STUDIES ON MASTITES IN SHEEP, CAUSED BY COXIELLA BURNETII

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ABSTRACT
Five enzootics of Coxiella burnetii – induced mass mastitis in sheep were discovered in Bulgaria. Employed were the methods of isolation and cultivation of Coxiella in chicken embryos (CE), cell cultures (CC), guinea pigs, mice, light and electron microscopy, direct immunofluorescence, complement fixation test and clinical studies. Five strains of C.burnetii were isolate from milk secretions. The strains were identified morphologically and serologically. The patogenetic properties of the isolates for CE, CC, guinea pigs, mice and rabbits were investigated. Serologically, 87 (56, 86%) of the examined 153 diseased sheep from five settlements in three districts were positive for coxiellosis with titres $\geq 1:128$ (41, 16%), $\geq 1:32$ (72, 74%) and a mean geometrical titre of 49. The observed C. burnetii mastites had as characteristic of mass outbrakts of clinically defined illness that showed the tendency of progressive symptoms decline, severe inflammation of the milk gland, disappearance of the milk secretion, and general intoxication leading to death of some animals.

Keywords: Coxiella burnetii, Q-fever, mastitis, sheep

Introduction
The Q-fever is an important ricketsial zoonosis of overall distribution, a large number of carriers and of diverse clinical manifestations in animals and in humans. The highly infectious causative agent Coxiella burnetii has a unique way of development, incompletely deciphered biology and pathogenesis and an established potential of an agent of bioterrorism threat. In animals, as differentiated from humans, the clinical symptoms of the coxiellosis are not studied sufficiently (1, 2, 3, 4, 5, 10, 13, 15, 16, 21, 22).

The udder of the domestic cattle is a frequent location for the reproduction of the C. burnetti, and the shedding of the pathogen with the milk is a well-known fact, demanding a focus from epizootiological and epidemiological point of view (1, 12, 18, 19, 24). Byrne (1) showed that the shedding of coxiella with the milk secretion of lactating cows can become chronic and remain for months and years. However, the clinical demonstration of the inflammation of the udder remains in the shadow of the epidemiological consequences of the dissemination of the agent in this way. Moreover, its shedding can be performed even when there is a lack of clinically manifested mastitis. In other cases, like in goats (8), the mastitis is seen as a part of the general clinical syndrome of the Q-rickettiosis, including degradation of the general state of the animals, fever, conjunctivitis, rhinitis, decreased or lack of appetite and very late miscarriage, the latter being a major symptom. Especially, in view of the fact that it is not clear what is the possible participation of the coxiellas in the etiology of the mastites in sheep.

We focused on the clinically manifested mastites in sheep as enzootics of defined, self developing diseases that affect a large number of animals.

The aim of the present work is to carry out virological, serological and clinical investigations of the coxiella infection in flocks of sheep affected by mass mastitis.

Materials and Methods
Sheep. Investigations were carried out on six sheep-breeding farms in three regions of the country, where several cases of mastites were established.

Materials. Milk secretions from clinically manifested mastites were used in the experiments for the isolations and the direct detection of the agent. The milk samples were obtained in a sterile way and had the volume of 3-10 ml. They were treated with antibiotics and were inoculated in the corresponding biological systems - CE, CC and laboratory animals. From the samples were obtained preparations which were observed directly through light, fluorescent and electron microscopy.

Chicken embryos. We infected 6-7 days old CE in the yolk sacs. The inoculations were carried out in sterile conditions, through the opening in the center of an air chamber. Each inoculation was microbiologically checked in ordinary broth and ordinary agar. The embryos were incubated at 37°C with the ovoscopy being carried out on a daily basis during the period of 15 days.

Cell cultures. We used the cell lines HEla and BHK-21 and a primary cell culture of chicken embryonic fibroblasts. The inoculations (milk escudates) were pre-treated by differential centrifugation (1500g / 15 min; 20000g / 90 min).

