Changes of the blood coagulation system of Holstein-Friesian heifers during the course of chlamydiosis

Przemysław Sobiech1*, Justyna Radwińska1, Ryszard Targoński2, Anara Eleusizowa3, Rauza Tuyakova3, Katarzyna Żarczyńska1, Agata Mitura4, and Anna Snarska1

1Department of Internal Diseases, Faculty of Veterinary Medicine, University of Warmia-Mazury in Olsztyn, Olsztyn, Poland
2Department of Cardiology, City Hospital, Olsztyn, Poland
3Department of Veterinary Sanitary, Faculty of Veterinary Sciences and Animal Husbandry, Kostanay State University, Kostanay, Republic of Kazakhstan
4Department of Cattle and Sheep Diseases, National Veterinary Research Institute in Pulawy, Pulawy, Poland

ABSTRACT
The objective of this study was to describe changes in the coagulation and fibrinolytic systems of Holstein-Friesian (h-F) heifers infected naturally with Chlamydia spp. in Kazakhstan. Blood coagulation and morphology tests were performed in 30 heifers, including 20 infected animals (experimental group I) and 10 clinically healthy and uninfected animals (control group II). In the laboratory tests, the following blood parameters were determined: prothrombin time (PT), activated partial thromboplastin time (APTT), thrombin time (TT), antithrombin III activity (ATIII), fibrinogen (FBG), D-dimer (D-D) concentrations, thrombocyte counts (PLT) and leukocyte counts (WBC). Decreased PT and APTT values in the blood plasma of heifers infected with Chlamydia spp. were indicative of intensified procoagulant activity in such an acute infection. Increased ATIII activity could point to the activation of the anticoagulant system to prevent excessive fibrin production in diseased animals. The results indicate that the hemostatic system was activated in heifers with symptoms of chlamydiosis, and regulatory mechanisms prevented the development of life-threatening conditions such as thrombosis or disseminated intravascular coagulation (DIC).

Key words: chlamydiosis, hemostasis, fibrinolysis, cattle

*Corresponding author:
Przemysław Sobiech, Assoc. Prof., Department of Internal Diseases, Faculty of Veterinary Medicine, University of Warmia-Mazury in Olsztyn, Oczapowskiego 14, 10-957 Olsztyn, Poland, Phone: +48 89 5233 487; E-mail: psobiech@uwm.edu.pl
**Introduction**

Chlamydiosis is caused by intracellular bacteria whose developmental cycle comprises two characteristic forms: the infectious elementary body (EB) and the non-infectious reticulate body (RB) (PAWLIKOWSKA and DEPTUŁA, 2006). *Chlamydiaceae* target specific hosts, and elementary bodies infect epithelial cells and monocytes/macrophages in many animal species and in humans (KERR et al., 2005). Chlamydia nomenclature and classification have undergone significant changes in recent years. EVERETT et al. (1999) recognized four families within the order *Chlamydiales: Chlamydiaceae, Parachlamydiaceae, Simkaniaaceae* and a nameless family containing the strain WSU 86-1044. The family *Chlamydiaceae* included two genera of *Chlamydia* (*C. muridarum, C. suis, C. trachomatis*) and *Chlamydophila* (*C. psittaci, C. pecorum, C. pneumoniae, C. caviae, C. felis, C. abortus*) (KRIEG et al., 2011). Chlamydia infections in cattle produce a broad spectrum of clinical symptoms, and they have been noted in many countries around the world, including Germany, Australia, China, Switzerland, Ireland and Sweden. The three most pathogenic Chlamydia species for cattle are *C. abortus, C. pecorum* and *C. psittaci*, where the first two are ubiquitous bacteria that are noted most frequently in cattle (GODIN et al., 2008). In cattle, the most characteristic clinical symptoms of chlamydiosis caused by *C. abortus* include reproductive disorders such as: miscarriage in the last trimester of pregnancy, endometritis, repeat breeding, vaginal infections, weak calf syndrome and perinatal mortality. *C. pecorum* causes infections that lead to pneumonia, bowel inflammations, conjunctivitis, polyarthritis, encephalomyelitis and urinary tract disease. Similarly to *C. abortus*, but to a lesser extent, *C. pecorum* can also contribute to metritis and reproductive dysfunctions (RODOLAKIS and MOHAMAD, 2010). *C. psittaci* has been isolated from cows that had miscarried and in studies of bulls (BOREL et al., 2006; KAUFFOLD et al., 2007). In cattle, chlamydia infections do not always produce symptoms, which significantly obstructs diagnosis and the determination of the spread of disease in the herd. The polymerase chain reaction (PCR) and ELISA tests can be used to effectively diagnose chlamydiosis.

