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Clinical and Pathoanatomical Aspects of Combined Chlamydial-Mycoplasma-Klebsiella Infection of Lambs

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Abstract

An autopsy of lambs that died during the first two days after birth was carried out. Typical clinical disease signs were refusal to milk, lethargy, rounded formations in the middle third part of the neck measuring 11×7×8 (cm), and death. An autopsy revealed an acute expansion and paralysis of the heart, as a result of which congested phenomena in the organs in the pulmonary circulation (pulmonary and cerebral edema) were established; hemorrhagic inflammation of the kidneys, lymphoidulitis of deep cervical and mesenteric lymph nodes, acute hemorrhagic enterocolitis. The death of the lambs was caused by heart paralysis. In the process of the autopsy, samples of pathological material (lymph nodes, heart, lungs, liver, kidneys, spleen, and brain) were taken aseptically. The samples were subjected to PCR-diagnostics and microbiological studies at the Research Laboratory of Biochemical, Molecular Genetic Research, and Breeding of Farm Animals of the Kuzbass State Agricultural Academy; they were studied for the presence of infectious agents that can be transmitted transplacentally. As a result of studies of the internal organs of lambs, the presence of the following microorganisms was established: *K. pneumoniae, Chlamydiaceae spp., Mycoplasma spp.* The ability of *K.pneumoniae* to grow under anaerobic conditions, with the production of hemolysin, was studied as well, which indicates the ability to penetrate and develop in eukaryotic cells, incl. in intrauterine conditions.

Keywords: Intrauterine infections, Mixed infections, *K. pneumoniae, Chlamydiaceae spp., Mycoplasma spp*

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Introduction

Intrauterine infections remain an important problem in obstetrics and gynecology because they affect the course of pregnancy and its outcomes, cause abortion, the birth of weak and unviable young animals, lead to intrapartum fetal death, and affect the rates of neonatal and postnatal morbidity and mortality (Aboud *et al*., 2019; AL-Shakhshir *et al*., 2019; Ostrovskaya, 2021).

Infections occurring in the pregnant body can lead to fetal systemic inflammatory response syndrome, which entails an increased risk of periventricular leukomalacia, cerebral palsy (Ayubi *et al*., 2021), and chronic lung disease (Thébaud *et al*., 2019).

Colonization of the mother's body and transmission of the pathogen to the fetus plays a key role in the pathogenesis of intrauterine infection. With the development of intrauterine infections, bacteria reach the amniotic fluid by ascending routes. Further aspiration of contaminated amniotic fluid occurs through the upper respiratory tract and pharynx, which leads to the development of intrauterine infection (Markevich & Apatenko, 1995; Russell *et al*., 2017). Further, the penetration of pathogens occurs through intact cell membranes, which leads to severe cases of intrauterine infections and/or abortions (Zaki *et al*., 2020). Abortion is a serious problem for pregnant sheep and results in large financial losses for small-scale livestock producers. This is a production limiting factor as it reduces the potential for restocking and milk production and increases the number of unproductive females kept in the herd for a long time. Thus, abortion in sheep significantly affects the food security and living standards of rural small farmers, sheep are an integral part of households, providing food, employment, and sources of income (Ali *et al*., 2019). Abortions of an infectious nature often occur with the development of intrauterine infection (Alemayehu, 2021).

The intrauterine infection has two different clinical aspects: a disease with early and late development of the clinical picture and manifests itself in the form of an enzootic outbreak. And also can proceed in the form of intestinal (enteric) and septic forms. Depending on the form of the infectious process, the localization of the pathogen (gastrointestinal tract, blood, and internal organs), clinical signs, and pathological picture are determined (Thadchanamoorthy & Dayasiri, 2020).