Guinea pigs. The experimental animals had a weight of 350-400g and they were initially investigated serologically to exclude spontaneous infection with C.burnetii. We performed intraperitoneal inoculations (2ml) with milk suspensions of yolk sacs of CE of the isolated strains.

White mice. We inoculated young white mice with weight of 7-8 g under ether anaesthetics performed in a nasal or
intracerebral way. The inoculums (5-10 % suspensions), were prepared from infected with strains yolk sacs. The applied dosages were 0.5-1ml (i.p.), 0.5 ml (nasal) and 0.1-0.5 ml (cerebral). We used native and concentrated suspensions. Recultivation of the coxiellas in the CE was achieved by means of infected material of the spleens and separately from the lungs of the experimental mice.

**Rabbits.** Here we applied intravenous infection of male rabbits with two strains that were isolated in yolk sacs of changed milk secretions of sheep at a dosage of 3 ml. In view of the inhibition of possible latent bacterial infections, we had treated the rabbits prior to the experiments with tetracycline perorally for the duration of three days. The inoculations were carried out one week later.

**Methodology for the indication and identification of C. burnetii.** In order to detect the coxiellas by light microscope we prepared smears of yolk sacs of CE, cell monolayer, pathologically changed parts of the parenchymatous organs of the laboratory animals and milk secretions. The preparations were stained by the classical methodology of Stamp, Macchiavello and Zdrodovskii, Golinevich. The displayed marked gammaglobuline and by electron microscopy (EM) – negative staining and ultrathin sections (7, 20).

**Sero-logy.** The serological investigations of sheep affected by mastitis were carried out by the direct method of complement fixation test with antigens of C. burnetii in 1st and 2nd phase. The total number of the animals that were investigated was 153. The obtained results were processed statistically.

**Clinical and epizootiological observations.** We registered mastitis in the affected flocks of sheep and the characteristic clinical display of the diseases and the data from the etiological investigations for coxiellosis. The laboratory animals infected by milk suspensions or passage material were observed clinically in the corresponding time frames.

**Results and Discussion**

**Serological Investigations**

The results from the serological investigations of sheep with mastitis are shown in Table 1. It can be seen that the general sero-positivity for C. burnetii in the examined 153 animals is 56.86%.

The first investigations for coxiellosis in sheep that showed mass inflammations of the udder were carried out in North-Eastern Bulgaria. We excluded microbiologically infectious agalaxis and infection with pathogenic bacteria prior to the investigations. We examined four flocks of sheep on a farm in Tutrakan and there we obtained various number of blood samples, a total number of 66. We established antibodies against C. burnetii (antigen in first-phase) in 30 sheep with heavy clinical form of mastitis. The distribution of the positive seroreagents was as follows: flock I – 2 (40%), flock II - 7 (38.88 %), flock III – 13 (52 %) and flock IV – 8 (44.44 %).

<table>
<thead>
<tr>
<th>Settlement</th>
<th>Tested samples (No)</th>
<th>Seropositive (No / %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tutrakan (Ss)</td>
<td>66</td>
<td>30 / 45.45 %</td>
</tr>
<tr>
<td>Tarnovtsi (Ss)</td>
<td>15</td>
<td>15 / 100 %</td>
</tr>
<tr>
<td>Dragalevtsi (Sofia - city)</td>
<td>17</td>
<td>11 / 64.7 %</td>
</tr>
<tr>
<td>Samokov (Sf)</td>
<td>20</td>
<td>15 / 75 %</td>
</tr>
<tr>
<td>Gutzal (Sf)</td>
<td>17</td>
<td>12 / 70.5 %</td>
</tr>
<tr>
<td>Ivaniiane ( Sofia city)</td>
<td>18</td>
<td>5 / 27.77 %</td>
</tr>
<tr>
<td>Total</td>
<td>153</td>
<td>87 / 56.86 %</td>
</tr>
</tbody>
</table>

The titres varied from 1:8 to 1:512. The higher levels were dominant (1:128 – 1:512) comprising 46.66% of the positive reactions. The average titre values (1:32 – 1:64) were 26.66%. The same share – 26.66% had also the low titres (1:8 – 1:16). This first serological data of coxiellosis in acute mastitis were solidly proven by isolations of C. burnetii from the heavily changed milk secretions of the sheep and by direct indication and identification of the agent by means of electron microscopy.

