

## Evaluation of contamination of cow milk with various conditionally pathogenic microflora for mastitis: genera *Staphylococcus*

I. V. Borovyc<sup>1</sup> , P. O. Davydenko<sup>1</sup> , O. M. Kulishenko<sup>1</sup> , V. V. Zazharskyi<sup>1</sup> ,  
M. L. Radzikhovskiy<sup>2</sup> , O. V. Dyshkant<sup>2</sup> , B. V. Gutyj<sup>3</sup> 

<sup>1</sup>Dnipro State Agrarian and Economics University, Serhiia Efremova Str., 25, Dnipro, 49600, Ukraine

<sup>2</sup>National University of life and environmental sciences of Ukraine, Heroiv Oborony Str., 15, Kyiv, 03041, Ukraine

<sup>3</sup>Stepan Gzhytskyi National University of Veterinary Medicine and Biotechnologies Lviv, Pekarska Str., 50, Lviv, 79010, Ukraine

### Article info

Received 29.09.2023

Received in revised form

30.10.2023

Accepted 31.10.2023

### Correspondence author

Mykola Radzikhovskiy

Tel.: +38-067-725-65-07

E-mail: [nickvet@ukr.net](mailto:nickvet@ukr.net)

2023 Borovyc I. et al. This is an open-access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.



### Contents

1. Introduction .....	24
2. Results and discussion .....	26
3. Conclusions .....	30
References .....	30

### Abstract

The food safety issue of livestock products concerning insemination by opportunistic and accompanying microflora has attracted increasing attention from specialists. The ever-increasing trade and export of dairy and meat products from Ukraine to the EU countries requires constant monitoring of inoculation with mesophilic-aerobic and facultatively anaerobic microorganisms and psychrophilic microorganisms, in particular the genera *Listeria* and *Yersinia*, which survive and multiply at low temperatures and in salted and fermented products and pose a threat to human health. Milk and lactic acid products make up a significant part of the diet of a modern person. Therefore, constant control of raw materials from dairy enterprises for the production of milk and directly at enterprises for its processing is a guarantee of food safety. At the stage of milk production, due to violations of technological cycles of production (mastitis and contamination with antibiotics) and the spread of several diseases in Ukraine (paratuberculosis, tuberculosis, leptospirosis, brucellosis), milk from a herd of dairy cows poses a significant threat to human health due to the danger of mastitis milk and milk containing the causative agents of tuberculosis, brucellosis, leptospirosis, and recently there are more and more reports about the association of bovine paratuberculosis and human short bowel syndrome (Crohn's disease) mainly among the young population of Europe, which may be due to extreme variability and adaptive capacity mycobacteria.

**Keywords:** milk and dairy products; mastitis; contamination; microbiological diagnostics; mesophilic aerobic and facultative anaerobic microorganisms; human health.

### Citation:

Borovyc, I. V., Davydenko, P. O., Kulishenko, O. M., Zazharskyi, V. V., Radzikhovskiy, M. L., Dyshkant, O. V., & Gutyj, B. V. (2023). Evaluation of contamination of cow milk with various conditionally pathogenic microflora for mastitis: genera *Staphylococcus*. *Ukrainian Journal of Veterinary and Agricultural Sciences*, 6(3), 24–31.

### 1. Introduction

Despite the progress of science, for many years, mastitis, especially of a hidden nature, remains one of the factors that negatively affect the growth of milk productivity of cows, goats, ewes, and rabbits and the sanitary quality of milk in farms of various forms of ownership (Gaurav et al., 2019; Qiu et al., 2021; Sabino et al., 2023). In addition, pathological inflammatory processes in the mammary gland are often found in rabbits (Hu et al., 2021).

According to foreign and domestic scientists' data, 7 to 80 % of cows suffer from mastitis (Idbeis & Khudor, 2019; Zazharskyi et al., 2019; Yue et al., 2021).

Significant economic losses caused by dairy farming due to this pathology are primarily due to a decrease in milk

productivity of cows and goats, culling of animals (30–35 %), and treatment costs.

In addition to economic losses, contamination of milk by pathogenic microorganisms, changes in the chemical composition, and physical and biochemical properties of milk, as a result of which the nutritional value is lost, affecting its quality and biological safety, is a threat. Drinking colostrum from animals suffering from mastitis to newborn animals can lead to gastrointestinal disorders and even death. When treating sick cows and goats, in most cases, preference is given to using antibiotics and sulfonamide drugs by intracis-ternal administration. The most damaging consequence of using antibiotics in treating cows suffering from mastitis is the presence of their residues in collected milk, which impairs its technological properties and harms human health.

Mastitis is an inflammation of the mammary gland that occurs in response to adverse environmental factors under reduced body resistance and complications from infection (Phophi et al., 2019; Kassich et al., 2022).

The most significant number of animals with inflammation of the mammary gland is registered after childbirth (33–89 %), due to diseases of the genital organs (25–60 %), and at the end of peak lactation (22–52 %). During drought, the number of animals with mastitis decreases moderately to 6 %, and due to violations of the rules of starting, unsatisfactory conditions of keeping, feeding, and lack of exercise, it increases to 80 % (Hoernig et al., 2016; Saad et al., 2019; Qiu et al., 2022).

The secretory tissue of the mammary gland of healthy animals does not contain microflora. Still, saprophytic bacteria (coccal flora, corynebacteria) that contaminate the first portions of milk are constantly present in the teat canal and milk cistern.

The infection most often enters the mammary gland through the mammary duct (galactogenic pathway), much less often – through the wounds of the mammary gland and teats (lymphogenic pathway), and even less often – through the bloodstream (hematogenic pathway) from other organs due to the development of an inflammatory process in them (endometritis, gastroenteritis), with infectious diseases (brucellosis, tuberculosis, leptospirosis). Contributes to the occurrence of the disease of retention of colostrum at the birth of a dead offspring, retention of litter, postpartum septic processes, and intoxication.

The incidence of the disease increases to 72% in autumn, winter, and spring and decreases to 7–14 % in summer. Subclinical mastitis is detected in 32–90 % and clinical – in 4–30 % of lactating females (Zhou et al., 2015; Zazharskyi et al., 2020).

