colony characteristics, motility, and biochemical reactions.

3. Results

From the WST out of 307 samples there are 246 repeat breeding cattle, 56(22.76%) showed no color, 147(59.75%) showed light yellow, 29(11.78%) showed Yellow, and 14(5.69%) showed Dark Yellow color with a normal, mild, moderate, and severe grade of infection. The control group (normal cyclic cattle) shows 52(85.24%) with no color change and 9(14.75%) shows light yellow with normal and mild grades of infection. (Table 2)

Table 2: Results of WST showing grades of infection based on color intensity between repeat breeders and normal cyclic (Control group) animals

Color Intensity	Grade of Infection	Repeat Breeding cattle		Control group	
		N	%	N	%
No color	(0) Normal	56	22.76%	52	85.24%
Light Yellow	+ Mild	147	59.75%	9	14.75%
Yellow	++ Moderate	29	11.78%	-	-
Dark Yellow	+++ Severe	14	5.69%	-	-
Overall		246	100	61	100

Out of 246 repeat breeding cattle, 147 show light yellow color, 29 show yellow color, 14 show dark yellow color, and 56 show no color change whereas, in the control group 52 show no color change, and 9 show light yellow color. (Figure 2)

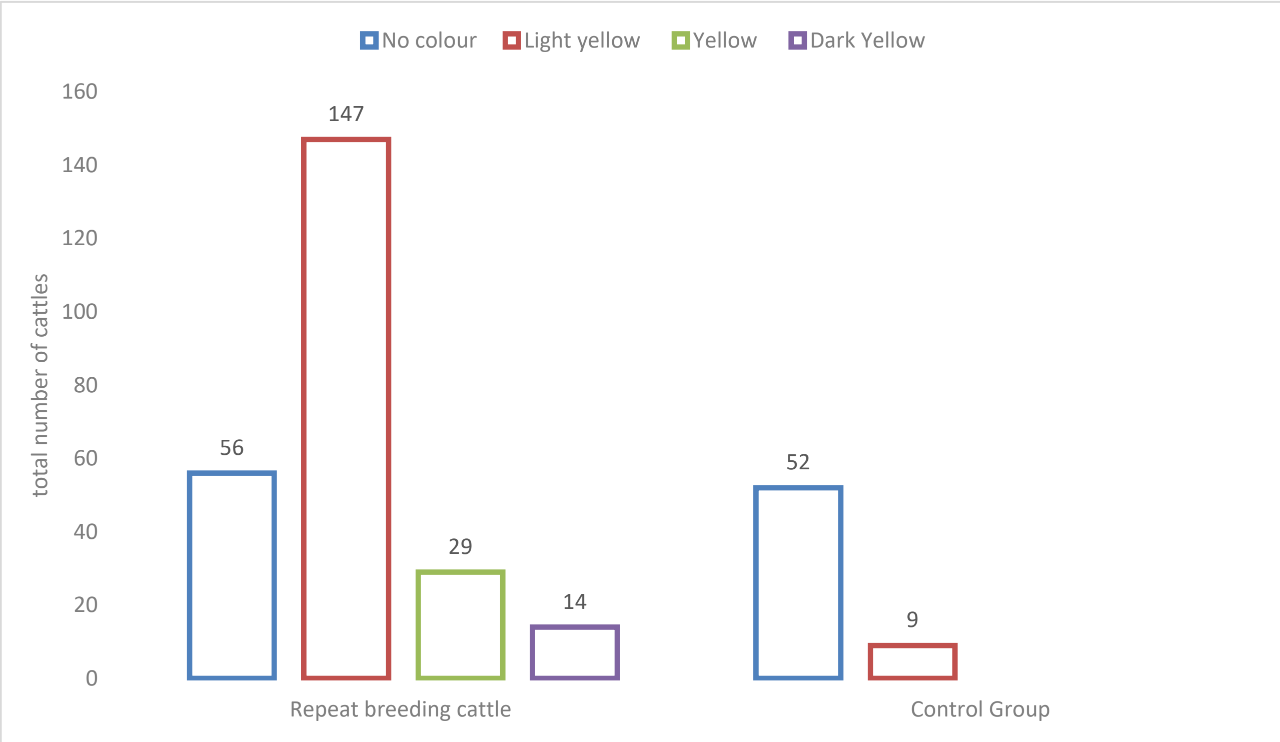

Figure 2: The above bar diagram represents the repeat breeding and control group of cattle about color intensity according to WST