Diseases with early appearance account for 80% of all the cases of intrauterine infections and consist in the development during the first week of life (in most cases within the first 24 hours) of bright clinical signs, with a predominance of symptoms of septic phenomena and pneumonia (Puopolo *et al*., 2019). Colonization of the mucous membrane of the lungs of the newborn is quickly replaced by the development of pneumonia. Violation of the barrier of the mucous membrane of the lungs leads to the penetration of the pathogen through the vessels and to the development of severe sepsis.

The late-appearing disease usually occurs between 1 week and 3 months postpartum and presents with meningitis as the predominant clinical syndrome. In this case, the entry of bacteria into the bloodstream is accompanied by invasion of the cerebrospinal fluid into the space, initiating an inflammatory reaction, which leads to the clinical picture of meningitis (Spellerberg, 2000).

Several scientists argue that pathogens of intrauterine infections can penetrate through active endocytosis into eukaryotic cells of various tissues and have the ability to survive inside these cells (Chiarot *et al*., 2018; De Gaetano *et al*., 2021), which is an important mechanism for penetration into various organs and tissues of the host. Inside the cells, bacteria are found in vacuoles. It has been established that the ability to penetrate eukaryotic cells correlates with the ability to induce an infectious process. At the same time, isolates isolated from the vagina or clinically healthy young animals are not capable of causing an infectious process (Armistead *et al*., 2020).

Various bacterial, viral, and, less commonly, fungal agents are associated with the main pathogenesis of intrauterine infections. Some of these frequently identified pathogens include *Chlamydia spp., Mycoplasma spp.,* Group B streptococci (Romero *et al*., 2007; Cappelletti *et al*., 2020).

The etiological factor of intrauterine infections is often methicillin-resistant Staphylococcus aureus, which causes inflammation of the membranes and vancomycin-resistant enterococcus (Romero *et al*., 2019; Siemens *et al*., 2020; Thadchanamoorthy & Dayasiri, 2020).

An etiological agent of intrauterine infections can be performed by from two to three nosological units to much larger species composition of pathogenic microorganisms act. Associated infection, as a rule, is acute in young animals of different types of farm animals. Etiological agents of mixed infections can be microorganisms of the same family or larger taxa and kingdoms and are represented by associations of pathogenic and opportunistic microorganisms (Belaya & Belaya, 1986).

It is often when representatives of microorganisms that cause associated infections include the following genera: *Escherichia, Citrobacter, Enterobacter, Proteus, Morganella, Klebsiella, Salmonella, Shigella, Yersinia, Edwardsiella, Serratia, Erwinia, Hafnia, Providencia, Pantoea, Pseudcostomoncus, Stapptohydella, Coxiella burnetii (C. burnetii), Chlamydia abortus (C.abortus), Brucella spp., Leptospira spp., Campylobacter fetus, Listeria spp.* and *Toxoplasma gondii (T.gondii) et al*. (Ning *et al*., 2017). Some authors describe combinations of pneumococcal infection with hemophilic, chlamydial, mycoplasma, legionella, or viral infections (Bachinskaya, 2000; Chuchalin *et al*., 2010; Shoar & Musher, 2020; Su *et al*., 2021).

Mycoplasmas have low virulence, which explains the absence of vivid clinical symptoms (Steel *et al*., 2005; Romero *et al*., 2006).

Various types of mycoplasmas are found in various pathological processes in small ruminants. In most cases, mycoplasmas affect the respiratory system, reproductive tract, cause severe septicemia, affecting internal organs. Mycoplasmas are capable of causing an infectious process in mammals exclusively in association with other microorganisms (Tolone *et al*., 2019). Several mycoplasma species have an immunosuppressive effect, which manifests itself in the selective suppression of cellular immunity. There is an assumption that the immunosuppression that develops in mycoplasmosis activates chlamydial infection. In association, these microorganisms act synergistically, promoting each other's reproduction (Prozorovskiy *et al*., 1995).