Among the forms of clinical mastitis, serous (2–26 %), catarrhal (3–46 %), and purulent-catarrhal (2–71 %) are most often diagnosed, and less often – fibrinous (2–4 %), hemorrhagic (2–4 %) and mixed (2 %). Most often (52–76 %), one part of the udder is affected; less often, two (14–35 %) and, only as an exception, three (5–13 %) or four (3–4 %) at the same time. According to literature data, 4 to 20 % of cows suffering from mastitis lose productivity due to irreversible atrophic processes in mammary gland tissues (Quintas et al., 2017; Bhakat, 2019; Das et al., 2022). There are also reports that mastitis can recur, but there are no exact data on the location and course of recurrent udder inflammation (Putz, 2020).

The development of the inflammatory process in the mammary gland occurs due to mechanical, physical (including thermal), chemical, and biological factors. In particular, the biological factor accounts for 85 % of all cases of mastitis (Avall-Jaaskelainen et al., 2021).

If the clinical form of mastitis gives reason to establish a diagnosis, it is challenging to diagnose subclinical mastitis without laboratory diagnostics. In samples of the secretion of the affected part of the udder, an increased number of blood cells, mainly leukocytes, changes in the chemical composition of milk, and bacteria pathogenic for animals are detected.

Milk always contains the enzymes catalase, reductase, diastase, lipase, etc. Their number depends on the degree of contamination of milk with microorganisms. An increase in enzymes in milk indicates an inflammatory process in the mammary gland caused by microbes. Therefore, for exam-

ple, catalase passes into milk from the cells of the mammary gland and is also produced by bacteria. Catalase content in milk varies. Fresh milk obtained from healthy animals contains little catalase. Its content increases in colostrum and milk obtained from animals with mastitis (abnormal milk) (Dai et al., 2023).

Milk obtained from animals suffering from mastitis contains substances that suppress the development of lactic acid bacteria. Harmful factors cause a violation of the nervous regulation of the udder, blood circulation, and trophic and smooth muscle functions of the milk ducts, cistern, and sphincter of the milk duct. This results in the stagnation of milk, a decrease in its bactericidal, bacteriostatic, and lysozyme effects, and an increase in pH. This contributes to the activation of the pathogenicity of microorganisms (Zhou et al., 2019; Zazharskyi et al., 2020).

The species composition of bacteria that play a role in the etiology of mastitis depends on the microbial landscape and the sanitary and hygienic condition of the farm or premises where animals are kept.

Biological factors can be specific microorganisms that cause infectious diseases (tuberculosis, brucellosis, foot and mouth disease, actinomycosis, smallpox, etc.); nonspecific microorganisms that cause mastitis (streptococci, staphylococci, enterobacteria, pseudomonads, corynebacteria, mycoplasmas, Candida fungi, Nocardia, Klebsiella, etc.) (Salaberri et al., 2016; Maramulla et al., 2019; Zaatout, 2022).

In up to 90 % of cases, mastitis is caused by streptococci and staphylococci. They can be its direct cause or a secondary factor in the inflammatory process caused by other factors. The occurrence of mastitis depends not only on the pathogenic agent and its potential ability to cause a pathological process but also mainly on the immunobiological reactivity of the animal's organism. Therefore, the same factor, including a microbial one, can cause different forms of mastitis (Friman et al., 2017; Zazharskyi et al., 2021).

There are many methods of laboratory diagnosis of mastitis (Pilegi Sfaciotte, 2019; Vieira et al., 2020; Fouad et al., 2022):

- physical and chemical methods (bromothymol test, phenolroth test, benzidine test, dimastine, mastidine, mastiprim test, Whiteside test; Draminski's device for determining subclinical mastitis and others);
- cytological methods – determination of the number of somatic cells (EKOMILK SCAN milk analyzer, microscopic method), leukocyte test, and determination of the number of leukocytes;
- biochemical (determination of enzymes: activity of catalase, lysozyme reductase (muramidase);
- bacteriological cultural method (classical);
- bacteriological express methods (swabs);
- accelerated identification of microorganisms using modern methods of detecting bacterial species (API BioMerieux, Vitek, HI-DIP, latex tests, Erba Lachema, and others);
- genetic and molecular methods for a specific pathogen (PCR, ELFA, ELISA).

For the diagnosis of subclinical mastitis, a count of leukocytes and somatic cells in milk, a bacteriological examination of the udder secretion, and the activity of catalase and lysozyme of milk is determined in laboratory conditions (Asadpour et al., 2021).

Bacteriological examination of milk makes it possible to establish the cause of mastitis and choose the best means of

treatment after determining the sensitivity of microorganisms to antimicrobial drugs (Zhang et al., 2017).

Bacteriological examination of milk from the affected quarter of the udder can identify the type of pathogen causing the udder infection (Padmaja & Halami, 2016).

Screening tests, such as bacteriological examination of a collective milk sample from the tank, allow monitoring of the herd for dangerous contagious mastitis pathogens and provide information on milking hygiene.

Other tests, such as the examination of litter or udder towels, can reveal potential risk factors for the development of mastitis or help control the management of this production area.

It is necessary to decide whether research will be conducted at an individual or a herd's level.

If at the individual animal level, it will be either a composite milk sample (when milk from all four quarters of the udder is mixed) or a sample taken from one affected lobe of the udder. It should be borne in mind that the test results of composite samples are sometimes difficult to interpret because, as a rule, many different microorganisms are isolated from such samples. If none of the isolated microbes are infectious, it will be impossible to determine whether the isolated agents are causing the infection or are simply contaminants (total microbial count).

Examination of composite samples can only be helpful for screening for contagious agents of mastitis. In most cases, examining a sample of milk taken from an individual quarter of the udder is most beneficial. However, suppose one is only interested in whether there are specific contagious pathogens in the herd (for example, *Staphylococcus aureus* or *Mycoplasma*), then you can choose an appropriate screening test. In that case, that is, precisely, conduct tests to detect certain microorganisms (Breen, 2020).