A total number of positive cases in Budhanilkantha Urban Municipality 9(3.65%), Chandragiri Urban Municipality 14(5.69%), Dakshinkali Urban Municipality 7(2.84%), Dakshinkali Urban Municipality 7(2.84%), Gokarneshwor Urban Municipality 28(11.38%), Kirtipur Urban Municipality 105(42.68%), Kageshwori Manahara Urban Municipality 12(4.47%), Kathmandu Metropolitan City 5(2.03%), Nagarjun Urban Municipality 5(2.03%), Shankharapur Urban Municipality 9(3.65%), Tarkeshwor Urban Municipality 7(2.84%), and Tokha Urban Municipality 45(18.29%). (Table 3)

Table 3: Total number of positive cases from 11 different municipalities

Municipality of Kathmandu District	Total number of positive cases	Percentage of positive cases
Budhanilkantha Urban Municipality	9	3.65%
Chandragiri Urban Municipality	14	5.69%
Dakshinkali Urban Municipality	7	2.84%
Gokarneshwor Urban Municipality	28	11.38%
Kageshwori Manahara Urban Municipality	12	4.47%
Kathmandu Metropolitan City	5	2.03%
Kirtipur Urban Municipality	105	42.68%

Nagarjun Urban Municipality	5	2.03%
Shankharapur Urban Municipality	9	3.65%
Tarkeshwor Urban Municipality	7	2.84%
Tokha Urban Municipality	45	18.29%
Total	246	100

Out of 307 samples collected, Kirtipur Municipalities show the highest percentage of positive cases 105 (42.68%), followed by Tokha Municipalities 45 (18.29%), and Gokarneshwor Municipalities 28 (11.38%), while Kathmandu Metropolitan City shows the lowest percentage of positive cases 5(2.03%), followed by Nagarjun Municipalities 5(2.03%). (Figure 3)

