THAI NGUYEN UNIVERSITY
UNIVERSITY OF AGRICULTURE AND FORESTRY

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STUDY ON SOME BIOLOGICAL CHARACTERISTICS OF *MYCOPLASMA, KLEBSIELLA, ESCHERICHIA COLI* BACTERIA ISOLATED FROM DAIRY COWS WITH MASTITIS AT TH FARM AND PREVENTION, TREATMENT MEASURES

Speciality: Veterinary Parasitology & Microbiology
Code: 9. 64. 01. 04

SUMMARY OF DOCTORAL DISSERTATION IN VETERINARY PARASITOLOGY AND MICROBIOLOGY

THAI NGUYEN - YEAR OF 2024

The doctoral dissertation was completed at:
UNIVERSITY OF AGRICULTURE AND FORESTRY –
THAI NGUYEN UNIVERSTITY

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The dissertation was defended in front of the institutional dissertation evaluation Committee
Meeting at: UNIVERSITY OF AGRICULTURE AND FORESTRY –
THAI NGUYEN UNIVERSTITY
On ...., day ...... month .... year 202......

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LIST OF SCIENTIFIC ARTICLES RELATED TO THE DISSERTATION

[1]. **Trần Trung Mỹ**, Lê Văn Thiện, Phạm Tuấn Hiệp, Đặng Xuân Bình. (2020), "Kết quả phân lập một số vi khuẩn gây bệnh viêm vú bò tại các trang trại bò sữa TH". *Tạp chí khoa học kỹ thuật Thú y*, XXVII(7), tr. 31 - 37.

[2]. **Tran Trung My**, Le Van Thien, Vu Duy Manh, Bui Thi Phuong My, Dang Thi Mai Lan, Dang Xuan Binh, Vu Minh Duc. (2023), "Antimicrobial resistance and molecular characterization of *Escherichia coli* isolated from bovine mastitis samples in Nghe An province, Vietnam". *Veterinary World*, 16(4), pp. 743 - 751.

[3]. **My Trung Tran**, Duc Minh Vu, Manh Duy Vu, My Thi Phuong Bui, Binh Xuan Dang, Lan Thi Mai Dang, Thien Van Le. (2023), "Antimicrobial resistance and molecular characterization of *Klebsiella* species causing bovine mastitis in Nghe An province, Vietnam", *Adv Vet Anim Res,* 10(1), pp. 132 - 143.

[4]. **Tran Trung My**, Le Van Thien, Dang Xuan Binh, Dang Thi Mai Lan, Vu Minh Duc. (2024), "Prevalence of bovine mastitis and the first detection of *Mycoplasma bovis* in Vietnam's dairy farms", *Suranaree J. Sci. Technol*. 30(6), pp. 020026(1-7). https://doi.org/10.55766/sujst-2023-06-e01836

INTRODUCTION

1. Urgency of the dissertation

In recent years, dairy farming has gradually shifted towards intensive, safe, environmentally friendly farming, applying science and technology, thereby increasingly contributing to stabilizing people's lives and socio-economic development. Milk and dairy products are classified as high-grade foods due to their complete nutritional and easy-to-digest properties. Since 2009, TH Group (hereinafter referred to as TH) has initiated a project on dairy farming and milk processing focusing on high technology in Nghe An province, thereby contributing to the shift in the proportion of liquid milk processed from reconstituted powdered milk from 92% (in 2008) to around 60% today.

Dairy farming in general and TH farms in particular are still facing many difficulties due to diseases that are affect to the health of cows, especially mastitis. Although mastitis does not kill many dairy cows, it causes great economic losses due to reduced milk production and quality, increased veterinary treatment costs, loss of cows, and increased herd replacement costs. In the US, the damage caused by mastitis in dairy cows is estimated at 2 billion dollars (Rollin et al., 2015)..

Based on the situation of mastitis at TH farms, with the aim of providing relevant scientific documents on the mastitis prevalance, the pathogenic role of *Mycoplasma, Klebsiella* and *E. coli* strains, as well as effective disease prevention and treatment, we conducted: "Study on some biological characteristics of *Mycoplasma, Klebsiella*, *Escherichia coli* bacteria isolated from dairy cows with mastitis at TH farms and prevention, treatment measures".

2. Objectives of the dissertation

- General assessment of the mastitis situation in dairy cows at TH farms.
- Determine the occurrence rate of *Mycoplasma bovis (M. bovis), Klebsiella* and *E. coli* bacteria in cows with and without mastitis.
- Determine some biological characteristics of 3 types of bacteria: *Mycoplasma bovis (M. bovis), Klebsiella* and *E. coli* isolated from dairy cows with mastitis at TH farms, Nghia Dan district, Nghe An province.

- Propose effective measures to help prevent and treat mastitis in dairy cows.

3. Scientific and practical significance of the dissertation

3.1. Scientific significance

Providing scientific documents on the disease situation and biological characteristics of *M. bovis, Klebsiella* and *E. coli* bacteria in dairy farming.

3.2. Practical significance

The results of the sduty are a practical basis for the disease, application of effective treatment and prevention measures for mastitis caused by *M. bovis, Klebsiella* and *E. coli* bacteria at TH farms.

4. New contributions of the dissertation

- Determined the rate of infection with mastitis in dairy cows in TH, the frequency and the incidence of mastitis according to different disease types.
- The first report on *M. bovis* bacteria causing mastitis in dairy cows in Vietnam.
- The prevalence rate, biochemical identification, molecular biological identification, antibiotic resistance characteristics, some virulence genes and antibiotic resistance of *M. bovis, Klebsiella* and *E. coli* bacteria causing mastitis in dairy cows in TH were determined.
- The disease prevention effectiveness of some new methods in Vietnam was evaluated.

5. Structure of the dissertation

The dissertation consists of 115 pages (excluding the list of references and appendices), 2 pages of introduction; 32 pages of overview; 19 pages of research content and methods; 60 pages of research results and discussion, 2 pages of conclusion and recommendations. The thesis consists of 46 tables, 4 charts and graphs, 21 color photos showing the results of the topic, 281 references (12 Vietnamese documents, 266 English documents and 3 documents from the Internet, of which 124 documents are from 2018 - present).

Chapter 1: BIBLIOGRAPHY

Mastitis is a disease that causes huge economic losses in dairy farming worldwide. Many studies on mastitis in dairy cows around the world show that the incidence rate ranges from 13.2% to 74.7%/year (Levison et al., 2016; Abebe et al., 2016), in Vietnam this rate ranges from 23.4% to 88.6% (Pham Bao Ngoc, 2003; Östensson et al., 2013).

Mastitis caused by *Mycoplasma* bacteria is increasingly nowaday, of which *M. bovis* is considered the most common species (George et al., 2007). The pathogenic properties of *M. bovis* that have been discovered to date include the ability to adhere to cells, produce toxins, invade cells, and resist antibiotics (Razin et al., 1998; Gautier-Bouchardon, 2018)..