The successful implementation of treatment and preventive measures in cases of mastitis in cows largely depends on a quick and correct determination of the etiology of inflammation of the mammary gland.

Milk is examined for general bacterial insemination to identify specific types of microorganisms as possible causative agents of mastitis and to study the sensitivity of milk microflora to antibiotics and other inhibitors (Chand et al., 2023).

However, the total microbial count (TMC) of bacteria measures the inoculation of milk with pathogenic and non-pathogenic bacteria of various species. This indicator mainly characterizes the conditions of obtaining milk and its sanitary quality (class) but does not provide a basis for establishing a diagnosis of mastitis, especially subclinical. ZMCH is determined in the case of sizeable bacterial contamination (not pure milk is suspected) by reductase samples and, in minor cases – by the bacteriological method (cup) (Tabis et al., 2022).

For the diagnosis of mastitis, at the first stage, research is carried out for the presence of pathogenic staphylococci, streptococci, and enterobacteria, including *Escherichia*, in milk or udder secretion, at the second stage – if the therapeutic effect is unsatisfactory – pseudomonads, *Mycoplasma*, corynebacteria, fungi of the *Candida* and *Nocardia* genera.

If it is necessary to detect the causative agents of anthrax, listeriosis, tuberculosis, brucellosis, and others in milk, use the relevant normative documents to diagnose these diseases.

It is challenging to diagnose subclinical mastitis since there are practically no noticeable clinical signs. Its essential feature is high bacterial contamination of milk and an increased content of somatic cells. Indicators above 100,000 cells in 1 cm<sup>3</sup> of milk more or less indicate mammary gland infection. The reason for the increase in the number of somatic cells is the increased decomposition of tissues caused by the infection, as well as the increased activity of white blood cells, which are involved in the process of fighting the pathogen. If the content of somatic cells in milk increases due to infection, then bacterial insemination also increases (Mahmmod et al., 2017).

## 2. Results and discussion

**Selection of samples for research.** If the animal was treated, then a bacteriological examination of the secretion of mammary glands (milk) should be carried out no earlier than 14 days after the last use of antibiotics or after the period of elimination of antibiotics from the body, which is indicated in the annotation to the drug.

Regardless of the type of sample, possible sources of contamination should be considered, and aseptic sampling techniques should be used.

**Selection of milk.** In animals, the udder, the lower abdominal wall, and the inner surfaces of the thighs are washed with household soap. Wipe the nipples, especially their tops, with a swab soaked in 70° alcohol. Wash and treat hands with an alcohol swab. The first milk spurts from the studied portion are milked into a separate container. Then, 10–15 cm<sup>3</sup> of experimental milk is milked into a sterile container (test tube, glass) so that the teats do not touch the container. It is closed with a sterile cork or lid, cooled, and sent to the laboratory for bacteriological examination.

Milk samples are delivered to the laboratory no later than 4 hours from their selection in containers that ensure a temperature of approximately no higher than eight °C (in thermoses, containers with ice or refrigerant, but not allowing them to freeze).

**Postmortem diagnosis** of mastitis takes place in small animals, fur animals, such as rabbits, to prevent the spread of infection in the herd/nest, in case of ineffective treatment, etc. A part of the affected mammary gland is selected under mandatory aseptic conditions and delivered to the laboratory with cold elements 4–6 hours from selection.

**Isolation of staphylococci.** Among the pathogenic staphylococci that can cause mastitis in animals, especially in the clinical form, is the more common *Staph. aureus*

**Selection.** To isolate staphylococci, the material is cultured on blood agar and other staphylococcal agar of one choice (blood agar with sodium chloride, milk-salt agar (MSA), yolk-salt agar (YSA), Baird Parker agar, salt agar with mannitol) – 0.1 cm<sup>3</sup> of udder secretion is rubbed with a spatula on the surface of the agar, pieces of affected gland tissue are sown by applying to the surface of the agar, as well as 0.1–0.2 cm<sup>3</sup> of udder secretion or a portion of mammary gland tissue in 5–7 cm<sup>3</sup> of salt broth.

The crops are incubated for 24–48 hours at 37 ± 1 °C.

When looking at cultures on blood agar, the growth of large (2–4 mm) convex colonies with a smooth, shiny, or matte surface of white or cream, yellow or lemon yellow color, surrounded by a transparent (β-hemolysis) or opaque zone (α-hemolysis), is taken into account. Sometimes there is double hemolysis (mixed) – pathogenic staphylococci.

Some strains of *Staphylococcus aureus* do not cause hemolysis of erythrocytes ( $\gamma$ -hemolysis) – non-pathogenic staphylococci.

On other differential agars, the cultural characteristics provided by the manufacturer of the medium and the enzymatic properties of staphylococci are taken into account. So, on Baird Parker agar – black shiny colonies with a lecithinase zone (the formation of a cloudy zone, corollas around the colonies); on agar with mannitol – on a red background of the medium, yellow colonies (fermenting mannitol) with lecithinase corollas.

On salt agar with mannitol – yellow colonies (fermentation of mannitol) on a red background of the medium.

On YCA, the colonies are cream with a zone of lecithinase around them (zone of enlightenment).

For further differentiation, part of the culture is transplanted onto a slanted non-selective nutrient agar.

**Confirmation of staphylococci.** From the salt broth, they are transplanted into one of the special dense media listed above.

Smears are made from the isolated colonies on agars; they are stained by Gram, and microscopy is carried out.

When Gram-positive cocci located singly, in clusters or tetrads, characteristic of staphylococci, are detected in smears, the selected culture is checked for catalase activity.

To determine catalase activity, colonies are ground in a drop of 3 % hydrogen peroxide solution applied to a glass slide, or a drop of 3 %  $H_2O_2$  is applied directly to a typical colony.

Staphylococci contain the enzyme catalase, which, upon contact with hydrogen peroxide, causes intense gas formation, which indicates a positive reaction.