On another farm (Tarnovtsi) in the same region, we discovered Q-rickettsial mastites again by the method of complex etiological diagnostics-serology, isolation of the agent and direct EM. The serological investigations were carried out twice during the period of 1 month. During the first investigation out of 15 animals tested, 14 (93.33%) were seropositive with a level of the individual titres and ratios of the average and low values, similar to the ones described above. The high titres did not exceed 1:128 and comprised 20% of the positive reactions. The repeated investigation after a period of 30 days established significant serological response in all 15 sheep. Specifically, we established 4 times increase of the titre in 4 animals, including the single seronegative sheep after examination of the first serum probe.

In a third investigation of mass acute mastitis in sheep (Dragalevtsi), the serological investigations were performed with antigens of C. burnetii in I and II phase. From a total of 17 serum samples, 11 (64.70%) reacted seropositively by first-phase anigen of not very high titre 1:32 – 2 samples, 1:16 – 5 samples, 1:8 – 4 samples, and 10 (58.82%) and – with second phase antigen: 1:256 – 1 samples., 1:128 – 2 samples., 1:64 – 2 samples., 1:32 – 4 samples., 1:8 – 1 sample.

These results show the participation of Coxiella burnetii as etiological agent of the mastitis, which was also proven by the isolation of coxiellas from the milk secretion.

Very convincing was the serological data of the coxiella-induced mastitis obtained from investigations on the two other farms, Samokov and Gutzal. There the seropositive sheep were 75% and 70.5% (see Table 1). We discovered coxiellas in the milk escudates (LM, direct isolation in CE and CC). Although, the reagents had a lower scale of action, the seropositive results...
of the last enzootics in Ivaniane, Sofia (2006/2007) were also significant.

The summarized data from the statistic processing of the seropositive results in three of the described enzootic mastitis is shown in Fig. 1. The distribution of the individual titres shows that the levels $> 1:128$ are 41.16% from the positive reactions, $> 1:32$ are 72.73%, and the mean geometric titre is 49%.

**Isolation and cultivation of Coxiella burnetii**

**Chicken embryos**

We isolated five strains from the animals with serologically proven Q-rickettsiosis and with direct detection of the agent in the milk secretions. We must underline that the available literature on the topic does not announce any similar isolations from sheep with acute mastitis as characteristic or independently developed clinical form of the disease. The isolations were achieved in yolk sacs of CE after inoculation of milk secretions, severely changed from the inflammation of the udder.

The process of isolation and cultivation of the infectious agent is shown in Table 2. It can be seen that the three strains show similar model of lethality of the KE.

In parallel with the advancement of the passages, the so-called ‘non specific lethality’ of the embryos decreases in the first 3-4 days after the inoculation. We observed a gradual increase of the magnitude of the specific lethality between the 6th and 14th days with the peak of this indicator reached on the 7th-12th days. The infectious titre of the strains for KE was $10^6$-$10^7$ ID50/ml.

The obtained isolates were identified morphologically through LM, EM and direct IF and serologically, through proving the antigen of *C. burnetii*. The infection with the *Coxiella* pathogen lead to very typical pathological changes in the chicken embryos and especially, in their yolk sacs, which became a useful indicator of the Q-rickettsial infection. In diligently stained preparations of yolk sacs we observed dot-like oval and spherical coxiellae, coloured in different shades of red, or in violet, and located in the cytoplasm of the endodermal cells in the shape of inclusions or with a diffusion distribution. Fig. 2 shows the light microscopic preparation (Stamp) of yolk sacs of CE infected by milk exudate of a sheep with Q-rickettsial mastitis.