Chlamydia is one of the leading causes of abortion in sheep and goats worldwide and is usually transmitted by ingestion or inhalation of dust, food, and/or water contaminated with aborted uterine fluid, placenta, and fetus. Enzootic abortion in sheep and goats occurs during the last 2-3 weeks of pregnancy. In addition, chlamydia infection causes stillbirth, premature lambing, and the birth of weak calves, which usually die immediately (Esmaeili *et al*., 2021; Fayez *et al*., 2021).

When combined infections appear, there is a decrease in the natural resistance of the organism under the influence of one pathogen, and active reproduction and long-term persistence of another, which is the reason for the severe course of the infectious process (Ning *et al*., 2017).

In the process of diagnosing diseases, the revealed associations of microorganisms can complicate the course of infectious diseases up to a lethal outcome, while individually these microorganisms are not so dangerous and quite easily curable (association of streptococci with salmonella). The diagnosis of co-infections is associated with the difficulties of a combined approach to the methods used (Espinosa *et al*., 2020). The sources of many authors reflect information on the most diverse combinations of infectious agents. Some authors resort only to bacteriological methods, others - to bacteriological and virological, the third group of researchers - to bacteriological and immunological, the fourth (rare) uses a combination of bacteriological, virological, and immunological research methods (Bachinskaya, 2000; Chuchalin *et al*., 2010; Shoar & Musher, 2020; Su *et al*., 2021).

Clinical signs of combined infections do not have specific symptoms and are diverse in their manifestation since they are not characterized by the sum of symptoms specific to each of their constituent mono-infections. The main clinical signs of the disease are loss of appetite, diarrhea turning into profuse, increasing

weakness, depression, rapid breathing and heartbeat, dehydration; often there is damage to the central nervous system (agitation, convulsions), sometimes pneumonia, arthritis; body temperature within normal limits, in some cases increased by 0.5-1°C, in the pre-agonal state it decreases below the norm.

Pathological changes in dead animals often have a picture of catarrhal or catarrhal-hemorrhagic gastroenteritis in the small intestine and cecum, with the formation of ulcers; often there are multiple punctate, banded, and spotted hemorrhages on the mucous membrane of the stomach, large and small intestines, under the spleen capsule, epi- and endocardium (valves); sometimes there is focal catarrhal pneumonia and pulmonary edema; liver dystrophy; regional mesenteric lymph nodes, as a rule, are enlarged, edematous, pink or cherry red on the cut; on opening the cranium - hyperemia of the blood vessels and edema of the brain tissue (Aleshkevich *et al*., 2017). The biological properties of pathogens, the state of the host's immunity, and the influence of the environment are of decisive importance in the emergence and development of mixed infections (Belaya & Belaya, 1986).

Multiple factors contribute to the development of mixed infections on farms:

1. Failure to comply with the technological, veterinary, and sanitary requirements of herd reproduction;
2. Violation of the regimes of keeping and feeding young animals;
3. Large overcrowding of livestock;
4. A large number of bystanders on the farm;
5. Open breeding system;
6. Introduction to the population of new animals (Santos *et al*., 2012; Hireche *et al*., 2016; Sun *et al*., 2020; Lima *et al*., 2021; Selim *et al*., 2021).

Materials and Methods

Epizootic data: in the last two months, 5 cases of stillborn lambs and 3 non-viable lambs that died during the first two days, of the Dorper + Romanov breed, were registered in the sheep breeding farm. All lambs were born from the same breeder. Lambs were conceived naturally.

The study included pathological material from 3 lambs born alive but died within the first 2 days after birth. The postmortem examination was performed in the autopsy room in daylight; an autopsy was carried out in the dorsal position, organs were removed by the method of complete evisceration. To take the material and establish the pathomorphological picture, an autopsy was performed using the method of isolated organ extraction. Samples of the brain, lungs, heart, lymph node, liver, spleen, and kidneys were taken for examination with sterile instruments. After sampling, the samples were placed in sterile containers for transportation to the Research Laboratory of Biochemical, Molecular Genetic Research and Breeding of Agricultural Animals of the FSBEI HE Kuzbass State Agricultural Academy, in which bacteriological studies were carried out, as well as studies for the detection of DNA of microorganisms of the genus *Mycoplasma* and *Chlamydiaceae*, using commercial kits (FBIS Central Research Institute of Epidemiology of Rospotrebnadzor).