In Kazakhstan, chlamydiosis poses a serious health risk in cattle and a significant challenge for local veterinary services. The experiment described was carried out in the Kostanay region, where 10-30% of cattle are diagnosed with chlamydia infections each year (IBRAGIMOW and ELEUSIZOWA, 2013). Chlamydiosis affects animals from various age groups, beginning from calves, to adult individuals. In the Kostanay region, the prevalence of chlamydiosis is relatively high. In 2010-2013, serological tests were performed on 205,000 animals and produced 31567 positive results.

The hemostatic abnormalities that accompany clinical manifestations of chlamydiosis in animals have not been studied to date. In human medicine, infrequent reports can be found on disseminated intravascular coagulation accompanying pneumonia caused by a
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chlamydia species (YANAGI et al., 2003). In view of the general scarcity of published data concerning the influence of chlamydiosis on hemostasis in cattle and the high incidence of chlamydiosis in cattle in Kazakhstan, the objective of this study was to determine the effect of infections caused by bacteria of the genus *Chlamydia* on coagulation and fibrinolysis in cattle.

**Materials and methods**

The tests were performed on 30 Holstein-Friesian heifers aged 9 to 12 months, reared in the region of Kostanay, northern Kazakhstan. All procedures followed animal care principles and were approved by the Ethics Commission for Animal Experiments. The experimental group (group I) comprised 20 heifers from a herd where clinical symptoms of chlamydiosis, including conjunctivitis, fertility disorders, monoarthritis, polyarthritis and mastitis, had been observed in calves and adult animals. The first clinical examination revealed symptoms of conjunctivitis, and, in some heifers, swelling of carpal and tarsal joints, lameness and decreased appetite. Clinical symptoms of chlamydiosis persisted throughout the study (two weeks after the first collection of blood samples). Chlamydiosis was confirmed by the results of the ELISA test. The control group (group II) was established to determine the reference ranges of parameters for statistical analyses, and it comprised 10 similarly aged and clinically healthy heifers that were regarded as free of chlamydial infections based on the results of the ELISA test.

Blood samples for blood morphology tests and blood coagulation tests were collected from the external jugular vein. Blood was sampled from heifers infected with *Chlamydia* spp. three times in weekly intervals.

Platelet (PLT) and leukocyte (WBC) counts were determined in 1 mL blood samples collected in test-tubes containing K₂EDTA. The contents were gently mixed by turning the test-tube upside down, and blood morphological parameters were determined within 60 minutes. Selected coagulation parameters were determined in platelet-poor plasma, obtained from blood collected in test-tubes containing 3.2% sodium citrate. Blood samples were thoroughly mixed and centrifuged immediately at 1500 × g for 15 minutes, and the resulting platelet-poor plasma was analyzed in a laboratory.

Platelet (PLT) and leukocyte (WBC) counts were determined in an ADVIA 2012i Hematology System analyzer (Siemens Healthcare Diagnostics Inc., USA) by flow cytometry and laser diffraction. Coagulation parameters - prothrombin time (PT), activate partial thromboplastin time (APTT), thrombin time (TT), antithrombin III activity (ATIII), fibrinogen (FBG) and D-dimer (D-D) concentrations were determined using the Technomedika APG2-02-P coagulometer (Electromechanical Company Ltd., Russia) and reagent kits supplied by the same manufacturer.
The ELISA test was performed to identify heifers infected with *Chlamydia* spp., as well as those free of chlamydia. The IDEXX Chlamydiosis Total Ab Test (formerly the CHEKIT *Chlamydompha abortus* Antibody ELISA Test Kit, IDEXX Laboratories) for identifying antibodies against *C. abortus* and *C. pecorum* in ruminants was also used (WILSON et al., 2009; 2012).