**Fig. 2.** Light microscopy. Q-rickettsial mastitis in sheep. *Coxiella burnetii* in a smear of yolk sac of chicken embryo, infected with milk exudates. Staining by Stamp. Magnification 1000x.

The electronmicroscopic indication and identification of the strains *C. burnetii* isolated in CE from milk secretions is possible already during the initial infection. In the ultrathin sections the *Coxiella* cells have oval, spherical or rod-like shape. Their sizes vary from 200 to 800 nm, and in some isolated cases up to 1500 nm. In the morphologically intact cells of the agent, one can observe a three-layered membrane and internal contents of plasma with granulated or fibre-like type and a nucleoid that is either compact or diffused (Fig. 3).

**Fig. 3.** Electron micrograph. Ultrathin section of yolk sac of chicken embryo infected with *Coxiella burnetii* – strain Tutrakan, isolated from milk secretion of a sheep with mastitis. Multitude of rickettsial cells. Magnification 12 000x.
Isolation and cultivation of *Coxiella burnetii* upon mastites in sheep

<table>
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<tr>
<th>FARMS</th>
<th>PASSAGES</th>
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<th>DEAD CE No</th>
<th>DAYS AND DAILY MORTALITY OF CE</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1-3</td>
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<td>4</td>
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<td></td>
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<td>19</td>
<td>1</td>
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<tr>
<td></td>
<td>III</td>
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<td>20</td>
<td>0</td>
</tr>
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<td>17</td>
<td>3</td>
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<tr>
<td>TOTAL</td>
<td>16</td>
<td>320</td>
<td>30</td>
<td>21</td>
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</table>

**Guinea pigs**

The intra-peritoneal inoculations of guinea pigs by milk suspensions lead to the infection of the animals that was demonstrated by high fever, degradation of the general state and 50% lethality. We discovered through pathomorphological examinations that the parenchymatous organs were affected, in the first place by significant splenomegaly. The experimentally induced coxiellosis was accompanied by the production of specific antibodies. Analogical results were obtained also during the infection by infected yolk suspensions, especially during the increase of the plurality of the infection achieved by prior concentration and purification with differential centrifugation and supercentrifugation. Therefore, the coxiellae isolated from milk of sheep with mastitis show significant pathogenicity for guinea pigs and demonstrated adaptive ability for cultivation in this way following intraperitoneal infection.

**White mice**

The experiments with young white mice included three schemes of inoculation with infectious suspensions of yolk sacs infected with the strains: i.p., nasal and intra-cerebral.

The inoculated in this way mice became infected in the majority of the cases and showed moderate fever and a slight degradation of the general condition. During autopsy (after euthanasia) between the 12th and 20th day, there was enlargement of the spleens and in preparations of them coxiellae were observed. With the spleen suspensions of the infected mice, we could successfully infect CE into the yolk sacs. We could judge that the *Coxiella* infection was indeed realized in the oligosympotmatic and in asymptomatic cases by the availability of sphenomegaly and the discovery of coxiellae in preparations of the spleens (LM, EM, IF) as well as by the establishment of specific antibodies in the serological reactions.

The nasal inoculations of strains of sheep with mastitis, isolated and cultivated in CE, were performed with two types of suspensions, native non-concentrated and concentrated and purified. The latter contained a very rich concentration of the infectious agent - 10⁸ – 10⁹. Both types of inoculums caused infection of the mice that had a different clinical course: latent inapparent or with a display of almost non-traceable signs in the native suspensions and visibly demonstrated general and respiratory symptoms for the concentrated. After autopsy between the 13th and 18th day we discovered that the agent was located primarily in the pneumatic parts of the lungs, but also in the enlarged to a various degree spleens. After intra-yolk inoculations of the suspensions of these organs, the coxiellae got recultivated in CE. The intracerebral inoculations of the mice with strains containing high concentration of the infection, caused significant degree of lethality between the 3rd and 9th days. The specific character of the lethality was confirmed by LM and EM of preparations from the cerebrum. We observed the availability of big intracytoplasmic inclusions of C. burnetii.
Pathomorphologically in the brain hyperemia and edema of the meninges were seen.