Klebsiella mastitis is characterized by pain and high infection rates in cows kept on wood products flooring (Gundogan, 2014; Massé et al., 2020). Factors contributing to the pathogenicity of this bacterium include the tissue envelope, LPS, OMP, HMV, adhesion, extracellular ion-binding complexes, growth, and antibiotic resistance (Schukken et al., 2012; Yang et al., 2019).

E. coli causes severe local and systemic mastitis, known as environmental mastitis, which can be fatal in severe cases (Burvenich et al., 2003). Pathogenic factors for mastitis detected in *E. coli* include adhesion factors, cell surface structures and antigens (LPS, CPS, EPS), endotoxins, serum resistance, extracellular ion-binding complexes (siderophore), and antibiotic resistance (Whitfield et al., 2015; Murinda et al., 2019; Caza and Kronstad, 2013; Poirel et al., 2012b).

Diagnosis of the disease usually applies the method of clinical examination combined with CMT testing, in addition to laboratory diagnosis. Disease prevention through comprehensive measures includes somatic cell control, nutrition, hygiene, milking, vaccination and biosafety (Pham Bao Ngoc, 2003; Faruk et al., 2020). The current trend of disease treatment focuses on early detection, treatment with supportive regimens, limiting the use of antibiotics, applying physical and herbal therapies (Pyorala, 2009; Pașca et al., 2017; Leitner et al., 2021).

Chapter 2. RESEARCH CONTENT AND METHODS

2.1. Subjects, time and location of study

2.1.1. Study subjects

- Dairy cows raised at TH farms.
- Bacterial strains of *Mycoplasma, Klebsiella* and *E. coli* isolated from milk samples of cows with mastitis.

2.1.2. Study location

The study was conducted at TH farms. Samples were analyzed at the laboratory of TH Dairy Food Joint Stock Company, Nghia Son Commune - Nghia Dan District - Nghe An Province.

2.1.3. Study time

The study was conducted from 2021 to 2023.

2.2. Study materials

2.2.1. Study samples

- Milk samples collected from cows suspected of having mastitis.
- DNA samples extracted from isolated bacterial strains.

2.2.2. Tools, equipment and chemicals

Use chemicals and tools necessary in microbiology and molecular biology research.

2.3. Study contains

2.3.1. Situation of mastitis in dairy cows at TH farms

2.3.1.1. Incidence of mastitis in dairy cows at farms

2.3.1.2. Situation of dairy cows with the disease by number of times

2.3.1.3. Situation of dairy cows with mastitis by disease type

2.3.2. Some biological characteristics of Mycoplasma, Klebsiella and E. coli bacteria isolated from mastitis dairy cows

2.3.2.1. Some biological characteristics of M. bovis bacteria

2.3.2.2. Some biological characteristics of Klebsiella bacteria

2.3.2.3. Some biological characteristics of E. coli bacteria

2.3.3. Research and propose measures to prevent and treat mastitis in dairy cows

2.3.3.1. Disease prevention measures

** Disease prevention by treating litter*

** Disease prevention by using teatX teat disinfectant before and after milking*

** Disease prevention by Rotatec J5 vaccine*

** Disease prevention by using teatseal teat sealant*

2.3.3.2. Evaluation of the effectiveness of some disease treatment regimens

2.3.3.3. Proposing comprehensive prevention and treatment measures

2.4. Study methods

2.4.1. Situation of mastitis in dairy cows at TH farms

2.4.1.1. CMT method for determining mastitis in dairy cows

CMT testing for individuals with suspected mastitis was performed according to the method described by Kandeel et al. (2018). The results were classified into 4 levels (Negative, Mild, Moderate and Severe).

2.4.1.2. Methods for determining the frequency of mastitis

Investigation (according to the method described by Nguyen Van Thien et al., 2002) of all cows with mastitis during the study period on the number of mastitis episodes during the study period.

2.4.1.3. Classification of mastitis by disease type

To classify mastitis into different disease types, we investigated and classified individual cows with mastitis into 3 different types (according to Adkins and Middleton, 2018).

2.4.2. Some biological characteristics of Mycoplasma, Klebsiella and E. coli bacteria isolated from mastitis dairy cows

2.4.2.1. Milk sampling method

Apply milk sampling method described by Adkins et al. (2017).

2.4.2.2. Chemicals and media

The chemicals and media used in this study are described in detail in Appendix 01.

2.4.2.3. Mycoplasma isolation method

Applying the procedure of the World Organization for Epidemiology (OIE, 2018) and the study of Alysia et al. (2018).

2.4.2.4. Klebsiella isolation method

Samples were tested according to Buchanan and Gibbon, (1974); Adkins et al. (2017).

2.4.2.5. E. coli isolation method

Samples were tested according to Buchanan and Gibbon, 1974;

TCVN 8400-16:2011; Adkins et al., 2017.

2.4.2.6. Method for determining some biochemical properties of Mycoplasma bacterial strains

Determine some of the following biochemical characteristics: glucose fermentation, arginine hydrolysis, tetrazolium decomposition

2.4.2.7. Method for determining some biochemical properties of Klebsiella and E. coli strains

Determine some of the following biochemical characteristics: indole production; fermentation of glucose, sucrose, lactose, H2S production, gas production; MR-VP reaction; citrate decomposition; oxidase reaction; mobility.

2.4.2.8. PCR method determines isolation results, some virulence and drug resistance genes

*** Determine results by PCR technique**

For *M. bovis*, the presence of the uvrC gene was determined.

For *Klebsiella* spp., the presence of the gyrA gene was determined.

For *E. coli*, the presence of the specific malB gene was determined.

Nucleotide sequences of the primers used are listed in Appendix 02.

*** Determination of virulence genes of bacterial strains**

M. bovis bacteria, the presence of Mbov2 and TrmFO genes related to adhesion ability was determined.

For *Klebsiella* spp., the presence of fimH, rmpA, magA, K1, K2, iroN, entB, and iutA genes was determined.

For *E. coli* bacteria, the presence of stx, stx2, F5, F41, eae, iroN, iutA genes was determined.

Nucleotide sequences of the primers used are listed in Appendix 02.

*** Identification of antibiotic resistance genes**

M. bovis bacteria identified the presence of rrs3 gene

Klebsiella bacteria identified the genes blaSHV, blaTEM, blaKPC, blaNDM, blaCTX-M-3 and blaIMP; sul1, sul2; tetA, tetB; DHFR-I; qnrA; acrAKp.

E. coli bacteria identified the genes tetA, tetB, sul1, sul2, blaSHV, blaKPC, DHFR-I, qnrA, blaOXA48.

Nucleotide sequences of the primers used are listed in Appendix 02.