The results of blood coagulation tests and selected hematological parameters were processed in the Statistica version 10.0 application (StatSoft Inc., USA). The significance of differences between sampling dates and groups was determined by ANOVA at P≤0.01.

**Results**

The mean values of selected hemostasis and blood morphology parameters are presented in Table 1. The results of laboratory analysis revealed a decrease in PT and APTT values in blood samples from the first collection. After 14 days, the above values became similar to those recorded in the control group. Plasma FBG levels were higher in heifers in group I than in group II throughout the experiment. Similarly to PT and APTT, AT III values were significantly higher in plasma samples from the first and second blood collection than from the third collection, and were higher in heifers in group I than in group II. D-dimer levels and PLT counts were similar in both healthy and infected heifers throughout the study. White blood cell (WBC) count was significantly higher in the heifers in group I than in group II.

Table 1. Mean values of selected coagulation and hematology parameters in heifers infected with *Chlamydia* spp. (group I) and in healthy animals (group II).

<table>
<thead>
<tr>
<th></th>
<th>PT (sec.)</th>
<th>APTT (sec.)</th>
<th>TT (sec.)</th>
<th>D-dimer (µg/L)</th>
<th>AT III (%)</th>
<th>FBG (g/L)</th>
<th>PLT (10⁹/L)</th>
<th>WBC (10⁹/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td></td>
<td></td>
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<td></td>
<td></td>
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</tr>
<tr>
<td>week</td>
<td>I</td>
<td>20.22±0.67</td>
<td>37.72±0.90</td>
<td>15.56±0.92</td>
<td>160.21±21.01</td>
<td>154.18±1.23</td>
<td>7.88±0.79</td>
<td>598.65±38.8</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>25.77±0.87</td>
<td>44.21±0.97</td>
<td>15.67±0.89</td>
<td>159.88±19.11</td>
<td>119.05±1.65</td>
<td>5.77±0.92</td>
<td>531.21±48.7</td>
</tr>
<tr>
<td>II</td>
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<td></td>
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</tr>
<tr>
<td>week</td>
<td>I</td>
<td>24.8±0.65</td>
<td>42.75±0.78</td>
<td>16.19±0.89</td>
<td>159.89±22.15</td>
<td>144.46±1.72</td>
<td>7.21±0.81</td>
<td>602.45±32.5</td>
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<tr>
<td></td>
<td>II</td>
<td>26.21±0.95</td>
<td>45.32±0.76</td>
<td>15.89±0.94</td>
<td>161.78±18.97</td>
<td>120.30±1.22</td>
<td>6.2±0.79</td>
<td>572.91±45.1</td>
</tr>
<tr>
<td>III</td>
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<td></td>
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</tr>
<tr>
<td>week</td>
<td>I</td>
<td>25.97±5.84</td>
<td>44.39±0.81</td>
<td>15.44±0.91</td>
<td>159.45±19.31</td>
<td>121.57±0.90</td>
<td>6.92±0.91</td>
<td>593.46±35.5</td>
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<tr>
<td></td>
<td>II</td>
<td>25.57±0.91</td>
<td>45.28±0.94</td>
<td>15.97±0.85</td>
<td>159.76±20.11</td>
<td>119.87±1.31</td>
<td>6.12±0.81</td>
<td>541.84±37.8</td>
</tr>
</tbody>
</table>

A - significantly different between sampling dates at P≤0.01; B - significantly different between experimental groups at P≤0.01.
Discussion