**Rabbits**

Three male rabbits were infected intravenously with 3 ml from strain Tutrakan, reproduced in yolk sacs of CE. An analogical scheme was applied to three other rabbits, injected with yolk suspensions from strain Samokov. The animals reacted by display of fever. The beginning of the fever was registered 24 hours after the inoculation and its peak on the 4th day. Specific CF-antibodies appeared on the 7th day (1:8-1:16) and they progressively increased to levels 1:256 – 1:512 between the third and fourth weeks. These results showed the rabbits, in general, less used in the laboratory practice of C. burnetii, are a suitable biological model for the investigations of Q-rickettsial infections of mastitis in sheep. We had already previously published etiological, clinical and pathomorphological data of the experimental Q-rickettsiosis in pregnant rabbits with coxiellae, isolated from miscarried sheep (6, 17). The high sensitivity of this type for infection with C. burnetii, leading frequently to stillbirths, has also been shown by Maurin and Raoult (11). The rabbits were used as an experimental animal model for the chronic Q-fever in humans (endocarditis) where the illness was simulated on animals by introducing a catheter in the left cardiac ventricle, leading to damage of the aortic heart valve and thrombotic vegetation. The follow-up inoculation of coxiellae leads to the localization of the agent in the place of the damage and to the development of Q-rickettsial endocarditis (13).

**Cell cultures**

We inoculated 48-hour old cell cultures of the type BHK-21 with strain Turnovtsi in the form of concentrated and purified yolk suspensions. On the 48th and on the 72nd hour we could discover in the cell monolayer (LM, direct IF) coxiella cells with satisfactory concentration. The majority of these were located in the cytoplasm, and some single bodies outside of the cells. Except the availability of the agent, we could establish in the infected cells some degenerative and destructive changes. Similar constatations were made during the infection with the strain of a primary cell culture of chicken embryonal fibroblasts. The experiments with another type of coxiella strain, reproduced in CE (Dragalevtsi) and applied as a highly concentrated suspension (10^7 – 10^8), showed good sensitivity towards the agent of the cell line HELA. These results showed that the cell cultivating method was useful also in the experiments of isolating and cultivating of C. burnetii from mastites in sheep, under the conditions that the methodological principle of inoculation of the initial material of high concentration and plurality of the infection is strictly followed (8).

**Direct electronic microscopic diagnostics of the Q-ricketsial mastites**

The method of negative staining was used. The investigated clinical materials – milk secretions in most cases were severely deformed as a result of the inflammation and actually, were escuates with changed consistency, colour and odor. We also investigated native milk suspensions and a priori concentrated and purified preparations. During the natural availability of coxiellae with high or satisfactory concentration, the agent was discovered without any difficulty in the initial non-treated milk secretions. When the concentration of the agent was lower, we applied the differential centrifugation and supercentrifugation methods, which secured its more successful detection in the obtained concentrated preparations. During EM observations of negatively stained suspensions, the coxielle were being discovered as ricketsial cells that has rod-like or oval shape and a size from 200-1500 nm.

**Clinical picture of the coxiella-induced mastites**

The observed by us enzootics of mass Q-ricketsial mastites started suddenly with quickly developing picture of high fever: hyperthermia (41.5 – 42°C) continuing for two days, increasing depressions, loss of appetite. The milk glands of the lactating animals showed inflammation processes demonstrated by significant temperature, redness, edema and painfullness. These phenomena affected the posture, walk and the movement of the sheep that now were cautious, stiff and spared. The milk secretion was quickly changing its normal color, quantity and odor, turning in the beginning into a serous-catarhral fluid, and later into a dirty-brown, badly smelling escudate. From the clinical start to the heavy inflammation processes, the quantity of the secretion was continuously decreasing until it stopped completely. Most often the udders can be affected bilaterally.

If quick action is taken in the initial phase of the illness, this can lead in many cases to the prevention of the heavy complications and to the slow recovery of the mammary gland, but usually the secretion of the milk would be restored during the next lactation season.