2.4.2.9. Method of studying antibiotic resistance of isolated bacterial strains using antibiotic-impregnated discs diffusion on agar

Apply the method described in CLSI M02-A11 (2012), M100 (2020). Use 19 antibiotics in this study as shown in Table 2.3.

2.4.2.10. Method for determining the ability to produce β-lactam antibiotic-degrading enzymes - ESBL

Apply the method described in CLSI M100-30th (2020).

2.4.2.11. Minimum Inhibitory Concentration (MIC) Method

Apply the procedure of Jay et al. (2021); Hannan (2000) with antibiotics oxytetracycline; florfenicol; tulathromycin; tylosin, tiamulin.

2.4.3. Research and propose measures to prevent and treat mastitis in dairy cows

2.4.3.1. Testing the effect of bedding treatment on mastitis incidence

Two bedding treatment methods (traditional biocomposting and rapid treatment via bedding recovery unit (BRU)) were tested on mastitis incidence at the experimental farm..

2.4.3.2. Testing the effect of teat disinfectant concentration on mastitis rate

Testing two concentrations (1:4 and 1:7) of teat disinfectant used in milking activities, TeatX (Deosan, New Zealand).

2.4.3.3. Rotatec J5 vaccine trial

Two trials were conducted at two farms, the first farm experimented on first-lactation cows, the second farm experimented on second-lactation cows.

2.4.3.4. Test of mastitis prevention in postpartum cows using teat sealant

Tested on a farm with Teatseal product (Zoestic, Australia). The experiment was conducted on 2 types of cows (cows preparing to give birth for the first time, cows giving birth for the second time), each type was conducted on 2 groups of animals: experimental group (using teat sealant) and control group (not using teat sealant).

2.4.3.5. Evaluation of the effectiveness of some treatment regimens

For cows with mastitis caused by *Klebsiella* and *E. coli*, treatment is only applied in moderate and severe cases. For mild cases, only non-antibiotic treatment is applied (regimen 1) or no treatment is applied but isolation and monitoring are carried out, treatment is only applied when the disease becomes severe. For moderate and severe cases, the choice of treatment regimen and preparation is randomly selected according to regimens 2 and 3 applied by the farm, depending on the actual production of the farm, no experiments are arranged.

For cows with mastitis caused by *Mycoplasma*, this study did not test the treatment regimen but culled.

2.4.3.6. Bacteria cure surveillance

A survey was conducted on all farms for cows with mastitis caused by *Klebsiella* and *E. coli* over a 2-year period. Milk sample collection and pathogen isolation were performed as described previously..

2.4.4. Data processing methods

Data were collected and synthesized using Excel application, statistical processing using Minitab 16 software and Excel application.

Chapter 3. RESULTS AND DISCUSSION

3.1. Mastitis situation in dairy cows at TH farms

3.1.1. Rate of mastitis in dairy cows in TH

Table 3.1 shows that the overall incidence of mastitis in dairy cows on farms is 34.8%/year.

The results of mastitis incidence in our study are consistent with the reports of Pham Bao Ngoc (2003), and Truong Quang et al. (2008) when reporting the infection rate between 36.1 and 39.8% per year in Hanoi, in Ethiopia by Tezera and Aman (2021) with a monthly clinical mastitis rate of 3.0% (36.0%/year) and also consistent with Fesseha et al. (2021) when reporting that the monthly mastitis rate was 3.6%.

3.1.2. Frequency of mastitis in dairy cows at TH farms

Table 3.2. 3.2 shows that out of a total of 6,992 cows with mastitis during the study period, the rate of cows with mastitis once was 4,699, accounting for 67.2%. This result gradually decreased (from 22.0% to 0.9%) as the number of mastitis episodes increased (from 2 to 6 times).

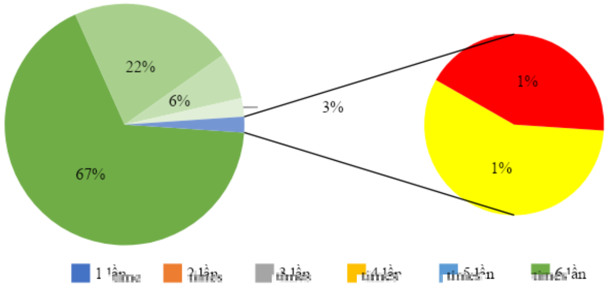

Figure 3.1: Frequency of mastitis in dairy cows on farms

In France, Lescourret et al. (1995) reported that the rate of cows with mastitis once was 68.5% and the rate of reinfection was 31.5%, of which 2 infections accounted for 24.6%, 3 infections accounted for 4.5%, and 4 infections accounted for 2.4%. In Germany, Wente et al. (2020) reported that the rate of dairy cows with mastitis once was 78.2% and the rate of reinfection was 21.8%.

Thus, it can be seen that the results of this study are consistent with the general trend in the frequency of mastitis in dairy cows..

3.1.3. Situation of dairy cows with mastitis according to disease type

Table 3.3 shows that the rate of cows with mild mastitis during the study period was 60.2%, this result for moderate mastitis was 30.5% and severe mastitis was 9.3%.

In the US, Oliveira et al. (2013) reported that the rate of cows with mild mastitis was 47.8% (279/583), moderate mastitis was 36.9% (215/583) and severe mastitis was 15.3% (89/583).

In Vietnam, Le Viet Bao et al. (2020) reported that cows with clinical mastitis in Ho Chi Minh City had 34.0% mild mastitis, moderate mastitis was up to 61.9% while the rate of inflammation in severe mastitis was 4.1%.

Thus, it can be seen that the types of mastitis have clear differences between studies.

3.2. Study on some biological characteristics of *M. bovis, Klebsiella* and *E. coli* bacteria isolated from dairy cows with mastitis at TH farms

3.2.1. Biological characteristics of M. bovis bacteria

3.2.1.1. Isolation of M. bovis from milk samples of mastitis and non-mastitis cows

Table 3.4: Results of isolation of M. bovis bacteria from mastitis and non-mastitis cows

Interpretation	**Mastitis**	**Non-mastitis**
Total samples	4.025	120
Number of positive samples	145	0
Rate (%)	**3,6**	**0**

For mastitis-affected cows, Timonen et al., (2017) reported a 17.2% isolation rate of *M. bovis*, Ashraf et al., (2019) reported 9.0%, García-Galán et al. (2020) reported 28%.

For non-mastitis-affected cows, Penterman et al. (2022) reported 2.2% while Gogoi-Tiwari et al. (2022) did not detect *M. bovis* from non-mastitis-affected cows.

3.2.1.2. Results of identification of some biochemical properties of isolated M. bovis bacteria

Table 3.5 shows that *M. bovis* bacteria are not able to hydrolyze arginine, do not ferment glucose but are able to decompose tetrazolium salts (Figure 3.1).

Nicholas and Ayling (2003) reported that *M. bovis* bacteria are able to decompose tetrazolium salts. Niu et al. (2021) reported that M. bovis bacteria are not able to ferment glucose, lactose; do not hydrolyze arginine.