Isolation of nucleic acids was carried out with a set of reagents for DNA extraction from biological material with the reagent "DNA-sorb-B", following the instructions for use. The total volume of the reaction mixture was 25 μl, including 10 μl of the isolated DNA sample.

Polymerase chain reaction (PCR) was carried out on a Tertsik amplifier (manufactured by *NPO DNA-Tekhnologiya*, Russia) at a temperature-time regime **(Table 1)**:

Table 1. Polymerase chain reaction (PCR)

Chlamydiaceae spp.	*Mycoplasma spp.*
1. Initial denaturation 95 ^{0}C – 5 min;	1. Initial denaturation 95 ^{0}C – 5 min;
2. 42 cycles (denaturation 95 ^{0}C – 10 sec., annealing 60^{0}C – 10 sec., elongation 72 ^{0}C – 10 sec.)	2. 41 cycles (denaturation 95 ^{0}C – 10 sec., annealing 61^{0}C – 10 sec., elongation 72 ^{0}C – 10 sec.)
3. 1 cycle – 72 ^{0}C – 60 sec.	3. 1 cycle – 72 ^{0}C – 60 sec.

The results of PCR-diagnostics were interpreted based on the presence or absence of a specific amplified DNA band on the electrophoretogram.

The bacteriological study was carried out by inoculating pieces of pathological material into peptone water, followed by cultivation at 37 °C for 24-72 hours. Then they were sub-cultured onto the sectors of the following plate media: meat-peptone agar with the addition of 5% sheep erythrocytes, Endo, Ploskirev, yolk-milk-salt agar, Enterokokkagar. Crops are grown at 37 °C for 24-72 hours. For the cultivation of anaerobic microorganisms, the inoculations were incubated at 37 °C for 18-24 hours in an anaerostat with CO_2 gas-generating bags.

From the anamnestic data, it is known that after birth, 3 lambs did not show activity, they did not have a sucking reflex. There was no treatment. The death occurred within 2-3 days after birth.

There was found the presence of the *Mycoplasma spp*. and *Chlamydiaceae spp.* DNA in 3 lambs. The length of specific amplified DNA fragments were: microorganisms of the genus Mycoplasma spp. - 509 bp, microorganisms of the family Chlamydiaceae spp. - 300 bp **(Figures 1 and 2)**.

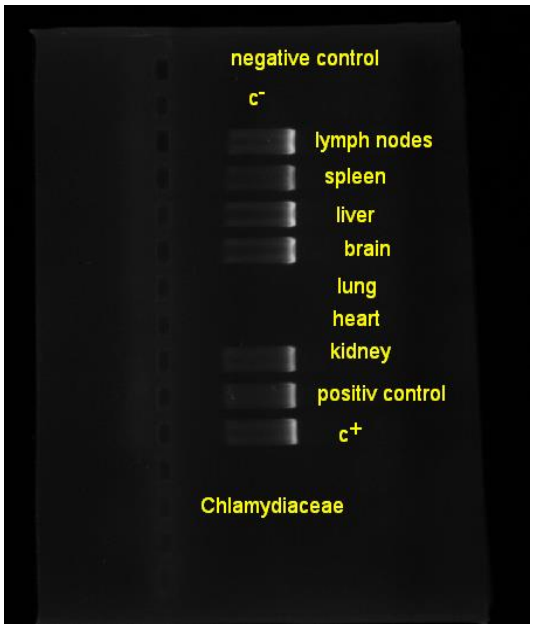

Figure 1. Picture of PCR-products of microorganisms of the family *Chlamydiaceae spp.* in an electrophoretic chamber