To differentiate staphylococci from micrococci, glucose fermentation is determined under anaerobic conditions. That is, after sowing the culture in a glucose medium, 2 cm<sup>3</sup> of sterile vaseline oil is layered on its surface. The crops are incubated at a temperature of  $37 \pm 1$  °C. The result is considered after 24 hours and, in the absence of fermentation, another 24 hours.

Micrococci and sometimes *St. saprophyticus* do not ferment glucose in anaerobic conditions, unlike other staphylococci.

**Determination of mannitol and maltose fermentation.** 3-to four drops of the tested broth culture of *Staphylococcus* or a loop of agar culture are sown in two test tubes with 5 cm<sup>3</sup> medium with mannitol and 2 test tubes with maltose. 2 cm<sup>3</sup> of sterile vaseline oil layered in one test tube of medium with mannitol and one with maltose to create anaerobic conditions. The crops are incubated at a temperature of  $37 \pm 1$  °C. The result is considered after 24 hours and, in the absence of fermentation, another 24 hours. A change in the indicator's color indicates the fermentation of mannitol or maltose.

For *Staphylococcus aureus*, fermentation of mannitol and maltose in anaerobic conditions, as well as in aerobic conditions, is a differentiating feature.

**Determination of pathogenic factors of staphylococci.** Hemolytic properties of staphylococci can be taken into account during primary growth on blood agar. However, because of the possibility of simultaneous growth on blood agar of other microflora, in the presence of which the hemolytic activity of staphylococci can be suppressed or, conversely, enhanced, as well as in the case of the presence of inhibitory substances (novocaine, sodium chloride) in the

composition of nutrient agar, it is recommended to carry out reseeded of isolated colonies on another plate with blood agar without inhibitors.

To determine the plasma-coagulating properties of staphylococci due to the presence of the coagulase enzyme, a reaction is performed with whole or dry diluted rabbit blood plasma (RBP).

For this, staphylococci colonies grown on non-selective ordinary agar (MPA, TSA) at  $37 \pm 1$  °C for 18–24 hours are used.

Rabbit blood plasma is diluted with a sterile physiological solution in a ratio of 1 : 5 and poured into Florinsky tubes of 0.5 cm<sup>3</sup>.

Commercial dry plasma is diluted according to the instructions for its use.

The staphylococcus culture is removed from the agar with a bacteriological loop and placed in tubes with diluted plasma.

For control, a physiological solution (negative test) is introduced into one test tube with plasma and a strain of plasma coagulant, for example, *St. aureus* (positive test). The results are taken into account every hour for 3 hours, then after 6 hours, and up to 24 hours (or according to the instructions of the plasma manufacturer), after which the final accounting of the results is carried out or according to the instructions for the use of plasma.

With a positive plasma coagulation reaction, a clot is formed that does not fall out of the test tube when it is tilted or floats in the plasma. If the result is negative, the plasma remains liquid and easily pours out of the test tube.

Non-pathogenic strains of staphylococci do not coagulate plasma. Streptococci of group D (enterococci) can also produce coagulase in small doses, so it is necessary to consider this during identification (by testing for catalase).

In the absence of coagulase-positive staphylococci during primary inoculation on dense media, colonies isolated from enrichment media are studied.

**Additional tests.** Determination of phosphatase activity of *Staphylococcus*. The isolated culture is sown in Petri dishes with a medium containing sodium phenolphthalein phosphate. The crops are incubated at a temperature of  $37 \pm 1$  °C for 24 hours, after which a few drops of a 10 % aqueous ammonia solution are placed on the cover of the cup, and the bottom of the cup is covered with agar on which the culture colonies are growing. Under the action of ammonia vapors, phosphatase-positive cultures acquire a pink hue, and phosphatase-negative cultures of staphylococci turn yellow or remain unchanged (Fig. 1).

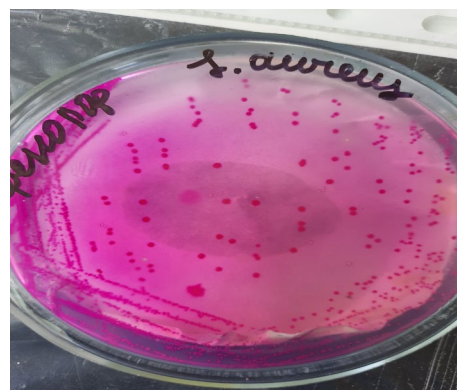


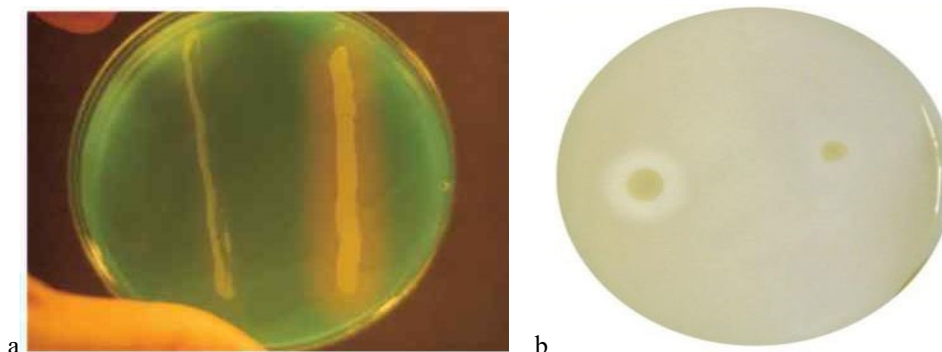
Fig. 1. Positive test for phosphatase

### Determination of DNase activity of staphylococci.

DNase is an enzyme of pathogenicity, which indicates the enterotoxigenicity of staphylococci. To determine it, a staphylococcus culture is sown in Petri dishes with a particular DNase medium.