Our results are similar to the above authors.

3.2.1.3. Identification of isolated Mycoplasma bovis strains using molecular biology techniques

Table 3.6 and Figure 3.2 show that 100% of these bacterial strains carry the uvrC gene, which has a size of 106bp, typical of *M. bovis* bacteria.

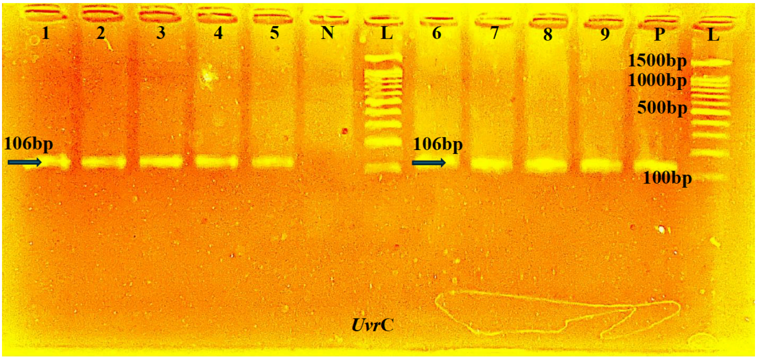

Figure 3.2: Electrophoresis results of PCR products to identify the uvrC gene (106bp) of M. bovis bacterial strains

(Note: DNA ladder (L), negative (N) and positive (P) control samples, test samples (1-9))

Our results are consistent with Niu et al. (2021) when they showed that applying PCR technique to determine the presence of uvrC gene with specific primer pair resulted in 100% of *M. bovis* strains carrying uvrC gene.

A PCR product sample was sent to Axil Scientific Pte Ltd (Singapore) laboratory for sequencing. The results confirmed that the PCR product was *M. bovis* bacteria.

3.2.1.4. Identification of some virulence genes of isolated M. bovis bacteria

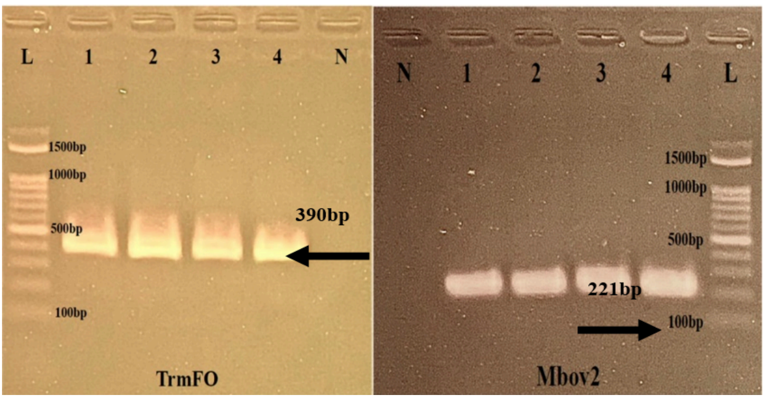

Figure 3.3: Electrophoresis results to identify the TrmFO (390bp) and Mbov2 (221bp) genes

(Note: positive sample (1-4), DNA ladder (L), negative control (N))

Table 3.7 and Figure 3.3 show that 100% of the strains carry 2 genes encoding adhesion factors, TrmFO with a size of 390bp and Mbov-2 with a size of 221bp. Guo et al. (2017) reported that the TrmFO gene is 98% similar to different *M. bovis* strains. Sachse et al. (2000) reported that the ISMbov2A gene segment plays a role in the adhesion of this bacterium. Li et al. (2011) reported that there are 6 elements of *M. bovis*, namely ISMbov1, ISMbov2, and ISMbov3, ISMbov4, ISMbov5, and ISMbov6.

3.2.1.5. Determination of antibiotic susceptibility of isolated M. bovis strains

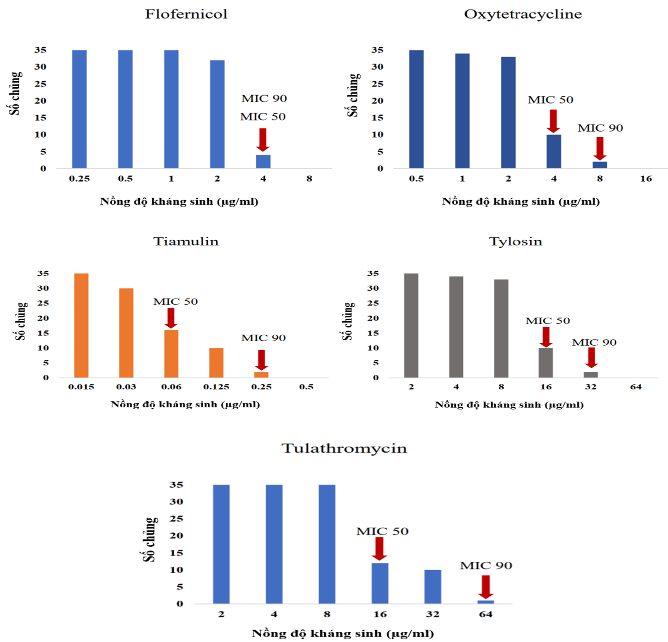

Figure 3.2 shows that the MIC90 values were 4; 8; 0.25; 32; and 64 µg/ml for florfenicol, oxytetracycline, tiamulin, tylosin, and tulathromycin, respectively. Compared to the cut-off values published by Hannan (2020), oxytetracycline, florfenicol, tulathromycin were moderately susceptible, and tylosin was resistant according to the cut-off values published by Ammar et al. (2022).

Our results were lower than those of Sulyok et al. (2014), Hata et al. (2019), Liu et al. (2020), and were relatively consistent with Barberio et al. (2016) on the tested antibiotics. For the antibiotic tulathromycin, our results were lower than those reported by Jelinski et al. (2020).

3.2.1.6. Identification of some antibiotic resistance genes in isolated M. bovis bacteria

100% of the tested bacterial strains carried the 508bp rrs3 gene. This result is consistent with the study of Amram et al. (2015); Niu et al. (2022) reported that single-point mutations in the rrs3 and rrs4 genes are the cause of bacterial resistance to aminoglycosides, fluoroquinolones, and tetracyclines.