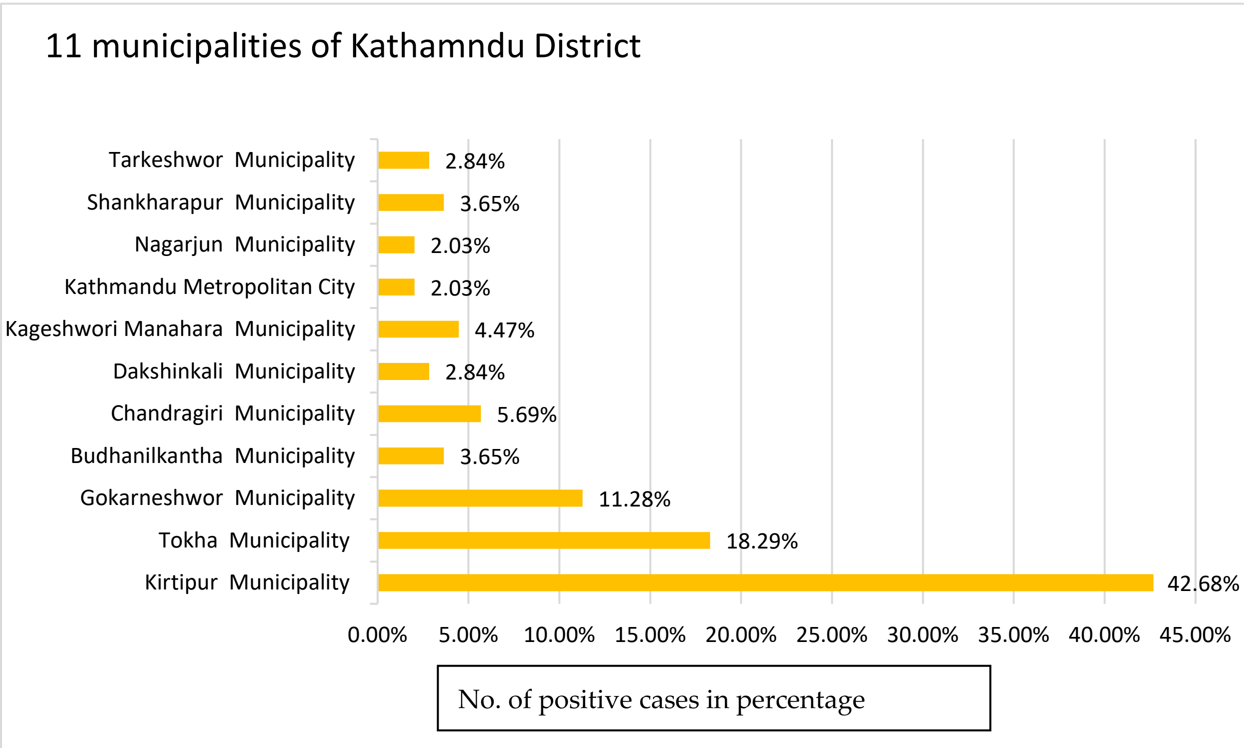

Figure 3: Total percentage of positive cases in Repeat breeding cattle of different Municipalities of Kathmandu

The sample which shows mild (+), moderate (++), and severe (+++) grades of infection from the WST were cultured. i.e., 190 samples were cultured. In bacterial culture, different bacteria were isolated i.e., *E.coli* 55(28.94%), *Pseudomonas spp.* 7(3.68%), *Staphylococcus spp.* 39(20.52%), *Streptococcus spp.* 26(13.68%), *Corynebacterium spp.* 10(5.26%), *Klebsiella spp.* 9(4.73%), and mix bacterial growth were 44(23.15%).*Pseudomonas spp.* 7(3.68%), *Klebsiella spp.* 9(4.73%), *Corynebacterium spp.* 10(5.26%), *E. coli* 55 (28.94%), *Staphylococcus spp.* 39(20.52%), *Streptococcus spp.* 26(13.68%), and mix bacterial growth were 44(23.15%) seen in (Figure 4).