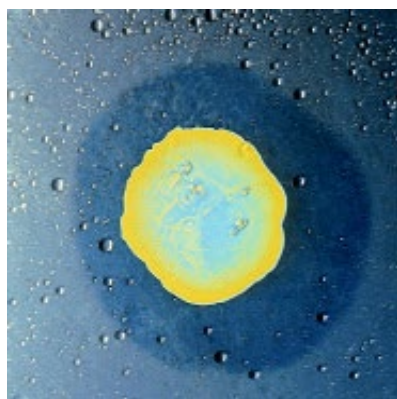
Sowing of cultures is carried out in one cup up to 12–16 cultures of staphylococci. Cultures are sown with strokes along the radius or in the form of plaques in the center of the square, placing a stencil drawn into squares under the cup. The crops are incubated at  $37 \pm 1$  °C for 24 hours.

At the end of cultivation, the culture cup is removed from the thermostat, and 5–7 cm<sup>3</sup> of 1-norm is poured into it. Of a hydrochloric acid solution and kept at  $22 \pm 2$  °C for 2–3 min. Then, the acid is carefully drained so as not to wash away the culture, and the results are considered—the appearance of lightening of the zone around plaque colonies or along the strokes (positive reaction). In the absence of zone illumination for up to 15 minutes, the reaction is adverse (Fig. 2).



**Fig. 2.** DNase activity of *St. aureus*: a – on DNase medium with toluidine blue; b – on the media of the “Himedia” brand, cat. No. M482

Lipolytic activity is determined on a medium with tributyrin. The culture is streaked with a bacteriological loop on agar with tributyrin. Incubation of crops at a temperature of  $37 \pm 1$  °C for 24–48 hours. Around the colonies of lipolytic microorganisms on the yellow cloudy medium, a zone of enlightenment is formed (due to the degradation of tributyrin) (Fig. 3).



**Fig. 3.** Lipolytic activity of *Staphylococcus aureus* on agar with tributyrin

The biological test makes it possible to determine the pathogenicity of staphylococcal cultures by parenteral (into the abdominal cavity) and paroral (per os) administration of the material.

**Option 1.** In the experiment, 24–48-hour cultures of staphylococci grown on MPA are used. With the help of a spatula or a spoon, agar is crushed and mixed with 10 ml of physiological solution to a homogeneous porridge-like consistency. Next, this mixture of agar culture is kept in a thermostat for 2 hours at a temperature of  $20 \pm 2.0$  °C. The mixture is centrifuged for 10 minutes at 1500–2000 rpm. 0.5–1.0 cm<sup>3</sup> of supernatant is used for infection.

**Option 2.** In this experiment, washing liquids of daily cultures of staphylococci are used. To do this, the colonies on the slanted MPA are washed with a sterile 0.85 % sodi-

um chloride solution (2–3 cm<sup>3</sup>). The washed culture is kept in a thermostat for 2 hours at a temperature of  $20 \pm 2.0$  °C. The infection dose of pathogenic staphylococcus culture is 0.5–1.0 cm<sup>3</sup>.

According to options 1 and 2, two or three white mice (body weight 14–18 g) are infected intraperitoneally. Animals are observed for up to 3 days. Usually, in the presence of enterotoxin in the culture, animals die within two days. Pathogenic staphylococci are considered if two or three infected mice have died.

**Option 3.** Detection of staphylococcal enterotoxin per os. As experimental animals, 2–3 heads of white mice (2–3 weeks old), young hamsters, young guinea pigs (weight 200 g), or kittens (1.5–2 months old) can be used). The experimental culture of the isolated *Staphylococcus* is grown in sterile milk (20–25 cm<sup>3</sup>) at  $37 \pm 1.0$  °C for 48–120 hours. Next, this milk culture is fed to laboratory animals on an empty stomach (10–15 cm<sup>3</sup> each to mice and hamsters, 15–20 cm<sup>3</sup> each to guinea pigs and kittens). If the animals do not eat, then it is necessary to feed everyone with a pipette or a spoon. A positive reaction is considered vomiting, which will occur after 30–60 minutes; sometimes, there is diarrhea and general prostration. Vomiting that appears after 5–10 minutes is nonspecific. Animals infected *per os* are observed for 4–5 hours. If the animals do not react during this period, the biological sample is considered harmful.

If necessary, the organs of dead mice are cultured on certain media.

**Quantitative accounting of *Staphylococcus aureus*.** Blood agar with novocaine or Baird Parker's agar is used to study collected milk for quantitative accounting of staphylococci. If it is necessary to consider the degree of contamination of only *Staphylococcus aureus*, use DNA-novocaine-agar or another selective medium designed to confirm *Staphylococcus aureus*.

A 1 : 100 dilution is prepared from the delivered milk samples in a sterile saline solution. 1 cm<sup>3</sup> of milk + 9 cm<sup>3</sup> of

physical r-nu = dilution 1:10, from it 1 cm<sup>3</sup> in 9 cm<sup>3</sup> of physical. r-n = 1:100.

0.1 cm<sup>3</sup> of milk diluted 1:100 is applied to the surface of blood agar with novocaine or DNA-novocaine additive (rec. 4, 5) and evenly rubbed with a spatula over the entire surface of the medium.

The sown cups are placed in a thermostat with the lids down and incubated at 37 ± 1 °C for 22–24 hours.

On novocaine-blood agar, a zone of hemolysis is expressed around the colony.

On DNA-novocaine agar, *St. aureus* grows in large round colonies with even edges.

Pour 5–7 cm<sup>3</sup> of 1-norm into cups with DNA-novocaine agar. The hydrochloric acid solution is then viewed in transmitted light. The detection of colonies surrounded by a zone of illumination with clear boundaries indicates the presence of *St. aureus*.

To determine the number of *Staphylococcus aureus* in 1 cm<sup>3</sup> of the studied milk, typical colonies are counted over

the entire surface of the medium, and the resulting number of colonies is multiplied by 10 (the volume of seed material is 0.1 cm<sup>3</sup>) and by the degree of dilution of the material (1:100).

For example, 0.1 cm<sup>3</sup> of milk diluted 1:100 is sown. As a result of counting, 15 colonies were obtained. 15 CFU x 10 parts x 100 div. = thus, 1 cm<sup>3</sup> of milk contains 15,000 microbial cells.