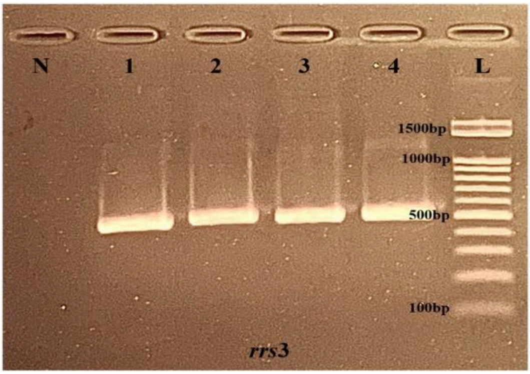

Figure 3.5: Electrophoresis results of PCR products to identify rrs3 gene (508bp)

(Note: positive sample (1-4), DNA ladder (L), negative control (N))

3.2.2. Some biological characteristics of Klebsiella bacteria causing mastitis in dairy cows

3.2.2.1. Results of isolation of Klebsiella bacteria from milk samples of cows with and without mastitis

Table 3.9: Results of isolation of *Klebsiella* bacteria from cows with and without mastitis

Interpretation	Mastitis	Non-mastitis
Total samples	4.025	120
Number of positive samples	848	4

Rate (%)	**21,1**	**3,3**

Klebsiella bacteria accounted for 21.1% of milk samples from cows with mastitis and 3.3% of milk samples from cows without mastitis.

For cows without mastitis, our results were lower than those of Nahar et al. (2021) who reported 30%.

For cows with mastitis, our results were higher than those of Nguyen Van Phat (2010) who reported 0.7%; Gao et al. (2017) who reported 13.0%; Dalanezi et al. (2020) who reported 7.9% of *Klebsiella*. However, Salauddlin et al. (2019) reported 62.5%.

3.2.2.2. Biochemical characterization of isolated Klebsiella bacteria

100% of the bacteria fermented glucose, sucrose, and lactose, and they also produced gas. *Klebsiella* bacteria did not produce H2S, were not mobile, did not produce indole, had a negative MR reaction, positive VP, were able to decompose citrate, and had a negative oxidase reaction, which was consistent with Rawy et al., (2020); He et al., (2022).

3.2.2.3. Identification of some Klebsiella strains isolated by molecular biology techniques

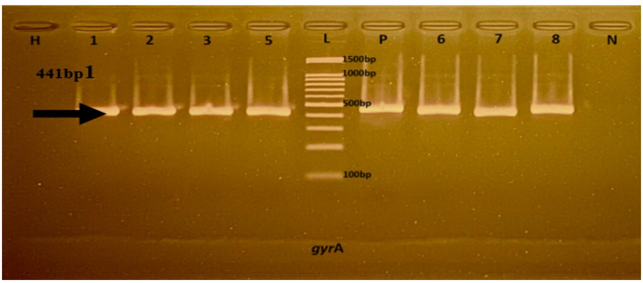

Figure 3.7: Electrophoresis results of PCR products identifying the gyrA gene (441bp) of *Klebsiella* spp..

(Note: DNA ladder (L), negative (N), H (PCR water) and positive (P) control samples, experimental samples (1-8))

100% of *Klebsiella* strains carried the characteristic 441bp gyrA gene. Fatima et al. (2021) reported that 30 isolates were assumed to be *K. pneumoniae* by culture, but only 21 strains were identified to carry the gyrA gene. Therefore, it is necessary to determine the isolation results and biochemical identification of *Klebsiella* bacteria by PCR technique to identify the gyrA gene.

3.2.2.4. Identification of some virulence genes of isolated Klebsiella bacteria

The proportion of strains carrying the K1 gene was 4%, fimH was 100% (688bp in size), entB gene was 98% (371bp in size), and no strains carried the K2, magA, rmpA, and iutA genes.

Cheng et al. (2021) reported that no *Klebsiella* strains carrying the K1 and K2 genes were detected, but 100% carried the entB gene, 12% iutA, 94% fimH, and 3% rmpA. Tianle et al. (2022) also reported the prevalence of fimH gene was 100%, iroN was 4.4%, and rmpA was 4.4%.

Thus, our results are similar to those of the above authors.

3.2.2.5. Determination of antibiotic susceptibility of Klebsiella strains

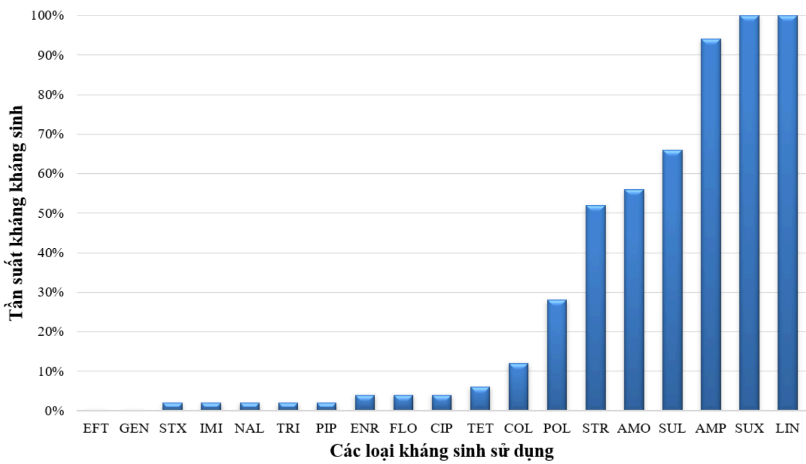

Figure 3.3: Antibiotic susceptibility of isolated *Klebsiella* strains

100% of *Klebsiella* bacteria were resistant to SUX and LIN, 94% were resistant to AMP, 66% were resistant to SUL. The resistance rates to AMO, STR, POL, COL, TET antibiotics were 56%, 52%, 28%, 12%, and 6%, respectively. There were 2/50 strains resistant to CIP, FLO, and ENR, followed by 1/50 strains resistant to PIP, TRI, NAL, IMI, and STX. No strains were resistant to GEN and EFT.

Massé et al. (2020) reported that 58% were completely susceptible to EFT, CIP, FLO, GEN, NAL, STR, STX, TET; the resistance rate to STR was 38%, and TET was 19%. Ali et al. (2021) reported that bacteria were resistant to LIN 98.0%; oxytetracycline 89.3%; AMP 86.1%; STR 67.2%; AMO 66.0%; norflorxacin 34.6%; ENR 11.7%; and GEN 8.5%.

We found GEN and EFT to be good options for the treatment of mastitis caused by *Klebsiella* bacteria.

3.2.2.6. Determination of multi-resistance of isolated Klebsiella strains

Table 3.14 shows that 94% of *Klebsiella* strains were multiresistant.

Our results are higher in Canada, Massé et al. (2020) reported a multiresistant rate of 2%, in China, Tianle et al. (2022) reported a rate of 19.1%, and a result of 43.9% was recorded by Wu et al. (2022).

Thus, although the results are different, the multiresistant *Klebsiella* strains causing mastitis are concernable.

3.2.2.7. Results of determining the ability to produce β-lactam antibiotic-degrading enzymes of isolated Klebsiella strains

The results showed that no *Klebsiella* strains were identified as having the ability to produce this enzyme (ESBL).

Nobrega et al. (2021) studied in Brazil and found that 98.2% of *Klebsiella* strains causing mastitis were not capable of producing ESBL.