Systemic hemostasis involves complex and mutually interacting processes and factors that aim to balance out procoagulant and anticoagulant tendencies. The results of this study indicated that infections caused by *Chlamydia* spp. increased coagulation factor levels in plasma, and stimulated vessel repair processes such as fibrinolysis. Bacteria and viruses can affect coagulation factors, mostly endothelial cells, both directly and indirectly through various proinflammatory mediators produced by immune cells. The blood coagulation cascade can be activated by bacteria, mostly Gram-negative bacteria and their endotoxins, such as *Histophilus somni* (MOMOTANI et al., 1985). Blood plasma parameters determined in the samples from the first collection point to hypercoagulation in heifers infected with *Chlamydia* spp. Due to the absence of variations in PLT counts, the recorded abnormalities could be associated with secondary rather than primary hemostasis. The above observation was validated by reduced PT and APPT, that describe the effectiveness of tissue factor and contact activation pathways, respectively. The authors were unable to find any publications describing changes in coagulation parameters induced by acute chlamydiosis. GOEIJENBIER et al. (2014) reported acute chlamydiosis in ferrets with flu, and observed prolonged PT and APTT that peaked on the third to the fourth day of the infection. The highest D-dimer values were recorded on the same dates. The described changes were indicative of DIC with pulmonary microembolism, which leads to respiratory distress syndrome and multiple organ dysfunction syndrome. In this study, blood parameters were analyzed from day 7, which implies that earlier stages of the infection could not be analyzed, but the obtained results are not indicative of DIC in the investigated period. A reduction in PT and APTT values points to higher procoagulant readiness. The above is probably not clinically significant because D-dimer concentrations were within the range of normal values. This could be attributed to the activating influence on the plasma coagulation system of selected cytokines, which are produced and/or released during infection. In animals infected with *Chlamydia* spp., mainly through secretions and excretions from diseased animals, bacteria enter epithelial cells, but their infectious forms can also attack monocytes and macrophages (PAWLIKOWSKA and DEPTUŁA, 2006). The activation of the leukocyte system in infected heifers was confirmed by the observed increase in WBC counts. Infected host cells release proinflammatory cytokines, including the CXC-chemokine ligand 1 (CXCL1, CXCL8), interleukin 8 (IL-8), CXCL16, granulocyte/ monocyte colony stimulating factor (GM-CSF), interleukin-1α (IL-1α), interleukin-6 (IL-6) and tumor necrosis factor (TNF) (RASMUSSEN et al., 1997; JOHNSON, 2004). Increased expression of the CC chemokine ligand 5 (CCL 5) and CXCL10 in epithelial cells intensifies the production of chemokines and interferons (INF): IFN-γ-α, IFN-β and IL-12 (MAXION and KELLY, 2002; JOHNSON, 2004). Interleukin 1 (IL-1), IL-6, IL-8 and IL-12 have both proinflammatory and procoagulant properties. The IL-6 plays the
most important role and exerts the most significant effects on the hemostatic system. It increases the expression of the tissue factor that activates the TF-VIIa complex in the coagulation system. Active forms of factors IX and X (IXa and Xa) are produced in the initiation phase. Factors IXa and VIIIa form the tenase complex, and factors Xa and Va produce the prothrombin complex on TF-presenting cells. The plasma coagulation system (amplification phase) is prepared for thrombin production (propagation phase). The IL-6 also increases the concentrations of FBG and factor VIII, which is one of the reasons why FBG is an acute phase protein (ZYMAŃSKA-CZERWINSKA and BEDNAREK, 2007). Thus, the increase in FBG levels recorded in heifers infected with Chlamydia spp. could not be directly linked with the synthesis of the fibrin network stabilizing intravascular clots, especially since clinical symptoms of vascular thrombosis were not observed in the analyzed animals. The observed increase in the FBG concentration was probably a marker of the acute chlamydiosis. The IL-6 plays an important role during the activation of the vascular endothelial cells that regulate hemostasis. Endothelial activation or dysfunction leads to the secretion of active compounds, both procoagulant factors such as the platelet-activating factor (PAF), and anticoagulant factors such as ATIII, thrombomodulin and tissue factor pathway inhibitor, which regulate hemostasis. Despite elevated plasma FBG levels, FBG was converted to fibrin at the same rate in infected and healthy heifers, as demonstrated by TT measured in seconds. This indicates that the processes inhibiting the plasma coagulation system were effective during thrombin production. Interestingly, ATIII values increased considerably in the group of infected heifers. In a study of pigs experimentally infected with Staphylococcus aureus, SOERENSEN et al. (2013) observed a drop in ATIII values in the progression of DIC. In our study, the increase in ATIII values points to an anticoagulant tendency, which is recorded during infections and convalescence. The observed changes in ATIII suggested that the anticoagulation system inhibited secondary hemostasis enzymes, because ATIII is the most powerful natural inhibitor of thrombin and factor Xa. To a lesser extent, ATIII also inactivates factors XIIa, XIa and IXa through the formation of inactive complexes that are quickly removed from circulation. Antithrombin III activity is probably also capable of inhibiting the TF-VIIa complex. In consequence, the results of coagulation tests in the group I heifers, performed on blood plasma samples from the third collection, were similar to those recorded in group II and described in the literature in healthy cattle (SOBIECH et al., 2005). Interestingly, D-dimer concentrations did not increase at successive sampling dates in group I or between groups. D-dimer, the smallest degradation product of cross-linked (by factor XIII) fibrin, is an indirect indicator of fibrinolytic activity. D-dimer concentration increases significantly in disorders that involve thrombin production, in particular inside blood vessels, such as DIC. In human medicine, D-dimer is one of the most important diagnostic and therapeutic indicators of DIC. Stable D-dimer levels in
infected heifers indicate that fiber clots were not produced in the blood vessels or organs where the fibrinolytic system could be activated.