If it is necessary to identify typical colonies of pathogenic *Staphylococcus*, the procedure according to clause 4.2 is carried out. Then, it is calculated according to the formula: the number of confirmed pathogenic staphylococci is divided by the number selected for confirmation and multiplied by the total number of typical colonies in the cup.

Differentiation of coccal microflora isolated from milk or udder secretion of cows is carried out based on the tests indicated in Fig. 4.

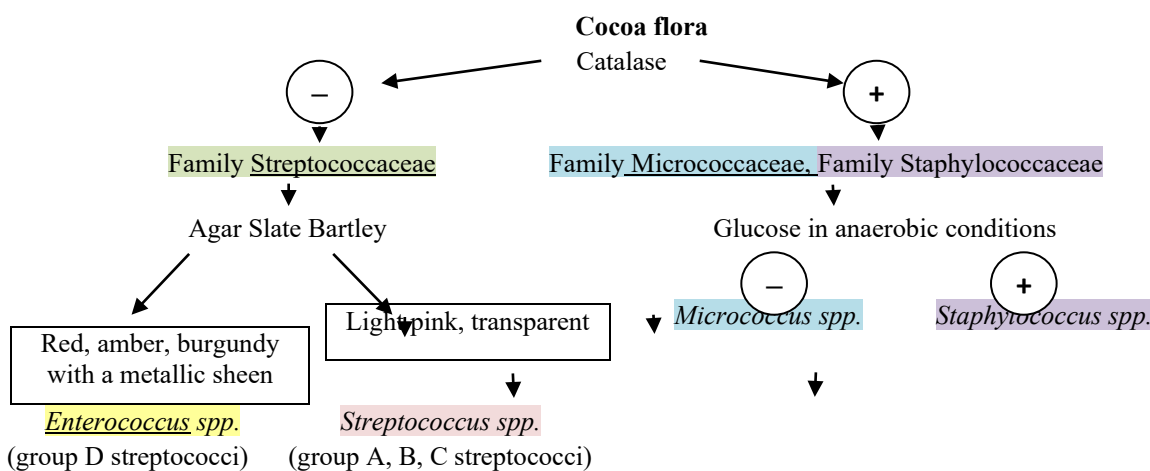


Fig. 4. Differentiation of coccal microflora

Table 1

The main differential signs of staphylococci, causative agents of mastitis

Features	<i>St. aureus</i>	<i>St. intermedius</i> *	<i>St. hemolyticus</i>	<i>St. hyicus</i>	<i>St. epidermidis</i>	<i>St. saprophyticus</i> **
Formation of pigment	+ (dogs -)	-	+/-	-	+	+
Catalase	+	+	+	- /+ less often	+	+
Plasmocoagulase (rabbit plasma)	+	+	-	+/-	-	-
Lecithinase	+	+	-	+	+/-	-
Mannitol in anaerobic conditions	+	-	-	-	- biotype 1,2,3; + biotype 4	- biotype 1,2; + biotype 3, 4
Maltose in anaerobic conditions	+	+ (without oxidation)	-	-	+ biotype 1; - biotype 2; ± biotype 3, 4	+ biotype 2,3,4; ± biotype 1
DNase	+	+	-	+	-	-
Hemolysis (β, α) (ram's blood)	+	+	+	-	+/-	-
Lipase	+	-	+	-	+	-
Phosphatase	+	+	-	+	+ biotype 1,2; - biotype 3, 4	-

Notes: "+" – positive reaction; "-" – negative reaction; +/- – varies; \* – representative of the staphylococci species only in animals; \*\* – a representative of the staphylococci species only in humans

**Accounting of results.** The main differential signs of staphylococci, the causative agents of mastitis, are listed in Table 1.

The milk of cows and goats suffering from mastitis most often contains staphylococci of the species *St. aureus* (*Staphylococcus aureus*), *St. haemolyticus* (*hemolytic Staphylococcus*), *St. epidermidis* (*epidermal Staphylococcus*).

Staphylococcus species *St. saprophuticus* is the causative agent of mastitis and can be detected in the milk of both healthy and sick cows due to its contamination. Hence, the determination of pathogenicity in white mice takes place.

Staphylococci of the species *St. aureus*, possessing pathogenicity enzymes: lecithinase, hemolytic activity, plasma coagulation property and/or DNase activity, and decomposing mannitol and maltose in anaerobic conditions, are classified as causative agents of mastitis.

### 3. Conclusions

Detection of opportunistic and pathogenic agents in the composition of dairy products is one of the priority tasks of modern microbiological practice to preserve the health of people and animals in the concept of one health

#### Conflict of interest

The authors declare that there is no conflict of interest.