Silva-Sanchez et al., (2021) reported that the majority of *Klebsiella* bacteria causing mastitis in dairy cows were multidrug-resistant and produced ESBL.

To our knowledge, this is the first screening study on the ability to produce ESBL of *Klebsiella* strains causing mastitis in dairy cows in Vietnam to date.

3.2.2.8. Results of determining some antibiotic resistance genes of isolated Klebsiella strains

100% of strains carried the 940bp acrAKp gene; 94% of strains contained the blaSHV gene, 8% blaNDM and no blaTEM, blaCTX-M-3, blaKPC, blaCTX-M-3 and blaIMP genes were detected in all strains tested; 4% of strains were found to carry the sul1 gene and 2% of strains were found to carry the sul2 gene; 100% carried the tetA gene and 0% tetB; qnrA.

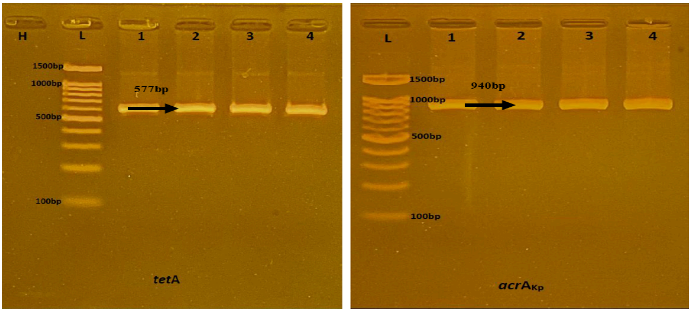

Figure 3.9: Electrophoresis results of PCR products to identify tetA and acrA_{Kp} genes

(Notes: tetA (1-4) 577bp, acrAKp (5-8) 940bp, negative control (H), DNA ladder (L))

Padilla et al. (2017) reported that AcrAb is associated with resistance to quinolones and some other antibacterial compounds, as well as host antimicrobial peptides.

Enferad and Mahdavi. (2021) reported that the proportions of blaCTX, blaSHV, and blaTEM genes were 62.5%, 42.5%, and 87.5%, respectively. Safia et al., 2013 did not detect the blaSHV gene but detected the blaTEM-1 gene at a rate of 2.2%. Yang et al. (2021) reported that in China, 16.7% carried the blaCTX gene, 68.2% blaSHV, and 30.3% tetA. Aslam et al. (2022) reported that *Klebsiella* causing mastitis in dairy cows had a sul1 gene ratio of 39% and sul2 gene ratio of 44%. Ahmed et al. (2021) reported that 14.3% of *Klebsiella* carried the qnrA gene.

3.2.3. Some biological characteristics of E. coli bacteria causing mastitis in dairy cows

3.2.3.1. Isolation of E. coli from mastitis and non-mastitis cow milk samples

Table 3.17: Results of *E. coli* isolation from cows with and without mastitis

Interpretation	Mastitis	Non-mastitis
Total samples	4025	120
Number of positive samples	306	0

Rate (%)	7,6	0,0

For cows without mastitis, Megersa et al. (2019) reported that 40% of samples isolated *E. coli*; Geletu et al. (2022) reported 9.6%.

For cows with mastitis, Pham Bao Ngoc (2003) reported that the *E. coli* isolation rate was 19.6%; Truong Quang et al. (2008) reported 30%; Nguyen Van Phat (2010) reported 2.5%. Results recorded in China ranged from 12.2% to 18.5% (Gao et al., 2017; Liu et al., 2018; Lan et al., 2020)

Thus, the results of this study show that the isolation rate of this bacteria on farms is lower than many previous reports.

3.2.3.2. Results of biochemical characterization of isolated E. coli bacteria

100% of *E. coli* bacteria produced indole; fermented glucose, sucrose and lactose; produced gas, did not produce H2S; were unable to use citrate; VP reaction was negative, MR was positive. Four *E. coli* strains were detected that were not motile. All of these biochemical characteristics of the isolated *E. coli* strains were similar to those reported by Adkins et al. (2017); Megersa et al. (2019).

3.2.3.3. Identification of some E. coli strains isolated by molecular biology techniques

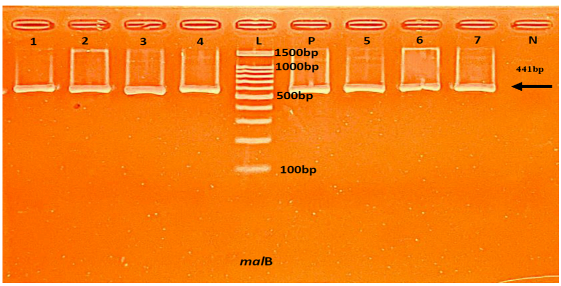

Figure 3.11: Electrophoresis results of PCR products identifying the malB gene (585bp) of *E. coli* bacteria

(Ghi chú: thang ADN (L), mẫu đối chứng âm (N) và dương (P), mẫu thí nghiệm (1-7))

Figure 3.11 shows that 100% of *E. coli* strains carry the malB gene with a characteristic size of about 585bp.

The results of this study are consistent with Bag et al. (2021) when stating that the isolation culture process using Eosin methylence blue agar to search for characteristic colonies followed by PCR confirmation of the presence of the malB gene and 16S rRNA gene sequencing is necessary to ensure accuracy..

3.2.3.4. Results of determining some virulence factors of isolated E. coli bacteria

Figure 3.12 shows that 2 strains carrying the eae gene (425bp) accounted for 4%, 1 strain carrying the iutA gene (302bp) accounted for 2%, and 3 strains carrying the stx2 gene (118bp) (6%).

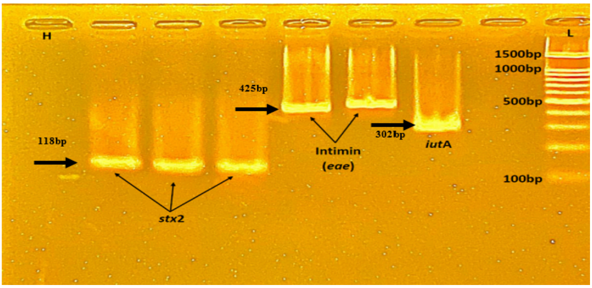

Figure 3.12: Electrophoresis results of PCR products identifying genes iutA (302bp), stx2 (118bp), eae (425bp)

(Note: positive sample (1), negative sample (2-4), DNA ladder (L), negative control (H - H2O))

Fernandes et al. (2011) found that 100% of *E. coli* strains carried the fimH gene and no strains carried the F5, F41, or eae genes. Ahmadi et al. (2020) reported that the proportion carrying the eae gene was 20.5%, of which 75% of the strains also carried the stx2 gene, and 25% also carried the stx1 and stx2 genes. Hamideh et al. (2021) reported that the bacterial strains carried the iroN and iutA genes at a rate of 14.0% and 12.1%, respectively.