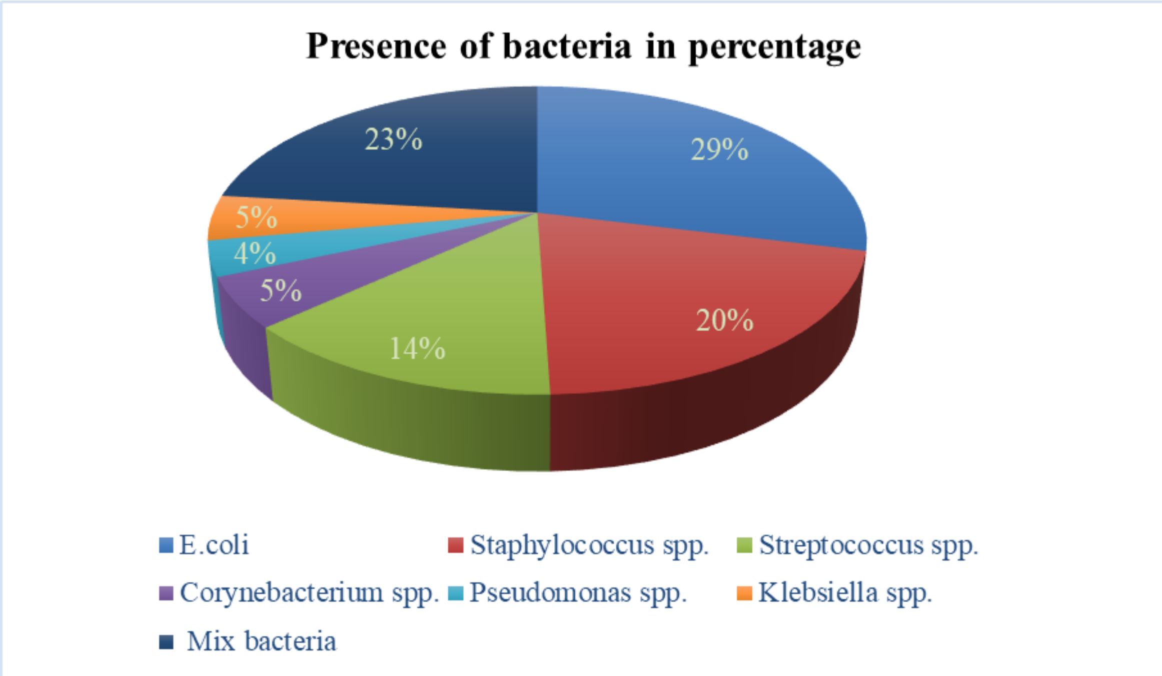

Figure 4: Total percentage of bacteria present in the culture of repeat-breeding cattle

4. Discussion

From the results of WST, it can be inferred that only 9(14.75%) animals in the control group showed infection and the remaining 52(85.24%) animals were free of infection; however, the majority of repeat breeding animals showed infection 190(77.22%) and only 56(22.76%) of animals were free of infection. Furthermore, on bacterial culture, we found *E.coli* 55 (28.947%), *Staphylococcus spp.* 39(20.526%), *Streptococcus spp.* 26(13.684%), *Corynebacterium spp.* 10(5.263%), *Pseudomonas spp.* 7(3.684%), *Klebsiella spp.* 9(4.736%), and mix bacterial growth were 44(23.157%).

This result is consistent with the results of the White Side Test in repeat breeder crossbred Jersey cows, which were recorded as 16.67, 66.67, 09.26, and 7.41 percent color pattern of no color, light yellow, yellow, and dark yellow type, respectively. These results were interpreted as negative, mild, moderate, and severe. The various bacteria that were isolated and cultured from repeat breeding cattle included *Staphylococcus spp*. 16 (21.05%), *E. coli* 14 (18.42%), *Bacillus spp*. 10 (13.16%), *Corynebacterium spp.* 10 (13.16%), *Pseudo-monas spp.* 8 (10.53%), *Proteus spp*. 8 (10.53%), *Klebsiella spp*. 6 (7.89%), and *Streptococcus spp*. 4 (5.26%). Of the 46 (76.67%) repeat-breeding cows, a single organism was identified, whereas mixed infections involving many types of organisms were found in 14 (23.33%) [9]

The results of the present study were more or less in agreement where the majority of aerobic bacteria (84.72%) are present in the cervical mucus of repeat-breeding cows with subclinical endometritis. *Staphylococcus aureus* isolates have been shown to make up the biggest proportion of all facultative anaerobic bacterial species isolates, followed by *E. coli*, *Streptococcus spp*., *Enterobacter spp*., *Proteus spp*., and *Pseudomonas spp.* [14].

The reported color reaction on WST in 92.85% of cervical mucus samples of normal animals indicating subclinical uterine infections which differ from our result where 77.22% were positive for WST in repeat breeding cattle [8]. The recorded color changes in 100% samples of cervical mucus in repeat breeding cows suffering from endometritis which differ from our result [15]. The variance in the infection rate between research may be attributable to the severity of the infections being studied, sample size and demographic variation, and the agro-climatic conditions in the regions where the studies were conducted.

The proportion of positive cases was highest in Kirtipur Municipalities, where it was 105 (42.68%), and lowest in Kathmandu Metropolitan City and Nagarjun Municipalities, where it was 5(2.03%) and 5(2.03%), respectively. Less sample collection and a smaller number of cattle being raised may be to reasons for the disparity in the results.

5. Conclusions

The findings from this study underscore the significant prevalence of nonspecific bacterial genital infections in repeat breeding cattle, as evidenced by the WST. With 77.22% of repeat breeders testing positive for infection, compared to a mere 14.75% in the control group, it is clear that these infections pose a major concern for cattle reproductive health. The predominance of specific bacterial pathogens, particularly E. coli and Staphylococcus spp., highlights the urgent need for targeted management strategies to mitigate their impact on fertility. Geographical analysis reveals notable disparities in infection rates across different municipalities, suggesting that local management practices, environmental conditions, and herd health protocols may significantly influence the occurrence of these infections. This variability emphasizes the importance of region-specific interventions and monitoring systems to enhance reproductive performance in cattle. The WST proves to be a valuable diagnostic tool for identifying subclinical endometritis in cattle, facilitating early intervention and potentially improving reproductive outcomes. By employing such a rapid and cost-effective test, farmers can make informed decisions regarding treatment and management, ultimately leading to better herd health and productivity.