#### References

- Asadpour, R., Zangiband, P., Nofouzi, K., & Saberivand, A. (2021). Differential expression of antioxidant genes during clinical mastitis of cow caused by *Staphylococcus aureus* and *Escherichia coli*. *Veterinarski Arhiv*, 91(5), 451–458. [\[Crossref\]](#) [\[Google Scholar\]](#)
- Avall-Jaaskelainen, S., Koort, J., Simojoki, H., & Taponen, S. (2021). Genomic analysis of staphylococcus aureus isolates associated with peracute non-gangrenous or gangrenous mastitis and comparison with other mastitis-associated *Staphylococcus aureus* isolates. *Frontiers in Microbiology*, 12, 688819. [\[Crossref\]](#) [\[Google Scholar\]](#)
- Bhakat, C. (2019). Use of herbal preparations in dry cow management against sub clinical mastitis – an alternative approach. *IndiaRxiv*. [\[Crossref\]](#) [\[Google Scholar\]](#)
- Breen, J. (2020). Dry cow environment management and mastitis control in dairy herds. *Livestock*, 25(5), 210–215. [\[Crossref\]](#) [\[Google Scholar\]](#)
- Chand, U., Priyambada, P., & Kushawaha, P. K. (2023). *Staphylococcus aureus* vaccine strategy: Promise and challenges. *Microbiological Research*, 271, 127362. [\[Crossref\]](#) [\[Google Scholar\]](#)
- Dai, Y., Wang, Z., Liu, H., & Liu, L. (2023). An improved feature selection algorithm for cow subclinical mastitis. *Research Square*. [\[Crossref\]](#) [\[Google Scholar\]](#)
- Das, A., Guha, C., Jana, P. S., Biswas, U., Kar, I., & Rana, T. (2022). Biomarker of clinical diagnosis and therapeutic management of subclinical mastitis in cow. *Exploratory Animal and Medical Research*, 12(2), 235–245. [\[Crossref\]](#) [\[Google Scholar\]](#)
- Fouad, E. A., Abd El-Razik, K. A., & Abdel-Rahman, E. H. (2022). Diagnosis of *Staphylococcus aureus* Infection in Bovine Mastitis using its Affinity Purified Fraction. *Advances in Animal and Veterinary Sciences*, 10(9), 1887–2089. [\[Crossref\]](#) [\[Google Scholar\]](#)
- Friman, M., Hiitio, H., Niemi, M., Holopainen, J., Pyorala, S., & Simojoki, H. (2017). The effect of a cannula milk sampling technique on the microbiological diagnosis of bovine mastitis. *The Veterinary Journal*, 226, 57–61. [\[Crossref\]](#) [\[Google Scholar\]](#)
- Gaurav, K., Nathiya, P., Diwakar, Bhati, T. & Kataria, A. K. (2019). Detection of Enterotoxin Genes (seg, seh and sei) in *Staphylococcus aureus* Milk Isolates from Cow with Subclinical Mastitis. *Journal of Animal Research*, 9(2), 275–280. [\[Crossref\]](#) [\[Google Scholar\]](#)
- Gotsulia, A. S., Zazharskyi, V. V., & Davydenko, P. O. (2018). Synthesis and antituberculosis activity of N'-(2-(5-((theophylline-7'-yl) methyl)-4-R-4H-1,2,4-triazole-3-ylthio)acetyl)isonicotinohydrazides. *Zaporizhzhia State Medical University*, 4(109), 578–583. [\[Crossref\]](#) [\[Google Scholar\]](#)
- Hoernig, K. J., Donovan, D. M., Pithua, P., Williams, F., & Middleton, J. R. (2016). Evaluation of a lysostaphin-fusion protein as a dry-cow therapy for *Staphylococcus aureus* mastitis in dairy cattle. *Journal of Dairy Science*, 99(6), 4638–4646. [\[Crossref\]](#) [\[Google Scholar\]](#)
- Hu, H., Fang, Z., Mu, T., Wang, Z., Ma, Y., & Ma, Y. (2021). Application of Metabolomics in Diagnosis of Cow Mastitis: A Review. *Frontiers in Veterinary Science*, 8, 747519. [\[Crossref\]](#) [\[Google Scholar\]](#)
- Idbeis, H. I., & Khudor, M. H. (2019). Detection Of Intracellular Adhesion Gene (Icaa And Icad) And Biofilm Formation *Staphylococcus Aureus* Isolates From Mastitis Milk Of Cow. *Kufa Journal For Veterinary Medical Sciences*, 10(1), 1–13. [\[Crossref\]](#) [\[Google Scholar\]](#)
- Kandeel, S. A., Ebied M. H., Arnaout, F. K., Galila, E. M. Megahed A. A., & Constable P. D. (2017). Clinical utility of two leukocyte esterase reagent strips for the cow-side diagnosis of subclinical mastitis in lactating dairy cattle. *Assiut Veterinary Medical Journal*, 63(155), 109–118. [\[Crossref\]](#) [\[Google Scholar\]](#)
- Kassich, V., Kasianenko, O., Zazharskyi, V., Yatsenko, I., & Klishchova, Z. (2022). Influence of Ionizing Radiation on the Allergic Reactivity of Tuberculosis-Infected Laboratory Animals. *Scientific Horizons*, 24(10), 17–27. [\[Crossref\]](#) [\[Google Scholar\]](#)
- Mahmmod, Y. S., Klaas, I. C., & Enevoldsen, C. (2017). DNA carryover in milk samples from routine milk recording used for PCR-based diagnosis of bovine *Staphylococcus aureus* mastitis. *Journal of Dairy Science*, 100(7), 5709–5716. [\[Crossref\]](#) [\[Google Scholar\]](#)
- Maramulla, A., Gadige, A., Kosapati, L., Bommu, S., Katta, P., & Keshamoni, R. (2019). Cow Side and Laboratory Tests for Diagnosis of Subclinical Mastitis in Cows. *International Journal of Current Microbiology and Applied Sciences*, 8(11), 2187–2205. [\[Crossref\]](#) [\[Google Scholar\]](#)
- Padmaja, R. J., & Halami, P. M. (2016). Taming C-terminal peptides of *Staphylococcus aureus* leukotoxin M for B-cell response: Implication in improved subclinical bovine mastitis diagnosis and protective efficacy in vitro. *Toxicon*, 119, 99–105. [\[Crossref\]](#) [\[Google Scholar\]](#)
- Phophi, L., Petzer, I.-M., & Qekwana, D. N. (2019). Antimicrobial resistance patterns and biofilm formation of coagulase-negative *Staphylococcus* species isolated from subclinical mastitis cow milk samples submitted to the Onderstepoort Milk Laboratory. *BMC Veterinary Research*, 15(1), 420. [\[Crossref\]](#) [\[Google Scholar\]](#)
- Pilegi Sfociotte, R. A. (2019). Formation of Biofilm by *Staphylococcus* Spp. in Bovine Mastitis *Staphylococci* Biofilm in Bovine Mastitis. *Journal of Dairy & Veterinary Sciences*, 10(2). [\[Crossref\]](#) [\[Google Scholar\]](#)