3.2.3.5. Results of antibiotic susceptibility testing of isolated E. coli strains

Figure 3.4 shows that *E. coli* bacteria were completely resistant to SUX and SUX (100%), 90% of strains were resistant to SUL. 32% to TET and POL; 38% to STR; 14% to AMP, COL, AMO and PIP; 10% to TRI and SXT, the resistance results to CIP, FLO and EFT were 4% of the strains; 1 strain (2%) was resistant to ENR, NAL acid, and IMI; no strain was resistant to GEN.

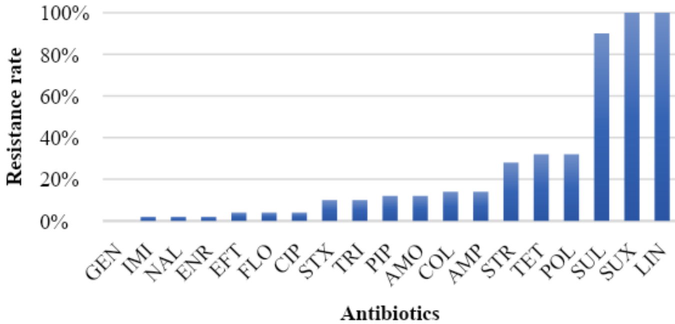

Figure 3.4: Antibiotic susceptibility of E. coli bacteria

Yu et al. (2020) research in China showed that this bacteria is resistant to penicillin 100%, oxacillin 98.8%; AMP 30.1%; AMO 25.3%; CIP 4.8%, ENR 4.8%; norfloxacin 4.8%; TET 12.0%; trimethorpim 22.9%; STR 13.3%; GEN 12.0%; LIN 98.8%.

Our results are relatively consistent with the above author..

3.2.3.6. Results of determining antibiotic resistance of isolated E. coli strains

There were 23 strains of multidrug-resistant *E. coli*, accounting for 46%.

Awosile et al. (2018) reported that the prevalence of multidrug-resistant *E. coli* causing mastitis in dairy cows was 12.7%; Bag et al., (2021) reported that in Bangladesh the result was 84.2%.

The results of this study differ from the above reports.

3.2.3.7. Determination of β-lactam antibiotic-degrading enzyme production of isolated E. coli strains

Non of *E. coli* strains were identified to be capable of producing this enzyme.

Our study results are similar to those of Saini et al. (2012), and in France by Dahmen et al. (2013). Meanwhile, in Greece it was 6.7% (Filioussis et al., 2020), in Egypt it was 100% (Ahmed et al., 2021).

To our knowledge, this is the first screening study on ESBL production of *E. coli* strains causing mastitis in dairy cows in Vietnam to date..

3.2.3.8. Results of determining some antibiotic resistance genes of isolated E. coli strains

Table 3.24 shows that *E. coli* bacteria causing mastitis in dairy cows carry 4 different genes including tetA (577bp) and tetB (634bp) (Figure 3.13), each gene has a rate of 12%, sul1 with a rate of 26% and sul2 is 30%, non of *E. coli* strains carrying genes such as blaSHV; blaOXA48; blaKPC; DHFR-I or qnrA were detected.

Lan et al. (2020) reported that the rate of *E. coli* bacteria causing mastitis in dairy cows carrying the tetA gene was 75% while tetB was not detected; Rana et al. (2022) reported the rates of 35.3% tetA, 20.6% tetB, 47.1% sul1, 32.4% sul2.

Genes encoding ESBL production and resistance to β-lactam antibiotics published worldwide include blaTEM; blaOXA; blaKPC from 6.4% to 98.7% (Ali et al., 2016; Yu et al., 2020; Lan et al., 2020; Song et al., 2020; Bag et al., 2021; Liu et al., 2021).

Yu et al. (2020) studied in China and found that the occurrence rate of DHFR-I gene was 14.5%, and no strains were found to carry the qnrA gene.

Thus, our results are consistent and relatively different from those of the above authors..

3.3. Research and propose measures to prevent and treat mastitis in dairy cows

3.3.1. Research on some measures to prevent mastitis in dairy cows

3.3.1.1. Testing the effect of litter treatment on mastitis incidence

The incidence of the disease at 30 days of the experimental group (LTN) was 2.0% and that of the control group (LDC) was 3.5% ($P > 0.05$); at 60 days, LTN was 2.6% and LDC was 4.6% ($P > 0.05$). However, at 90 days of the experiment, the incidence of the disease in LTN was lower than that in LDC, and this difference was statistically significant ($P < 0.05$).

Our results are consistent with Fournel et al. (2019b) reported that the rapid composting process reduced the *E. coli* count from 1.0 × 105 to 2.0 × 101, and *Klebsiella* from 3.2 × 104 to 4.0 × 102 bacteria/g after 24 hours, thereby significantly reducing the incidence of mastitis in dairy cows..

3.3.1.2. Testing the effect of teat disinfectant concentration on mastitis incidence in dairy cows

The incidence of mastitis in LTN1 was lower than that in LTN2, but not significantly ($P > 0.05$). At 60 or 90 days of the experiment, the incidence of disease in LTN1 was significantly lower than that in LTN2 (2.7% vs. 8.2% at 60 days, 2.1% vs. 7.6% at 90 days of the experiment) ($P < 0.05$).

There are no similar studies to compare, but some studies have evaluated the effectiveness of using disinfectants before milking without affecting the incidence of mastitis (Singh et al., 2019); Fitzpatrick et al., 2021).

Thus, according to the results of this experiment, the use of disinfectants before and after milking, as well as the concentration of chemicals, has an impact on the incidence of disease.

3.3.1.3. Rotatec J5 vaccine trial

Vaccination of first-lactation heifers and second lactation cows reduced the incidence of mastitis at 30, 60 or 90 days of follow-up compared to LDC ($P > 0.05$). At 30 days, there was no difference in the incidence of mastitis between the two groups. At 60 and 90 days, LTN significantly reduced the incidence of moderate mastitis compared to LDC ($P < 0.05$), but there was no difference between mild and severe mastitis ($P > 0.05$).

Wilson et al. (2007); Gurjar et al. (2013) reported that vaccination did not reduce the incidence of disease, but did reduce the severity of the disease. Gentilini et al. (2012) reported that the vaccinated group had a lower incidence of clinical mastitis compared to the unvaccinated group. Sánchez-Castro et al. (2023) reported that vaccinated cows yielded 163.9 kg more than unvaccinated cows over a 305-day period.

Thus, our results suggest that J5 vaccination reduces disease severity in lactation cows in the first and second lactation.

3.3.1.4. Test of Teatseal nipple sealant for mastitis prevention in dairy cows

LTN had a mastitis rate of 1.3%, LDC 6.0% in the first 30 days ($P < 0.05$). In the period of 30 - 60 days, although LTN was lower in quantity than LDC, this difference was not significant ($P > 0.05$).