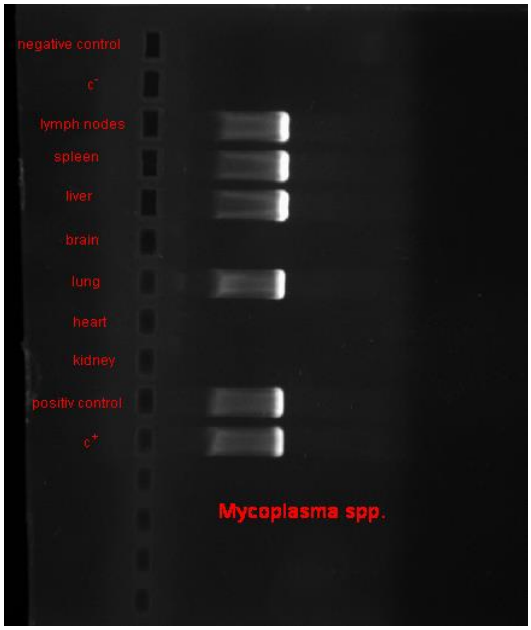

Figure 2. Picture of PCR-products of microorganisms of the family *Mycoplasma spp*. in an electrophoretic chamber

The bacteriological method established in 3 lambs the presence of a pure culture of *K.pneumoniae*, showing hemolytic activity: in the lymph node, spleen, liver, brain, lungs, heart, and kidneys.

In these 3 lambs from all internal organs, during incubation of crops under anaerostat conditions, the presence of growth of a culture of pure *K.pneumoniae* with hemolytic properties on 5% blood agar was established.

When analyzing the sensitivity of the isolated culture to antibiotics, resistance to: tetracycline, doxycycline, gentamicin was established; and sensitivity to: norfloxacin, levofloxacin, ciprofloxacin, ampicillin was established as well.

As a result of the autopsy of the dead lambs, the following pathological picture was recorded:

Deep cervical lymph nodes (anterior, middle, and posterior) are not delimited, combined into volumetric formations (on the right - 3×2.5 cm, on the left - 5×3 cm), dark cherry color, with an uneven surface, dense consistency, on the cut - dark in color, strongly filled with blood. The blood vessels feeding these formations are enlarged and filled with blood. The rest of the lymph nodes were unchanged.

The abdominal organs are located anatomically correctly; there is no free fluid in the abdominal cavity. The peritoneum is red, moist, without damage. The omentum is yellow-pink, contains a small amount of fat. The omentum vessels are poorly filled.

There is no free fluid in the chest cavity. The pleura is thin, transparent, without overlaps, colored red.

Lungs are not sleeping, bright pink. The tissue is light doughy consistency, the cut surface is moist, juicy, shiny. The dark red pieces of the lung float hard in the water. Pieces are of light bright pink color, they float on the surface.

The integrity of the bursa is not compromised; the pericardial cavity contains not a large amount of fluid. The blood vessels of the pericardium are overflowing with blood. The heart sac is white, not transparent.

The heart is cone-shaped, greatly enlarged. Coronary vessels are filled with non-clotted blood. The atria contain blood clots, and the right and left ventricles are filled with blood clots. The ratio of the thickness of the right and left ventricles is 1:2. The epicardium is smooth, shiny, cherry red; the bicuspid valve is not thickened, without hemorrhages. The heart muscle is elastic, dark cherry color.

Large and medium-sized blood vessels contain a significant amount of non-clotted blood and blood clots. The inner surface of large blood vessels is smooth, shiny, and ivory.

The spleen is of normal size, gray-red, and fine-grained. Scraping from the incision surface is scanty, semi-liquid, dark red.

The liver is enlarged; the liver tissue is elastic, red-brown from the surface, and on the cut, full-blooded. A significant amount of non-coagulated blood is released from the blood vessels.

The kidneys are slightly increased in size, jelly-like consistency, dark cherry color; the capsule can be easily removed. The surface of both buds is smooth and shiny. When cut, there is no clear distinction between cortical and medulla, all of the same consistency and color. A significant amount of blood drains from the incision surface.