- Putz, E. J., Palmer, M. V., Ma, H., Casas, E., Reinhardt, T. A., & Lippolis, J. D. (2020). Case report: characterization of a persistent, treatment-resistant, novel *Staphylococcus aureus* infection causing chronic mastitis in a Holstein dairy cow. *BMC Veterinary Research*, 16(1), 336. [\[Crossref\]](#) [\[Google Scholar\]](#)
- Qiu, M., Feng, L., Zhao, C., Gao, S., Bao, L., Zhao, Y., Fu, Y., & Hu, X. (2022). Commensal *Bacillus subtilis* from cow milk inhibits *Staphylococcus aureus* biofilm formation and mastitis in mice. *FEMS Microbiology Ecology*, 98(7), 1–12. [\[Crossref\]](#) [\[Google Scholar\]](#)
- Qiu, M., Zhao, C., Feng, L., Gao, S., Hu, X., Fu, Y., & Zhang, N. (2021). Commensal *Bacillus* From Cow Milk Inhibits *Staphylococcus Aureus* Biofilm Formation and Mastitis in Mice. *Research Square*, 1–25. [\[Crossref\]](#) [\[Google Scholar\]](#)
- Quintas, H., Margatho, G., Rodriguez-Estevéz, V., Jimenez-Granado, R., & Simoes, J. (2017). Understanding Mastitis in Goats (II): Microbiological Diagnosis and Somatic Cells Count. *Sustainable Goat Production in Adverse Environments: Volume I*, 335–358. [\[Crossref\]](#) [\[Google Scholar\]](#)
- Saad, W., Abd El-Tawab, A., & Nabih, A. (2019). Bacteriological and Molecular diagnosis of Most Common Bacteria Causing subclinical Mastitis in Cow. *Benha Veterinary Medical Journal*, 37(2), 28–32. [\[Crossref\]](#) [\[Google Scholar\]](#)
- Sabino, Y. N. V., Cotter, P. D., & Mantovani, H. C. (2023). Antivirulence compounds against *Staphylococcus aureus* associated with bovine mastitis: A new therapeutic option? *Microbiological Research*, 271, 127345. [\[Crossref\]](#) [\[Google Scholar\]](#)
- Salaberry, S. R. S., Saldenber, A. B. S., Zuniga, E., Gonsales, F. F., Melville, P. A., & Benites, N. R. (2016). Microbiological analysis and sensitivity profile of *Staphylococcus* spp. in sub-clinical mastitis of dairy goats. *Arquivo Brasileiro de Medicina Veterinária e Zootecnia*, 68(2), 336–344. [\[Crossref\]](#) [\[Google Scholar\]](#)
- Tabis, A., Gonet, M., Schubert, J., Miazek, A., Nowak, M., Tomaszek, A., & Bania, J. (2022). Analysis of enterotoxigenic effect of *Staphylococcus aureus* and *Staphylococcus epidermidis* enterotoxins C and L on mice. *Microbiological Research*, 258, 126979. [\[Crossref\]](#) [\[Google Scholar\]](#)
- Vieira, T. B., Almeida, R., Jesus, I. B., Freitas, F., Kemper, R. T., Rizek, C. F., & Castro, B. G. (2020). Microbiological and molecular aspects of *Staphylococcus aureus* isolated from bovine mastitis in West-Central Brazil. *Scientific Electronic Archives*, 14(1), 87. [\[Crossref\]](#) [\[Google Scholar\]](#)
- Yue, T., Xie, S., Meng, K., Liu, S., Pan, Y., Qu, W., Liu, Z., & Chen, D. (2021). Dosage Regimen Formulation and Therapeutic Effect Evaluation of Cyadox Nanosuspension Against Dairy Cow Mastitis Caused by *Staphylococcus aureus*. *Current Drug Delivery*, 18(7), 965–974. [\[Crossref\]](#) [\[Google Scholar\]](#)
- Zaatout, N. (2022). An overview on mastitis-associated *Escherichia coli*: Pathogenicity, host immunity and the use of alternative therapies. *Microbiological Research*, 256, 126960. [\[Crossref\]](#) [\[Google Scholar\]](#)
- Zazharskyi, V., Parchenko, M., Fotina, T., Davydenko, P., Kulishenko, O., Zazharskaya, N., & Borovik, I. (2019). Synthesis, structure, physicochemical properties and antibacterial activity of 1,2,4-triazoles-3-thiols and furan derivatives. *Voprosy Khimii i Khimicheskoi Tekhnologii*, 6, 74–82. [\[Crossref\]](#) [\[Google Scholar\]](#)
- Zazharskyi, V., Parchenko, M., Parchenko, V., Davydenko, P., Kulishenko, O., & Zazharska, N. (2020). Physicochemical properties of new S-derivatives of 5-(5-bromofuran-2-yl)-4-methyl-1, 2, 4-triazol-3-thiols. *Voprosy Khimii i Khimicheskoi Tekhnologii*, 6, 50–58. [\[Abstract\]](#) [\[Google Scholar\]](#)
- Zazharskyi, V., Bigdan, O. A., Parchenko, V. V., Parchenko, M. V., Fotina, T., Davydenko, P., Kulishenko, O., Zazharskaya, N., & Borovik, I. (2021). Antimicrobial Activity of Some Furans Containing 1,2,4-Triazoles. *Archives of Pharmacy Practice*. 12(2), 60–65. [\[Crossref\]](#) [\[Google Scholar\]](#)
- Zhang, X., Hu, X., & Rao, X. (2017). Apoptosis induced by *Staphylococcus aureus* toxins. *Microbiological Research*, 205, 19–24. [\[Crossref\]](#) [\[Google Scholar\]](#)
- Zhou, K., Wang, X., Chen, D., Yuan, Y., Wang, S., Li, C., Yan, Y., Liu, Q., Shao, L., Huang, L., Yuan, Z., & Xie, S. (2019). Enhanced treatment effects of tilmicosin against *Staphylococcus aureus* cow mastitis by self-assembly sodium alginate-chitosan nanogel. *Pharmaceutics*, 11(10), 524. [\[Crossref\]](#) [\[Google Scholar\]](#)
- Zhou, X., Yang, C., Li, Y., Liu, X., & Wang, Y. (2015). Potential of berberine to enhance antimicrobial activity of commonly used antibiotics for dairy cow mastitis caused by multiple drug-resistant *Staphylococcus epidermidis* infection. *Genetics and Molecular Research*, 14(3), 9683–9692. [\[Crossref\]](#) [\[Google Scholar\]](#)