Lipopolysaccharide (LPS) - an endotoxin found in the cell wall of *Chlamydia* spp. could initiate the tissue factor coagulation pathway, activate factor XII to trigger the contact activation pathway, and activate prothrombin and factor X. The LPS could also activate secondary fibrinolysis with the involvement of inflammatory immune mediators (RADWIŃSKA et al., 2012).

The results of this study indicate that *Chlamydia* spp. infections influenced the plasma coagulation system in heifers. In view of reports suggesting that *Chlamydia* spp. infections could contribute to vascular injury, endothelial damage, atherosclerosis and cardiovascular diseases in humans (GUPTA and LEATHAM, 1999; GATTONE et al., 2001), more extensive research related to animal models is needed in this area.

**References**


SAŽETAK

Cilj je ovog istraživanja bio opisati promjene u koagulacijskom i fibrinolitičkom sustavu holštajnsko-frizijske pasmine junica prirodno zaraženih vrstanima roda **Chlamydia** spp. u Kazahstanu. Testovi grušanja krvi i morfološki testovi provedeni su na 30 junica od čega 20 zaraženih (pokusna skupina) i 10 klinički zdravih nezaraženih životinja (kontrolna skupina). Uporabom laboratorijskih testova određeni su sljedeći krvni pokazatelji: protrombinsko vrijeme, aktivirano parcijalno tromboplastinsko vrijeme, trombinsko vrijeme, aktivnost antitrombina III, fibrinogen, koncentracije D-dimera (D-D), broj trombocita i broj leukocita. Smanjene vrijednosti protrombinskog vremena i aktiviranog parcijalnog tromboplastinskog vremena junica zaraženih vrstanima **Chlamydia** spp. bile su indikacija za pojačano prokoagulacijsku aktivnost kod takve akutne infekcije. Povećana aktivnost antitrombina III mogla bi upućivati na aktivaciju antikoagulacijskog sustava da bi se spriječila prevelika proizvodnja fibrina u oboljelih životinja. Rezultati upućuju na zaključak da je hemostazni sustav bio aktiviran u junica sa znakovima klamidioze te da su regulacijski mehanizmi spriječili razvoj po život opasnih stanja kao što je tromboza ili diseminirana intravaskularna koagulacija.

**Ključne riječi:** klamidioza, hemostaza, fibrinoliza, govedo