Rumen: empty, gray-pink mucous membrane, has a pronounced papillary structure. Grid: empty. From the side of the mucous membrane, it has a cellular structure, light pink. Book: contains a small amount of mucus, the mucous membrane is smooth, shiny, has a lamellar structure. Abomasum: Contains a small amount of thick, semi-frothy mucus. The mucous membrane is pink.

Duodenum: the mucous membrane is smooth, moist, shiny, bright pink, not swollen; there are roll-like folds. Contents - a small amount of gray-pink mucus. Jejunum: The mucous membrane is bright pink, almost red; slightly swollen. It contains a small amount of liquid mucus. Ileum: the mucous membrane is smooth, shiny, moist, reddened, in places gray-pink, not much swollen. It contains a small amount of green semi-liquid mucus. The mesenteric vessels are filled with non-coagulated blood. The mesenteric lymph nodes are slightly enlarged, elastic, dark red, moist on the cut. The cecum: the mucous membrane is not much reddened, smooth, shiny, not swollen. It contains a small amount of green slime. Colon: the mucous membrane is bright pink, smooth, shiny, reddened in places. It contains a small amount of greenish mucus. The rectum is empty.

Brain: the medulla is elastic, bright pink; there is not a lot of free fluid. The fundus of the cerebral ventricles is white, smooth, shiny, and moderately moist. The choroid plexus is heavily filled with blood.

Pathological diagnosis: anemia, hemorrhagic lymphadenitis, acute congestive hyperemia of the lungs, acute expansion and paralysis of the heart, acute congestive hyperemia of the liver, hemorrhagic inflammation of the kidneys, mesenteric lymph node lymphoidulitis, acute hemorrhagic enterocolitis, cerebral edema, meningitis. The death of the lamb was caused by heart paralysis.

Results and Discussion

Because of the studies, a hypothesis arises about the pathogenesis of mixed mycoplasma-chlamydial-klebsiella infection, based on laboratory tests and pathological anatomical pictures.

Considering the clinical state of animals after birth and early death, there is an assumption about the transplacental transmission of the infectious agent. Laboratory tests support the intrauterine infection hypothesis.

Microorganisms of the genus *Mycoplasma spp.* have an immunosuppressive effect, which activates chlamydial and Klebsiella infections, and in combination with microorganisms of the family *Chlamydiaceae spp.* and *K.pneumoniae* caused a vivid clinical picture and rapid death of young animals.

The acute course of infection and the development of inflammatory processes in the lymph nodes, kidneys, and gastrointestinal tract in the perinatal period are facilitated by the enzymatic properties of *K.pneumoniae*, which has acquired the ability to ferment hemolysin under anaerobic conditions. Against the background of sepsis and systemic intoxication of the body, which led to an acute expansion of the heart and congestion in the lungs and brain, this pathological picture was formed.

In addition to the pathogenic properties characterized by the production of hemolysin, *K.pneumoniae* acquired resistance to 40% of the antibiotics used in this study, as well as in the treatment of diseases caused by Gram-negative rods of the family *Enterobacteriaceae*.

The presence of resistance to antibiotics, together with hemolytic properties and the ability to grow and multiply under anaerobic conditions, indicates a long persistence of the pathogen in the herd and its adaptation to unfavorable environmental conditions (antibiotics, disinfectants), which served as selective factors for the selection and reproduction of the most pathogenic strains of microorganisms.

Conclusion

Combined chlamydial-mycoplasma-klebsiella infection in lambs is characterized by lethargy, lack of sucking reflex, and early death of young animals, within 2 days after birth. A striking pathological sign is inflammation of the deep cervical lymph nodes, aseptic condition, and acute enlargement and paralysis of the heart. Heart paralysis provided congestion in the pulmonary circulation, which caused edema of the brain and lungs, and the septic condition - congestive hyperemia of the liver and acute hemorrhagic inflammation of the kidneys.

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