Implications for South Asia

These findings are particularly relevant for South Asian nations, such as Nepal and India, where agriculture and dairy production are vital components of the economy. The high prevalence of reproductive infections in cattle can lead to significant economic losses due to reduced milk yield and fertility. By utilizing the WST, farmers can quickly identify infected animals and implement appropriate management strategies, thus enhancing milk production and overall herd health. Improving reproductive health in dairy cattle not only increases milk availability, which is crucial for food security, but also supports the livelihoods of millions of smallholder farmers dependent on dairy for their income. Enhanced reproductive efficiency can contribute to the sustainability of dairy farming in these regions, ensuring that farmers can meet the growing demand for dairy products while maintaining the health of their livestock. Future research should focus on elucidating the relationship between specific bacterial pathogens and reproductive failure, as well as exploring effective management practices to reduce the incidence of genital infections in cattle populations. These efforts will be crucial in advancing cattle health and productivity, ultimately contributing to sustainable livestock management practices that benefit both farmers and the broader agricultural economy in South Asia. By prioritizing the health of livestock, these nations can secure a more resilient agricultural framework capable of withstanding economic and environmental challenges.

Supplementary Materials (Figures)

Figure S1: Collection of cervical mucus for sample

Figure S2: Laboratory Examination of samples

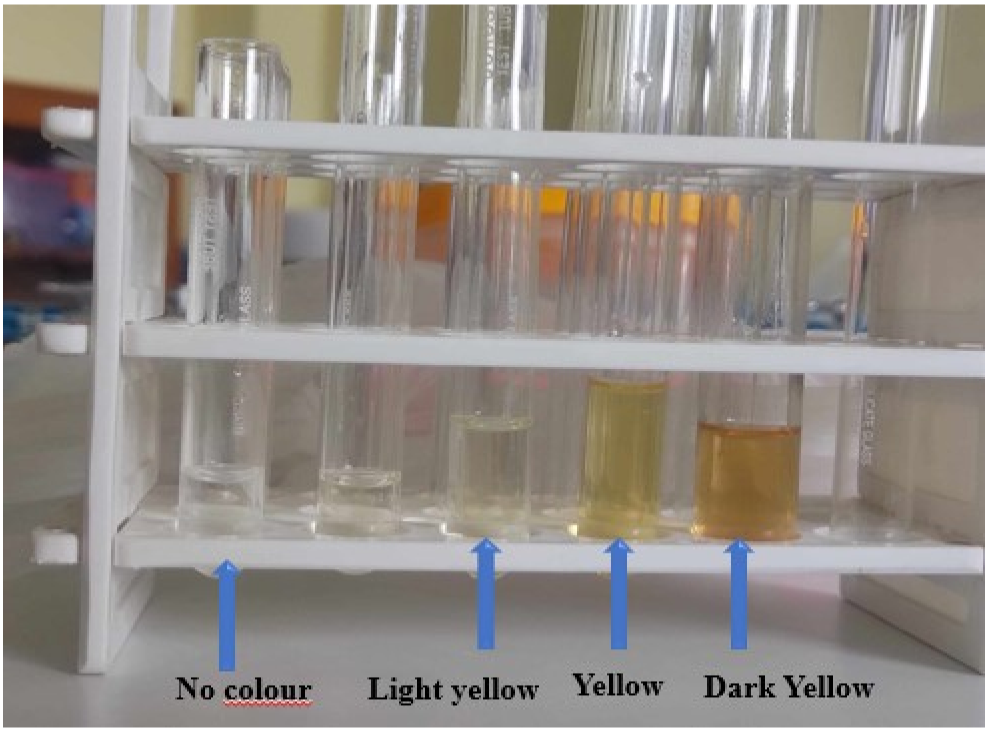

Figure S3: Changes in colour after WST

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