One mandatory initial condition in order to start an adequate etiotropic therapy is to state the etiological diagnosis. The application of antibiotics for the group of the tetracylines as intra-mammar infusions can be effective only in the beginning stage of the coxiella mastitis, and if the therapeutic concentration is applied for a period longer than 10-14 days. A better effect can be reached both in the early as well as the later, more advanced stages of the disease, by combined application of antibiotics as injections or internally and intra-mammarly. It is convenient to use the tetracyclines that have a prolonged active period (72h-24h) such as Tetravet, Oxytetracyclin 20%, etc.

When the medical intervention by antibiotics is delayed or when the latter treatment is ineffective, the inflammation process is deepened and the udder turns dark-red to a blue-violet colour. The Q-ricketsial infection in our experiments took a generalised development and some of the animals died. After recovering from the disease of the most heavily affected animals, the secretory function of their udder was not recovering or was remaining incomplete. This physiological incompleteness and
the related to it economical justification lead to removal of the animals from produce and reproduction.

The results shown here underline the Coxiella mastitis as a clinical form of the Q-rickettsiosis in sheep as a disease of important agricultural and zoonic significance. The coxiellae mastitis are part of the diverse possible showings of the disease among the domestic animals (8, 9).

The obtained results are a basis for enlarged directed search towards the definition of Coxiella burnetii etiology upon inflammations of the udder in sheep, especially when there is availability of sero-epizootiological data for Q-fever in a given region or village.

Conclusions

- We isolated 5 strains Coxiella burnetii from milk secretions of sheep with mass acute mastitis as a specified independently developing clinical form of the Q-rickettsiosis. The isolations of these strains described in the present work are the first to be published in the literature.
- The adapted to chicken embryos Coxiella burnetii strains are pathogenic for guinea pigs when they are infected intraperitoneally. The animals react with clinical symptoms, hystopathological changes (splenomegally) and serological response.
- The coxiellae from clinical material (milk escudates) of sheep with acute mastitis, show obvious pathogenicity for guinea pigs during the intra-peritoneal method of inoculation, leading to a generalised infection with 50% lethality, enlargement of the spleen and the production of specific antibodies.
- The cultivated in chicken embryos C. burnetii strains from sheep with mastitis are pathogenic for young white mice during intraperitoneal and nasal inoculation. The intraperitoneal infection leads to fever and splenomegaly, and the nasally-induced infection to pneumonia, oligosymptomatic or asymptomatic course.
- The infectious milk escudates of the diseased sheep are pathogenic for young white mice after intraperitoneal and nasal inoculations and cause clinical symptoms and patomorphological changes, analogous of the described for infections with strains of CE.
- The adapted for reproduction in chicken embryos strains have pathogenic action for rabbits following intravenous inoculation.
- The cell culture method of cultivation of C. burnetii from mastitis in sheep in the lines HELA, BHK-21 and the primary cell culture of chicken embryonal fibroblasts, is suitable for application when is based on the methodological principle of inoculation of the output material with high concentration and plurality of the infection.
- The electronmicroscopic methods are effective for the direct detection of the pathogen in milk secretions of sheep with mastitis (negative staining) and for morphological indication and identification of the isolated strains in chicken embryos, cell cultures and laboratory animals (negative staining and ultrathin sections).
- During the serological investigations of 153 sheep originating from 5 settlements in 3 regions, and affected by acute mastitis, the number of the positive for coxiellosis was 87 (56.86%). The number of the seropositive animals varied from 27.77% to 100%.
- The individual complement-fixing titres of the sick animals with levels ≥ 1:128 comprised 41.16% of the seropositive reactions ≥ 1:32 – 72.73% and the mean geometric titre was 49.
- During observation of the enzootics from Coxiella burnetii mastitis in sheep we established a tendency towards progressive complications of the clinical development in a large number of animals, with the development of acute inflammatory processes, cease of the milk secretion and general intoxication.

REFERENCES