The use of Teatseal in LTN resulted in a significantly lower mastitis rate in the first 30 days compared to LDC (P = 0.045), with similar results in the next 30 - 60 days (P = 0.050).

Freu et al. (2020) reported that drying cows with antibiotics combined with teat sealants reduced the risk of mastitis in cows up to 60 days in milk, and the risk of mastitis caused by common pathogens compared to cows dried with antibiotics alone. Thus, these two experiments show that the use of teat sealants is effective in reducing the incidence of mastitis in cows, and this result is consistent with other authors' previous publications.

3.3.1.5. Control and improvement of SCC in subclinical mastitis

The proportion of cows with SCC greater than 300,000 in 2021 and 2022 was 15%, and in 2023 it was 14%.

Al-harbi et al. (2021) reported that the proportion of cows with mastitis on farms with SCC greater than 300,000 accounted for 9.3%. In this study, no correlation was established between cows with SCC greater than 300,000 and the incidence of mastitis. However, the above results show that maintaining a low proportion of cows with SCC greater than 300,000 can minimize the risk of mastitis.

3.3.2. Evaluation of the effectiveness of some treatment regimens for mastitis in dairy cows caused by Klebsiella and E. coli

3.3.2.1. Results of some treatment regimens for mastitis

Regimen 1 (no antibiotics) gave good results for mild mastitis, with a cure rate of up to 85.7%. Regimen 2 for moderate mastitis was 63.3%. Regimen 3, applied to severe mastitis, had the worst treatment results, with a cure rate of 45.9%.

Goulart and Mellata (2022) stated that antibiotic treatment of mastitis caused by *Klebsiella, E. coli* and other coliform bacteria is not necessary. Supportive therapies such as fluid resuscitation, anti-shock, anti-inflammatory should be the first choice.

Thus, it can be seen that this result is consistent with the above author.

3.3.2.2. Treatment results of clinical mastitis according to disease types

The recovery rate of cows with mastitis caused by *Klebsiella* and *E. coli* bacteria was very high (84.5% and 90.0%) for mild inflammation, and this result gradually decreased when cows had moderate inflammation (59.3% and 73.1%) to severe inflammation (40.7% and 54.8%).

Sugiyama et al. (2022) reported that cows with severe mastitis caused by *Klebsiella* and *E. coli* had a clinical cure rate ranging from 52.8% (*Klebsiella*) to 86% (*E. coli*).

Thus, our results are consistent with the above authors.

3.3.2.3. Bacteria cure surveillance results

Cows with mastitis caused by *Klebsiella* had a pathogen cure rate of 31.9%, and a rejection rate of 27.7%; this result for *E. coli* was 46.9% and 19.1%. The microbiological cure rate of cows with mastitis caused by *E. coli* was higher than that of *Klebsiella* (P = 0.001). The rejection rate of cows with mastitis caused by *Klebsiella* was higher than that of *E. coli* (P = 0.031).

Suojala et al. (2010) reported that the microbiological cure rate of cows with mastitis caused by *E. coli* was up to 90.5%. Fuenzalida and Ruegg (2019a) reported that the microbiological cure rate of cows with mastitis caused by *Klebsiella* was up to 74%, the rejection rate of cows with mastitis caused by *Klebsiella* was 22.0%, and 7.0% for *E. coli.*

Thus, our results are different from those of the above authors..

3.3.3. Comprehensive measures to prevent and treat mastitis in dairy cows

We propose a comprehensive disease prevention and treatment for bovine mastitis including: using rapidly fermented separator (BRU) as bedding, chemicals at a concentration of 1:4 (TeatX), injecting J5 vaccine, using teat sealant (Teatseal) to dry milk, checking the number of SCC cells for early treatment, separating suspected diseased cows for CMT testing. Treatment is based on the disease: mild and moderate, do not treat immediately but take samples to the laboratory, isolate and monitor, treat according to the results (*E. coli, Klebsiella*: based on the condition of the cow to treat with non-antibiotic supportive regimen (infusion, anti-shock, anti-inflammatory) if necessary, if clinical symptoms become severe, use regimen 2 or 3; *M. bovis* is eliminated). Severe form, take samples and treat immediately with regimen 3.

CONCLUSIONS AND RECOMMENDATIONS

1. Conclusions

Dairy cows raised on TH farms have an average annual incidence of mastitis of 34.8%, with a frequency of 1 to 6 times. Most of the cows with mastitis are mild and moderate, accounting for 90.7%, and severe cases account for 9.3%.

M. bovis, Klebsiella, and *E. coli* bacteria isolated from milk samples of cows with mastitis have biological and chemical characteristics typical of the breed and species; have typical pathogenic factors:

M. bovis bacteria do not appear in milk samples of healthy cows, but account for 3.6% in cows with mastitis; 100% carry the uvrC gene and biochemical characteristics; 100% carry the 3 virulence genes Mbov2, TrmFO, and rrs3. Of the 5 tested antibiotics, only tiamulin is still susceptible.

Klebsiella bacteria isolated from healthy cow milk samples accounted for 3.3% and 21.1% of cows with mastitis; 100% carried the gyrA gene and biochemical characteristics; were resistant to 17 tested antibiotics at a rate of 2% - 100%, sensitive to ceftiofur and gentamicin; did not produce ESBL enzymes; 94% were multi-resistant; carried 3 genes encoding virulence factors at a frequency of 4.0% - 100%, and 6 genes related to antibiotic resistance at a rate of 2.0% - 100%.

E. coli bacteria were not isolated from healthy cow milk samples but accounted for 7.6% of milk samples from mastitis cows; 100% carried the malB gene and biochemical characteristics; were resistant to 18 tested antibiotics at a rate of 2% - 100%, sensitive to gentamicin; 46% were multi-resistant; did not produce ESBL enzymes; carrying 3 genes encoding virulence factors with frequencies from 2.0% - 6.0%, 4 genes related to antibiotic resistance with rates from 12.0% - 30.0%.

Effective disease prevention measures include: treating bedding with BRU, using the correct concentration of teat disinfectants, using Rotatec J5 vaccine, teat sealants in dry milk therapy. In addition, ensuring hygiene and periodically monitoring the number of SCC cells are also good measures in controlling the incidence of mastitis in dairy cows.

Treatment regimens according to the disease type, combining antibiotics with supplementives (electrolytes, energy supply) are effective in treating mastitis caused by *Klebsiella* and *E. coli* in severe cases with a cure rate of 45.5 to 85.6%. The microbiological cure rate of mastitis caused by *Klebsiella* is lower than that of *E. coli*, this difference is significant.

2. Recommendations

(1) Continue to study other characteristics of mastitis causing bacteria in this study as well as other pathogens..

(2) Continue to study of new treatment regimens with antibiotics identified to have high susceptibility to pathogenic bacteria.

(3) Research on losses caused by mastitis on dairy farms, especially in large-scale farming conditions.